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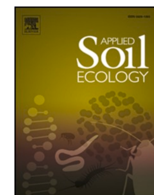

Research paper

Unveiling the impact of human urine fertilization on soil bacterial communities: A path toward sustainable fertilization

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ABSTRACT

Using human urine as a crop fertilizer has sparked interest due to its potential benefits, but its application requires an understanding of how urine can affect soil functions and microbial communities. This study aims at elucidating the response of soil bacterial communities to fertilization with human urine. To this end, a spinach crop was fertilized with 2 different doses of a source-separated and stored human urine (170 kg N ha^{-1} + 8.5 kg P ha^{-1} and 510 kg N ha^{-1} + 25.5 kg P ha^{-1}) and compared with a synthetic fertilizer treatment (170 kg N ha^{-1} + 8.5 kg P ha^{-1}) and a water treatment without fertilization. The experiment was conducted in four soil tanks in greenhouse conditions, according to a randomized block scheme. We assessed urine and soil bacterial composition at the beginning and the end of the experiment that we compared to soil and plant properties to understand the drivers in bacterial composition changes. After 12 months of storage, urine had a depleted microbiome but still contained few common strains of urine or faeces. Overall, soil bacterial communities were resistant to urine fertilization with only 3 % of the taxa impacted. However, urine fertilization increased the relative abundance of nitrifying and denitrifying groups compared to the synthetic fertilizer implying that more N_2O and NO could be emitted when fertilizing with urine. The urine's high salt concentration had little discernible effect on the bacterial community. In a broader context, this experiment provides evidence that one-year-stored urine can be applied to a plant-soil system without negatively impacting soil bacterial communities in the short term.

1. Introduction

Recycling human urine as a fertilizer has been identified these past years as a real opportunity to enhance agricultural sustainability, reduce wastewater pollution, wastewater energy consumption, and decrease dependency on synthetic fertilizer, among other benefits (Karak and Bhattacharyya, 2011; Wald, 2022). Fresh urine is composed of 95 % water and the 5 % remaining are amino compounds (such as urea or creatinine), organic anions and inorganic salts making it a source of bioavailable nutrients and micronutrients for plant growth (Akpan-Idiok et al., 2012; Pradhan et al., 2009; Rumeau et al., 2023).

In addition, stored urine is considered “safe” for fertilization application as urine contains low numbers of microorganisms compared to faeces (< 10^4 CFU mL^{-1} of total bacteria in the urinary tract compared to 10^{11} CFU g^{-1} in the colon) (Pearce et al., 2014), and can be safely collected through source separation systems (Lienert and Larsen, 2010). Moreover, storing urine for several months, with the resulting increase in its pH value (about 9 versus 6.5 for fresh urine) and its free ammonia concentration induced by ureolysis, is considered sufficient to inactivate most human pathogenic bacteria (World Health Security, 2006; Xu et al., 2022) and breakdown extracellular DNA (Goetsch et al., 2020). Therefore, source-separation of urine followed by a storage process further reduces risks of disease transmission. Disseminating this knowledge led to a favourable opinion among farmers and civilians regarding the consumption of food grown with human urine (Andersson, 2015; Simha et al., 2021) emphasizing further the importance of validating the safety of this practice for human and soil health.

While the risk of pathogen contamination has been well studied, very little research has been conducted on the effect of human urine on soil microbial community structure or diversity. Yet, bacteria are sensitive to environmental changes and could therefore be affected by urine application in several ways. For instance, the high concentration of

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ammonium in stored urine could either stimulate nitrifying communities (Hartmann et al., 2013; Mahmood and Prosser, 2006), or on the contrary, inhibit their activity because of toxic levels of ammonia in high pH soils (Clough et al., 2003). Additionally, urine application, by enhancing nitrification and volatilization, could significantly acidify soils (Bolan et al., 1991; Raza et al., 2021). This acidification could potentially modify soil microbial communities, as soil pH is a key determinant of bacterial community structure (Karimi et al., 2018; Poulsen et al., 2013). Furthermore, urine fertilization can increase soil salinity (Neina, 2013), potentially leading to a decrease in bacterial diversity and the emergence of halotolerant taxa (Zhao et al., 2020). Finally, the concentration of carbon (C) and micronutrients (Fe, Cu, Mn, Zn) in urine could stimulate microbial growth (Orwin et al., 2010) and could modify the bacterial community structure, as a recent study has highlighted the role of micronutrients in shaping soil microbial community structure (Peng et al., 2022).

To our knowledge, the existing studies on the effect of urine fertilization on the soil microbiome either used synthetic urine (Nunan et al., 2006), lime-treated urine (Roy et al., 2022) or animal urine when the study was based on a pasture field (Hartmann et al., 2013; Orwin et al., 2010). The main findings suggest an overall resistance of microbial communities to urine application but small shifts were observed (Roy et al., 2022). However, the question remains on the amplitude and significance of these shifts when using stored human urine. It is therefore crucial to assess and ensure that this practice will not hamper soil bacterial diversity, a pillar for plant production, nutrition and defence (Khmelevtsova et al., 2022; Lugtenberg and Kamilova, 2009).

In order to investigate the effects of human urine application on soil bacterial communities, a greenhouse experiment was conducted on an agricultural soil supporting a spinach crop (*Spinacia oleracea*). Spinach was chosen because of its relatively high nitrogen (N) requirements (170 kg N ha^{-1} under optimal growth conditions, Frerichs et al., 2022), short growth cycle, moderate sensitivity to salts (Langdale et al., 1971) and tolerance to urine application (Kutu et al., 2011; Sangare et al., 2015). The spinach crop was fertilized with two different doses of toilet-diverted and stored human urine. Their effects were compared with those of a synthetic fertilizer treatment, as well as a water treatment with no fertilization. We measured bacterial community composition that we analysed in regard with previously published data on plant growth, soil physico-chemical properties (pH, conductivity, nutrients) and soil microbial biomass (Rumeau et al., 2023). The hypotheses were that 1) stored human urine would have a depleted microbiota, 2) the taxonomic composition of the soil bacterial communities would remain relatively resistant to the addition of urine, although 3) urine fertilization would favour bacteria involved in nitrification and salt tolerance.

2. Material and methods

2.1. Treatments and experimental design

The plant experiment was carried out in four soil tanks, each with a soil surface of 0.94 m^2 (0.85 m × 1.10 m) and a depth of 50 cm. These tanks were each divided into four experimental units and placed within a tunnel greenhouse exposed to natural sunlight in Montpellier, France (43°38′51.05"N, 3°52′26.13″E). The detailed experiment is related in Rumeau et al. (2023). Briefly, the soil used was a loamy clay soil rich in carbonates (45 %) with a pH of 8.7, and spinach plants (*Spinacia oleracea* L.) were cultivated in soil tanks from May to July 2020. We compared four treatments: two different doses of human urine (U1 = urine dose *x* 1; U3 = urine dose *x* 3) with a synthetic fertilizer (F) and a water control (W). The doses applied were: 170 kg N ha^{-1} + 8.5 kg P ha^{-1} (U1 and F), 510 kg N ha^{-1} + 25.5 kg P ha^{-1} (U3), and 0 kg N ha^{-1} + 0 kg P ha^{-1} (W). N and phosphorus (P) in the F treatment were added as ammonium nitrate (NH_4NO_3) and potassium phosphate (KH_2PO_4) respectively. U1 and F are recommended N doses for spinach, while U3 triples this dose, amplifying urine effects and approaching the recommended P dose. Each treatment was applied to one experimental unit (i.e. quarter of tank) containing eight spinach plants (Fig. S1). Tank quarters were separated from each other by 30 cm deep aluminium sheets resulting in a total of 16 experimental units (four treatments × four tanks or replicates) were set up. The treatments were fractionated into 6 applications. Before addition, each fertilizer was diluted in 6 L of water and poured on the entire surface of the four experimental units per treatment. Irrigation was conducted with sprinklers located above the tanks and controlled with tensiometers placed at 15 cm depth in the soil to maintain the soil moisture around field capacity.

2.2. Urine sampling and composition

The urine used in this experiment was collected by the start-up EcoSec (Montpellier, France, https://ecosec.fr/), which specializes in manufacturing source-separating toilets. While a gravity system in these toilets facilitates the separate collection of urine and faeces, it does not completely prevent the risk of cross-contamination with faeces. Urine originated from male toilets during a festival day and was stored for a year in an opaque and airtight container in order to sanitize the effluent according to World Health Organization (WHO) recommendations (World Health Security, 2006). Prior to the experiment, the urine was analysed for potential pathogen indicators after reception (fresh urine) and later after six months of storage. Sampling was consistently done after stirring the urine for 1 min to ensure homogenisation. Analyses included total coliforms, Enterococci, sulphite-reducing anaerobic bacteria, and F-specific RNA bacteriophage quantification. The analyses were performed by the laboratory ABIOLAB-ASPOSAN (Montpellier, France, www.asposan.fr). The specific methods used for the determination of each microbial indicator can be found in Table S1 (Supplementary data).

Right before the start of the experiment, four samples of stored urine (12-month-old) were analysed for bacterial community composition by metabarcoding using a 16S rRNA gene marker. Each urine DNA sample was extracted from 20 mL of stored urine by the ADNid laboratory (Montpellier, QUALTECH group, http://www.adnid.fr/). After urine centrifugation, the pellet was lysed mechanically with garnet beads (3 × 30s) and chemically with a solution of SDS (2 %), NaCl 100 mM, EDTA 100 mM and Tris-HCl 100 mM (pH = 8) at 70 °C for 30 min as previously described (Dequiedt et al., 2011). Crude DNAs were purified on silica membrane-based columns (NucleoSpin Soil column, Macherey Nagel). The 16S rRNA region amplification, sequencing and read data analysis were performed following the same procedure as the soil DNA samples (see Section 2.3 for method description). The urine's chemical composition is given in Rumeau et al. (2023). Briefly, the urine contained 4341 mg L^{-1} of ammonium, 206 mg L^{-1} of inorganic P and 57 mg L^{-1} of organic carbon. Furthermore, it contained Cl^- (3575 mg L^{-1}), Na^+ (1290 mg L^{-1}), SO_4^{2-} (1229 mg L^{-1}) and K^+ (1108 mg L^{-1}).

2.3. Soil prokaryotic community structure

In each experimental unit ($n = 16$), four subsamples of soil from the 0–10 cm layer were collected between the plant rows and were combined to create a single final sample for each unit, resulting in four replicates per treatment (Fig. S1, Supplementary data). This was done at the beginning of the experiment before sowing (T0), and at the end of the experiment, 38 days after T0 (TF). Samples for bacterial analyses were stored at −20 °C until analysis.

2.3.1. Soil DNA extraction, amplification and sequencing

Soil DNA extraction and sequencing of bacterial 16S rRNA hypervariable regions were performed by the ADNid laboratory. Soil DNA was extracted and purified as mentioned above for urine, except than garnet beats were replaced by silica beads (MP Biomedicals). PCR was performed using the universal primers V3-V4 of the 16S RNA genes for prokaryotes A519F (5′-CAGCMGCCGCGGTAA-3′) and 806R (5′-

GGACTACNNGGGTATCTAAT-3′), a hot-start DNA polymerase (Type-it QIAGEN) and 10 ng of template DNA. PCR products were then purified using AMPure magnetic beads (Agencourt) and tagged using the Nextera XT DNA Library Prep Kit ((Illumina, Inc., San Diego, USA) in a second PCR reaction. After purification, the resulting PCR amplicons were pooled together at 15 μg/μL in a final library. The DNA library was sequenced using an Illumina MiSeq generating 2 × 250 bp reads and V3 chemistry (Illumina, Inc., San Diego, USA). Sequencing proceeded in two runs, each including blank samples (DNA extraction, PCR).

2.3.2. Sequence data processing

Paired Illumina MiSeq reads were quality filtered and main bio informatic processing was performed by the ADNid Laboratory. Demultiplexed amplicon data were processed using the automated pipeline FROGS 3.2 (Find Rapidly OTU with Galaxy Solution) developed by INRAE (Escudié, 2018). This pipeline consists first in several cleaning steps (trimming and filtering). Then, sequences were clustered by OTUs (operational taxonomic units) using the SWARM v2 method with a maximum number of differences between sequences in each aggregation step of 1 (Mahé et al., 2015). Chimeric sequences were removed using the tool VSEARCH and the command uchime_denovo. To determine bacteria and archaea taxonomic affiliations, OTUs were aligned using BLASTn against the SILVA SSU database version 138 Rref NR99 inferred in FROGS. Blank controls considered as contaminants were removed from the dataset. An OTU table with 11,711 to 56,471 validated sequences per soil sample was generated from the FROGS pipeline and processed in R v4.3.1 using the Phyloseq v1.22.3 package (McMurdie and Holmes, 2013) to analyse data and the diversity of microbial communities. In order to compare each soil sample at T0 and TF, the high-throughput sequencing result were normalized to the sample with the lowest total counts (11,711 reads). The effective numbers of species (ENS) based on α-diversity indices were quantified using three diversity quantifiers (Number of OTUs, exponential of Shannon index and the inverse of the Simpson index) (Jost, 2006). β-Diversity was measured with the different metrics available (i.e. Bray-Curtis dissimilarity, Jaccard distance, weighted and non-weighted UniFrac distance).

2.3.3. Prediction and quantification of key genes and metabolic pathways

PICRUST2 integrated in the FROGS pipeline (Escudié et al., 2018, FROGS version 4.0.1, PICRUST version 2.4.1) on the MIGALE facility was used to predict the relative abundance of functional genes from the 16S rRNA gene marker. Briefly, the pipeline places each sequence on a reference phylogenetic tree and predicts the relative abundance of each functional gene using the KEGG database (where a KO number corresponds to a gene) and then categorizes genes into metabolic pathways. Finally, the KOs involved in N, P cycling and salt tolerance were selected based on the literature (Albright et al., 2019; Kim et al., 2019; Liu et al., 2018) and the KEGG database (https://www.genome.jp/kegg) were isolated. A total of 19 genes involved in N cycling were identified and categorized by reactions (1 to 10) (Coskun et al., 2017). 41 genes involved in P cycling were categorized by function (P starvation, P transport, C—P lyase, P solubilisation, phosphatase, phytase). And nine genes were identified to be involved in salinity stress tolerance, encoding for osmoprotectant transporters, porins, osmolarity sensors, and synthesis of proline and trehalose (i.e. osmoprotectant molecules). All these genes with their corresponding KEGG numbers are listed in Table S2.

2.3.4. Co-occurrence network

We computed correlation-based networks of community composition at bacterial OTU resolution to compare putative bacterial relationships and nuanced diversity changes in response to the four treatments. Before constructing the network, the OTU table was filtered to keep the OTUs that represent at least 0.5 % of reads in at least one sample. Networks were constructed using the Spearman correlation calculated from the filtered OTU table using the vegan package in R (Kolaczyk, 2009). Significantly related OTUs with a correlation coefficient above 0.8 were selected to construct the co-occurrence network. Network properties were then calculated with the *igraph* package in R (Csárdi et al., 2023). The number of nodes, the number of edges, the number of clusters, the modularity and the 5 top keystone species were used to compare the different networks. The connectance, used as an index of network stability, was computed by dividing the number of edges (established relationships) by the square of the number of nodes (possible inter-relations).

2.4. Statistical analyses

Statistical analyses were carried out with Rstudio software (version 4.1.0, R Core Team, 2021). Significant difference in taxa abundance at multiple taxonomic levels (phylum, family, OTU) between treatments were detected using Kruskal-Wallis tests (Kruskal and Wallis, 1952) followed by a Dunn tests when necessary (Dunn, 1964). Groups that had a relative abundance below 0.001 % in all samples were removed prior to this analysis. All the families that were significantly affected by treatments were then extracted and the natural logarithms of response ratios (lnRR) between the urine treatment (U1 and U3) and the synthetic fertilizer treatment (F) were calculated to quantify the magnitude and direction of urine's effect. The response ratio and its standard error (SE) were calculated via Eqs. (1) and (2).

$$lnRR = ln\left(\frac{\overline{x_1}}{\overline{x_F}}\right) \tag{1}$$

$$SE\ (lnRR) = \sqrt{\frac{{s_1}^2}{{n_1}^2 * \overline{x_1}^2} + \frac{{s_F}^2}{{n_F}^2 * \overline{x_F}^2}} \tag{2}$$

where $\overline{x_1}$ and $\overline{x_F}$ denote the observed means of respectively the urine treatments (U1 or U3) and the synthetic fertilizer treatment (F), s_1 and s_F denote the standard deviations, and n_1 and n_F the sample sizes of the two groups.

The percentage of change from T0 to TF in each treatment and the Orwin Wardle resistance index (Maynaud et al., 2019; Orwin and Wardle, 2004) were computed on the α-diversity indices and the main 19 phyla abundances. The resistance index quantifies the stability of the bacterial community structure after a disturbance (i.e. fertilization treatments) (Eq. (3)).

$$Resistance\ (\%) = \left(1 - \frac{2|D_F|}{(C_F + |D_F|)}\right) \times 100 \tag{3}$$

where D_F is the difference between the control treatment (C_F) and the fertilized treatment at TF. Resistance is bounded between −100 and + 100 where a resistance value of +100 indicates that the treatment had no effect.

Furthermore, a PERMANOVA (permutational multivariate analysis of variance) test was used to identify any treatment or block effect on the Bray-Curtis dissimilarity. The Bray-Curtis dissimilarity was further visualized through various NMDS analyses, incorporating environmental variables or depicted trajectories from T0 to TF to illustrate the temporal evolution of bacterial community composition.

3. Results

3.1. Urine microbial analyses

The urine samples exhibited few to indistinguishable OTUs, as sequencing reads resembled those in the technical control obtained after DNA extraction and sequencing using only solutions. The control and urine samples had fewer numbers of taxa (977 and 1048 OTUs respectively) compared to soil samples (2700 OTU on average before rarefaction) (Table S3), which where distinct from soil samples, showing

highly divergent communities (Fig. S2). Urine samples shared 66 % of their OTUs with the control, while soil samples shared only 2 % for (Table S3). Only 46 OTUs were common to all four urine samples, and a total of 515 OTUs were found in at least two samples. Among these 515 urine OTUs, the main taxa detected at the order level were *Peptostreptococcales-Tissierellales* (17 %), *Pseudomanadales* (17 %), *Burkholderadiales* (15 %), *Cardiobacteriales* (14 %), *Lactobacillales* (11 %), and *Bacillales* (9 %) (Fig. S2C). At the genus level, *Pseudomonas* (15 %) *Ignatzschineria* (14 %) and *Paenalcaligenes* (14 %) were dominant followed by *Tissierella* (9 %) and *Atopostipes* (8 %). Notably, neither *Enterobacteriaceae* (that includes *Escherichia* and coliforms) nor *Enterococcaceae* (that includes *Enterococcus*), were detected (data not shown). Moreover, the microbial contamination analyses performed on fresh urine and six-month-old urine revealed a decrease of all microbial indicators with time. Fresh urine contained coliforms and enterococci (indicators of faecal contamination) but after six months of storage in an airtight container, coliform became undetectable (<1 CFU 100 mL^{-1}) and enterococci decreased from >8.3 10^8 MPN 100 mL^{-1} to 23,981 MPN 100 mL^{-1}). Sulphite-reducing bacteria and faecal bacteriophage indicators also exhibited reduced levels after this storage period (Table S1, Supplementary data).

3.2. Soil microbial community structure

3.2.1. Community structure before applying fertilizer

At the beginning of the experiment, before applying any treatment, the soil microbiome mainly comprised *Proteobacteria* (33 %), *Acidobacteria* (20 %) and *Bacteriodota* (12 %) (Fig. 1). At T0, there was a clear separation of the soil microbial community structure between the four blocks as evidenced by the PERMANOVA (Table 1) and the NMDS where samples from blocks 4 and 5 clustered in the upper right part (Fig. 2) and samples from block 1 clustered in the upper left part. This separation was mainly explained by initial soil phosphate, nitrate content and soil pH (Fig. 2).

Table 1
PERMANOVA analyses on the Bray-Curtis distance comparing the effect of treatment, block and time at T0 and TF. An asterisk next to the *p*-value indicates a significant effect (*** $P < 0.001$).

	Bray-Curtis p-value	
Factors	T0	TF
Treatment	0.9781	0.2866
Block	>0.001***	>0.001***
Time	>0.001***	

3.2.2. Community structure after fertilization treatments

At TF, the soil microbiome was predominantly composed of *Proteobacteria* (46 %), *Acidobacteria* (14 %) and *Actinobacteria* (8 %). At the phylum level, only the *Nitrospirota* phylum was significantly more abundant in the U1 treatment compared to the water control (Fig. 1B). The *Nitrospirota* phylum also showed the lowest resistance value (relative to W treatment) for U1 and U3 (−16.9 % and − 9.7 % respectively, Table S4). With the exception of Nitrospirota and Latescibacterota phyla, all other phyla displayed positive resistance values (7–88 %) in response to the treatments with nine of them above 50 %. Furthermore, resistance to urine and synthetic fertilizer was similar across the 19 phyla averaging +50 % (Table S4).

The co-occurrence networks were also similar between the four treatments, characterized by an average number of nodes of 48 ± 2, a number of edges of 898 ± 54 and a connectance index of 0.387 ± 0.005 (Fig. S3).Several of the top five keystone species were similar across treatments. Species of *Shingomonas* and *Dongia* were detected as keystone species in W, F and U1 and *Ellin6055* was detected in both U1 and F. However, the U3 treatment featured two distinct top keystone species of *Nitrospira* and *Nitrosospira*, unlike the other treatments (Fig. S3). Additionally, the presence of *Nitrospirota* (including the genus type *Nitrospira)* (Oren and Garrity, 2021), was noticed within the main supergroups or phyla forming the co-occurrence network of F and U1 (Fig. S3).

Finally, while there was no significant difference between

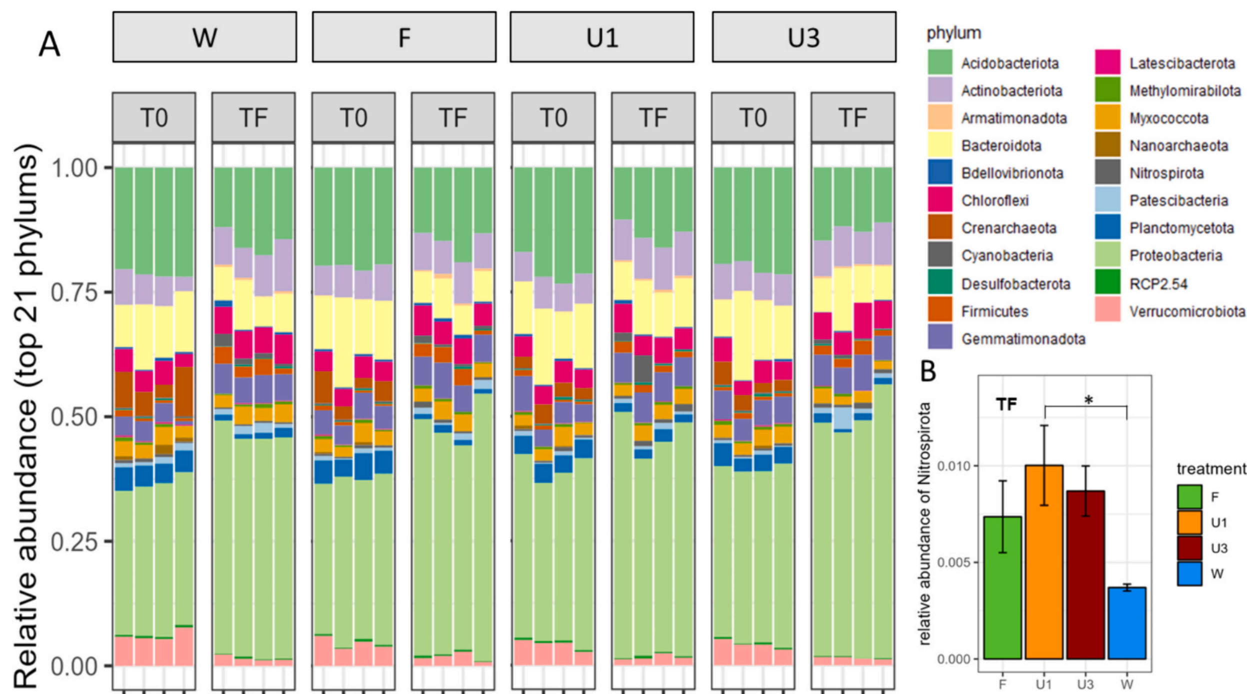

Fig. 1. A) Phylum abundances in soil samples collected in the four treatments at the beginning (T0) and the end of the experiment (TF). B) Relative abundance of the Nitrospirota phylum for the four treatments at TF (only phylum whose relative abundance was significantly affected by treatments). Error bars represent standard errors between the four replicates and the asterisk above the bar plot indicates a significant difference between treatments ($p < 0.05$).

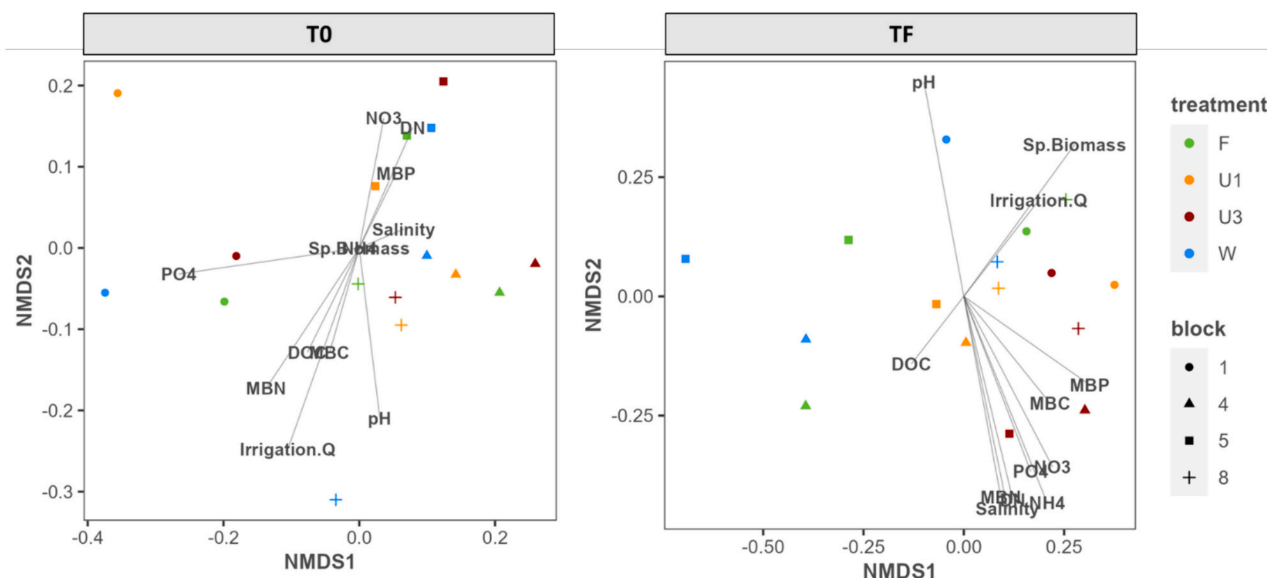

Fig. 2. Non-metric multidimensional scaling (NMDS) of the samples at T0 on the left and of samples at TF on the right, including 12 fitted environmental variables according to Rumeau et al., 2023. The more impacting variables are shown: PO4 (soil phosphate), salinity (electric conductivity), NO3 (soil nitrate), NH4 (soil ammonium), soil pH (measured in water), Spinach shoot biomass (Sp.Biomass), Dissolved organic carbon (DOC), Dissolved nitrogen (DN), Microbial biomass carbon, nitrogen, phosphorus (MBC, MBN, MBP) and moisture (soil gravimetric moisture). NMDS stress is 0.12 and 0.11 for T0 and TF respectively.

treatments, diversity measured by effective number of species (ENS) indices was slightly higher in the urine treatments, especially the number of OTUs (+37 % and + 47 % in U1 and U3 respectively) leading to a lower resistance (based on number of OTUs) in these two treatments (Table 2). The β-diversity index (i.e. Bray-Curtis distance) did not reveal any effect of treatments at TF but revealed a block effect ($P < 0.05$) (Table 1, Fig. 1).

3.2.3. Community structure shift from T0 to TF

The diversity of the soil bacterial communities, estimated by the numbers of OTUs and the exponential of the Shannon index, decreased significantly from T0 to TF (Table 2). Additionally, the soil community composition changed from T0 to TF, shifting the relative abundance of the main bacterial phyla (Fig. 1A). Among the phyla affected, the relative abundance of *Planctomycetota, Acidobacteria* and *Verrucomicrobiota* decreased significantly and the abundance of *Proteobacteria, Actinobacteria* and *Firmicutes* increased significantly from T0 to TF (Fig. 1A). At the family level, 50 % of the taxa ($n = 280$) shifted significantly between T0 and TF. Particularly, the relative abundances of *Acetobacteraceae*, *Azospirillaceae*, *Bacillaceae*, *Burkholderiaceae, Paenibacillaceae* and *Pseudomonadaceae* increased by a factor ranging from 4 to 40 (Fig. S4).

Furthermore, the metabolic pathway prediction showed a strong difference between T0 and TF in predominant metabolic pathways. At T0, the predominant pathways were mainly biosynthesis pathways (pentose phosphate, aromatic compounds, nucleotide and amino acid biosynthesis) and C1 compound degradation, while at TF, the predominant pathways were degradation pathways (specialized metabolites, carbohydrate, inorganic and organic nutrient metabolism) except for secondary metabolites and fatty acids biosynthesis (Fig. S5).

Trajectory analyses revealed that from T0 to TF, samples treated with U3 followed similar directions converging toward the upper-left part of the NMDS plot (Fig. 3). This trajectory may have been driven by higher soil mineral N, P, salt contents and lower soil pH in U3 (Fig. 2). In contrast, samples under W, F, and U1 treatments did not exhibit a clear pattern. Furthermore, block effects caused blocks 1 and 8 to diverge from blocks 4 and 5 (Fig. 3), with high spinach shoot biomass more closely related to blocks 1 and 8 (Fig. 2).

Table 2
Effective numbers of species based on α-diversity indices (Number of OTUs, exp.(Shannon) and Inverse Simpson (mean ± se)) calculated on the soil bacterial communities at the beginning (T0) and the end of the experiment (TF), change in α-diversity from T0 to TF, and resistance standardized by the W treatment. Different uppercase letters indicate differences between T0 and TF (p-value <0.05) while no significative differences were detected between treatments.

α-Diversity	W	F	U1	U3
Number of OTUs				
T0	2337 ± 112 A	2373 ± 99 A	2222 ± 69 A	2299 ± 98 A
TF	1194 ± 282 B	1285 ± 225 B	1641 ± 105 B	1762 ± 241 A
Change from T0 to TF (%)	−47 ± 15	−47 ± 8	−26 ± 6	−24 ± 9
Resistance (%)	–	74 ± 15	37 ± 23	30 ± 16
Exp(Shannon)				
T0	759 ± 63 A	796 ± 68 A	735 ± 57 A	783 ± 80 A
TF	503 ± 121 B	535 ± 77 B	632 ± 39 B	594 ± 95 B
Change from T0 to TF (%)	−27 ± 24	−33 ± 7	−12 ± 9	−23 ± 10
Resistance (%)	–	62 ± 14	44 ± 24	34 ± 7
Inverse Simpson				
T0	170 ± 30 A	195 ± 28 A	211 ± 29 A	200 ± 28 A
TF	176 ± 56 A	203 ± 40 A	225 ± 19 A	168 ± 52 A
Change from T0 to TF (%)	42 ± 63	10 ± 22	13 ± 15	−10 ± 22
Resistance (%)	–	38 ± 20	26 ± 29	21 ± 10

3.3. Key nutrient cycling taxa and gene prediction abundance

The effect of treatments was further analysed by Kruskal-Wallis tests on the relative abundances at different levels of classification at TF. It revealed on average 3 % of dissimilarities between treatments (1.1 % at OTU level, 2.6 % at family level, and 4.7 % at the phylum level) (Table S5). Fig. 4 illustrates the response ratio of taxa expressing different abundances between the synthetic fertilizer treatment and the urine treatments. Most taxa are in higher proportion in the urine treatments (RR = 1.40 on average) than in the F including *Nitrosomonas* and *Nitrobacter* which showed a further enhancement in U3 compared to U1 (Fig. 4). Interestingly, *Sneathiellaceae* responded very strongly to the U3 (RR = 7) treatment but not to the U1 treatment (RR = 0) (Fig. 4).

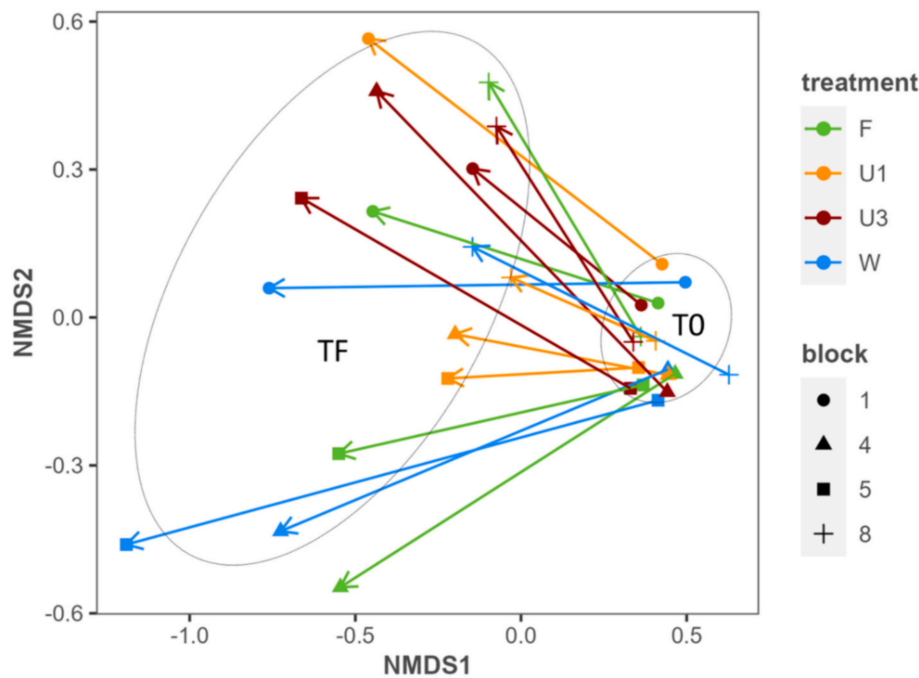

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination plots of soil microbial community structure based on OTUs with the trajectory of each sample composition from T0 to TF, indicated by arrows starting at T0 and ending at TF.

Furthermore, the prediction of functional gene abundance revealed a significant effect of treatment on 53 % of identified N cycling genes, 10 % of identified P cycling genes and none of the identified salt tolerance genes. According to the prediction, ammonium oxidation genes (*hao* and *amoA*) and nitrite/nitric oxide reductase genes (*norC*, *norB* and *nirK*) were more abundant in the U3 and/or U1 treatment than in F and W ($P < 0.05$) (Fig. 5). While *hao* and *amoA* increased proportionally with the amount of NH_4^+ applied (Fig. S6), *norC*, *norB* and *nirK* genes showed similar relative abundances between U1 and U3 (Fig. 5). Genes involved in the dissimilatory nitrate reduction pathway (DNRA) were usually lower in the urine treatments with the only significant difference observed for *narG* between W and U3 (Fig. 5). Urease genes (*ureA, ureB, ureC*) were all more abundant in W compared to U3 ($P < 0.05$) (Fig. 5). The majority of P cycling genes showed no significant changes between treatments (Table S6). However, several genes were significantly more abundant in the urine treatments. Among them, *appA*, encoding for a phytase, was potentially more abundant in U3 and U1 than in W and F and two phosphatase genes (*pho*N and *pho*A) were approximately twice more abundant in U3 than in the other treatments ($P < 0.05$) (Table S6). Finally, none of the genes identified to be involved in salinity stress tolerance was affected by the treatments (Table S7).

4. Discussion

4.1. Temporal shift in the microbial community composition

The soil bacterial community shifted from T0 to TF in all samples in our experiment. This shift could be due to environmental conditions (e.g. soil moisture) but is most likely driven by plant presence. Growing spinach on these soils probably exerted a selective pressure on soil bacterial communities as it decreased bacterial diversity. Multiple studies have shown that plants shape bacterial communities of their rhizosphere by releasing root exudates (Grunert et al., 2019; Lei et al., 2019). Furthermore, in our experiment, we observed that plant presence promoted several taxa i.e. *Acetobacteraceae*, *Azospirillaceae*, *Burkholderiaceae, Bacillaceae*, *Paenibacillaceae*, *Pseudomonadaceae* that potentially shelter plant growth-promoting rhizobacteria (PGPR) promoting plant defence, hormone modulation and nutrient acquisition in plants (Backer et al., 2018; Delaporte-Quintana et al., 2020). The spinach crop may have also increased the abundance of copiotroph bacteria, such as Firmicutes (Stone et al., 2023), known for thriving in nutrient-rich environments with faster growth rates. This was supported by the metabolic pathway prediction by PICRUST2 suggesting that bacteria able to degrade and utilize nutrients were favoured over others at the end of the experiment. This shift occurred regardless of the fertilization treatment, which supports previous research indicating that the plant host has a greater influence on the soil bacterial community structure than fertilization treatments (Grunert et al., 2019). This study shows that this pattern remains consistent even when the fertilizer is human urine.

4.2. Effect of urine fertilization on the bacterial community structure

In accordance with the WHO recommendation (World Health Security, 2006), storing urine over a period of at least six months results in a reduction of microbial contaminant indicators concurring with the low level of bacteria detected in this study. Urine microbiome was relatively depleted after 12 months of storage, with only 46 OTUs found systematically in urine samples and 515 OTUs founds in at least two samples. This result is in line with a study finding that storing urine for 82 days reduced bacterial diversity to about 130 OTUs (defined at 97 % similarity) (Lahr et al., 2016). During storage, urea hydrolysis increases the pH of urine causing the death of most bacteria and pathogens (Chandran et al., 2009; Höglund et al., 1998), explaining the absence of E.coli and enterococci after storage. Nonetheless, our results suggest that some bacteria commonly found in urine or faeces such as species of *Lactobacillales (Atopostipes),* species of *Peptostreptococcales-Tissierellales (Ignatzschineria) and species of Paenalcaligenes* that include pathogenic strains like *Ignatzschineria indica* and *Paenalcaligenes hominis* may survive (Devane et al., 2023; Lee et al., 2020; Pearce et al., 2014; Snyder et al., 2020). However, none of these pathogenic OTUs appeared to have been transferred to the soil (data not shown). Our findings, together with prior research, confirm that storing urine is an effective method for mitigating the risk of introducing bacterial pathogens although its effectiveness might not be total.

Urine applications modified soil chemical properties by reducing soil pH (likely through ammonia volatilization and nitrification) and

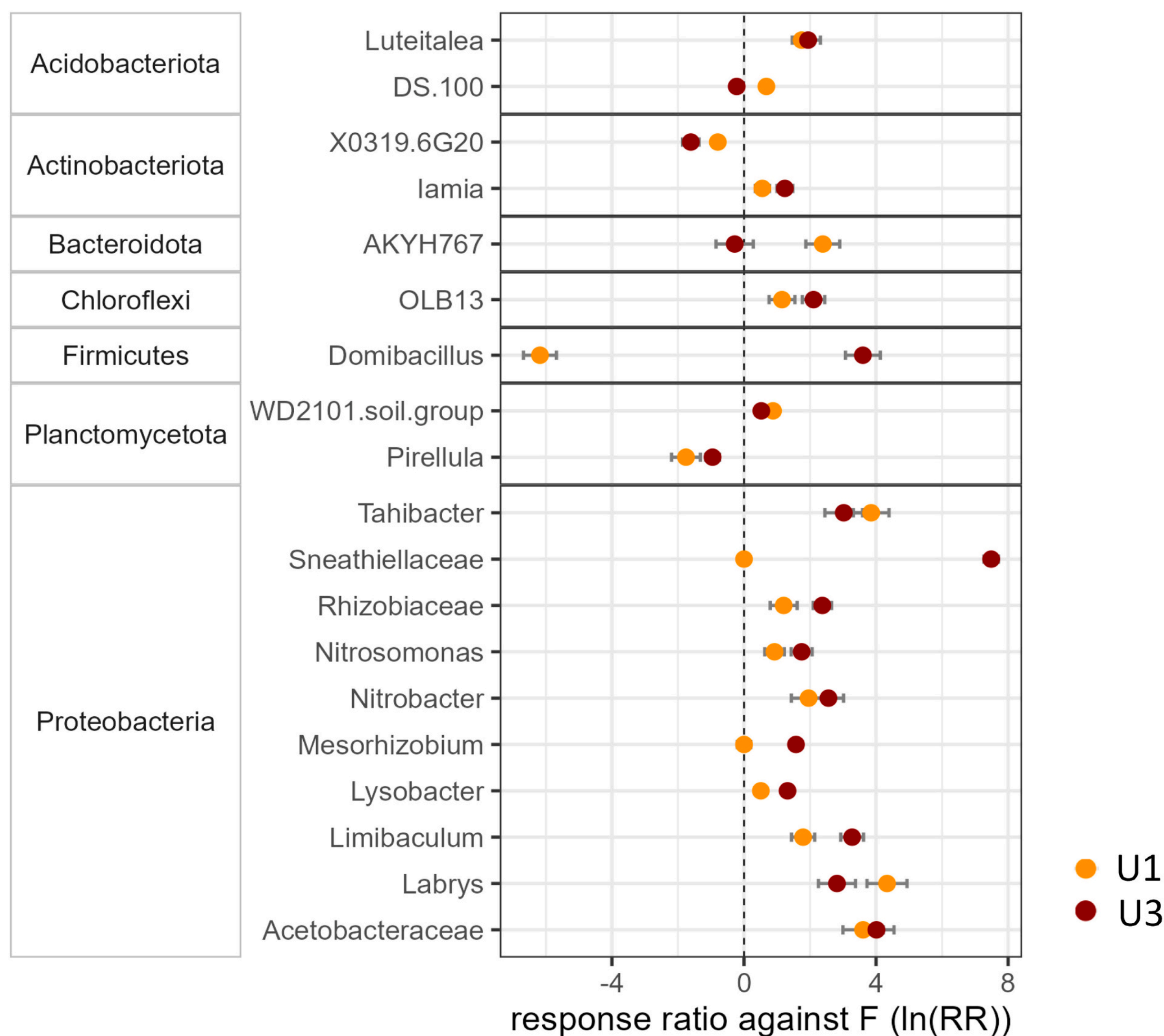

Fig. 4. Response ratio (lnRR, Eq. 1) showing the 19 taxa with significant differences between fertilization treatments (Kruskal Wallis, $p < 0.05$). The response ratio was computed for the two urine treatments relative to the synthetic fertilizer treatment (F).

increased soil conductivity and soil mineral nitrogen content (Rumeau et al., 2023). Despite these changes, the soil bacterial community structure was similarly resistant to urine as it was to synthetic fertilizer even under excessive dose of urine application that only showed a slight and non-significant divergence in community structure. Furthermore, α and β-diversity indices were not significantly altered by the addition of urine and only around 3 % of the bacterial taxa showed significant differences in their abundances between treatments. Together these results indicate that the overall diversity and composition of the bacterial community remained largely unaffected after the addition of urine. Our findings align with previous studies investigating synthetic urine and lime-treated urine, which also reported no significant shifts in soil bacterial communities (Nunan et al., 2006; Roy et al., 2022). However, these studies did detect minor changes in community structure which could have been hindered in our study by the divergence in bacterial composition between blocks, introducing variability between replicates. This block effect observed at T0 could have arisen from slight differences in plant growth performance and/or irrigation intensity between blocks in previous experiments. This effect persisted until the end of our study, with the higher performance of plants in blocks 1 and 8 amplifying or sustaining this initial block effect. Nevertheless, our study is in line with previous studies reporting that pH modulation after urine application was the main driver in bacterial composition changes (Rooney et al., 2006; Singh et al., 2009). Yet, on our calcareous soils, pH only decreased by 0.2 units under excessive urine application (U3) potentially explaining the slight divergence in community structure in this treatment compared to the normal dose of urine (U1). pH modulation was less pronounced compared to experiments on acidic soils (Neina, 2013) due to carbonates' pH buffering properties (Luo et al., 2015), potentially limiting bacterial community changes following urine application.

4.3. Effects of urine on nutrient cycling and bacteria involved in salt tolerance

Urine fertilization primarily influenced the abundance of taxa potentially associated with nutrient cycling and more especially those involved in N cycling. Among the 3 % of the taxa impacted by fertilization, around 30 % shelter species associated with N cycling. By providing N solely as ammonium, urine significantly increased the relative abundance of nitrifiers (*Nitrosomonas*, *Nitrobacter, Nitrospirota*) (Gee et al., 1990) and the predicted nitrification-related genes (*hao, amoA*) with a stronger effect under a high dose, compared to a synthetic fertilizer (NH_4NO_3) and reduced the predicted urease-related genes (*ureA, ureB, ureC*). Higher nitrification activity agrees with previous studies (Mahmood and Prosser, 2006; Orwin et al., 2010) and with the

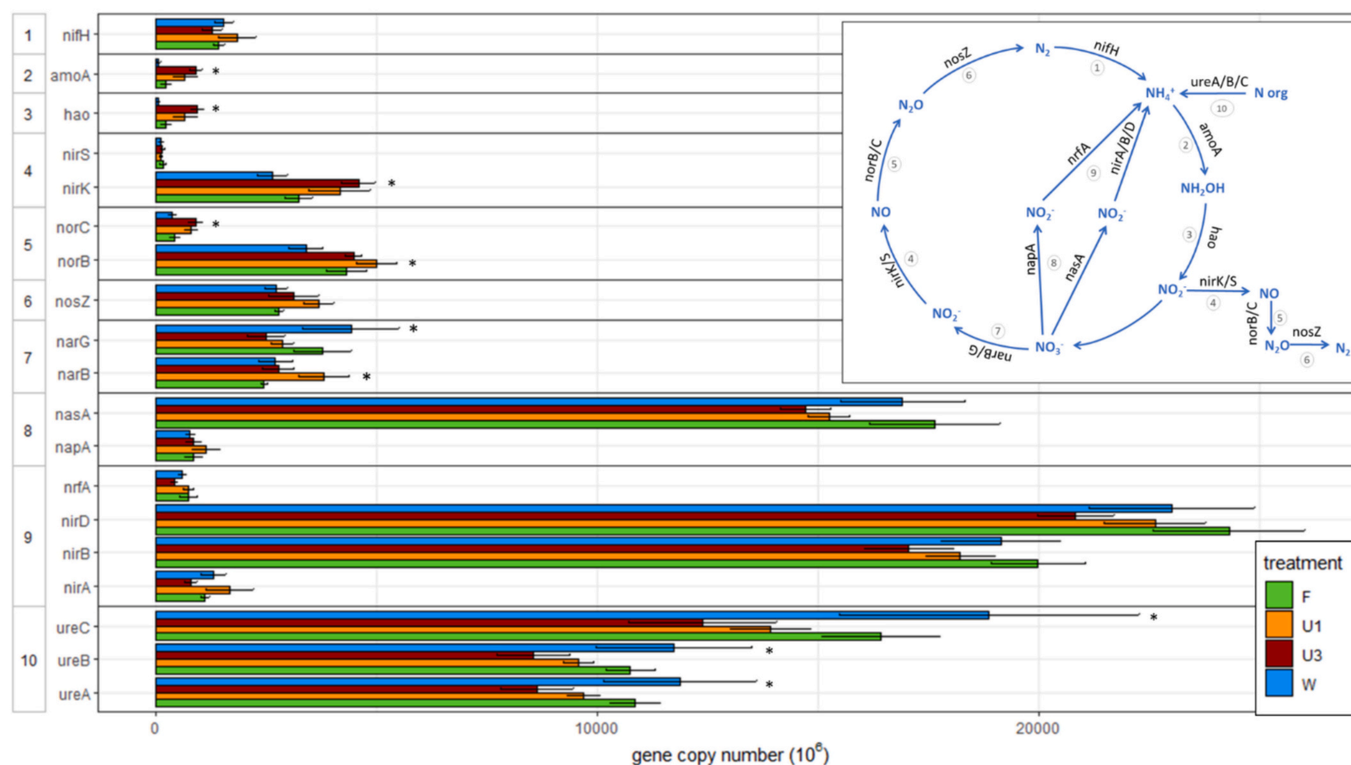

Fig. 5. Nitrogen cycling gene abundance prediction from 16S RNA gene metabarcoding by the PICRUST2 pipeline. All genes are classified by reactions from 1 to 10 (i.e. refer to the N cycle). Asterisks indicate a significant difference between treatments ($P < 0.05$) (nar: nitrate reductase, nir: nitrite reductase, nor: nitric oxide reductase, nas: assimilatory nitrate reductase, nos: nitrous oxide reductase, nrf: membrane-bound formate dependent nitrite reductase, nap: periplasmic nitrate reductase, nif: nitrogenase, hao: hydroxalamine oxidoreductase, amo: ammonia mono-oxygenase, ure: urease).

rapid nitrification (within ten days) observed in this experiment (Rumeau et al., 2023).

Yet, both urine treatments increased to a similar extent the abundance of genes involved in nitrite and nitric oxide reduction (*norB, norC* and *nirK*) (Coskun et al., 2017) compared to the synthetic fertilizer (NH_4NO_3) contradicting our assumption that synthetic fertilizer, by providing nitrate, would lead to higher N_2O emissions. This was further supported by the higher abundance of *Acetobacteraceae*, *Pseudomonadaceae* and *Rhizobiales,* some members of which were originally known as N fixers (Peix et al., 2015; Reis and Teixeira, 2015; Wolińska et al., 2017) and more recently recognized for their nitrite reduction abilities (abundant groups of *nir*K or *nir*S denitrifying communities) in the absence of symbiotic plants (Fang et al., 2021; Li et al., 2019). High nitrification activity in urine treatments might have led to a transient build-up of nitrite (Clough et al., 2003; Orwin et al., 2010) leading to the production of NO and N_2O. In contrast, in the synthetic fertilizer treatment, the applied nitrate was probably taken up by plants, reducing potential N_2O losses (Rumeau et al., 2023). This is consistent with experiments on animal urine patches which commonly report high N_2O losses from urine patches (Clough et al., 2020; Williams et al., 1998). Interestingly, while nitrification related genes were more abundant in U3 compared to U1, NO and N_2O production related genes were similarly present in both treatments. This suggests that NO and/or N_2O losses may not be proportional to the application rate of urine. A similar observation was reported when testing various concentrations of cattle urine on N_2O emissions (De Klein et al., 2014) indicating that N_2O losses may rather depend on other factors such as soil moisture and C availability (Peng et al., 2011). Consequently, by promoting nitrification, urine fertilization potentially leads to higher emissions of NO and N_2O than ammonium nitrate fertilizer. This emphasizes the need for further research into assessing N_2O emissions from human urine in comparison with similar ammonium-based fertilizers, as well as exploring potential mitigation strategies.

In contrast, urine fertilization had a limited impact on predicted genes associated with P cycling, affecting only a small fraction (13 %) of the identified P cycling genes. Nevertheless, the higher abundance of phytase-producing bacteria (*appA*) and phosphatase producing bacteria (*phoN* and *phoA*) suggests a potential rise in organic P mineralization (derived from urine or soil) thereby increasing P bioavailability (Liu et al., 2022).

Despite the high level of electrical conductivity in the U3 treatment (Rumeau et al., 2023), the bacterial community did not exhibit signs of salinity stress as bacterial diversity was not reduced. This contrasts with expectations in a saline soil, where a reduction in bacterial diversity is anticipated (Zhang et al., 2019; Zhao et al., 2020). Furthermore, no response was observed in the predicted salinity-related genes mitigating salts' impact. None of the most common salt-tolerant bacteria genera (i. e. *Bacillus*, *Pseudomonas, Novosphingobium, Moraxella, Vibrio,* etc.*)* (Sharma et al., 2015; Van Gerrewey et al., 2021; Vives-Peris et al., 2018) responded to treatments further supporting this observation. Nevertheless, four taxa which exhibited higher abundances in the urine treatments, could shelter moderately halotolerant species: *Sneathiella* (marine bacteria) (Austin, 2014) *Limibaculum halophilum* (2 % NaCl) (Shin et al., 2017), *Lysobacter aestuarii* (0–7 % NaCl) (Jeong et al., 2016) and *Mesorhizobium* (0–3 % NaCl) (Laranjo and Oliveira, 2011). These findings suggest that urine salt content may selectively favour a small number of halotolerant bacteria without impeding the survival of non-halotolerant bacteria. However, it is important to note that long-term fertilization with urine may potentially build-up soil salinity and thus alter soil bacterial community structure more significantly, as it can be observed under long-term fertilization trials with mineral fertilizers (Shen et al., 2016). Therefore, it is important to consider the potential long-term effects of urine fertilization on soil salinization.

5. Conclusion

This study provides evidence that soil bacterial communities resist urine fertilization as effectively as they do synthetic fertilization, even under high application doses of urine that affect soil pH and salinity. A soil legacy effect and crop growth were found to have a greater impact on the community structure than fertilization itself. However, urine fertilization increased the relative abundance of nitrifying and, denitrifying groups compared to a synthetic fertilizer, implying that more N oxide gases could be emitted when fertilizing with urine. This should, however, be confirmed through direct gas flux measurements. Overall, this experiment indicates that stored urine can be safely applied to a plant-soil system without negatively impacting the soil microbiome. This study encourages further research on the long-term effects of urine fertilization, specifically addressing nitrous gas production and salinity accumulation and its subsequent effects on both the soil microbiome and plant physiology.

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Ethical approval

Not applicable.

Consent to participate

All authors gave their consent to participate in the conception of this paper.

Consent to publish

All authors read and approved the final manuscript.

CRediT authorship contribution statement

Manon Rumeau: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Chiara Pistocchi:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Nassim Ait-Mouheb:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Claire Marsden:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Brigitte Brunel:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization.

Declaration of competing interest

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

Data availability

Raw sequencing data has been deposited in NCBI's Sequence Read Archive (SRA) under the BioProject PRJNA1081346 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1081346). Other datasets are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2024.105471.

References

Akpan-Idiok, A.U., Udo, I.A., Braide, E.I., 2012. The use of human urine as an organic fertilizer in the production of okra (Abelmoschus esculentus) in South Eastern Nigeria. Resour. Conserv. Recycl. 62, 14–20. https://doi.org/10.1016/j.resconrec.2012.02.003.

Albright, M.B.N., Timalsina, B., Martiny, J.B.H., Dunbar, J., 2019. Comparative genomics of nitrogen cycling pathways in Bacteria and Archaea. Microb. Ecol. 77, 597–606. https://doi.org/10.1007/s00248-018-1239-4.

Andersson, E., 2015. Turning waste into value: using human urine to enrich soils for sustainable food production in Uganda. J. Clean. Prod. 96, 290–298. https://doi.org/10.1016/j.jclepro.2014.01.070.

Austin, B., 2014. The family Sneathiellaceae. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes. Springer, Berlin Heidelberg, Berlin, Heidelberg, pp. 637–639. https://doi.org/10.1007/978-3-642-30197-1_262.

Backer, R., Rokem, J.S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., Subramanian, S., Smith, D.L., 2018. Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Front. Plant Sci. 9, 1473. https://doi.org/10.3389/fpls.2018.01473.

Bolan, N.S., Hedley, M.J., White, R.E., 1991. Processes of soil acidification during nitrogen cycling with emphasis on legume based pastures. Plant and Soil 134, 53–63. https://doi.org/10.1007/BF00010717.

Chandran, A., Pradhan, S.K., Heinonen-Tanski, H., 2009. Survival of enteric bacteria and coliphage MS2 in pure human urine. J. Appl. Microbiol. 107, 1651–1657. https://doi.org/10.1111/j.1365-2672.2009.04353.x.

Clough, T.J., Sherlock, R.R., Mautner, M.N., Milligan, D.B., Wilson, P.F., Freeman, C.G., McEwan, M.J., 2003. Emission of nitrogen oxides and ammonia from varying rates of applied synthetic urine and correlations with soil chemistry. Soil Res. 41, 421. https://doi.org/10.1071/SR02105.

Clough, T.J., Cardenas, L.M., Friedl, J., Wolf, B., 2020. Nitrous oxide emissions from ruminant urine: science and mitigation for intensively managed perennial pastures. Curr. Opin. Environ. Sustain. 47, 21–27. https://doi.org/10.1016/j.cosust.2020.07.001.

Coskun, D., Britto, D.T., Shi, W., Kronzucker, H.J., 2017. How plant root exudates shape the nitrogen cycle. Trends Plant Sci. 22, 661–673. https://doi.org/10.1016/j.tplants.2017.05.004.

Csárdi, G., Nepusz, T., Müller, K., Horvát, S., Traag, V., Zanini, F., Noom, D., 2023. igraph for R: R Interface of the igraph Library for Graph Theory and Network Analysis. https://doi.org/10.5281/ZENODO.7682609.

De Klein, C.A.M., Luo, J., Woodward, K.B., Styles, T., Wise, B., Lindsey, S., Cox, N., 2014. The effect of nitrogen concentration in synthetic cattle urine on nitrous oxide emissions. Agric. Ecosyst. Environ. 188, 85–92. https://doi.org/10.1016/j.agee.2014.02.020.

Delaporte-Quintana, P., Lovaisa, N.C., Rapisarda, V.A., Pedraza, R.O., 2020. The plant growth promoting bacteria Gluconacetobacter diazotrophicus and Azospirillum brasilense contribute to the iron nutrition of strawberry plants through siderophores production. Plant Growth Regul. 91, 185–199. https://doi.org/10.1007/s10725-020-00598-0.

Dequiedt, S., Saby, N.P.A., Lelievre, M., Jolivet, C., Thioulouse, J., Toutain, B., Arrouays, D., Bispo, A., Lemanceau, P., Ranjard, L., 2011. Biogeographical patterns of soil molecular microbial biomass as influenced by soil characteristics and management: biogeography of soil microbial biomass. Glob. Ecol. Biogeogr. 20, 641–652. https://doi.org/10.1111/j.1466-8238.2010.00628.x.

Devane, M.L., Taylor, W., Dupont, P.-Y., Armstrong, B., Weaver, L., Gilpin, B.J., 2023. Exploring the bacterial Community in Aged Fecal Sources from dairy cows: impacts on fecal source tracking. Microorganisms 11, 1161. https://doi.org/10.3390/microorganisms11051161.

Dunn, O.J., 1964. Multiple comparisons using rank sums. Technometrics 6, 241–252. https://doi.org/10.1080/00401706.1964.10490181.

Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., Maman, S., Hernandez-Raquet, G., Combes, S., Pascal, G., 2018. FROGS: find, rapidly, OTUs with galaxy solution. Bioinformatics 34, 1287–1294. https://doi.org/10.1093/bioinformatics/btx791.

Fang, X., Zheng, R., Guo, X., Fu, Q., Fan, F., Liu, S., 2021. Yak excreta-induced changes in soil microbial communities increased the denitrification rate of marsh soil under warming conditions. Appl. Soil Ecol. 165, 103935 https://doi.org/10.1016/j.apsoil.2021.103935.

Frerichs, C., Key, G., Broll, G., Daum, D., 2022. Nitrogen fertilization strategies to reduce the risk of nitrate leaching in open field cultivation of spinach (*Spinacia oleracea* L.) #. J. Plant Nutr. Soil Sci. 185, 264–281. https://doi.org/10.1002/jpln.202100275.
Gee, C.S., Pfeffer, J.T., Suidan, M.T., 1990. *Nitrosomonas and Nitrobacter* interactions in biological nitrification. J. Environ. Eng. 116, 4–17. https://doi.org/10.1061/(ASCE)0733-9372(1990)116:1(4).
Goetsch, H.E., Love, N.G., Wigginton, K.R., 2020. Fate of extracellular DNA in the production of fertilizers from source-separated urine. Environ. Sci. Technol. 54, 1808–1815. https://doi.org/10.1021/acs.est.9b04263.
Grunert, O., Robles-Aguilar, A.A., Hernandez-Sanabria, E., Schrey, S.D., Reheul, D., Van Labeke, M.-C., Vlaeminck, S.E., Vandekerckhove, T.G.L., Mysara, M., Monsieurs, P., Temperton, V.M., Boon, N., Jablonowski, N.D., 2019. Tomato plants rather than fertilizers drive microbial community structure in horticultural growing media. Sci. Rep. 9, 9561. https://doi.org/10.1038/s41598-019-45290-0.
Hartmann, A.A., Barnard, R.L., Marhan, S., Niklaus, P.A., 2013. Effects of drought and N-fertilization on N cycling in two grassland soils. Oecologia 171, 705–717. https://doi.org/10.1007/s00442-012-2578-3.
Höglund, C., Stenström, T.A., Jönsson, H., Sundin, A., 1998. Evaluation of faecal contamination and microbial die-off in urine separating sewage systems. In: Water Sci. Technol. Water Quality International '98, 38, pp. 17–25. https://doi.org/10.1016/S0273-1223(98)00563-0.
Jeong, S.E., Lee, H.J., Jeon, C.O., 2016. Lysobacter aestuarii sp. nov., isolated from estuary sediment. Int. J. Syst. Evol. Microbiol. 66, 1346–1351. https://doi.org/10.1099/ijsem.0.000884.
Jost, L., 2006. Entropy and diversity. Oikos 113, 363–375. https://doi.org/10.1111/j.2006.0030-1299.14714.x.
Karak, T., Bhattacharyya, P., 2011. Human urine as a source of alternative natural fertilizer in agriculture: a flight of fancy or an achievable reality. Resour. Conserv. Recycl. 55, 400–408. https://doi.org/10.1016/j.resconrec.2010.12.008.
Karimi, B., Terrat, S., Dequiedt, S., Saby, N.P.A., Horrigue, W., Lelièvre, M., Nowak, V., Jolivet, C., Arrouays, D., Wincker, P., Cruaud, C., Bispo, A., Maron, P.-A., Bouré, N.C. P., Ranjard, L., 2018. Biogeography of soil bacteria and archaea across France. Sci. Adv. 4, eaat1808 https://doi.org/10.1126/sciadv.aat1808.
Khmelevtsova, L.E., Sazykin, I.S., Azhogina, T.N., Sazykina, M.A., 2022. Influence of agricultural practices on bacterial community of cultivated soils. Agriculture 12, 371. https://doi.org/10.3390/agriculture12030371.
Kim, K., Samaddar, S., Chatterjee, P., Krishnamoorthy, R., Jeon, S., Sa, T., 2019. Structural and functional responses of microbial community with respect to salinity levels in a coastal reclamation land. Appl. Soil Ecol. 137, 96–105. https://doi.org/10.1016/j.apsoil.2019.02.011.
Kolaczyk, E.D. (Ed.), 2009. Statistical Analysis of Network Data: Methods and Models, Springer Series in Statistics. Springer, New York, New York, NY. https://doi.org/10.1007/978-0-387-88146-1.
Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47, 583–621. https://doi.org/10.1080/01621459.1952.10483441.
Kutu, F.R., Muchaonyerwa, P., Mnkeni, P.N., 2011. Complementary nutrient effects of separately collected human faeces and urine on the yield and nutrient uptake of spinach (*Spinacia oleracea*). Waste Manag. Res. J. Sustain. Circ. Econ. 29, 532–539. https://doi.org/10.1177/0734242X10374900.
Lahr, R.H., Goetsch, H.E., Haig, S.J., Noe-Hays, A., Love, N.G., Aga, D.S., Bott, C.B., Foxman, B., Jimenez, J., Luo, T., Nace, K., Ramadugu, K., Wigginton, K.R., 2016. Urine bacterial community convergence through fertilizer production: storage, pasteurization, and struvite precipitation. Environ. Sci. Technol. 50, 11619–11626. https://doi.org/10.1021/acs.est.6b02094.
Langdale, G.W., Thomas, J.R., Littleton, T.G., 1971. Influence of soil salinity and nitrogen fertilizer on spinach growth. J. Rio Grande Valley Hort. Soc. 25, 61–66.
Laranjo, M., Oliveira, S., 2011. Tolerance of Mesorhizobium type strains to different environmental stresses. Antonie Van Leeuwenhoek 99, 651–662. https://doi.org/10.1007/s10482-010-9539-9.
Lee, K.-E., Kim, J.-K., Han, S.-K., Lee, D.Y., Lee, H.-J., Yim, S.-V., Kim, D.-H., 2020. The extracellular vesicle of gut microbial Paenalcaligenes hominis is a risk factor for vagus nerve-mediated cognitive impairment. Microbiome 8, 107. https://doi.org/10.1186/s40168-020-00881-2.
Lei, S., Xu, X., Cheng, Z., Xiong, J., Ma, R., Zhang, L., Yang, X., Zhu, Y., Zhang, B., Tian, B., 2019. Analysis of the community composition and bacterial diversity of the rhizosphere microbiome across different plant taxa. MicrobiologyOpen 8. https://doi.org/10.1002/mbo3.762.
Li, H., Zhang, Y., Wang, T., Feng, S., Ren, Q., Cui, Z., Cao, C., 2019. Responses of soil denitrifying bacterial communities carrying nirS, nirK, and nosZ genes to revegetation of moving sand dunes. Ecol. Indic. 107, 105541 https://doi.org/10.1016/j.ecolind.2019.105541.
Lienert, J., Larsen, T.A., 2010. High acceptance of urine source separation in seven European countries: a review. Environ. Sci. Technol. 44, 556–566. https://doi.org/10.1021/es9028765.
Liu, J., Cade-Menun, B.J., Yang, J., Hu, Y., Liu, C.W., Tremblay, J., LaForge, K., Schellenberg, M., Hamel, C., Bainard, L.D., 2018. Long-term land use affects phosphorus speciation and the composition of phosphorus cycling genes in agricultural soils. Front. Microbiol. 9, 1643. https://doi.org/10.3389/fmicb.2018.01643.
Liu, X., Han, R., Cao, Y., Turner, B.L., Ma, L.Q., 2022. Enhancing phytate availability in soils and phytate-p acquisition by plants: a review. Environ. Sci. Technol. 56, 9196–9219. https://doi.org/10.1021/acs.est.2c00099.
Lugtenberg, B., Kamilova, F., 2009. Plant-growth-promoting Rhizobacteria. Annu. Rev. Microbiol. 63, 541–556. https://doi.org/10.1146/annurev.micro.62.081307.162918.
Luo, W.T., Nelson, P.N., Li, M.-H., Cai, J.P., Zhang, Y.Y., Zhang, Y.G., Yang, S., Wang, R. Z., Wang, Z.W., Wu, Y.N., Han, X.G., Jiang, Y., 2015. Contrasting pH buffering patterns in neutral-alkaline soils along a 3600 km transect in northern China. Biogeosciences 12, 7047–7056. https://doi.org/10.5194/bg-12-7047-2015.
Mahé, F., Rognes, T., Quince, C., de Vargas, C., Dunthorn, M., 2015. Swarm v2: highly-scalable and high-resolution amplicon clustering. PeerJ 3, e1420. https://doi.org/10.7717/peerj.1420.
Mahmood, S., Prosser, J.I., 2006. The influence of synthetic sheep urine on ammonia oxidizing bacterial communities in grassland soil: analysis of ammonia oxidizing bacterial communities. FEMS Microbiol. Ecol. 56, 444–454. https://doi.org/10.1111/j.1574-6941.2006.00070.x.
Maynaud, G., Baudoin, E., Bourillon, J., Duponnois, R., Cleyet-Marel, J.-C., Brunel, B., 2019. Short-term effect of 915-MHz microwave treatments on soil physicochemical and biological properties. Eur. J. Soil Sci. 70, 443–453. https://doi.org/10.1111/ejss.12769.
McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217.
Neina, D., 2013. Short-term effects of human urine fertiliser and wood ash on soil pH and electrical conductivity 12.
Nunan, N., Singh, B., Reid, E., Ord, B., Papert, A., Squires, J., Prosser, J.I., Wheatley, R.E., McNicol, J., Millard, P., 2006. Sheep-urine-induced changes in soil microbial community structure: heterogeneity in upland grassland microbial community structure. FEMS Microbiol. Ecol. 56, 310–320. https://doi.org/10.1111/j.1574-6941.2006.00072.x.
Oren, A., Garrity, G.M., 2021. Valid publication of the names of forty-two phyla of prokaryotes. Int. J. Syst. Evol. Microbiol. 71 https://doi.org/10.1099/ijsem.0.005056.
Orwin, K.H., Wardle, D.A., 2004. New indices for quantifying the resistance and resilience of soil biota to exogenous disturbances. Soil Biol. Biochem. 36, 1907–1912. https://doi.org/10.1016/j.soilbio.2004.04.036.
Orwin, K.H., Bertram, J.E., Clough, T.J., Condron, L.M., Sherlock, R.R., O'Callaghan, M., Ray, J., Baird, D.B., 2010. Impact of bovine urine deposition on soil microbial activity, biomass, and community structure. Appl. Soil Ecol. 44, 89–100. https://doi.org/10.1016/j.apsoil.2009.10.004.
Pearce, M.M., Hilt, E.E., Rosenfeld, A.B., Zilliox, M.J., Thomas-White, K., Fok, C., Kliethermes, S., Schreckenberger, P.C., Brubaker, L., Gai, X., Wolfe, A.J., 2014. The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. mBio 5, e01283-14. https://doi.org/10.1128/mBio.01283-14.
Peix, A., Ramírez-Bahena, M.H., Velázquez, E., Bedmar, E.J., 2015. Bacterial associations with legumes. Crit. Rev. Plant Sci. 34, 17–42. https://doi.org/10.1080/07352689.2014.897899.
Peng, Q., Qi, Y., Dong, Y., Xiao, S., He, Y., 2011. Soil nitrous oxide emissions from a typical semiarid temperate steppe in inner Mongolia: effects of mineral nitrogen fertilizer levels and forms. Plant and Soil 342, 345–357. https://doi.org/10.1007/s11104-010-0699-1.
Peng, Z., Liang, C., Gao, M., Qiu, Y., Pan, Y., Gao, H., Liu, Y., Li, X., Wei, G., Jiao, S., 2022. The neglected role of micronutrients in predicting soil microbial structure. Npj Biofilms Microbiomes 8, 103. https://doi.org/10.1038/s41522-022-00363-3.
Poulsen, P.H.B., Magid, J., Luxhøi, J., de Neergaard, A., 2013. Effects of fertilization with urban and agricultural organic wastes in a field trial – waste imprint on soil microbial activity. Soil Biol. Biochem. 57, 794–802. https://doi.org/10.1016/j.soilbio.2012.02.031.
Pradhan, S.K., Holopainen, J.K., Heinonen-Tanski, H., 2009. Stored human urine supplemented with wood ash as fertilizer in tomato (Solanum lycopersicum) cultivation and its impacts on fruit yield and quality. J. Agric. Food Chem. 57, 7612–7617. https://doi.org/10.1021/jf9018917.
R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
Raza, S., Zamanian, K., Ullah, S., Kuzyakov, Y., Virto, I., Zhou, J., 2021. Inorganic carbon losses by soil acidification jeopardize global efforts on carbon sequestration and climate change mitigation. J. Clean. Prod. 315, 128036 https://doi.org/10.1016/j.jclepro.2021.128036.
Reis, V.M., Teixeira, K.R. dos S., 2015. Nitrogen fixing bacteria in the family *Acetobacteraceae* and their role in agriculture: nitrogen fixing Acetobacteria. J. Basic Microbiol. 55, 931–949. https://doi.org/10.1002/jobm.201400898.
Rooney, D., Kennedy, N., Deering, L., Gleeson, D., Clipson, N., 2006. Effect of sheep urine deposition on the bacterial community structure in an acidic upland grassland soil. Appl. Environ. Microbiol. 72, 7231–7237. https://doi.org/10.1128/AEM.00926-06.
Roy, M.A., Sharma, S., Braunius, K.P., Ajmani, A.M., Keyser, A.D., Butler, C.S., Reckhow, D.A., Dhankher, O.P., 2022. Lime-treated urine improves sunflower growth without shifting soil bacterial communities. Appl. Soil Ecol. 178, 104575 https://doi.org/10.1016/j.apsoil.2022.104575.
Rumeau, M., Marsden, C., Ait-Mouheb, N., Crevoisier, D., Pistocchi, C., 2023. Fate of nitrogen and phosphorus from source-separated human urine in a calcareous soil. Environ. Sci. Pollut. Res. 30, 65440–65454. https://doi.org/10.1007/s11356-023-26895-5.
Sangare, D., Sou/Dakoure, M., Hijikata, N., Lahmar, R., Yacouba, H., Coulibaly, L., Funamizu, N., 2015. Toilet compost and human urine used in agriculture: fertilizer value assessment and effect on cultivated soil properties. Environ. Technol. 36, 1291–1298. https://doi.org/10.1080/09593330.2014.984774.
Sharma, A., Singh, P., Kumar, S., Kashyap, P.L., Srivastava, A.K., Chakdar, H., Singh, R. N., Kaushik, R., Saxena, A.K., Sharma, A.K., 2015. Deciphering diversity of salt-tolerant bacilli from saline soils of eastern indo-gangetic plains of India. Geomicrobiol J. 32, 170–180. https://doi.org/10.1080/01490451.2014.938205.

Shen, W., Ni, Y., Gao, N., Bian, B., Zheng, S., Lin, X., Chu, H., 2016. Bacterial community composition is shaped by soil secondary salinization and acidification brought on by high nitrogen fertilization rates. Appl. Soil Ecol. 108, 76–83. https://doi.org/10.1016/j.apsoil.2016.08.005.

Shin, Y.H., Kim, J.-H., Suckhoom, A., Kantachote, D., Kim, W., 2017. Limibaculum halophilum gen. nov., sp. nov., a new member of the family Rhodobacteraceae. Int. J. Syst. Evol. Microbiol. 67, 3812–3818. https://doi.org/10.1099/ijsem.0.002200.

Simha, P., Barton, M.A., Perez-Mercado, L.F., McConville, J.R., Lalander, C., Magri, M.E., Dutta, S., Kabir, H., Selvakumar, A., Zhou, X., Martin, T., Kizos, T., Kataki, R., Gerchman, Y., Herscu-Kluska, R., Alrousan, D., Goh, E.G., Elenciuc, D., Głowacka, A., Korculanin, L., Tzeng, R.V., Ray, S.S., Niwagaba, C., Prouty, C., Mihelcic, J.R., Vinnerås, B., 2021. Willingness among food consumers to recycle human urine as crop fertiliser: evidence from a multinational survey. Sci. Total Environ. 765, 144438 https://doi.org/10.1016/j.scitotenv.2020.144438.

Singh, B.K., Nunan, N., Millard, P., 2009. Response of fungal, bacterial and ureolytic communities to synthetic sheep urine deposition in a grassland soil: perturbation of soil microbial community structure by urine. FEMS Microbiol. Ecol. 70, 109–117. https://doi.org/10.1111/j.1574-6941.2009.00731.x.

Snyder, S., Singh, P., Goldman, J., 2020. Emerging pathogens: a case of Wohlfahrtiimonas chitiniclastica and Ignatzschineria indica bacteremia. IDCases 19, e00723. https://doi.org/10.1016/j.idcr.2020.e00723.

Stone, B.W.G., Dijkstra, P., Finley, B.K., Fitzpatrick, R., Foley, M.M., Hayer, M., Hofmockel, K.S., Koch, B.J., Li, J., Liu, X.J.A., Martinez, A., Mau, R.L., Marks, J., Monsaint-Queeney, V., Morrissey, E.M., Propster, J., Pett-Ridge, J., Purcell, A.M., Schwartz, E., Hungate, B.A., 2023. Life history strategies among soil bacteria—dichotomy for few, continuum for many. ISME J. 17, 611–619. https://doi.org/10.1038/s41396-022-01354-0.

Van Gerrewey, T., El-Nakhel, C., De Pascale, S., De Paepe, J., Clauwaert, P., Kerckhof, F.-M., Boon, N., Geelen, D., 2021. Root-associated bacterial community shifts in hydroponic lettuce cultured with urine-derived fertilizer. Microorganisms 9, 1326. https://doi.org/10.3390/microorganisms9061326.

Vives-Peris, V., Gómez-Cadenas, A., Pérez-Clemente, R.M., 2018. Salt stress alleviation in citrus plants by plant growth-promoting rhizobacteria Pseudomonas putida and Novosphingobium sp. Plant Cell Rep. 37, 1557–1569. https://doi.org/10.1007/s00299-018-2328-z.

Wald, C., 2022. The urine revolution: how recycling pee could help to save the world. Nature 602, 202–206. https://doi.org/10.1038/d41586-022-00338-6.

Williams, P.H., Jarvis, S.C., Dixon, E., 1998. Emission of nitric oxide and nitrous oxide from soil under field and laboratory conditions. Soil Biol. Biochem. 30, 1885–1893. https://doi.org/10.1016/S0038-0717(98)00052-2.

Wolińska, A., Kuźniar, A., Zielenkiewicz, U., Banach, A., Izak, D., Stępniewska, Z., Błaszczyk, M., 2017. Metagenomic analysis of some potential nitrogen-fixing bacteria in arable soils at different formation processes. Microb. Ecol. 73, 162–176. https://doi.org/10.1007/s00248-016-0837-2.

World Health Security, 2006. Who guidelines for the safe use of wastewater, excreta and greywater - Policy and regulatory aspect.

Xu, K., Lu, J., Hu, L., Li, J., Cheng, S., Zheng, M., Wang, C., 2022. Pathogens inactivation in nutrient recovery from urine: a review. Front. Environ. Sci. 10, 1056019 https://doi.org/10.3389/fenvs.2022.1056019.

Zhang, K., Shi, Y., Cui, X., Yue, P., Li, K., Liu, X., Tripathi, B.M., Chu, H., 2019. Salinity is a key determinant for soil microbial communities in a desert ecosystem. mSystems 4, e00225-18. https://doi.org/10.1128/mSystems.00225-18.

Zhao, Q., Bai, J., Gao, Y., Zhao, H., Zhang, G., Cui, B., 2020. Shifts in the soil bacterial community along a salinity gradient in the Yellow River Delta. Land Degrad. Dev. 31, 2255–2267. https://doi.org/10.1002/ldr.3594.