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RESEARCH ARTICLE

Antibiotics commonly applied in animal farming can alter soil biotic effects on plant functioning and responses to drought stress

Louisa C. Neubauer · **Mark van Kleunen** ·
Rutger A. Wilschut

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Abstract
Background Antibiotic pollution, caused by extensive antibiotic applications in industrial farming, threatens human and animal health, but may also affect plant functioning. However, it remains unknown whether antibiotics affect plant performance directly, or indirectly via effects on soil biota. Potential soil-community-mediated antibiotic effects may be most pronounced under abiotic stress, as plant-stress alleviation by soil biota may be compromised.
Methods We conducted a greenhouse experiment to examine how two commonly applied antibiotics (tetracycline and amoxicillin) alter the performance of six grassland plant species in the presence and absence of soil biota. We also assessed whether antibiotic effects on plant performance are stronger under drought stress.
Results Antibiotic presence did not affect plant biomass, but high tetracycline concentrations reduced positive soil microbial-community effects on biomass allocation to roots. Furthermore, high amoxicillin concentrations enhanced the positive soil-community effect on chlorophyll A fluorescence kinetics (a performance index), while limiting the soil biotic drought stress-alleviating effect on leaf chlorophyll concentration. Nevertheless, most antibiotic effects were antibiotic type- and dose-dependent, differed among species and depended on soil-community presence.
Conclusion Our study shows that antibiotics at realistic environmental concentrations can alter plant-soil interactions in plant-species-specific ways and

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L. C. Neubauer (✉) · M. van Kleunen · R. A. Wilschut
Ecology Group, Department of Biology, University of Konstanz, Constance, Germany
e-mail: louisa.neubauer@unibe.ch

L. C. Neubauer
Institute of Plant Sciences, University of Bern, Bern, Switzerland

M. van Kleunen
Zhejiang Provincial Key Laboratory of Plant Evolutionary Ecology and Conservation, Taizhou University, Taizhou, China

M. van Kleunen
Zhejiang Key Laboratory for Restoration of Damaged Coastal Ecosystems, Taizhou University, Taizhou, China

R. A. Wilschut
Department of Nematology, Wageningen University and Research, Wageningen, The Netherlands

therefore may eventually affect natural plant community composition.

Keywords Environmental pollution · Plant-soil interactions · Antibiotics · Drought · Drought-stress amelioration

Introduction

Natural systems are increasingly exposed to multiple aspects of global change (Lewis & Maslin 2015), leading to reduced biodiversity and altered ecosystem functioning (Rillig et al. 2019; Speißer et al. 2022). Nevertheless, interactive effects of global change drivers on natural communities are poorly understood (Rillig et al. 2019; Speißer et al. 2022). Climate change and environmental pollution are major drivers of global change, resulting in weather extremes (*e.g.* droughts) as well as the accumulation of pollutants in terrestrial and aquatic ecosystems (Steffen et al. 2005; United Nations Environmental Programme 2021). One source of environmental pollution is the extensive use of antibiotics in industrial farming, which mostly enter natural ecosystems through the application of manure in agricultural fields (Burkhardt et al. 2005; Stoob et al. 2007). Antibiotic concentrations in natural ecosystems may even further increase given that the use of antibiotics is predicted to increase by 60% until 2030 (van Boeckel et al. 2015).

The presence of antibiotics in the environment has gained awareness due to the threat it poses to animal and human health through the increased antibiotic resistance of pathogens (Heuer et al. 2011). However, antibiotic pollution may also affect natural plant communities, as antibiotics can alter primary plant productivity and shift the composition of soil communities, microbes as well as other soil organisms (Adomas et al. 2013; Gomes et al. 2017; Minden et al. 2017). Yet, while impacts of antibiotics on plant performance are likely to be at least partly caused by changing interactions between plants and soil biota, it is unknown how antibiotics affect the impacts of soil biota on plant performance.

Research on antibiotic impacts on plant performance has focused on direct effects. Typically, antibiotics act phytotoxically (Adomas et al. 2013; Minden et al. 2017; Wei et al. 2009), as they can negatively affect plant biomass (Wei et al. 2009), inhibit root growth (Adomas et al. 2013), reduce chlorophyll content, or induce oxidative stress (Gomes et al. 2017). However, such antibiotic effects on plant performance are dose-dependent and may even be positive at a low dose (Migliore et al. 2010; Minden et al. 2017). Furthermore, antibiotic effects on plant performance vary among plant species (Batchelder 1982; Liu et al. 2009; Minden et al. 2017), suggesting that antibiotics could cause shifts in plant community composition. However, to understand impacts of antibiotics on plant communities, it is key to also consider possible indirect effects, which may be mediated by changes in soil communities.

The release of antibiotics into the environment may alter the structure and functioning of soil microbial communities. Antibiotic pollution has been shown to alter the biomass and relative abundances of different soil microbial groups (Kotzerke et al. 2011; Thiele-Bruhn & Beck 2005; Zhao et al. 2023). Antibiotic pollution especially reduces bacterial abundance and diversity (Cerqueira et al. 2020; Zhao et al. 2023). Such shifts may subsequently lead to reduced soil nutrient cycling (Hammesfahr et al. 2008; Thiele-Bruhn & Beck 2005), which may alter plant growth. Antibiotics can also negatively affect non-target organisms, including earthworms and nematodes (Vangheel et al. 2014; Zhao et al. 2022), which are known to affect plant performance in diverse ways (Wilschut & Geisen 2021; Xiao et al. 2018). However, whether antibiotic-driven changes in soil communities indeed translate to altered plant responses remains unknown.

Changes in soil community composition caused by antibiotic pollution may be especially relevant for plant performance under stressful conditions. Drought is one of the most prominent abiotic stressors in natural systems, typically causing a reduced primary productivity and shifts in plant community composition (Ciais et al. 2005; Kardol et al. 2010). In response to drought, plants often invest in their root system – at the expense of aboveground tissue growth – to maximize nutrient and water acquisition (Eziz et al. 2017; Poorter et al. 2012) and attract beneficial soil biota to alleviate stress (Williams & de Vries 2020). Under drought, soil biota may enhance plant biomass allocation to roots (Ruppert et al. 2024). Moreover, especially drought-adapted bacteria benefit the plants by alleviating stress through provisions of stress-reducing hormones and metabolites (Ortiz et al. 2015). Finally, mycorrhizal fungi can enhance antioxidant enzyme activity, thereby reducing oxidative stress and promote water use efficiency (Ortiz et al. 2015). As such, soil biota play a key role as promotors of plant performance during drought (de Vries et al. 2018). Examining how antibiotics affect the positive impacts of soil community presence on plant responses to drought stress may help to understand natural plant community dynamics in regions with intensive farming.

In this study, we set up a greenhouse experiment to examine how antibiotics alter the performance of grassland plant species in the presence and absence of soil biota, as well as to assess whether potential antibiotic effects on plant performance are stronger under drought (Fig. 1). We tested the effects of two commonly used antibiotics, tetracycline and amoxicillin, which vary in their mode of action (Bird 1994; Chopra & Roberts 2001), and applied them at concentrations typically detected in agricultural soils, as well as at tenfold higher concentrations, which still fall within the range of concentrations found in the environment. We tested the hypotheses that antibiotics 1) directly negatively affect plant performance, particularly when applied at high doses, 2) strengthen the negative impacts of drought on plant performance, and 3) alter soil-community effects on plant performance, most notably by reducing the drought-stress ameliorating effect of soil biota on plant performance. As measures of plant performance, we examined plant biomass, the chlorophyll A-based Performance Index as a measure of plant stress and leaf chlorophyll content as a measure of plant nutrient uptake. Additionally, we examined how plant biomass allocation responses to drought were modified by antibiotics exposure and the presence of a soil community.

Materials and Methods

Study species and pre-cultivation

To test our hypotheses, we used six native European grassland species (*Bromus hordeaceus* L., *Cerastium*

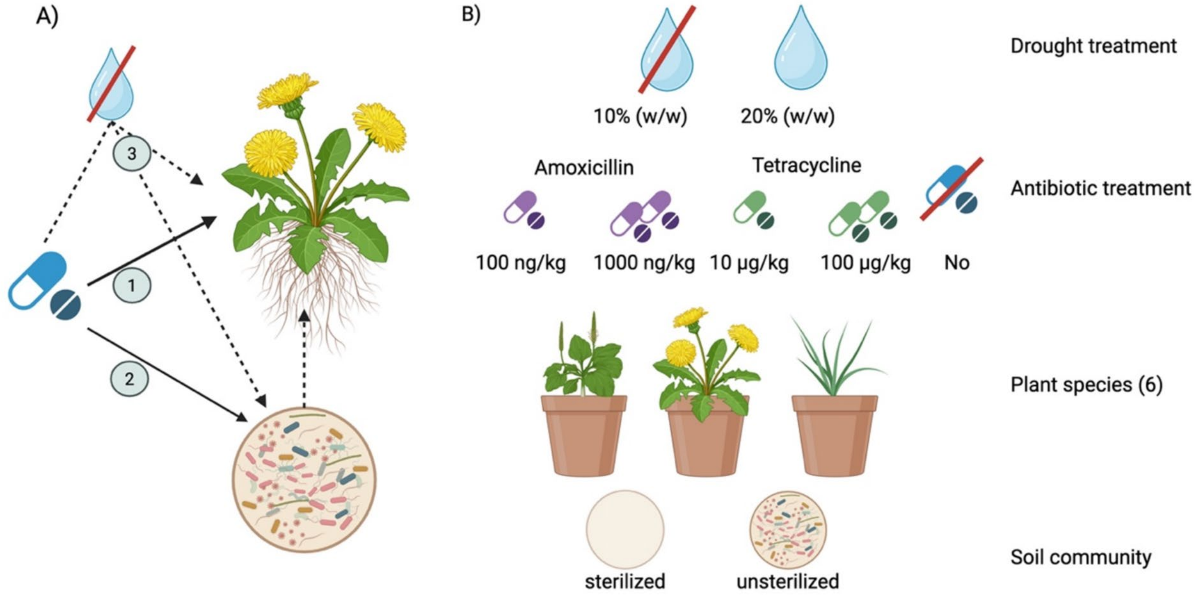

Fig. 1 (**A**) Conceptual overview of antibiotics impact on plants. First, antibiotics may affect plant performance directly (1). Second, antibiotics might alter soil-community effects on plant performance (2). Third, antibiotics may modify the impacts of drought on plant performance directly or indirectly, i.e. by reducing the drought-stress alleviating effect of soil biota (3). (**B**) Schematic overview of the experimental design. We examine how two different antibiotics in low and high concentrations alter the performance of 6 natural grassland plant species in the presence and absence of the soil community and whether it modifies plant performance under drought. Created in BioRender. Neubauer, L. (2025)https://BioRender.com/z20v865

fontanum Baumg., *Leontodon hispidus* L., *Lolium perenne* L., *Plantago lanceolata* L., *Prunella vulgaris* L., Sup. Table 1). The selected species all commonly occur in (semi-)natural grasslands and are thus likely to become exposed to antibiotic pollution from adjacent agricultural areas. The seeds were either supplied by the Botanical Garden of the University of Konstanz or purchased from Rieger-Hofmann GmbH (Sup. Table 1). Seeds were sown on 10 January 2022 and 17 January 2022, based on previous examinations of their germination time, ensuring that all transplanted seedlings would be in a comparable developmental stage (Sup. Table 1). We sowed the seeds in germination pots (TEKU® TK1214, Pöppelmann GmbH & Co.KG, Lohne, Germany) filled with potting soil (Einheitserde® CL P, Einheitserde Co., Sinntal-Altengronau, Germany), and transferred them to a climatized greenhouse with temperatures ranging from 14 °C (night) up to 22 °C (day), and a day-night rhythm of 12:12 h.

Experimental overview

To examine the direct and soil-community-mediated impact of antibiotics on plant performance under ambient and drought conditions, we conducted a greenhouse experiment at the Botanical Garden of the University of Konstanz (WGS84: 47.691952 N, 9.179672 E). In our experiment, we examined the effects of soil-community presence (sterilized vs. unsterilized), soil moisture (control vs drought), and five antibiotic treatments (two types of antibiotics in two concentrations and a control treatment without antibiotics) (Fig. 1). In total, we planned to establish 600 1.5 L pots (6 species × 2 soil treatments × 2 moisture treatments × 5 antibiotic treatments × 5 replicates), but due to a shortage of soil we only established four replicates for plants grown in sterilized soil without antibiotics under normal watering conditions, resulting in a total of 594 pots.

Soil treatments

To establish our soil-community treatment, we collected field soil from a semi-natural grassland area on a sandy loam soil (WGS 84: 47.6915195 N, 9.1791881 E) and sieved it through a 15-mm mesh to remove roots and stones but maintaining soil communities. Half of the field soil was sterilized by autoclaving (Type FVS, Federgari Autoclavi SpA, Albuzzano Italy) for one hour at approximately 140 °C. This was repeated after 48 h. We acknowledge that autoclaving may have increased the availability of certain nutrients in the soil due to the breakdown of microbial cells (Anderson & Magdoff 2005) and therefore cannot rule out that some effects attributed to soil-community absence are explained by changes in soil abiotic conditions. However, we did not find evidence for a generally positive effect on plant biomass (see Results section). We then separately mixed the sterilized and unsterilized field soil with a 1:1 (volume-based) mixture of sand (Quarzsand, size: 0.3–0.8 mm, hellbeige, Lose MF: 20,201, Emil steidle GmbH & Co. KG., Sigmaringen, Germany) and vermiculite (Vermiculite®, size: 0.3–0.8 mm, Sprockhövel, Germany) in a ratio 1:1. Prior to pot filling, we determined the moisture contents of both soil mixtures by drying five ~ 20 g soil samples at 105 °C for 24 h. Thereafter, circular 1.5 L pots (Soparco, 1.5 L SMH3 (2415), Condé-sur-Huisne, France) were filled with 1030 g dry weight soil mixture, allowing soil-weight-based watering treatments later on. On 31 January 2022 and 1 February 2022, we transplanted single, similar-sized seedlings of the six species to the centre of the pots which were then transferred to a greenhouse (light settings as described above) and positioned according to a randomized block design with five replicate blocks. As initial plant size often predicts eventual plant biomass variation (e.g., Ruppert et al. 2024), on 14 February 2022, we estimated the initial leaf area of all individual plants by counting the number of leaves, excluding cotyledons, measuring the width and length of the largest leaf using a digital calliper and multiplying the area of the largest leaf by the total number of leaves per seedling.

Antibiotic treatments

After three weeks of seedling growth, during which we allowed the plants and soil microbial communities to establish and acclimatize, we applied the antibiotic treatments. Pots were either treated with a low or high concentration of one of the two antibiotics tetracycline (T3258, Sigma-Aldrich Chemie GmbH, Germany) and amoxicillin (A8523, Sigma-Aldrich Chemie GmbH, Germany) or left untreated. Tetracycline and amoxicillin were the most frequently used antibiotics for livestock in 2019 (EMA 2021) and differ in their mode of action. Tetracycline belongs to the tetracycline family that inhibits protein synthesis, thereby decelerating bacterial growth (Chopra & Roberts 2001). Amoxicillin belongs to the class of beta-lactam antimicrobials, which cause lysis of the cell wall, thereby destroying the bacterium (Bird 1994). The selected concentrations fall within the range detected in the field. We established our low-concentration treatment based on the lower end of concentrations detected in the field and established a high-concentration treatment based on a tenfold increase of the low-concentration treatment. Tetracycline concentrations in European agricultural soils can range from below 5 µg/kg to over 200 µg/kg, with some cases reaching levels in the mg/kg range, however typical concentrations are below 100 µg/kg, (Graumintz & Jungmann 2021; Hamscher et al. 2002). Amoxicillin is detected in concentration from below 10 ng/L to above 1000 ng/L in the environment and irrigation waters (Graumintz & Jungmann 2021). Therefore, the plants were treated with one of the following concentrations: no antibiotics, 10 µg/kg dry soil or 100 µg/kg dry soil for tetracycline, and 100 ng/kg dry soil or 1000 ng/kg dry soil for Amoxicillin. The antibiotics were applied once: amoxicillin on 23 February 2022, and tetracycline on 24 February 2022. Both antibiotics were diluted in tap water and 100 mL of the dilution was applied. We also applied the same amount of tap water to the control group not treated with antibiotics.

Drought treatment

In the first weeks of the experiment, pots were regularly watered from above to ensure sufficient water availability. To examine how possible antibiotic-induced impacts on soil community development affected plant responses to drought, we then started a four-week drought treatment, seven weeks after seedling transplantation and four- weeks after antibiotic application (14 March 2022). During this drought treatment, we reduced soil moisture in half of the pots from 20% (w/w) (control) to 10% (w/w) (drought) by stopping the watering until the soil weight was reduced to the required minimum weight. We selected these soil moisture levels based on earlier experiments that showed significant performance declines between 20 and 10% soil moisture (e.g. Wilschut & van Kleunen 2021). During this period, soil moisture was maintained and adjusted by weighing and watering each pot individually every other day. After four weeks of drought, the soil moisture was recovered to 20% (w/w).

Harvest

We harvested the plants one week after the end of the drought treatment, so that plants in total grew for 12 weeks. We first clipped shoots directly above the soil surface, after which they were dried at 70 °C for at least 72 h and weighed. We then separated roots from the soil, and after washing them, they were dried and weighed in the same way as the shoots.

Performance index and chlorophyll concentration

In addition to measuring biomass as an indicator of plant performance at the end of the experiment, we performed non-destructive measurements during the experiment to gain information about the physiological and nutritional state of the plant. We measured the chlorophyll A fluorescence kinetics (O-J-I-P), which is indicative of plant performance (Stirbet & Govindjee 2011; Strasser et al. 2000), using a FluorPen FP100 (PSI, spol.sr.o, Czech Republic) (Banks 2017; Kalaji et al. 2016). We determined this 'performance index' (PI(abs)) for three out of the five replicates (replicate blocks 1, 3 and 5), with one measurement per plant. We also measured the light absorbance of leaves, which is indicative of chlorophyll content and nitrogen contents of leaves (Ling et al., 2011; Süß et al., 2015) using the SPAD-502Plus (KONICA MINOLTA, INC., Japan). To do this, we measured three out of five replicates (replicate blocks 1, 3 and 5) and averaged values of two leaves, each with three measurements, per plant, thereby incorporating variation among measurement positions as well as age-dependent chlorophyll content in the leaf (Hikosaka et al. 1994). We took the chlorophyll A fluorescence and SPAD measurements three times over the course of the experiment: 1) prior to the soil moisture treatment with antibiotics already applied (21 March 2024 and 15 March 2024, respectively), 2) at the end of the soil moisture treatment (05 April 2024 and 06 April 2024), and 3) after re-wetting (17 April 2024 and 18 April 2024). We performed measurements for both parameters on healthy leaves that were big enough to fill out the devices' area of measurement. In addition, we standardized measurements within species by always selecting mature, similar-sized leaves, and excluded cotyledons, as well as very young or old leaves, from the measurements.

Statistical analysis

All statistical analyses were performed in R version 4.2.0 (R Core Development Team 2022). In general, linear mixed-effect models using the lme function (nlme package, V3.1–152; (Pinheiro et al. 2021)) were used to analyse treatment effects on biomass, relative root weight (i.e. root weight divided by total plant weight), the performance index as an indicator of stress, and SPAD measurements as an indicator of leaf nitrogen content. In order to analyse the impacts of the different antibiotic treatment levels, we constructed a contrast matrix describing the main aspects of the antibiotic treatments in order to statistically test the following effects: presence of antibiotics (present vs absent), type of antibiotic (amoxicillin vs tetracycline), amoxicillin concentration (low vs. high), and tetracycline concentration (low vs. high).

Prior to the analysis of treatment effects on total biomass, we $\log_{10}$-transformed the total biomass data to improve the normality and homoscedasticity of the residuals. To account for effects of initial size differences among plant individuals, we calculated initial leaf area. Overall, we created two sets of linear mixed effects models. The first set of models examined the general effects of our treatments on the response variables by including 'plant species' as a random intercept term. In the second set, we specifically examined variation in species responses by including 'plant species' and its interactions with the treatments as fixed terms. The 'block' term was included as random effect, while 'soil treatment', 'soil moisture', the four contrasts, as well as all interactions of soil treatment and soil moisture with each other and with each contrast were included as fixed effects. When modelling total biomass and relative root weight, we added 'initial leaf area' as covariate in the model. Finally, we modelled the additional measurements – SPAD and performance index – separately for each of the three dates. In the latter analyses, we included the 'block' term as fixed term instead of a random term since we only measured three replicates. For all models, we tested the significance of fixed effects with the 'Anova' function ('car' package; (Bates et al. 2007)), using Wald Chi-squared tests for mixed effects models and F-tests for basic linear models.

Results

Biomass production

Our model testing overall treatment effects on plant biomass indicated that soil treatment and soil moisture interactively affected total plant biomass (Table 1), but that this effect was independent of antibiotic treatments (Fig. 2b). Drought reduced plant biomass both in sterilized and unsterilized soil (Fig. 2a). However, this effect was more negative (−68%; based on untransformed data) in sterilized than in unsterilized soil (−22%) (Fig. 2a). Notably, plant biomass on average was higher in unsterilized than in sterilized soil (Fig. 2a). When we included species as fixed term, we found that interactive effects of soil community and soil moisture on total plant biomass varied across species, but did not depend on antibiotic treatments (Sup. Figure 1, Sup. Table 3). Specifically, drought appeared to negatively affect the biomass of *Leontodon hispidus* and *Prunella vulgaris* under sterilized but not under unsterilized conditions, while such a pattern was not found for the other species, which were negatively affected by drought irrespective of soil-community presence (Sup. Figure 1). Overall, the grass species *Lolium perenne* and *Bromus hordeauceus* showed a positive biomass response to soil sterilization, exhibiting increased biomass but also the strongest negative effect on biomass caused by drought, whereas herbs displayed a negative response, with a reduction in biomass following sterilization (Sup. Figure 1).

Biomass allocation

Our model examining general treatment effects on root weight ratio did not indicate overall effects of the antibiotic treatment, nor an overall difference between tetracycline and amoxicillin application. However, the model indicated that plant biomass allocation to roots was affected by the tetracycline concentration, and that this effect depended on the interactive effect of soil moisture and soil treatment (Table 2). Relative biomass allocation to roots was higher (+4.60%)

Table 1 Summary of linear mixed-effect models for total biomass and root weight ratio. Significant results ($p < 0.05$), based on type-II Wald Chi-squared tests, are shown in bold

	Log(Total biomass)			Root weight ratio		
Fixed effects	χ^2	Df	Pr(> χ^2)	χ^2	Df	Pr(> χ2)
Initial size	75.557	1	**< 0.001**	7.151	1	**0.007**
Presence of soil community (S)	145.669	1	**< 0.001**	13.844	1	**< 0.001**
Soil moisture (M)	52.035	1	**< 0.001**	24.950	1	**< 0.001**
Antibiotic presence (A)	0.085	1	0.771	0.000	1	0.987
Type of antibiotic (T-A)	2.535	1	0.111	1.266	1	0.261
Amoxicillin concentration (A-C)	0.111	1	0.738	2.225	1	0.136
Tetracycline concentration (T-C)	0.004	1	0.947	0.183	1	0.668
S*M	6.711	1	**0.010**	2.726	1	0.099
S*A	1.173	1	0.279	0.006	1	0.940
S*T-A	0.054	1	0.816	0.477	1	0.490
S*A-C	0.053	1	0.818	0.690	1	0.406
S*T-C	0.004	1	0.949	0.557	1	0.456
M*A	0.030	1	0.863	1.385	1	0.239
M*T-A	0.133	1	0.716	0.041	1	0.840
M*A-C	0.041	1	0.839	1.269	1	0.260
M*T-C	0.376	1	0.540	0.452	1	0.502
S*M*A	0.837	1	0.360	0.424	1	0.515
S*M*T-A	0.999	1	0.318	0.158	1	0.691
S*M*A-C	0.511	1	0.475	0.001	1	0.973
S*M*T-C	0.046	1	0.830	4.571	1	**0.033**
Random effects	SD			SD		
Block	0.089			0.027		
Species	0.812			0.064		

under high tetracycline concentrations than under low tetracycline concentrations in control soil moisture conditions in unsterilized soil, but not in sterilized soil (Fig. 3a). However, when exposed to drought, biomass allocation to roots was lower (−3.96%) under high than under low tetracycline concentrations in unsterilized, but not in sterilized soil (Fig. 3a). Overall, relative biomass allocation to roots appeared to be higher in unsterilized than in sterilized soils. In contrast to the impacts of tetracycline, we did not find any significant effect of amoxicillin concentration on the root weight ratio (Fig. 3b).

Our model examining variation in species' relative biomass allocation responses showed that plant biomass allocation species-specifically depended on amoxicillin concentrations, and that this effect depended on the soil treatment (Sup. Figure 2, Sup. Table 3). Most notably, in sterilized soil, *Leontodon hispidus* and *Lolium perenne* plants allocated more biomass to roots when exposed to high amoxicillin concentrations than when exposed to low amoxicillin concentrations, while the opposite effect was observed for *Cerastium fontanum* and *Plantago lanceolata* (Sup. Figure 2). These effects were not observed in unsterilized soil. Furthermore, drought affected biomass allocation to roots in a species-specific way, and this effect depended on the soil treatment (Sup. Figure 2, Sup. Table 3). Most notably, in sterilized soils, drought negatively affected the root weight ratio in case of *Bromus hordeaceus, Cerastium fontanum and Lolium perenne,* while these species showed rather weak negative or positive root weight ratio responses to drought in unsterilized soil (Sup. Figure 2). Oppositely, drought positively affected root weight ratios of *Leontodon hispidus* and *Plantago lanceolata* in sterilized soil, but negatively affected their root weight ratios in unsterilized soil.

Fluorescence measurements

Prior to the start of the drought treatment, the chlorophyll A fluorescence, indicative of physiological

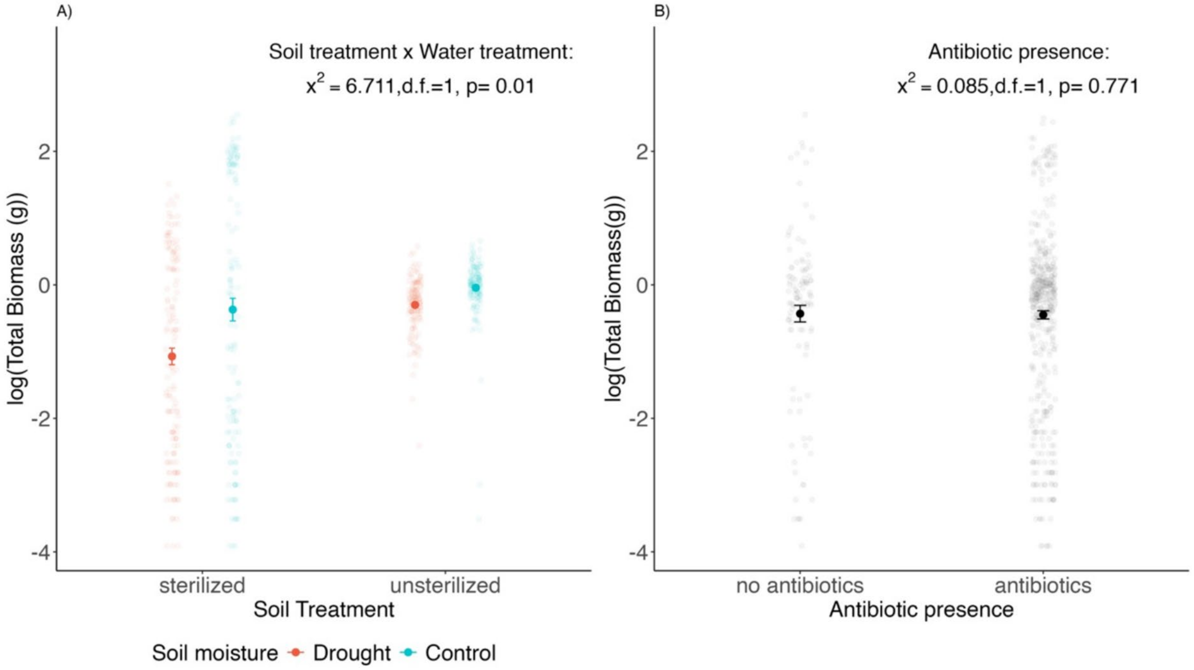

Fig. 2 Average plant biomass (log-transformed) responses of European native grassland plant species to the soil treatment (sterilized vs. unsterilized soil) and soil moisture (color-coded) (**A**) and to the presence of antibiotics (**B**). Large dots and whiskers represent mean values ± standard errors, while lighter dots show data distribution

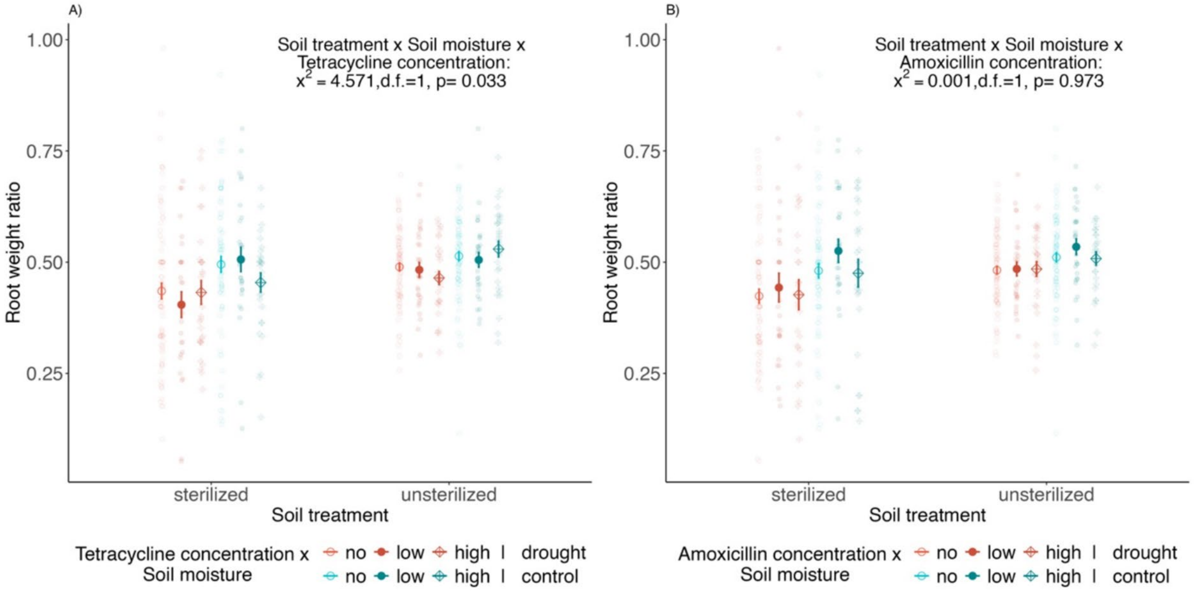

Fig. 3 Plant root weight ratio (root biomass/total biomass) responses to the soil treatment, soil moisture (colour-coded) and antibiotic concentration – tetracycline (**A**) and amoxicillin (**B**) (shape-coded). Bold dots and whiskers indicate means ± standard errors, while lighter points show the raw data distribution. Although not included in the statistical analysis, the values for plants not treated with tetracycline are plotted as a reference

stress experienced by the plants, differed between plants treated with different amoxicillin concentrations, but this effect depended on soil sterilization (Fig. 4A, Sup. Table 4). An increased amoxicillin concentration decreased the performance index by 24.49% in sterilized soil, but increased the physiological performance index by 17.75% in unsterilized soil (Fig. 4A). Yet, we did not find such an effect for the application of varying tetracycline concentration (Sup. Table 4). When including individual species responses in the model, soil sterilization was revealed to affect the physiological performance index in species-specific ways (Sup. Figure 3A, Sup. Table 5). The performance index of *Leontodon hispidus*, *Plantago lanceolata,* and *Prunella vulgaris* was higher in unsterilized soil, while that of the other three species was unaffected by soil sterilization.

Measurements at the end of the drought treatment showed that drought affected the chlorophyll A fluorescence, but this effect depended on soil sterilization (Fig. 4B, Sup. Table 6). Drought reduced the physiological performance index in unsterilized and sterilized conditions (Fig. 4B), but this effect of drought was stronger for plants growing in sterilized soil (−44.26%) than for plants growing in unsterilized soil (−1.30%).

Measurements done after soil re-wetting revealed differences in the chlorophyll A fluorescence between plants previously subjected to drought and plants continuously growing under control soil moisture conditions, but this effect depended on soil sterilization (Fig. 4C, Sup. Table 8). In sterilized soil, the physiological performance index of plants previously experiencing drought was 24.49% lower in comparison

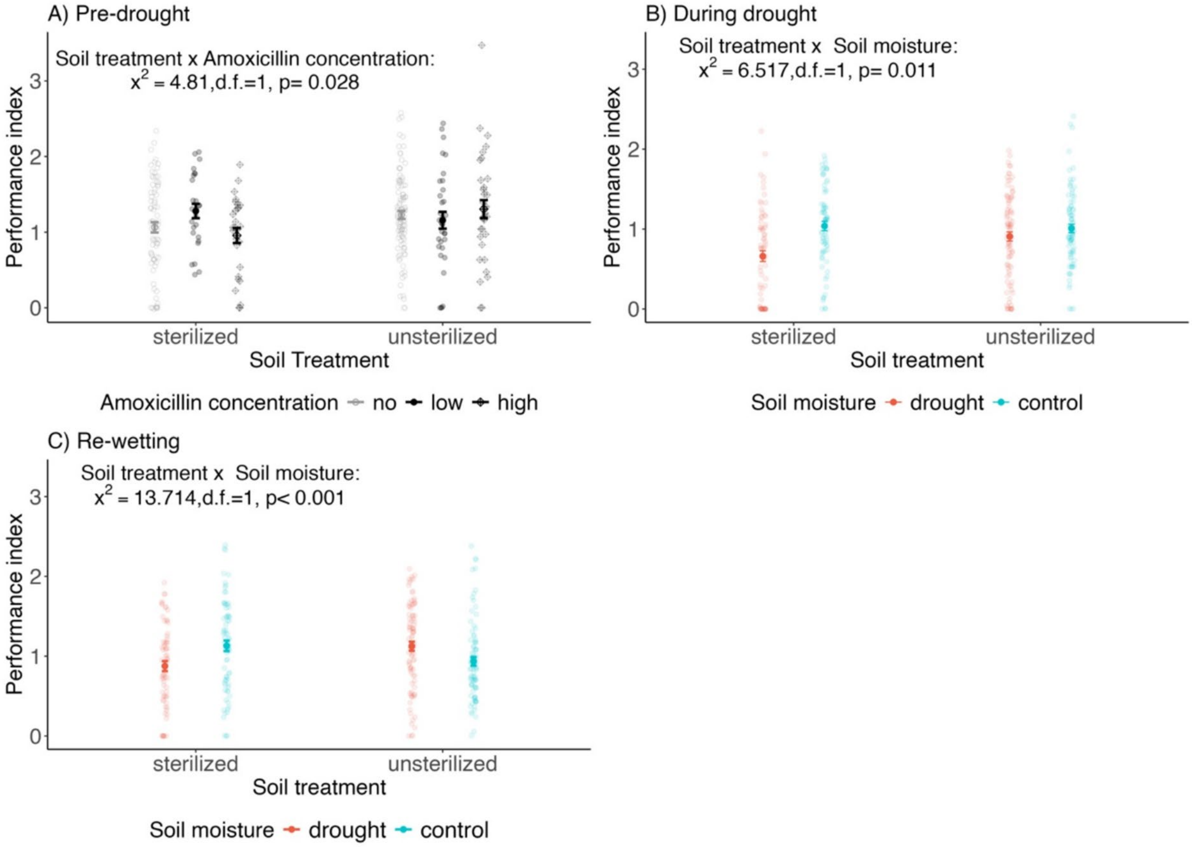

Fig. 4 Performance index response to the soil treatment and amoxicillin concentration (shape-coded) prior to drought (**A**) as well as the response to soil treatment and soil moisture (colour-coded) during drought (**B**) and with re-wetting (**C**). Dots and whiskers indicate means ± standard errors, while lighter dots show raw data distribution. Although not included in the statistical analysis, the values for plants not treated with amoxicillin (A) are plotted as a baseline-guide

to plants which grew under control soil moisture throughout the experiment. In unsterilized soil, the re-wetting led to a 17.75% higher physiological performance index for plants previously experiencing reduced soil moisture compared to plants that experienced control soil moisture conditions throughout the experiment (Fig. 4C). Plants treated with different amoxicillin concentrations also exhibited differences in the physiological performance index, but these differences depended on the previous soil-moisture treatment and were plant-species specific (Sup. Figure 3B, Sup. Table 9).

SPAD values

Prior to the start of the drought treatment, plants exposed to high amoxicillin concentrations on average showed 4.85% lower SPAD values, indicating reduced chlorophyll and N concentrations, than plants treated with a low amoxicillin concentration (Fig. 5A, Sup. Table 4). However, no such effect was observed with varying tetracycline concentrations (Sup. Table 4). Moreover, prior to the drought treatment, plants grown in sterilized soil on average exhibited 15.47% higher SPAD values than plants grown in unsterilized soil (Fig. 5A, Sup. Table 4). Examination of individual species' responses indicated that soil sterilization affected SPAD values in species-specific ways (Sup. Figure 4A, Sup. Table 5). *Bromus hordeaceus*, *Lolium perenne*, *Plantago lanceolata* and *Prunella vulgaris* showed lower SPAD values when grown in unsterilized soil, while the SPAD values of *Leontodon hispidus* and *Cerastium fontanum* were not affected by soil sterilization.

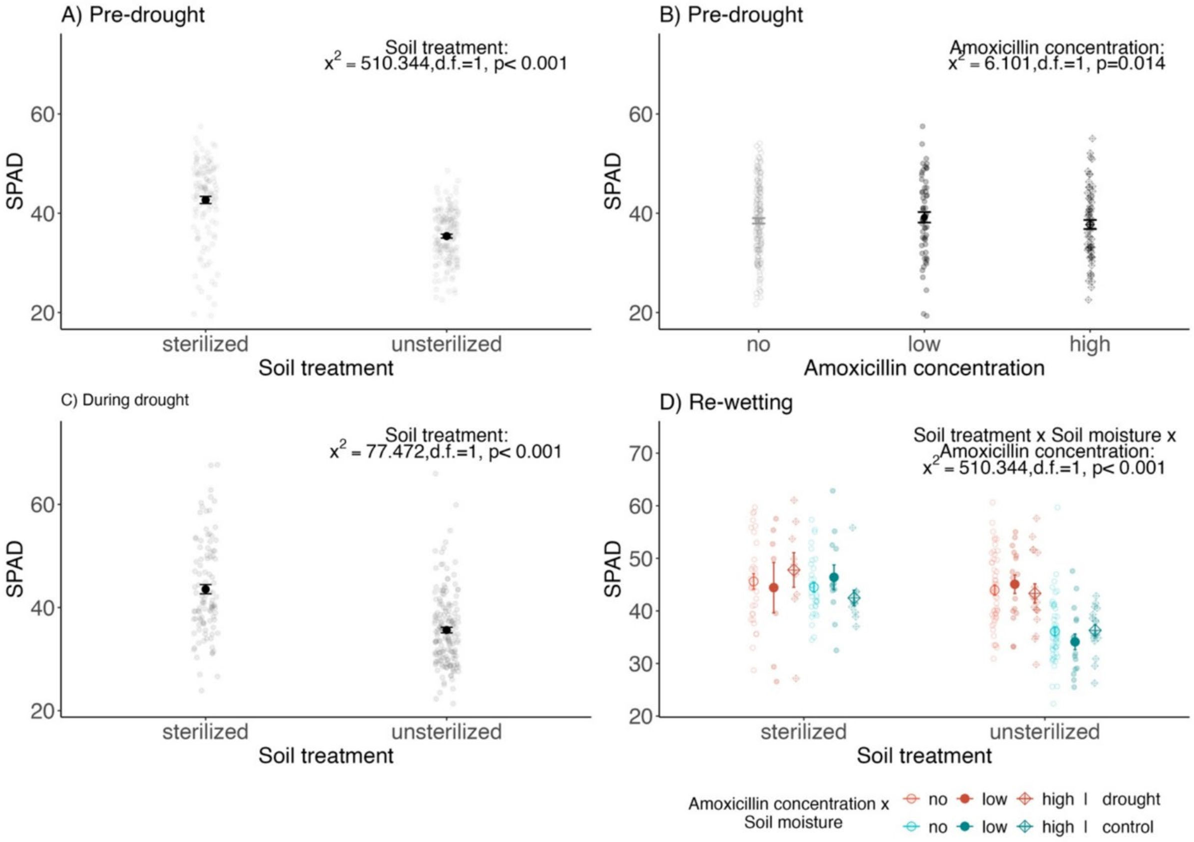

Fig. 5 SPAD responses to the soil treatment (**A**) and to amoxicillin concentrations prior to drought (**B**), to the soil treatment during drought (**C**), and to the combined effects of amoxicillin concentration (shape-coded) and previous drought (colour-coded) (**D**), as measured during the re-wetting phase. Dots and whiskers indicate means ± standard errors, while lighter dots show raw data distribution. Although not included in the statistical analysis, the values for plants not treated with amoxicillin (B, D) are plotted as a baseline-guide

During drought, soil sterilization overall positively affected SPAD values (Fig. 5B, Sup. Table 6). However, plant SPAD values were also shown to species-specifically depend on the interactive effect of drought and soil sterilization (Sup. Figure 4B, Sup. Table 7). For example, drought increased the SPAD values of *Bromus hordeaceus* and *Plantago lanceolata* in sterilized soil, while it decreased SPAD values of the other species. Further, SPAD values also differed between plants treated with different amoxicillin concentrations, and this effect depended on soil sterilization, although in species-specific ways (Sup. Figure 4C Sup. Table 7). In sterilized soils, a low amoxicillin concentration increased, and a high concentration decreased SPAD values in *Lolium perenne*, *Plantago lanceolata* and *Prunella vulgaris*, while the opposite effect was found for *Cerastium fontanum* and *Leontodon hispidus*.

After rewetting, SPAD values depended on the interactive effect of amoxicillin concentration, the previous drought treatment and soil sterilization (Fig. 5D, Sup. Table 8). Notably, in unsterilized soil, the previous drought treatment increased SPAD values. However, this increase was stronger for plants treated with low amoxicillin concentrations than for plants treated with high amoxicillin concentrations. Contrastingly, in sterilized soil, neither drought nor amoxicillin concentrations appeared to affect SPAD values. Subsequent analysis of species responses also indicated that amoxicillin effects on SPAD values interactively depended on soil sterilization and the previous soil-moisture treatment but showed that these effects also acted species-specifically (Sup. Figure 4D, Sup. Table 9), with *Prunella vulgaris* and *Lolium perenne* showing the most distinctly different responses to treatment combinations. In contrast to amoxicillin, there were no detectable effects of tetracycline on SPAD values after rewetting.

Discussion

Previous research has shown that antibiotics can directly affect plant performance and alter soil community composition (Hammesfahr et al. 2008; Migliore et al. 2010; Minden et al. 2017; Thiele-Bruhn & Beck 2005). Our study adds to these findings by showing that antibiotics affected relative biomass allocation and chlorophyll-based performance indicators differently in the presence of a soil community compared to in the absence of a soil community. In addition, these antibiotic effects on plant functioning depended on whether plants were exposed to drought stress and varied among species.

We expected that antibiotics would exert a negative direct impact on plant performance (Hypothesis 1), with higher concentrations having a more pronounced effect. Contrary to our expectation, neither under control soil moisture conditions nor under drought, antibiotic exposure affected plant biomass. Possibly, direct negative effects on plant biomass, which have been shown for various crop species (Adomas et al. 2013; Batchelder 1982; Liu et al. 2009; Migliore et al. 2010), only occur at concentrations much higher than those found in natural systems. In case of the other measured proxies for plant traits – root weight ratio (resource allocation), chlorophyll A fluorescence (i.e. physiological performance index) and SPAD values (as indicator for leaf chlorophyll and nitrogen content) – our results showed that higher doses of specific antibiotics often caused stronger plant responses when the soil community was absent (i.e. sterilized soil). Firstly, in the absence of a soil community, plant exposure to high tetracycline concentrations reduced root weight ratios compared to plant exposure to low tetracycline concentrations. Moreover, in the absence of a soil community, plants exposed to high amoxicillin concentrations showed a lower physiological performance index and a reduced leaf chlorophyll concentration than plants exposed to low amoxicillin concentrations. On the other hand, high tetracycline concentrations had a positive effect on the physiological performance index, independent of soil community presence, but only at the end of the experiment. The late appearance of this tetracycline concentration effect suggests that some plant responses induced by antibiotics may become prominent only after a certain time period, but possible mechanisms underlying this delayed plant response remain unknown. Combined, these results indicate that especially high antibiotic concentrations may exert direct negative impacts on plant functioning, but also that the exact effects depend on the type of antibiotic that plants are exposed to. Notably, in previous studies demonstrating negative direct effects of antibiotics on crop species performance plants were exposed to much higher concentrations than those detected in soil systems (Adomas et al. 2013; Batchelder 1982; Liu et al.

2009; Migliore et al. 2010). Our study shows that the examined European grassland plant species already show changes in functioning when exposed to antibiotic concentrations that lie well within the range detected in the environment. As such, our results suggest that natural grassland plant species may be particularly sensitive to antibiotics, but experiments directly comparing responses of crop- and natural grassland plant species are needed to confirm this.

As antibiotics have been shown to alter soil community composition (Cycoń et al. 2019; Hammesfahr et al. 2008; Pino-Otín et al. 2022), we expected that antibiotic effects on plant performance depend on soil-community presence (i.e. unsterilized soil) (Hypothesis 3). Whereas the exposure to antibiotics did not affect plant biomass, the other plant traits showed dose-dependent responses to specific antibiotic types in the presence of soil biota, but no responses to overall antibiotic exposure. Firstly, in response to high tetracycline concentrations, plants allocated more biomass to roots in presence of a soil community, but less biomass in absence of a soil community, than when exposed to a low tetracycline concentration. This result may suggest that high tetracycline concentrations released plants from a soil-community mediated suppression of root growth (Franco et al. 2020), or stimulated biomass allocation to roots to compensate for the possible suppression of belowground nutrient cycling by microbes (DeVries et al. 2015; DeVries & Zhang 2016). Secondly, in presence of a soil community, plants exhibited a higher performance index indicating reduced stress levels when exposed to high amoxicillin concentrations than when exposed to low amoxicillin concentrations. In line, in presence of a soil community, high amoxicillin concentrations positively affected leaf chlorophyll content, indicating increased nitrogen uptake, and suggesting that soil N cycling was not hampered by this antibiotic treatment. This dose-dependency of antibiotic effects on variables indicative of physiological plant performance might result from the effectiveness of the antibiotic and the susceptibility of plant-associated soil organisms. Indeed, previous studies already indicated that antibiotic identity and concentration are important determinants of the impacts of antibiotics on soil community composition and plant health (Migliore et al. 2010; Minden et al. 2017; Pino-Otín et al. 2022; Thiele-Bruhn & Beck 2005). Our results add to these results, by highlighting that antibiotics affect soil-community impacts on plant physiology and resource allocation in dose- and type-dependent ways**.** Nevertheless, our analysis of plant-species-specific responses also indicates that soil-community-mediated effects of antibiotics on plant traits (e.g., root weight ratio) vary among plant species, suggesting that antibiotic pollution may cause changes in plant community composition. While molecular examinations of soil community responses to the applied antibiotic treatments went beyond the scope of our project, we emphasize the necessity to explore such responses in future projects, in order to understand the mechanisms underlying indirect antibiotic effects on plant performance.

While drought negatively affected plant biomass, this negative impact was partly mitigated when a soil community was present. These results suggest an important role of beneficial soil biota, such as beneficial bacteria and fungi (de Vries et al. 2023, 2018; Eziz et al. 2017; Ortiz et al. 2015), which have been shown to be able to alleviate drought stress by sustained provisioning of nutrients and water, and by releasing stress-reducing hormones (Eziz et al. 2017; Poorter et al. 2012). We expected that this drought-stress alleviating soil-community effect would be compromised by antibiotic exposure (Hypothesis 2 & 3), but did not find evidence for this in case of biomass responses. However, in the presence of a soil community, exposure to the high tetracycline concentration reduced the positive effect of soil-community presence on biomass allocation to roots observed in drought-exposed plants, providing some support for our hypothesis. This reduced positive soil-community effect on biomass allocation to roots upon drought stress possibly reflects a negative effect of the high tetracycline concentration on beneficial soil organisms, e.g. drought-stress-ameliorating bacteria (de Vries et al. 2018; Ortiz et al. 2015). These type- and dose-dependent effects of antibiotics further suggest that antibiotic effects likely vary with the susceptibility of the soil community to antibiotic impacts. More importantly, these results suggest that some types of antibiotic pollution, through changes in the soil community, can alter plant responses to drought stress.

In line with the positive effects on biomass production and root biomass allocation of drought-exposed plants, soil-community presence also alleviated negative drought effects on the performance index. Interestingly, after re-wetting, drought-exposed plants exhibited a higher performance index than plants continuously experiencing control soil-moisture conditions, but this was not the case for plants grown in the absence of the soil community. Indeed, beneficial soil biota support plant health not only during, but also after drought, through the provisioning of nutrients, water or stress-reducing metabolites (de Vries et al. 2018; Ortiz et al. 2015; Poorter et al. 2012). In the presence—but not in the absence—of a soil community, plants growing in soils that were previously exposed to drought exhibited much higher leaf chlorophyll concentrations than plants that had experienced continuous control soil-moisture conditions. This result further emphasizes the important role of soil communities for plant recovery after drought. High concentrations of amoxicillin, however, appeared to partly compromise this positive effect of soil-community presence on post-rewetting leaf chlorophyll content, while they positively affected leaf chlorophyll content of plants that continuously experienced control soil-moisture conditions. In line with observed responses of plant biomass and biomass allocation, these results show that antibiotics may indirectly benefit plants under benign environmental conditions, but may compromise beneficial effects of soil communities on plant performance under stressful conditions.

In our experiment, plants growing in sterilized soil on average performed less well than plants growing in the presence of a soil community. While high SPAD values of plants growing in sterilized soil indicate an increase in nutrients due to sterilization (Powlson & Jenkinson 1976), plant total biomass, root weight ratio and physiological performance index were reduced in sterilized soil. Overall, our results therefore highlight the importance of beneficial soil biota for plant performance (van der Heijden et al. 2016; Weidner et al. 2015). Yet, our results also demonstrate that the effect of soil sterilization depends on plant species, as both grass species, *B. hordeaceus* and *L. perenne*, responded positively to soil sterilization. Possibly, the differences in performance responses between the examined grasses and forbs may reflect the former group's low dependency on arbuscular mycorrhizal fungi (Romero et al. 2023), which was also shown in a study using field soil from the same site (Ruppert et al. 2024). Associated with this limited dependence on mycorrhizal fungi, it is likely that the grass species directly benefitted from the increased nutrient levels that are typically associated with soil autoclaving, but that were not measured in this study.

As a general response to drought, we expected plants to invest relatively more resources in roots than in shoots in order to maximize water and nutrient uptake (Eziz et al. 2017; Poorter et al. 2012). However, in our study the plants invested less—rather than more—biomass into the roots in response to drought. Likely, the use of relatively small-sized pots (1.5 L) may have limited plants to show a plastic response to drought when compared to plants growing in the field (Wilschut & van Kleunen 2021). It must nevertheless be noted that the presence of a soil community appeared to ameliorate this negative drought effect on the root weight ratio (Fig. 2, Sup. Table 3), again highlighting the important role of soil biota under drought. In absence of a soil community, the negative drought effect on plant biomass allocation to roots was especially prominent when plants were also exposed to low concentrations of tetracycline, while a less negative drought effect occurred when plants were exposed to high concentrations of tetracycline. Similarly, in plants previously exposed to drought and grown in the absence of a soil community, high concentrations of amoxicillin overall appeared to increase leaf chlorophyll concentrations when compared to plants treated with low amoxicillin concentrations, although plant responses appeared to be highly species-specific. To better predict plant responses to antibiotic pollution, future research should aim to determine how antibiotic impacts on plant performance depend on plant traits, for example those related to the root economics spectrum (Bergmann et al. 2020).

Conclusion

In conclusion, our results indicate that two commonly used antibiotics, applied in concentrations within the range detected in the environment, did not directly affect the biomass of six European grassland plant species. When faced with drought stress, soil-community presence supported plant performance and relative biomass allocation to roots, but this increased biomass allocation to roots was inhibited by high concentrations of tetracycline. Our results therefore suggest that antibiotics can cause shifts in communities of plant-associated biota, of which the functional consequences depend on the abiotic environment. We nevertheless observed that plant species differed in their responses to the applied treatments, suggesting that antibiotic pollution may eventually alter plant performance and the plant-community composition. Future studies should therefore aim to understand variation in these responses among plant species, for example by studying the relationship between antibiotic-induced changes in soil community composition and observed plant performance responses of plants with different traits. Altogether, our study highlights the potential impacts of currently detected levels of terrestrial antibiotic pollution on natural plant communities.

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Authors contribution L.C.N conceived the main idea for the study and designed the experiment with inputs from R.A.W and M.v.K. L.C.N performed the greenhouse experiment, analyzed the data and wrote the manuscript with additional input from R.A.W and M.v.K during all stages.

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Data availability Upon acceptance of our manuscript, we will upload the data associated with the study to Figshare and provide the link/doi here.

Declarations

Competing interest The authors declare no competing interests.

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