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The Effect of Synthetic Cow Urine on Microorganisms and Their Potential Phosphorus Mining Activity in Rhizosphere and Bulk Soil

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ABSTRACT

Background and aims: Soil microbes adapt to varying nutrient inputs by modifying their utilization strategies, with root exudates and animal urine serving as key carbon (C) and nutrient sources in grasslands. Nonetheless, the specific mechanisms by which cow urine and root exudates influence microbial community and resource utilization remain elusive. This study investigates the differential effects of cow urine on microbial biomass, community composition and enzymatic activity in C-rich (rooted) and C-poor (vegetation-free) soil.

Methods: A laboratory experiment was conducted using synthetic cow urine applied to a Vertic Cambisol soil, either densely rooted with *Dactylis glomerata* (C-rich) or vegetation-free (C-poor). Destructive sampling occurred on days 1, 4 and 14. Microbial biomass (MBC, MBN and MBP), phospholipid fatty acids, DNA and enzymatic activities (acid phosphatase, β-glucosidase) were analysed to assess microbial responses.

Results: DNA content was twice as high in C-rich rooted as in C-poor bulk soil. In C-poor soil, urine strongly reduced DNA-to-MBC (−166%, day 14) and MBP-to-MBC (−23%, day 14), while increased MBN-to-MBC (+23% day 14). After 14 days of urine addition, phosphatase-to-β-glucosidase ratios increased from 0.76 to 1.1 in C-rich but declined in C-poor soil. In C-poor bulk soil, urine shaped the microbial community, whereas in C-rich soil, root exudates dominated. Abundance of bacterial fatty acids increased from 2% to 22% over 14 days.

Conclusions: In C-rich soil, urine stimulated microbial growth through C and nutrients availability from both roots and urine, thereby shifting enzymatic activity towards P mining. In contrast, microbial communities in C-poor soil exhibited restrained growth and stable stoichiometric ratios, adapting to low nutrient supply. These findings highlight that microbial responses to urine input varied between rooted and bulk soil, emphasizing their relevance in soil microbial ecology.

1 | Introduction

Grasslands cover more than 40% of the Earth's land surface, providing diverse habitats for soil organisms (Bardgett and Van Der Putten 2014; Decaëns 2010). These soil communities are fundamental to ecosystem functioning as they drive nutrient cycling and regulate soil fertility (Oliver and Gregory 2015; Wall et al. 2015). Grassland management practices – such as applying cow dung and urine as fertilizers or mowing intensity – can increase nutrient input or removal (Haynes and Williams 1999) and control carbon (C) supply to soils (Huang et al. 2021; Hütsch et al. 2002; Jones et al. 2009). These practices affect soil nutrient availability and reshape microbial community composition, thereby affecting grassland ecosystem productivity (Koranda et al. 2011; Pantigoso et al. 2023). Our understanding of the underlying mechanisms by which grassland management practices impact the soil microbiome and its functionality is not yet sufficiently known to make generic predictions on management thresholds and their effects. This calls for an increased effort to identify the key controlling parameters for soil element cycles and grassland productivity.

In grazed grasslands, livestock influences soil nutrient dynamics primarily through dung and urine deposition (Breidenbach et al. 2022; Zhang et al. 2021). Among these, cow urine is especially important, as it creates highly concentrated nutrient hotspots (Bertram et al. 2012). Its input, either alone or in combination with plant presence, substantially affects soil microbial communities. This response is driven by the plasticity of microbial biomass stoichiometry and the potential phosphorus (P) mining activity in relation to shifts in soil C, nitrogen (N) and P pools (Chen et al. 2016; Francioli et al. 2016; Jiang et al. 2021).

In grazed systems, urine patches are sparsely and heterogeneously distributed, covering only 4%–29% of a pasture area (Moir et al. 2011). Such spatial heterogeneity in nutrient deposition generates distinct stoichiometric patterns, a characteristic feature of grazing activity in grasslands (McIntire and Hik 2002; Olofsson and Oksanen 2002). Urine typically contains N concentrations of 2–20 g N L^{-1} (Betteridge et al. 1986; Bristow et al. 1992), while P is primarily excreted in ruminants' dung, with urine containing less than 0.1 g P L^{-1} (Betteridge et al. 1986; Knowlton and Herbein 2002). Consequently, the scarcity of P and other trace elements in cow urine may limit microbial growth and nutrient cycling in rooted soil beneath urine patches. Whether the increasing competition between microorganisms and plants for available P resources at urine patches affects microbial functions remains to be elucidated (Bertram et al. 2012). The C, N and P ratios of microbial biomass may provide valuable insights into the nutritional status and growth constraints of the soil microorganisms (Chen et al. 2019).

Although numerous studies targeted urine deposition on grasslands (Bol et al. 2004; Haynes and Williams 1999; Orwin et al. 2010), the majority have focused on soil chemistry and gaseous emissions. Far less attention has been given to the effects of cow urine on microbial stoichiometry and the subsequent consequences for the microbial community structure and functioning. In this context, our study links microbial stoichiometry and P-mining enzyme activity to nutrient availability. This approach reveals underlying mechanisms by which grazing-driven nutrient inputs regulate soil functioning and shape microbial community composition. Such understanding is essential for developing sustainable grassland management strategies that enhance soil fertility, maintain productivity and support ecosystem resilience under changing environmental conditions.

To address these knowledge gaps, we conducted a laboratory experiment using temperate grassland soils. We compared the effects of cow urine application on densely rooted soils – where rhizodeposits served as an energy and C source, with unrooted (bulk) soils. Synthetic cow urine was applied to simulate the nutrient inputs (i.e., N and P) provided by natural cow urine under field conditions. We assessed changes in soil microbial biomass stoichiometry by measuring microbial C, N and P biomass contents as well as microbial abundance through deoxyribonucleic acids (DNA) analysis. In addition, acid phosphatase and ß-glucosidase enzyme activities were measured, along with phospholipid-derived fatty acids (PLFAs), to identify links between microbial community structure and microbial element demands driving distinct enzyme activities.

This integrated approach provides a mechanistic understanding of microbial strategies for C and nutrient utilization under contrasting stoichiometric conditions, that is, bulk vs. rooted soils. We hypothesized that (1) microbial biomass C: N: P stoichiometry and DNA content are more strongly influenced by biotic factors (e.g., C-rich rooted soil compared to C-poor bulk soil) than by urine addition; (2) in the absence of urine, plant presence exerts a stronger effect than P-limitation on the acid-phosphatase-to-ß-glucosidase ratio, which serves as a proxy for potential organic P mining to cope with elevated C availability; and (3) urine addition triggers shifts in microbial community structure in both bulk and rooted soils.

2 | Materials and Methods

2.1 | Soil Samples and Experimental Design

Soil samples were collected from a long-term grazing experiment site located in Relliehausen, Lower Saxony, Germany (51°46′50″ N, 9°41′55″ E), at an elevation of 265–340 m asl. The site has been managed without the use of fertilizers, pesticides or cutting for more than 16 years. It receives an average annual precipitation of 806 mm and has a mean annual temperature of 8.9°C (Tonn et al. 2018). The soil is classified as Vertic Cambisol, with a texture comprising 5% sand, 87% silt and 8% clay.

Topsoil (0–10 cm) was collected and thoroughly homogenized in the laboratory. The soil was then portioned into pots, each containing 1.5 kg (dry weight), and maintained at 50% moisture for 33 days prior to planting. Approximately 50 seeds of *Dactylis glomerata* were sown in the plant-treatment pots, where they germinated and grew for two months. Due to the abundance and fineness of the roots, all soil from planted pots was regarded as rhizosphere soil, whereas soil from unplanted pots served as bulk soil. The topsoil had a pH of 5.9 ($CaCl_2$) and contained total C, N and extractable P (calcium–acetate–lactate extraction) concentrations of 37.7 g kg^{-1}, 3.3 g kg^{-1} and 65 mg kg^{-1}, dry soil,

respectively (Tonn et al. 2018). All visible roots and stones were removed before processing. The samples were then homogenized, and 1500 g of each sample was weighed into 1000-mL plastic jars. Preincubation was carried out at 18°C for three weeks at 50% of the soil´s water holding capacity (WHC). Four replicates were prepared for each treatment (rooted and bulk soil) and for each of the three sampling time points.

2.2 | Plant Materials and Growth Conditions

Seeds of *D. glomerata L.* were sown directly into pots and cultivated in a growth chamber designed to simulate the climatic conditions of Relliehausen. The chamber maintained a 12 h photoperiod, with an average temperature of 21°C ± 1°C and a relative air humidity of 60% ± 5%. Lighting was provided by 243 W light-emitting diodes (LEDs) (Kind LED Growth Lights, California, USA), delivering a constant light flux density of approximately 600 µmol m^{-2} s^{-1}. Soil moisture was maintained at 50% of WHC using distilled water for the duration of the experiment.

The roots and shoots were freeze-dried and finely ground using a Retsch MM200, ball mill (Retsch Haan, Germany). For the determination of total C and N contents, 50 mg of the milled samples was weighed into tin capsules (IVA, Meerbusch, Germany). The samples were then analysed using a 2000 CHNS/O Elemental Analyser (Thermo Fisher Scientific, Cambridge, UK).

2.3 | Cow Urine Application and Plant Harvesting

Synthetic cow urine was prepared as outlined by Frase et al. (1994) and Clough et al. (2003) (see Table S1 for details). After 45 days of plant growth, synthetic cow urine (5 L m^{-2}) was applied to both sets of pots – those with and without plants. Overall, with this amount of cow urine, we have added 12.8 mg K g^{-1} soil, 8.8 mg N g^{-1} soil, 0.08 mg P g^{-1} soil and 0.42 mg S g^{-1} soil. Control was maintained using water instead of urine. The pots were harvested at 1, 4 and 14 days after urine application. These time points were selected to investigate the effects of the urine on the uptake and reallocation of key elements (C, N and P) into microbial cells (day 1); their incorporation into microbial cellular compounds (e.g., PLFA and DNA) and enzymatic transformation processes (day 4); and microbial turnover or cycling (day 14) in both bulk and rooted soils. At each sampling time, plant shoots were cut at the base, and the roots were manually removed from the soil, washed and weighed together with the shoot. The roots and shoots were then freeze-dried separately for further analysis.

The first sampling, conducted one day after urine application, was designed to capture the initial stages of element reallocation and microbial activation, with microorganisms anticipated to have transitioned from the lag phase (>4–13 h) to the growth phase (Blagodatskaya et al. 2014). The second sampling, four days post-application, was chosen to capture the end of the active microbial growth phase and the onset of nutrient incorporation because microorganisms begin the transition from active growth to a starvation phase (Blagodatskaya and Kuzyakov 2013). The final sampling, 14 days post-application, corresponded to the cycling stages of C, N and P turnover within the soil microbial community.

2.4 | Soil Microbial Biomass Analysis

The extractable microbial biomass C and N was estimated using the chloroform fumigation–extraction method (Vance et al. 1987). Briefly, 10 g sieved fresh subsample was fumigated in a chloroform atmosphere in a desiccator for 24 h before the extraction, whereas the other 10-g subsample was extracted directly with 40 mL of a 0.05 M K_2SO_4 solution. The samples were shaken continuously for 1 h at 150 rpm on a horizontal reciprocating shaker (Laboratory shaker, GFL 3016). After shaking, the supernatant was filtered using Sartorius filters 110 mm. C and N contents were measured in the extracts on a C/N analyser (Multi N/C 2100, Analytik Jena, Germany). The microbial C and N biomass was then calculated by determining the differences in extracted C and N between the fumigated and non-fumigated samples corrected by the extraction factor 0.45 and 0.54, respectively (Brookes et al. 1985; Joergensen and Mueller 1996; Wu et al. 1990).

Anion exchange resin membranes (AEM, AMI-7001 membrane, Membranes International), with a reactive area of 18.75 cm^2 per strip, were used to extract available phosphate from soil extracts. Briefly, 50 mL centrifuge tubes were used for 2 g of soil samples. The samples were extracted with 30 mL of deionized water with the AEM on a horizontal shaker (150–170 rpm) for 24 h at room temperature. Each sample comprised a fumigated and unfumigated subsample. Directly prior to extraction, 0.3 mL of 1-hexanol was added to the fumigated subsample to lyse the cells. After 24 h of shaking, the membranes were removed and rinsed three times with deionized water to remove any soil particles. The membranes were then transferred to a new 50-mL tube filled with 45 mL 0.25 M HCl and shaken for 3 h to desorb the P from the anion exchange membrane into solution. Phosphate in the fumigated and unfumigated extracts was determined by the malachite green colorimetric method using an Implen NanoPhotometer NP80 at 610 nm wavelength. Standards were also prepared to obtain a calibration curve and transform measured units to the phosphate concentrations. Microbial biomass P (MBP) was determined as the difference between phosphate content in fumigated and unfumigated samples. We did not apply a correction coefficient because they are highly soil specific for Pmic, vary greatly between soils and were not determined in our experiment, but we instead worked with a spike standard for which we corrected. The ratios for MBC-to-MBN, MBP-to-MBC and MBP-to-MBN were calculated on a mass basis.

2.5 | Phospholipid Analysis

Phospholipids were extracted according to a modified method after Bligh and Dyer (1959), Frostegard et al. (1993) and purified by solid phase extraction (Dippold and Kuzyakov 2016). Briefly, 6 g of soil and a Bligh-and-Dyer extraction solution consisting of chloroform, methanol and citrate buffer (1:2:0.8, v:v:v, pH 4.0) were used, followed by the addition of more chloroform and citric acid buffer to obtain a two-phase system for the extraction and elution of total polar lipids. Prior to extraction, 25 µg of internal standard 1 (IS1) phosphatidylcholine dinonadecanoic acid (phospholipid 19:0) was added. The phospholipids were eluted from the silica column by adding 30 mL of methanol following chloroform and acetone to remove neutral and glycolipids, respectively. In order to prepare the samples for the measurement on the gas

chromatograph, the fatty acid chains were cleaved off by basic hydrolysis with NaOH in MeOH (100°C for 10 min); thereafter, derivatization to fatty acid methyl esters (FAMEs) with MeOH catalysed by BF_3 (80°C for 15 min) was performed. 15 µL of the tridecanoic acid methyl ester was added as internal standard 2 (IS 2) to the FAMEs. In analogy, the external standards made up of the 28 fatty acids given in Supplementary Table S7 containing the internal standard 1 were prepared with total fatty acid contents of 1, 4.5, 9, 18, 24 and 30 µg, respectively, and they were measured alongside the samples. FAME concentrations were measured on a gas chromatograph–mass spectrometer (GC-MS, GC 7820 with a MS 5977B, Agilent, Waldbronn, Germany) with a 30 m DB5-MS column coupled to a 15-m DB1-MS column, in scan mode. First, all analyte peak areas in one sample were normalized to the IS2 peak, then the relative peak areas of every sample were converted into µg of analyte using a linear regression model of the IS2-normalized peak areas derived from the six-external standard. Based on the area of the 25 µg of amended IS1, the recovery rate for every sample was determined and used to correct the measured masses of the FAMEs. Lastly, the amount was normalized on the soil dry weight exposed to extraction.

2.6 | Enzyme Assays

Extracellular enzyme activities were measured using fluorogenic substrates containing 4-methylumbelliferyl (MUF) (e.g., for acid phosphatase: 4-methylumbelliferyl-phosphate and for β-glucosidase: 4-methylumbelliferyl β-D-glucoside) (German et al. 2011). Briefly, 0.5 g of fresh soil was extracted with 50 mL of sterile deionized water on a horizontal shaker for 30 min, followed by sonication of the soil solution (40 J s^{-1}; 2 min). A 50-µL aliquot of the soil slurry was added to each well of a black polystyrene 96-well microtiter plate (Brand, Germany). Then, 50 µL of 2-(N-morpholino) ethanesulfonic acid (MES) buffer and 100 µL of the MUF substrate for β-glucosidase or for acid phosphatase were added. Substrate concentrations for BG and ACP followed the Michaelis–Menten series: 0, 5, 10, 15, 20, 25, 50 and 100 µmol L^{-1}. Fluorescence was measured after 0, 1, 2 and 3 hours using a microplate fluorometer (Victor^3 1420–050 Multi label Counter, PerkinElmer, USA) with excitation and emission filters set at 355 and 460 nm, respectively (Razavi et al. 2015). Enzyme activities were calculated in nmol g^{-1} h^{-1} from the regression slopes of the MUF standards for three analytical replicates after a 3 h incubation (Razavi et al. 2015). Activity data were fitted to the Michaelis–Menten equation to produce nonlinear saturation curves, yielding the kinetic parameters V_{max} (i.e., maximum rate of enzymatic activity under optimal substrate conditions) and K_m (i.e., the half-saturation constant, an indicator of substrate affinity).

2.7 | Soil Microbial Total DNA Extraction and Quantification

Soil microbial total DNA was extracted using the FastDNA™ SPIN Kit, following the manufacturer's instructions (MP Biomedicals, Solon, OH, USA). In a nutshell, 0.5 g of fresh soil was placed into a Lysing Matrix E tube containing 122 µg of MT buffer and 978 µL of sodium phosphate buffer. The soil was homogenized in a FastPrep bead beater for 40 s at 6.0 m/s. The samples were then centrifuged at 14,000 × g for 15 min to remove cell debris. To the supernatant, 250 µL of protein precipitation solution was added and mixed thoroughly by manual shaking. After a second centrifugation at 14,000 × g for 5 min, the resulting supernatant was combined with 800 µL of Binding Matrix suspension. This mixture was transferred to a SPIN filter inside a reaction tube and centrifuged to remove the filtrate. The DNA on the filter was then washed with 500 µL of SEWS-M solution, followed by centrifugation to dry the filter. Finally, 550 µL of DES solution was added to the filter and centrifuged to elute the DNA into a 2-mL collection tube placed beneath the SPIN filter. The DNA concentration in the extract was measured using an Implen NanoPhotometer NP80 at 260 nm. We calculated DNA-to-MBC ratios as a community trait that is indicative of the degree of replicative microbial growth to assess the physiological state and growth strategies of soil microbial communities (Mason-Jones et al. 2023).

2.8 | Statistical Analysis

All statistical analyses and graphical representations were performed in R version 4.1.2 (R Core Team 2021). Linear model analysis was conducted using the lme4 package, with sampling time (1 day, 4 days and 14 days) as the first factor, soil type (C-poor bulk soil (without plants) and C-rich rooted soil (with plants)) as the second factor, and nutrient addition (control with water vs. synthetic cow urine) as the third factor, as well as their interactions with each measured variable. Values in the figures and tables represent means of four replicates ± standard error of the mean (mean ± SEM). Outliers were screened using the Nalimov test. Data were tested for normality (Shapiro–Wilk test, $p > 0.05$) and homogeneity of variance (Levene test, $p > 0.05$) prior to analysis of variance (one-way ANOVA) to examine differences in each variable between paired sampling points and (three-way ANOVA) to examine the effect of three factors as well as their interaction. Data that did not meet normality were log-transformed. Post hoc comparisons (Tukey HSD; $p < 0.05$) were performed using the Multcomp package. PLFAs were categorized into microbial groups based on factor analysis with principal component extraction (refer to Supplementary Table S3 for factor loadings of the PLFA fingerprint). Fatty acids that were either ubiquitous (e.g., unsaturated, straight-chain fatty acids) or close to the detection limit were excluded from the factor analysis. The classified data were cross-referenced with literature on individual fatty acids to identify the functional groups of microorganisms (Zelles 1997).

3 | Results

3.1 | Total Plant Biomass

On day 14, plants treated with urine exhibited a 71% increase ($p < 0.05$, $F = 27.1$) in shoot biomass compared to the control. Shoot biomass consistently exceeded root biomass in both the urine-amended and control treatments throughout the study period (Table 1). Notably, in the urine-treated group, root biomass decreased by 20% between day 1 and day 4. Conversely, the control group recorded the highest root biomass across all samples on day 14. The C-to-N (C:N) ratio of root ($p < 0.05$, $F = 22.6$) and shoot ($p < 0.05$, F = 13.5) biomass was higher in the control- compared to the urine-treated plants (Table 2).

TABLE 1 | Effect of cow urine on shoot and root biomass measured after destructive harvest (1, 4 and 14 days after urine application) without (control) and with urine application.

Plant biomass						
	1 Day	4 Days	14 Days	1 Day	4 Days	14 Days
Shoot biomass (g)				**Root biomass (g)**		
Control	2.1 ± 0.2^{a}	2.5 ± 0.2^{a}	2.8 ± 0.1^{a}	1.2 ± 0.1^{ab}	1.2 ± 0.1^{ab}	2.1 ± 0.4^{b}
Cow urine	2.3 ± 0.2^{a}	3.1 ± 0.2^{a}	4.8 ± 0.4^{b}	1.2 ± 0.2^{ab}	1 ± 0.1^{a}	1.5 ± 0.2^{ab}

Note: Different letters indicate significant differences between treatments at each time point ($p < 0.05$). Data represent mean ± standard error ($n = 4$).

TABLE 2 | Effect of cow urine on the C:N ratio of shoot and root biomass measured after destructive harvest (1, 4 and 14 days after urine application) without (control) and with urine application.

Plant C:N ratio						
	1 Day	4 Days	14 Days	1 Day	4 Days	14 Days
Shoot biomass				**Root biomass**		
Control	20.0 ± 0.7^{a}	22.6 ± 0.7^{a}	23.6 ± 0.7^{a}	47.2 ± 4.9^{a}	46.9 ± 4.9^{a}	42.6 ± 4.9^{a}
Cow urine	9.8 ± 0.7^{b}	6.8 ± 0.7^{b}	6.7 ± 0.7^{b}	26.3 ± 4.9^{b}	26.2 ± 4.9^{b}	27.1 ± 4.9^{b}

Note: Different letters indicate significant differences between treatments at each time point ($p < 0.05$). Data represent mean ± standard error ($n = 4$).

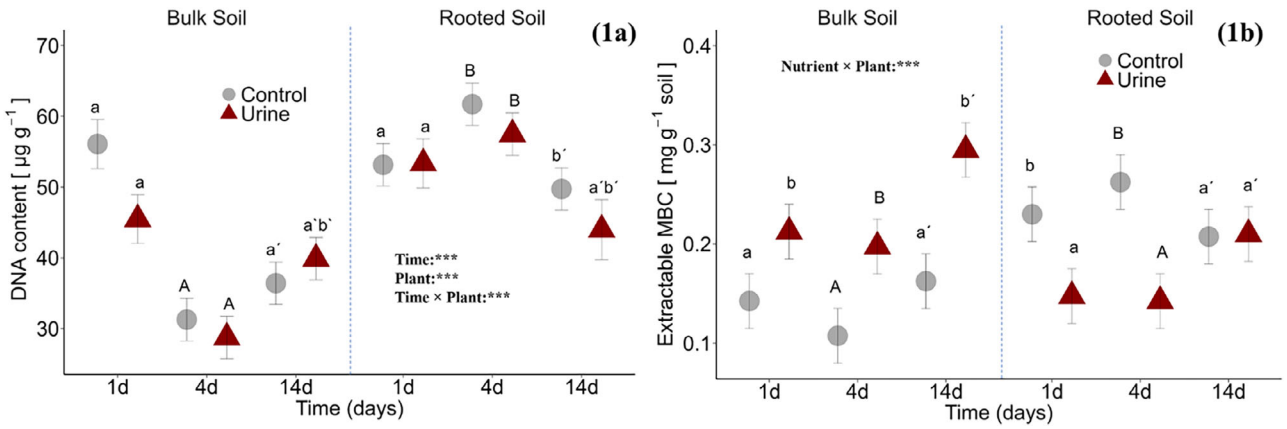

FIGURE 1 | DNA content (a) and MBC (b) measured after destructive harvest (1, 4 and 14 days after urine application) in C-poor bulk soil (left) and C-rich rooted soil (right) without (control in grey) and with urine application (in red). Letters indicate significant differences between treatments at each time point ($p < 0.05$). Whiskers represent the standard errors of means for each treatment ($n = 4$). The result of the ANOVA test for the three factors – time, nutrient and plant – is presented on each graph. The combinations of these factors are indicated as significant based on the *p*-values obtained. Significance levels are represented as follows: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

3.2 | DNA Content and Microbial Biomass

The soil DNA content was enhanced in the presence of plants and depended on the sampling time ($p < 0.001$, $F = 20.5$), whereas the effect of urine addition was relatively low (Figure 1a). On day 4, the DNA content – with and without urine – was approximately 100% higher ($p < 0.001$, $F = 64.4$) in rooted compared to bulk soil (Figure 1a). At the beginning of the incubation, the MBC content in bulk and rooted soil with urine application was 0.21 and 0.14 mg C g^{-1}, respectively. In bulk soil under urine application, MBC values were significantly higher ($p < 0.001$, $F = 26.6$) by 37% on day 4, and by 28% on day 14 compared to rooted soil (Figure 1b).

To assess the physiological state and growth strategies of soil microbial communities, we calculated the DNA-to-MBC ratios. Notably, these ratios with urine application were significantly higher in rooted soil ($p < 0.01$, $F = 28.4$) on both day 4 and day 14, with an increase of 166% compared to bulk soil. This trend was not observed in the urine-free control treatments, where the ratio remained stable over time regardless of plant-derived C input. Interestingly, in bulk soil with urine application, the

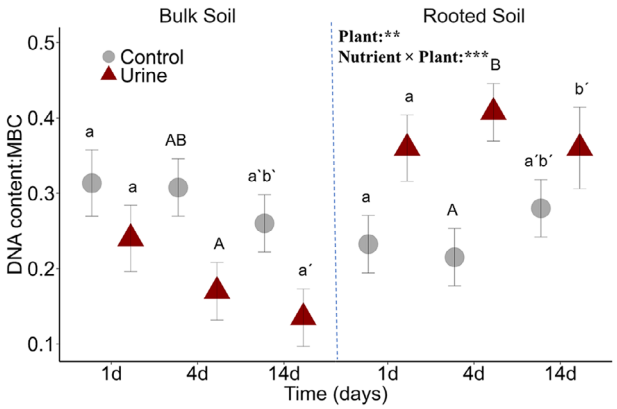

FIGURE 2 | The ratio of soil microbial DNA content to MBC measured after destructive harvest (1, 4 and 14 days after urine application) in C-poor bulk soil (left) and C-rich rooted soil (right) without (control in grey) and with urine application (in red). Letters indicate significant differences between treatments at each time point ($p < 0.05$). Whiskers represent the standard errors of means for each treatment ($n = 4$). The result of the ANOVA test for the three factors – time, nutrient and plant – is presented on each graph. The combinations of these factors are indicated as significant based on the p-values obtained. Significance levels are represented as follows: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

DNA-to-MBC ratios sharply decreased with ongoing incubation (Figure 2).

3.3 | C, N and P in Soil Microbial Biomass and Their Stoichiometry

At the beginning of the incubation (day 1), the MBN and MBP values in bulk soil with urine application were 0.05 mg N g^{-1} and 0.9 mg P g^{-1}, respectively (Table 3; Table S1). In rooted soil with urine, these microbial element pools accounted for 0.02 mg N g^{-1} and 0.5 mg P g^{-1}, respectively. Furthermore, MBN was significantly higher ($p < 0.05$, $F = 9.2$) on both day 4 and day 14 in bulk soil compared to rooted soil with urine (Table 3). In contrast, neither of the three factors – time, nutrients (urine application) or plants (the distinction between bulk vs. rooted soil) – affected MBP content (Supplementary Table S1).

In urine-amended soil, MBN-to-MBC ratios were slightly higher in bulk soil than in rooted soil (Figure 3a). Without urine, however, this ratio was significantly higher ($p < 0.01$, $F = 13.03$) in bulk soil than in rooted soil on days 4 and 14, with an increase of 23%. Similarly, in non-amended control soil, the MBP-to-MBC ratio was significantly higher ($p < 0.001$, $F = 16.2$) in bulk vs. rooted soil on day 4 (Figure 3b). In contrast, neither of the three factors – time, nutrients (i.e., urine application) or plants (i.e., the distinction between bulk and rooted soil) – significantly affected this ratio (Supplementary Figure S1). However, the MBN-to-MBC ratios in bulk soil followed a similar pattern to MBP-to-MBC ratios, suggesting parallel effects on both microbial nutrient ratios.

3.4 | Enzyme Activity, Substrate Affinity and Their Ratios

Maximal acid phosphatase activity was higher ($p < 0.0001$, $F = 10.5$) in rooted soil without urine application than with it (Figure 4a) and progressively increased with plant age. Interestingly, urine application did not immediately affect phosphatase activity in bulk soils. Additionally, the acid phosphatase K_m value, indicating substrate affinity, was higher ($p < 0.01$, $F = 5.8$) on day 4 in bulk soil compared to rooted soil, regardless of urine application (Figure 4c).

In bulk soil with urine addition, β-glucosidase activity steadily decreased from day 1 to day 14. In contrast, in rooted soil, β-glucosidase activity successively increased over the same period (Figure 4b). The K_m value of β-glucosidase showed no significant response to nutrient addition (Figure 4d).

The phosphatase-to-β-glucosidase ratio showed no significant response to urine application in either rooted or bulk soil. However, in rooted soil without urine, the ratio displayed an increasing trend, leading to significantly higher ratios on days 4 and 14 ($p < 0.0001$, $F = 14.8$). Specifically, the ratio in rooted soil increased from 0.76 ± 0.1 to 1.1 ± 0.1 compared to bulk soil (Figure 5). In bulk soil specifically under urine application, the ratio gradually declined over time, decreasing from 0.98 ± 0.2 to 0.75 ± 0.1.

3.5 | Microbial Community Composition in Bulk and Rooted Soil Depending on Urine Application

To compare the microbial community composition across treatments, we conducted a principal component analysis (PCA), of which the first two axes explained 81.7% of the variation in the PLFA fingerprint in bulk soil (Figure 6a) and 69.1% of the variation in the rooted soil (Figure 6b). PCA factor loading and assignments of PLFA to microbial functional groups were used to identify microbial fatty acid groups (Gram-positive 1 bacteria, Gram-positive 2 bacteria, Gram-negative bacteria, Actinobacteria

TABLE 3 | Extractable MBN measured after destructive harvest (1, 4 and 14 days after urine application) without (control) and with urine application.

Extractable MBN (mg g^{-1})						
	1 Day	4 Days	14 Days	1 Day	4 Days	14 Days
Bulk soil				**Rooted soil**		
Control	0.02 ± 0.01^{a}	0.04 ± 0.01^{AB}	0.05 ± 0.01$^{a´b´}$	0.05 ± 0.01^{ab}	0.03 ± 0.01^{B}	0.03 ± 0.01$^{c´}$
Cow urine	0.05 ± 0.01^{b}	0.05 ± 0.01 ^{A}	0.05 ± 0.01$^{a´}$	0.02 ± 0.003^{a}	0.02 ± 0^{B}	0.03 ± 0.01$^{b´c´}$

Note: Different letters indicate significant differences between treatments at each time point ($p < 0.05$). Data represent mean ± standard error ($n = 4$).

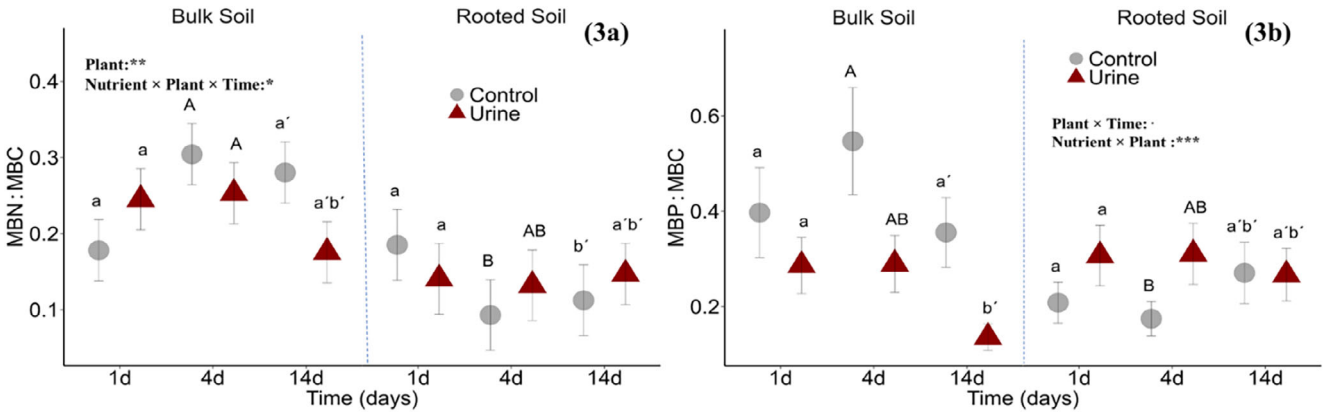

FIGURE 3 | MBN:MBC ratio (a) and MBP:MBC ratio (b) measured after destructive harvest (1, 4 and 14 days) after urine application in C-poor bulk soil (left) and C-rich rooted soil (right) without (control in grey) and with urine application (in red). Letters indicate significant differences between treatments at each time point ($p < 0.05$). Whiskers represent standard errors of means for each treatment ($n = 4$). The result of the ANOVA test for the three factors – time, nutrient and plant – is presented on each graph. The combinations of these factors are indicated as significant based on the p-values obtained. Significance levels are represented as follows: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

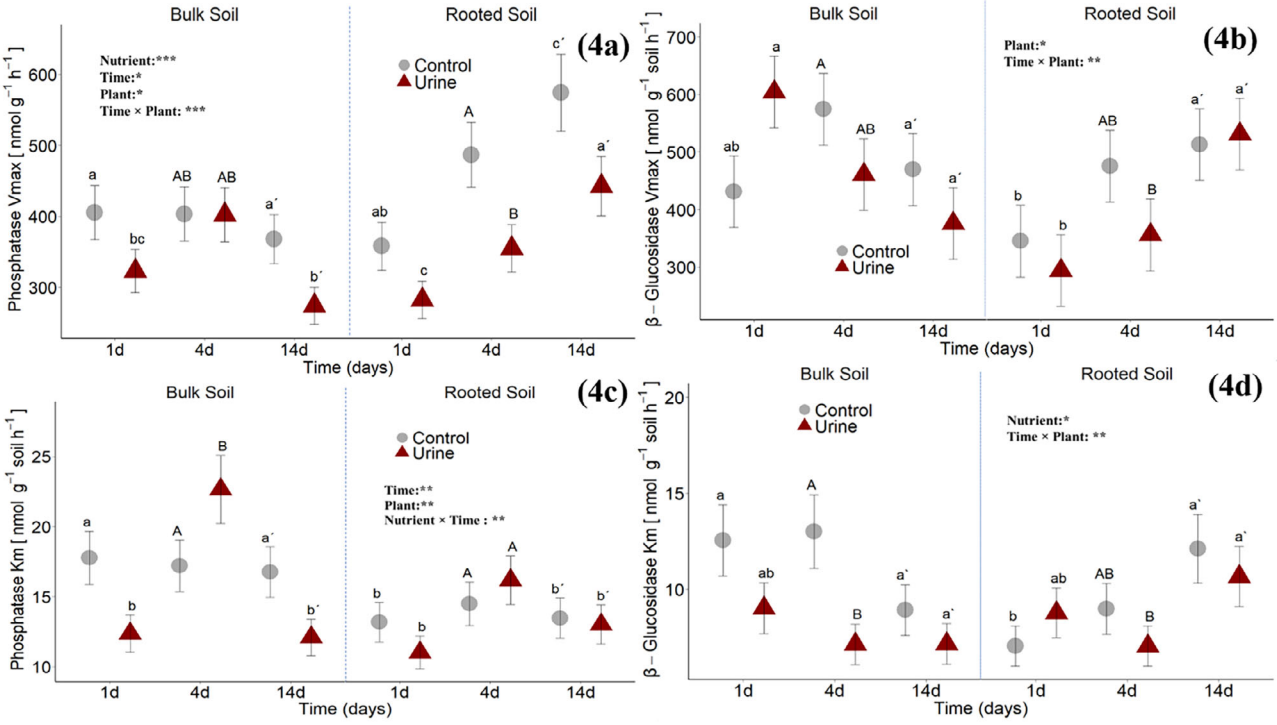

FIGURE 4 | Phosphatase V_{max} activity (a), β-Glucosidase V_{max} activity (b), Phosphatase K_m (c) and β-Glucosidase K_m (d) measured after destructive harvest (1, 4 and 14 days after urine application) in C-poor bulk soil (left) and C-rich rooted soil (right) without (control in grey) and with urine application (in red). Letters indicate significant differences between treatments at each time point ($p < 0.05$). Whiskers represent standard errors of means for each treatment ($n = 4$). The result of the ANOVA test for the three factors – time, nutrient and plant – is presented on each graph. The combinations of these factors are indicated as significant based on the p-values obtained. Significance levels are represented as follows: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

1, Actinobacteria 2, protozoans, fungi and AM – arbuscular mycorrhiza fungi), whose contributing fatty acids are listed in Supplementary Table S5.

In bulk soil, the microbial community was significantly influenced by urine input, which led to an increase in Gram-positive 1 bacteria, Gram-negative bacteria, Actinobacteria 2 and, to a lesser extent, arbuscular mycorrhizal fungi compared to soil without urine addition. In untreated soil, Gram-positive 2 bacteria, Actinobacteria 1, saprotrophic fungi and protozoans were more dominant. This difference in microbial community structure between control and urine-amended soils was consistent on days 4 and 14 of the incubation (Figure 6a) but had not yet developed on day 1 (Supplementary Figure S6).

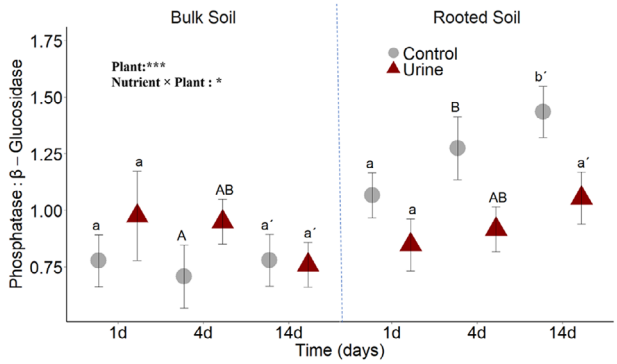

FIGURE 5 | The ratio of phosphatase-to-β-glucosidase measured after destructive harvest (1, 4 and 14 days after urine application) in C-poor bulk soil (left) and C-rich rooted soil (right) without (control in grey) and with urine application (in red). Letters indicate significant differences between treatments at each time point ($p < 0.05$). Whiskers represent standard errors of means for each treatment ($n = 4$). The result of the ANOVA test for the three factors – time, nutrient and plant – is presented on each graph. The combinations of these factors are indicated as significant based on the p-values obtained. Significance levels are represented as follows: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

In the PCA biplot of the rooted soil, the effect of urine application was not evident. This suggests that root presence and associated rhizodeposition were the dominant factors shaping the microbial community, surpassing the influence of additional amendments like urine. Despite an increase in plant biomass from days 4 to 14, there was no detectable shift in the microbial community composition in the PLFA fingerprint.

The bacteria-to-fungi ratio was significantly influenced by both urine treatment ($p < 0.001$, $F = 72.26$) and the presence of plants ($p < 0.001$, $F = 24.39$), with values ranging from 1.2 and 2.9 across all treatments. In bulk soils, this ratio was significantly higher on day 1 under urine treatment compared to rooted soils. Bacterial abundance (in mg per g dry soil) increased markedly over time, rising from approximately 2% on day 1 to 22% by day 14 (Supplementary Table S6).

4 | Discussion

4.1 | Activation of the Soil Microbiome in Response to Labile Rhizodeposition of Carbon and Cow Urine Application

In C-poor bulk soils, microorganisms did not undergo growth by multiplication (i.e., no increase in DNA) following urine application. Instead, an increase in soluble cell C accumulation was evident (Figure 1b), which was not accompanied by an accumulation of other elements (Figure 3). The decreasing ratio of microbial DNA-to-MBC in urine-amended bulk soil over the course of the incubation demonstrates the low and successively decreasing C substrate availability as a limiting factor for replicative growth. Instead, urine-activated microbes rather accumulate C in their cells (Figure 2). Mason-Jones et al. (2023) suggest that microorganisms, depending on the relative availability of C versus nutrients, may invest a substantial amount of C in storage compounds rather than using it for replication, specifically under nutrient-limited conditions. Besides, starving cells may still also follow an extremely slow growth strategy to survive (Gray et al. 2019), which we could hardly have detected with our methodological approach. Conversely, in C-rich rooted soils, urine addition reduced C accumulation of soluble C in cells (Figure 1). This led to a relatively higher ratio of microbial DNA-to-MBC in the rooted soils, indicating the microbial use of the labile C deposited by plants as building blocks for various cellular components (Gunina and Kuzyakov 2015) and thus for replicative microbial growth (Figure 2), a phenomenon typical for rhizosphere conditions (Blagodatskaya et al. 2014). This underlines the dominating effect of the plant–microbial interaction in the rhizosphere over the effect of urine addition (Meyer et al. 2019). It is also possible that rapid nutrient absorption by plants could have depleted urine-derived resources in soil, leading to a limited stimulatory effect of urine on microbial growth (Bertram et al. 2012). This interpretation is confirmed by the ~40% reduction of MBC in rooted compared to bulk soil during the 14-day incubation (Figure 1b), suggesting that plants outcompete microorganisms for urea-derived nutrients (Kuzyakov and Xu 2013), restricting microbial biomass accumulation.

A substantial urine-derived nutrient uptake by plants is also supported by a decrease in root biomass after urine amendment from days 1 to 4 (Table 1). This suggests that the high urea levels in cow urine (Reimer et al. 2024) offer a highly available N source that makes further root growth and development for N uptake less important (Mantelin and Touraine 2004). Such a decrease in root biomass in plants with urine application may also reduce the nutrient foraging potential to acquire nutrients other than N as well as water (Fort et al. 2015; Zhang et al. 2023). By day 14, root biomass without urine was even 40% greater than that with urine (Table 1), pointing to extensive root foraging for N (Junaidi et al. 2018). Thus, applying urine enhanced plant productivity, boosting above-ground but decreasing below-ground biomass compared to without urine amendment.

The MBN-to-MBC ratio was higher in bulk than in rooted soils, indicating that urine application to bulk soils alleviated N limitation by increasing the N supply for microorganisms. Conversely, in rooted soils, that ratio remained stable regardless of urine application, confirming the conclusion that microorganisms compete strongly with plants for N in intensively rooted temperate grasslands (Liu et al. 2016). The C-to-N ratio of plants was significantly higher in soils not treated with urine (Table 2). This further confirms that plants compete more strongly for urine-derived N than do microbes. In long-term grassed pasture soils, this leads to the conclusion that grassland plants are N-limited – but that microbes are not. Plants rapidly take up as much N as possible, thereby outcompeting the soil microbiome. This is evidenced by the stable MBN-to-MBC ratio observed in rooted soil irrespective of urine addition throughout the incubation period. Accordingly, microorganisms do not exhibit severe N deficiency (Figure 3).

4.2 | Effect of Cow Urine on Enzymatic Nutrient Mining Depending on Microbial Stoichiometry

In C-rich rooted soil, with and without urine addition, the maximum activity (V_{max}) of acid phosphatase increased sharply

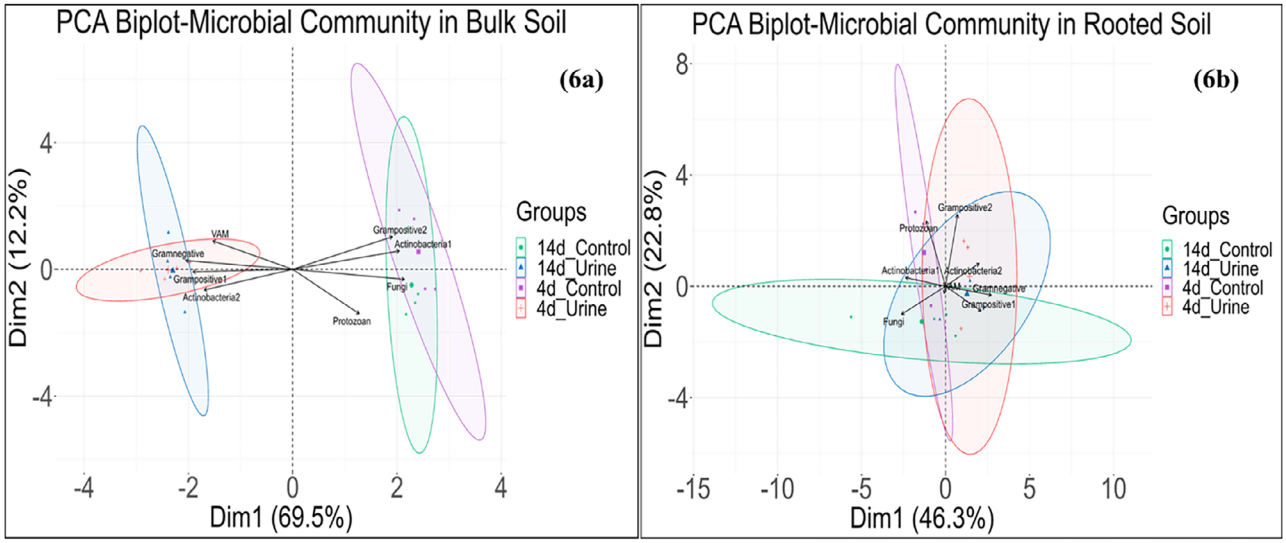

FIGURE 6 | PCA results for PLFAs measured microbial community after destructive harvest (4 and 14 days after urine application) in C-poor bulk soil (a) and C-rich rooted soil (b) without (control in purple and green), and with urine application (in red and blue).

with plant age compared to C-poor bulk soils (Figure 4a). This supports the concept of rhizodeposit-driven enzymatic activities (Ma et al. 2021). Furthermore, urine stimulated the production of β-glucosidase from days 1 to 14 in C-rich rooted soils (Figure 4b). This supports the idea that the upregulation of C-acquiring enzymes occurs in response to higher N accessibility (Stock et al. 2019), based on two processes: (1) microorganisms require N to synthesize enzymes and (2) N supply shifts the stoichiometric ratio of resource availability and thus increases the demand for C for microbial growth and metabolism (Allison and Vitousek 2005; Saiya-Cork et al. 2002; Sinsabaugh and Follstad Shah 2012). Several N addition experiments have shown that stoichiometric controls are crucial in mitigating microbial C limitation by enhancing C-acquiring enzymatic activity following N amendments (Spohn 2016; Yuan et al. 2019).

Additionally, cow urine did not exhibit consistent effects on the affinity of both acid phosphatase and β-glucosidase (Figure 4c, d), demonstrating that cow urine does not induce the production of different sets of enzymes. However, the substrate affinity of P-acquiring enzymes at day 4 was significantly higher in rooted compared to bulk soil with urine application. This illustrates that competition of plants for both N and P may occur in rooted soils, driving microbes to mine for low-concentrated P sources (Mason et al. 2021).

A higher ratio of acid phosphatase-to-β-glucosidase activity was observed in the rhizosphere during ongoing plant growth (and thus nutrient uptake). This reflects ongoing microbial elemental demand due to high microbial activity under increasing P deficiency (Kuzyakov and Blagodatskaya 2015; Wei et al. 2019). This phenomenon was observed under both urine-amended and non-urine-amended conditions (Figure 5), suggesting that urine does not compensate for reduced P availability in the rhizosphere during growth of plants. In summary, root exudation apparently has a more pronounced impact on the stoichiometry of available resources (and thus on microorganism's C vs. nutrient mining) than urine addition has (Figure 5).

4.3 | Effect of Cow Urine on Microbial Community Composition

Cow urine had a significant impact on the microbial community composition in C-poor bulk soils (Figure 6), where it notably increased the abundance of Gram-positive and Gram-negative bacteria (Supplementary Table S6). These groups are known to be often C-limited in C-poor bulk soils (Fanin et al. 2019; Zheng et al. 2021) and thus more actively benefited by the C and nutrient sources of the urine (Figure 6a). Bacteria are more strongly affected by urine application than fungi and protozoans in bulk soils (Figure 6a).

Plant roots have a pronounced effect on the composition of the microbial community, exceeding that of urine by far. Any potential effect of urine, as observed for the C-poor bulk soils, is eliminated by the presence of roots. The potential explanations are that plants effectively use the urine-derived nutrients (Figure 6b) or that root exudates in addition to C sources also contain enzymes, mucilage and many secondary metabolites (flavonoids, phenolics, peptides, etc.), which, along with many other rhizosphere factors, exert a major influence on the microbial community.

In bulk soils, urine treatment had a rapid effect on soil microbial communities, inducing a significant increase in the bacteria-to-fungi ratio already on day 1 compared to rooted soils (Table 4). In the bulk soil, however, no substantial shifts in microbial community structure due to urine addition were observed at the first sampling time, requiring at least 4 days to become evident (Supplementary Figure S6). Accordingly, the 14-day incubation period in our study was sufficient for microbial communities to acclimatize to urine and most likely exploit its nutrients. Beyond causing a shift in microbial community composition, this also implies a metabolic and/or ecophysiological switch involving the release of altered quantities or qualities of extracellular enzymes.

In bulk soils, the bacteria-to-fungi ratio was significantly higher on day 1 under urine treatment compared to rooted soils (Table 4).

TABLE 4 | Effect of cow urine on the bacteria-to-fungi ratio measured after destructive harvest (1, 4 and 14 days after urine application), without (control) and with urine application.

Bacteria/Fungi						
	1 Day	4 Days	14 Days	1 Day	4 Days	14 Days
Bulk soil				**Rooted soil**		
Control	1.2 ± 0.2^{a}	1.9 ± 0.1^{AB}	$1.8 \pm 0.2^{a´b´}$	1.9 ± 0.2^{a}	1.5 ± 0.1^{A}	$1.4 \pm 0.1^{a´}$
Cow urine	2.9 ± 0.2^{b}	2.8 ± 0.2^{C}	$2.8 \pm 0.2^{c´}$	2.1 ± 0.2^{a}	2.2 ± 0.2^{BC}	$2.2 \pm 0.2^{b´c´}$

Note: Different letters indicate significant differences between treatments at each time ($p < 0.05$). Data represent mean ± standard error ($n = 4$).

This suggests that increased N availability relative to C enhances bacterial dominance (Wang and Kuzyakov 2024) with a risk of promoting nitrification and denitrification processes (Hu et al. 2023). However, many changes in the microbial community structure require more than 24 h. Therefore, the observation on day 1 showing the immediate effect of urine addition on the microbial community structure may not solely be related to the urine inputs and may not represent the complete alteration. Nevertheless, effects observed on days 4 and 14 show that even if plants finally outcompete microorganisms to some extent in the use of their nutrients, microorganisms are adapting by changing the community structure.

This study demonstrates a close link between microbial community structure and strategies to adapt elemental stoichiometry in temperate pasture soils treated with cow urine, both in C-poor bulk and C-rich rooted soils. A major finding is that the effects of urine on microbial community composition are completely overridden by root exudates, which also implies that urine patches in these grasslands play a lesser role in microbial stoichiometry. This highlights that C supply via exudates, but not the input of urine, predominantly defines the microbial communities and their physiological adaptations in grassland soils.

5 | Conclusions

The urine application and the presence of rhizodeposits both have relevant implications for grassland C cycling and their microbial communities. Urine patches, which are rich in N but low in P, lead to stoichiometric imbalances in bulk soil, which do not affect the extractable cytosol compounds of microbial cells but alter microbial growth behaviour. In rooted soils, the DNA-to-MBC ratio increased, pointing towards enhanced replicative microbial growth utilizing root exudate C and urine-derived nutrients. Urine caused P deficiency as indicated by an increase in the acid-phosphatase-to-β-glucosidase activity ratio to enhance P mining from soil organic sources, which went along with a shift in the microbial community structure. However, the effects of urine on microbial community composition are completely overridden by root exudates, highlighting that C availability in the rhizosphere predominantly defines the microbial communities present in rooted soils. The distinction between bulk and rooted soils highlights the importance of spatial heterogeneity and emphasizes the need to consider both soil compartments separately. This, in turn, complicates potential upscaling approaches of element cycling because the ratio of rooted to non-rooted soils in spatial correlation with urine-amended patches is an essential information for robust generalization of the consequences of urine application for microbial functions in soils.

Author Contributions

Y.D., M.K., C.B. and M.D. designed the experiment. M.K., L.F., O.M. and Y. D. collected and M.K. analysed the data. M.K. wrote the manuscript. N.B., C.B., T.P. and M.D. helped with writing, editing and finalizing the manuscripts. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. They will be published in an open-access format with the finalization of the project BA 6982/1-1.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: jpln70032-sup-0001-SuppMat.pdf.