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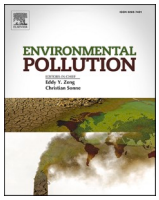

Long-term organic fertilization shields soil prokaryotes from metal stress while mineral fertilization exacerbates it☆

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ABSTRACT

Metal contamination in agricultural soils threatens prokaryote dynamics essential for soil health and crop productivity. Yet, whether fertilization in the long-run affects their resilience to metals remains unclear. This study examined the biogeochemical impacts of realistically low-dose applications of cadmium, zinc, and lead in soils subjected to 119 years of non-fertilization, mineral-fertilization (NPK), organic-fertilization (manure), or combined mineral-organic fertilization. Amended metals remained in the mobile fraction with the order: mineral < unfertilized < mineral + organic < organic, mirroring the effects on soil prokaryotes. In both unfertilized and mineral-fertilized soils, 16S rRNA gene copy numbers declined by 30 % upon metal addition, but recovery timing differed: in unfertilized soil, recovery began after three days, whereas in mineral-fertilized soil, numbers declined until day seven before recovering. This coincided with an increase in metal-resistant taxa, particularly in mineral-fertilized soil, with 10 significantly affected OTUs, and to a lesser extent in unfertilized soil, with 5 affected OTUs. Carbon-, nitrogen-, and phosphorus-mining enzyme activities increased 50–100 % in mineral-fertilized soils, suggesting enhanced nutrient acquisition to mitigate metal toxicity. In contrast, organic-fertilized soil hosted stable enzymatic activities and microbial copy numbers with minimal community shifts (1 affected OTU), indicating greater resistance to metal amendment. Combined mineral-organic fertilization stabilized copy numbers and enzymatic activity upon metal amendment, but 8 OTUs were affected, including specialized nutrient cyclers, suggesting increased availability of previously adsorbed NPK cations. Our findings indicate that organic fertilization shields prokaryotes from metal stress, while mineral fertilization exacerbates it, highlighting the benefits of organic practices for maintaining soil health and productivity.

1. Introduction

Fertilizers are applied to meet food demands, but at the same time they drastically manipulate ecosystems (Erisman et al., 2013; Stoate et al., 2009). Fertilizers are divided into mineral-based (inorganic salts) and carbon-based organic types. Many studies, including "Long-Term Experiments" (LTE) have examined their impacts on crop production, nutrient cycling, biota, and overall environment (Berti et al., 2016). Large-scale LTE field experiments lasting over 20 years, provide ecologically relevant insights into soils that have biogeochemically adapted to different treatments (Rasmussen et al., 1998). The Static Fertilization Experiment in Bad Lauchstädt (SFE), founded in 1902, is among the oldest LTEs investigating fertilizer impacts.

SFE and other LTEs demonstrate that organic-fertilized soils have higher soil pH, organic carbon (OC) content, nitrogen content, and biodiversity than mineral-fertilized soils (Diacono and Montemurro, 2011; Francioli et al., 2016; Luo et al., 2018). While chemical soil health indicators have long been established, biological indicators like

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prokaryotes are increasingly recognized as drivers of agroecosystem processes, including nutrient cycling, decomposing soil organic matter (SOM), defining soil structure and moisture, inhibiting plant diseases, and promoting plant productivity (Lehmann et al., 2020). These functions can be impaired when the stability of the soil prokaryotic community is disrupted, which can be assessed through prokaryotic resistance and resilience - resistance being the ability to withstand disturbance, and resilience the recovery rate (Shade et al., 2012).

One notable disturbance in agricultural soils is metals' omnipresence and fresh input (FAO & UNEP, 2021). Metals like cadmium (Cd), lead (Pb), and zinc (Zn) are found in mineral and organic fertilizers (Lopes et al., 2011; Nziguheba and Smolders, 2008), leading to consistent metal inputs into agricultural soils (Rodríguez-Eugenio et al., 2018). Cadmium and Pb have no established biological functions and cause toxicity even at environmental relevant concentrations (Tchounwou et al., 2012), disrupting the prokaryotic functioning of nucleic acids, proteins/enzymes, and cell membranes (Igiri et al., 2018). Although Zn is an essential micronutrient for prokaryotes, excess levels cause toxicity by preventing the uptake of other micronutrients, which ultimately inhibits prokaryotic growth (McDevitt et al., 2011).

Once in soil, metals interact with soil components through adsorption, precipitation, and complexation, reducing their mobility (Caporale and Violante, 2016; Park et al., 2011). As a result, only a fraction of total soil metals—those dissolved or loosely adsorbed—remain mobile and capable of interacting with soil biota (Kim et al., 2015). When exposed to toxic metals, prokaryotes employ defense mechanisms such as metal detoxification, efflux, and cellular repair to withstand metal toxicity (Lemire et al., 2013). These processes demand additional energy and nutrients, which can disrupt other physiological processes, such as cellular nutrient acquisition, respiration or reproduction, ultimately affecting soil health (Azarbad et al., 2016). Substantial metal stress can affect community composition, as less-adapted prokaryotes are replaced by more tolerant groups, changing the entire prokaryotic community (Li et al., 2017).

Despite studies on fertilization effects on prokaryotic communities and metal mobility, prokaryotic resistance and resilience to metals under different fertilization regimes remain underexplored. Here, we evaluated (i) changes in metal mobility and (ii) prokaryotic resistance and resilience upon metal amendment in differently fertilized soils. For that, we sampled soils from the SFE, in which the prokaryotic community and geochemistry have adapted over 119 years to different fertilization practices. Fertilization included mineral fertilization (application of NPK salts), organic fertilization (farmyard manure), a no-fertilization treatment, and a combination of both mineral and organic fertilization (Table S1). The study is conducted on agricultural chernozem soil, one of the most fertile and suitable soils for farming (Altermann et al., 2005), with toxic metal contents at the lower end of European soils (Table S2; European Environment Agency, 2022). These differently fertilized soils were amended with low amounts of Cd, Zn, and Pb, concentrations mimicking realistic scenarios for low-dose contamination events such as fertilizer application (Atafar et al., 2010; Latare et al., 2014; Wang et al., 2008). We hypothesized that long-term organic fertilization promotes greater prokaryotic resilience and resistance to metal inputs than mineral fertilization. We assume this is attributed to an increased metal-immobilization via higher pH, SOM complexation, and richer prokaryotic life in organic-fertilized soils. Our results identify which fertilization practices best stabilize prokaryotic communities against low-dose metal disturbances.

2. Material and methods

2.1. Study site and soil collection strategy

To investigate the impact of fertilization practices on the resilience of the soil prokaryotes to Cd, Zn, and Pb inputs, a soil that has undergone 119 years of various long-term fertilization treatments was used. This long period of consistent fertilization practices allowed the prokaryotes to adapt to the respective types of fertilization. The Haplic Chernozem (described in detail by Altermann et al. (2005)) was obtained from the SFE, located in Bad Lauchstädt, central Germany (11°53′E, 51°24′N), and has been running since 1902. According to the USDA, the soil is classified as silt loam. This long-term experiment investigates the effects of various organic and mineral fertilization methods on crop yield and quality and their impact on soil properties that determine fertility (a detailed description is provided by Blair et al. (2006)). The total experimental area of 4 ha was divided into eight stripes, of which the management on stripes 2, 3, 6, 7 have remained almost unchanged since 1902 (Merbach and Schulz, 2013). Each stripe was separated into 18 plots, each with a different fertilization treatment (Fig. S1A), thus, lacking randomization and being devoid of real biological replicates like many long-term experiments (Grosse et al., 2020). Crops were simultaneously cultivated in the four strips, following a yearly rotational sequence that comprises winter barley (*Hordeum vulgaris*), silage maize (*Zea mays*), and winter wheat (*Triticum aestivum*).

For this study, soils of four fertilization treatments were chosen: unfertilized, organic (farmyard manure), mineral (NPK), and organic + mineral (farmyard manure + NPK) (Table S1). The fertilizers and their respective application rates were provided in Table S1. Due to varying fertilizer application rates, total nutrient input differed by treatment. Therefore, mobile nutrient levels were additionally measured throughout the experiment. Stripe 2 was not sampled and included in our study due to a higher pH than the other three stripes. Considering that metal mobility highly depends on soil pH (Alloway, 2013), we assumed that this pH among replicates would lead to artifacts. Consequently, soils collected from stripes 3, 6, and 7 served as this study's replicates. Fresh soil samples were taken from the SFE in October 2021 before fertilizer application and seeding. Although there are no signs of soil carryover between the plots in the experiment, as a precaution only the central area of each selected plot was chosen to minimize the impacts of neighboring plots. This was achieved by taking three soil samples at 6.5 m intervals along a horizontal transect in the central area of each plot (Fig. S1B). The top 20 cm of soil was excavated with a hand shovel at each point, pooled by volume into one composite sample in a sterile container, air-dried, and sieved to 2 mm. Between samplings of different plots, equipment was cleaned in water and wiped with 80 % ethanol. Soil was stored in the dark at room temperature until the soil incubation proceeded.

2.2. Experimental setup

Adaptive biogeochemical processes in the soils were investigated at native and elevated concentrations of Cd, Zn, and Pb (Table S2). To implement the elevated Cd, Zn, Pb regime, half the soil of each fertilization treatment was amended with 0.2 ppm_w Cd, 33.3 ppm_w Zn and 4.8 ppm_w Pb. Notably, while referred to as "elevated", the concentrations of Cd, Zn, and Pb applied in this study are commonly found in European soils and lie within the lower range of European soils (European Environment Agency, 2022). We used these low amounts to study the prokaryotes' realistic response to contamination, avoiding extreme scenarios. Cd, Zn, and Pb were added in the form of analytical-grade chlorides into the soil ($CdCl_2 \bullet 2.5H_2O$ (Alfa Aeser, Germany); $ZnCl_2$ (Thermo Fisher Scientific, Germany); $PbCl_2$ (Alfa Aeser, Germany)). For the native metal regime, $CaCl_2$ ($CaCl_2 \bullet 2H_2O$ (Merck KGaA, Germany)) was added to balance ionic strength between the two metal regimes and account for adverse chlorine impacts on biota (Sahab et al., 2021). Amendments for both the native ($CaCl_2$) and elevated (Cd, Zn, Pb chlorides) metal regimes were prepared using tap water. The pH was adjusted to 6.1–6.2 using HCl followed by autoclaving of both amendments.

To perform the soil incubations, we modified the MicroResp™ system (The James Hutton Institute, 2024). Using a filling device (MicroResp™, Scotland), autoclaved 96-deep-well plates (Thermo Fisher

Scientific, Germany) were filled with soil (600 μL ≈ 0.85 g of soil in each well) following a block design scheme depicted in Fig. S2. Afterward, tap water containing the above-described amendments with $CaCl_2$ (native metal regime) or Cd, Zn, $PbCl_2$ (elevated metal regime) was irrigated to soils to obtain a 20 % gravimetric water content. The 20 % gravimetric water content was determined by weighing the empty plate, then the plate with dry soil. The amount of water needed to achieve 20 % gravimetric water content by weight was calculated based on the weight of dry soil. To reduce water loss and minimize water content fluctuations, plates were sealed with Breath-Easy seals (Sigma-Aldrich, Germany). Plates were kept in the dark at 28 °C for 24 days and rotated daily in the incubator to minimize incubator position biases in temperature and air draft. Plates were weight-checked daily to replenish evaporated water with autoclaved tap water, which was collected on a single day, autoclaved, and used for irrigation throughout the experiment. Three soil replicates were sampled on days 3, 7, and 24 of incubation by extracting all soil from the desired wells of the 96-deep-well plates using a sterile spatula and aliquoted for respective biogeochemical analyses described below. If an analysis required more than the amount from a single well, additional wells were sampled and thoroughly mixed before analysis. Additionally, on day 0 of the experiment, initial samples were prepared. For each treatment, 8 g of dry soil was mixed with 1.6 mL (20 % gravimetric water content) of above described amendments and aliquoted for respective biogeochemical analyses. The soil samples were flash-frozen in dry ice and stored at −20 °C for analyzing $CaCl_2$-extractable metals, organic carbon (OC) and total nitrogen (TN). For soil prokaryotes analysis soil samples were stored at −80 °C. For enzymatic activity assays, samples were stored at 4 °C for 24 h before processing. Electrical conductivity and pH were determined immediately after collection of the soil samples.

2.3. Soil geochemical analyses

Electrical conductivity and soil pH were measured in triplicate in a mixture of 1 g fresh soil and 5 mL ultra-pure water. Electrical conductivity was determined after 5 min in the settling solution at room temperature, and soil pH was determined after 24 h (SD335 Multi-Parameter Meter, Lovibond, Germany). Total soil metal contents were quantified in triplicates with aquaregia by extracting 0.5 g of 105°C-dried soil with 1.2 mL of 65 % nitric acid (HNO_3, analytical grade) and 3.7 mL of 37 % HCl (analytical grade) in a microwave digester (Mars6, CEM, USA) with a 15-min ramp phase to 15 min at 180 °C, followed by a 30 min cool-down phase (Melaku et al., 2005). Blanks and a reference soil standard (RTC SQC-001, Sigma Aldrich, Germany) were run simultaneously for quality checks on extraction effectiveness. Extracts were filtered through a paper filter (Whatman qualitative filter paper, Grade 1) and diluted in ultra-pure water for elemental contents quantification with inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7900, USA), using the He mode. Each sample was run with an ICP-MS internal standard mix (part number: 5188–6525, Agilent, USA) to trace internal drifts. Mobile (dissolved and loosely adsorbed) elements were approximated from 1.5 g of wet soil by a 15-min extraction with 10 mL of 0.01 M $CaCl_2$ at room temperature (Fang et al., 2021; Houba et al., 2000). Extracts were filtered through 0.22 μm filters (Minisart® high flow, Sartorius, Germany), which were pre-washed with ultra-pure water (to remove filter-associated carbon). Mobile metal and nutrient fractions were quantified by ICP-MS as described above. Moreover, $CaCl_2$-extractable OC and TN were quantified with a TOC analyzer (High TOC II, Elementar, Elementar Analysensysteme GmbH, Germany). Inorganic carbon was removed by acidifying the samples with 2 M HCl prior to analysis. Data is obtained from biological triplicates and normalized to the dry weight of the soil (105°C-dried soil).

2.4. Soil enzyme assays

The bulk activities of six hydrolytic soil enzymes were determined using a fluorometric assay based on the hydrolysis of 4-methylumbelliferone (4-MUB)-coupled substrates (German et al., 2011). 4-MUB-β-D-cellobioside, 4-MUB-β-D-glucoside, 4-MUB-sulfate, 4-MUB-N-acetyl-β-D-glucosaminide, 4-MUB-phosphate (Sigma Aldrich, Germany) and 4-MUB-β-D-xyloside (Merck KGaA, Germany) at saturation concentrations were used for analyzing activities of enzymes involved in the carbon (β-glucosidase, cellobiohydrolase, xylosidase), nitrogen (N-acetylglucosaminidase), sulfur (sulfatase), and phosphorus (phosphatase) cycles, respectively.

Substrates were combined with soil suspension (0.3 g of soil stored at 4 °C for 24 h mixed with 50 mL of 50 mM Tris buffer of pH = 5, followed by sonication for 5 min) in 96-well-microplates for fluorescence-based assays (Thermo Fisher Scientific, Germany) and incubated for 1 h at 28 °C. Each plate also included blanks for Tris buffer, soil suspension, and substrates, along with a calibration curve in the presence of buffer and a second calibration curve in the presence of soil suspension (referred to as the quenched standard curve). After the incubation, the enzyme reaction was terminated by adding 30 μL of 1 M NaOH per well. After 5 min, fluorescence was determined at extinction of 360 nm and emission of 465 nm (TECAN GENios Plus, TECAN Group AG, Germany). The bulk enzymatic activities were calculated according to German et al. (2011) and normalized to cellular enzymatic activities by dividing them by the 16S rRNA gene copy number, approximated for cell numbers, measured in the same sample (measured as described below).

2.5. Prokaryotic respiration

Prokaryotic respiration was measured on days 1, 2, 4, 8, 11, 17, 21, and 23 using the MicroResp™ assay. CO_2 emissions per g of dry soil were summed to calculate cumulative CO_2 over the incubation period. Details were presented in Supplementary Methods 1.

2.6. DNA extraction, quantitative PCR and 16S rRNA metagenome sequencing

To analyze bacterial and archaeal quantity and taxonomic diversity, DNA was extracted following Lueders et al. (2004), using 0.6 g of wet soil. Details of DNA quality control and quantification and follow-up microbiome analysis are presented in Supplementary Methods 2. Prokaryotic 16S rRNA gene copy numbers were quantified by qPCR and normalized to dry soil weight. Metagenome libraries were sequenced to reconstruct the 16S rRNA gene using the Mapping-Assisted Targeted-Assembly for Metagenomics pipeline. Due to low archaeal OTU counts, the analysis focused primarily on bacteria.

2.7. Data analyses, statistical evaluation and data visualization

We performed statistical analysis and plotting using R (R Core Team, 2021) and RStudio (RStudio Team, 2022). Two-way ANOVAs were conducted for each metal to assess the effects of fertilization and metal regime on $CaCl_2$-extractable metal concentrations averaged over incubation time. Log_2 transformations of metal concentrations were applied to address non-normal distributions and meet ANOVA assumptions. Post-hoc pairwise comparisons were performed using estimated marginal means (EMMs) from the emmeans package (Russell, 2021) with Tukey's adjustment for multiple comparisons. Compact letter displays were generated using package multcomp (Hothorn et al., 2008). Linear mixed-effects models using lme4 package (Bates et al., 2014) were used to account for repeated measures over time, treating replicate as a random effect. Additionally, to compare the immobilization capacity of amendment metals across soils with different fertilization practices, the contribution of $CaCl_2$-extractable metals from amendments was calculated as shown in Eq. (1). First, extractable metal concentrations from amendments were obtained by subtracting native soil metal concentrations from those in elevated samples. Second, the percentage of extractable metals from amendments was determined by normalizing to

the added metal concentrations and multiplying by 100 %:

$$\text{Mobile fraction}\,(\%) = \frac{c_{\text{elevated soil}}\left(\text{ng}\,g^{-1}_{\text{dry soil}}\right) - c_{\text{native soil}}\left(\text{ng}\,g^{-1}_{\text{dry soil}}\right)}{c_{\text{amendment}}\left(\text{ng}\,g^{-1}_{\text{dry soil}}\right)} \cdot 100\% \quad \text{Eq. 1}$$

Prokaryotic community composition was conducted separately. Linear models were fitted for each operational taxonomic unit (OTU) to assess differences based on metal regime at specific time points (Liu et al., 2022). Least-squares means were calculated, and p-values were adjusted using the false discovery rate (FDR). OTUs with p-values <0.1 were retained, allowing less stringent thresholds given the study's low metal concentrations.

A principal components analysis was performed to illustrate the relationships between soil prokaryotic and geochemical variables using a 95 % confidence interval for ellipses around treatment centroids. Parameters included prokaryotic number, cellular enzymatic activity, Shannon diversity, mobile metals, mobile organic carbon, and mobile nitrogen with averaged across timepoints during incubation, pH and electrical conductivity from the end of incubation, and cumulative respiration.

Data visualizations were created using the tidyverse (Wickham et al., 2019), ComplexHeatmap (Gu et al., 2016) and factoextra (Kassambara and Mundt, 2020) R packages.

3. Results

3.1. Changes in soil metal dynamics

The initial total Cd concentration in native soils ranged between 0.2 and 0.3 ppm_w across differently fertilized soils (Table S2), which increased due to Cd amendment by an average of 0.2 ppm_w. In unfertilized soils, the $CaCl_2$-extractable Cd was 1.7 ± 0.4 ng Cd•$g^{-1}_{dry\ soil}$ on average across incubation time, which increased 2.7-fold to 4.7 ± 0.8 ng Cd•$g^{-1}_{dry\ soil}$ upon Cd amendment (Fig. 1A, time-resolved data in Fig. S3). For mineral-fertilized soils, $CaCl_2$-extractable Cd increased 4.9 times from 2.9 ± 0.4 to 14.3 ± 3.7 ng Cd•$g^{-1}_{dry\ soil}$. Organic-fertilized soils had less extractable Cd with 0.8 ± 0.0 ng Cd•$g^{-1}_{dry\ soil}$, but this increased 3.4 times to 2.8 ± 0.1 ng Cd•$g^{-1}_{dry\ soil}$ under Cd amendment. For combined mineral and organic-fertilized soils $CaCl_2$-extractable Cd increased 3.0-fold from 1.0 ± 0.1 to 3.1 ± 0.5 ng Cd•$g^{-1}_{dry\ soil}$ upon Cd amendment.

The initial total Zn concentration in native soils ranged from 40.9 to 66.1 ppm_w (Table S2). The amendment of Zn increased this by an average of 33.3 ppm_w. In unfertilized soils, $CaCl_2$-extractable Zn was 96.5 ± 35.7 ng Zn•$g^{-1}_{dry\ soil}$, increasing 4.0-fold to 383.1 ± 111.4 ng Zn•$g^{-1}_{dry\ soil}$ under amended Zn (Fig. 1B, time-resolved data in Fig. S3). Mineral-fertilized soils saw a 26.3-fold increase from 59.6 ± 4.3 to 1564.7 ± 501.1 ng Zn•$g^{-1}_{dry\ soil}$ under Zn amendment, while organic-fertilized soils experienced a 2.1-fold increase from 95.1 ± 20.6 to 202.6 ± 7.3 ng Zn•$g^{-1}_{dry\ soil}$ in $CaCl_2$-extractable Zn following Zn amendment. Combined mineral and organic fertilization soils had a 4.3-fold increase from 48.6 ± 1.3 to 210.3 ± 52.1 ng Zn•$g^{-1}_{dry\ soil}$ under Zn amendment.

The initial total Pb concentration in native soils ranged from 21.6 to 29.9 ppm_w, with Pb amendment increasing this by an average of 4.8 ppm_w (Table S2). However, no significant differences in $CaCl_2$-extractable Pb were observed across fertilization regimes, with $CaCl_2$-extractable Pb remaining around 10 ng•$g^{-1}_{dry\ soil}$ for all treatments (Fig. 1C, time-resolved data in Fig. S3).

Soil pH and electrical conductivity differences were primarily attributed to fertilization practices rather than metal amendments (Table S3). Initial pH in the native metal regime followed this order: mineral-fertilized, unfertilized, combined mineral, and organic-fertilized, with the highest in organic-fertilized soils. By the experiment's end, pH decreased by approximately 0.1 units across all soils, with metals having no impact. Initial electrical conductivity increased in the following order: unfertilized, mineral-fertilized, organic-fertilized, combined mineral and organic-fertilized soils. By the experiment's end, conductivity rose significantly in all soils, with metals not affecting conductivity.

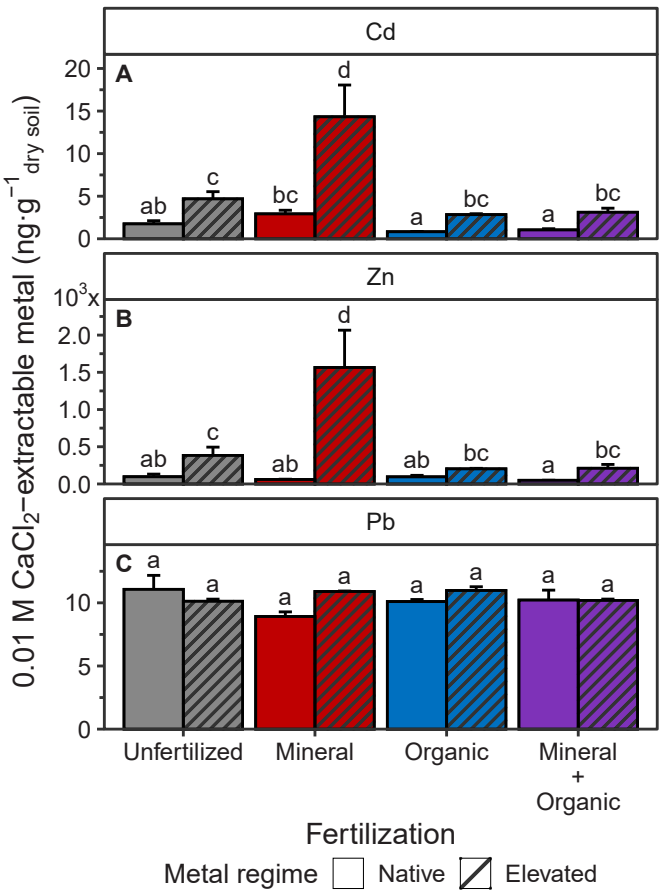

Fig. 1. Mobile metal fraction in soils with different historical fertilization practices under two metal regimes. Averaged 0.01 M $CaCl_2$-extractable Cd (top), Zn (middle), and Pb (bottom) were quantified across incubation 4 timepoints (days 0, 3, 7, to 24) in agricultural soils fertilized for 119 years under four practices: Unfertilized (grey), mineral (red), organic (blue), mineral + organic (purple) fertilization. Soils either contained their native metal contents (plain bars) or were exposed to elevated metal levels of +0.2 ppm_w Cd, +33.3 ppm_w Zn, +4.8 ppm_w Pb relative to background values (striped bars). Three biological replicates; mean values ± standard errors were compared with each other using 2-way ANOVA. Different lowercase letters denote groups with significantly different means within each metal (Tukey's HSD, α = 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Changes in soil nutrient cycling

Soil exoenzymatic activities were subtly but consistently affected by Cd, Zn, and Pb amendment across fertilization regimes at both bulk and cellular levels (Figure S4, Figure S5). To analyze specific prokaryotic responses to metal amendments, cellular rather than bulk enzymatic activities are discussed. Generally, cellular enzyme activities declined over time, except for the mineral-fertilized native soil, where it increased at the experiment's end by a single replicate (Fig. S5). Metal amendment did not alter general cellular activity patterns but influenced timing and extent. Cellular enzyme activities were highest in mineral-fertilized and unfertilized soils until day 7 before stabilizing, while they remained rather stable in both organic-fertilized soils. For simplicity of presentation, we focus on one example of each enzyme category in the main manuscript, with the C-degrading enzyme

xylosidase, the N-degrading enzyme N-acetylglucosaminidase, and the P-solubilizing enzyme phosphatase (Fig. 2); the other C-degrading enzymes depict similar trends and are presented in the supplement (Fig. S5).

Of all the tested carbon-cycling enzymes, xylosidase was most affected by metal amendment (Fig. 2A and B), with unfertilized and mineral-fertilized soils experiencing a 1.6 and 1.7-fold activity increase on day 3, respectively, while both organic-fertilized soils remained stable. Cellular N-acetylglucosaminidase activity in metal-amended soils was also most affected on day 3 (Fig. 2C and 2D), with the highest 1.4-fold activity increases in all fertilized soils, but the organic-fertilized soil. Cellular phosphatase activity rose the most in mineral-fertilized soil upon metal amendment (Fig. 2E and 2F), showing a 2.1-fold increase on day 3 compared to the native soil counterpart. Phosphatase in the unfertilized soil sank promptly after metal amendment, while both organic-fertilized soils responded barely.

Soil respiration was unaffected by long-term mineral fertilization but doubled in organic-fertilized and tripled in combined mineral and organic-fertilized soils compared to the unfertilized soil (Fig. S6). The amendment of metals consistently did not affect CO_2 emissions from soils. Mobile nutrient levels, including OC, TN, P, and K, were highest in both organic-fertilized soils but unaffected by metal amendment except for OC and TN, which increased in the mineral-fertilized soils until day 7 (Fig. S7).

3.3. Changes in soil prokaryotic community

Initial prokaryotic 16S rRNA gene copy numbers in unfertilized, mineral-fertilized, organic-fertilized, and combined mineral and organic-fertilized soils were $6.5 \pm 1.1 \times 10^8$, $1.3 \pm 0.2 \times 10^9$, $2.5 \pm 0.2 \times 10^9$, $2.1 \pm 0.4 \times 109$ $g^{-1}_{dry\ soil}$, respectively (Fig. 3). Gene copy numbers increased during incubation and stabilized to different degrees and time points. In unfertilized and mineral-fertilized soils, stabilization occurred by day 7 after a step-increase, while combined mineral and organic-fertilized soils stabilized by day 3 and organically-fertilized soils gradually increased. Final stable 16S rRNA gene copy numbers ranged between 3-5x10^9 $g^{-1}_{dry\ soil}$ across all fertilization regimes.

Metal amendment slowed and reduced the increase in 16S rRNA gene copy numbers, depending on the treatment. In unfertilized soils, a 0.7-fold decrease occurred by day 3 before stabilizing by day 7 (Fig. 3A). In mineral-fertilized soils, the largest difference was on day 7, with a 0.7-fold reduction in 16S rRNA gene copies (Fig. 3B). In organic-fertilized soils, metal amendment decreased 16S rRNA gene copy numbers 0.6-fold on day 2 followed by a 1.2-fold increase by day 7 (Fig. 3C). In organic mineral-fertilized soils, stable 16S rRNA gene copies were reached by day 3, with a 0.9-fold decrease compared to the native soil counterpart (Fig. 3D).

No clear patterns emerged in relative bacterial OTU abundance or beta diversity due to increased mobile metals (Fig. S8), though bacterial

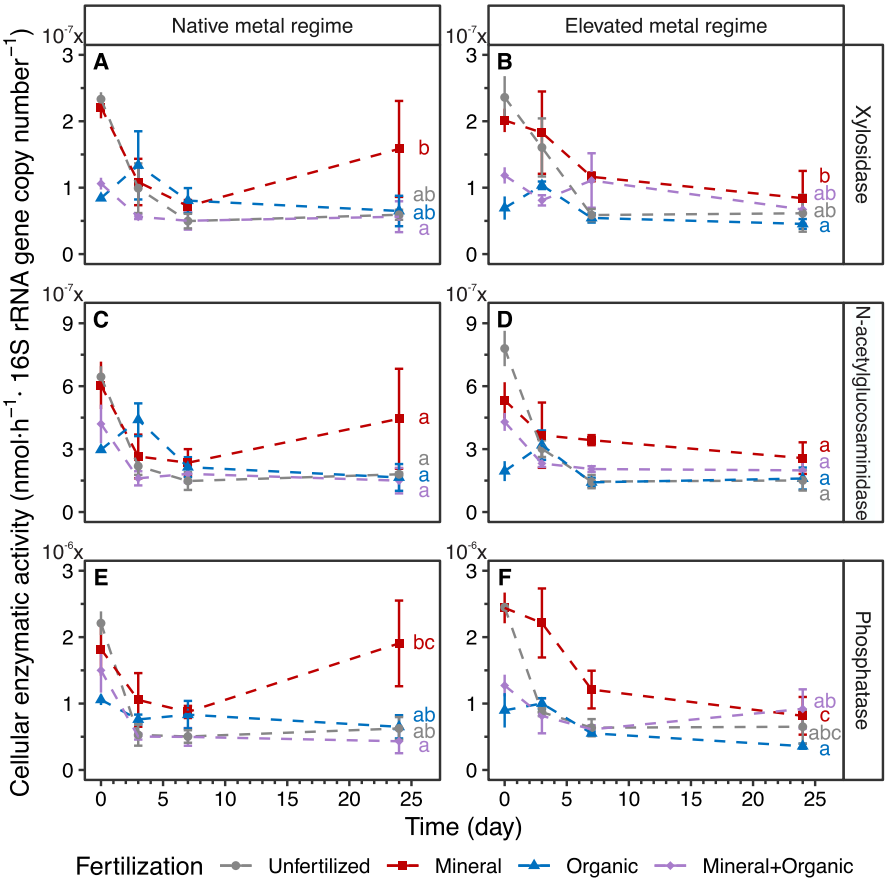

Fig. 2. Cellular enzymatic activity ratio in soils with different historical fertilization practices under two metal regimes. The activities of five exoenzymes were quantified in agricultural soils fertilized for 119 years under four practices: Unfertilized (grey), mineral (red), organic (blue), mineral + organic (purple). Soils either contained their native metal contents (left) or were exposed to elevated metal levels of +0.2 ppm_w Cd, +33.3 ppm_w Zn, +4.8 ppm_w Pb relative to background values (elevated, right). Cellular enzymatic activities are presented as the ratio of bulk enzymatic activities divided by 16S rRNA gene copy number. Three biological replicates, mean values ± standard errors were compared with each other using linear mixed model with repeated measures. Different lowercase letters denote groups with significantly different means within each metal regime (Tukey's HSD, $\alpha = 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

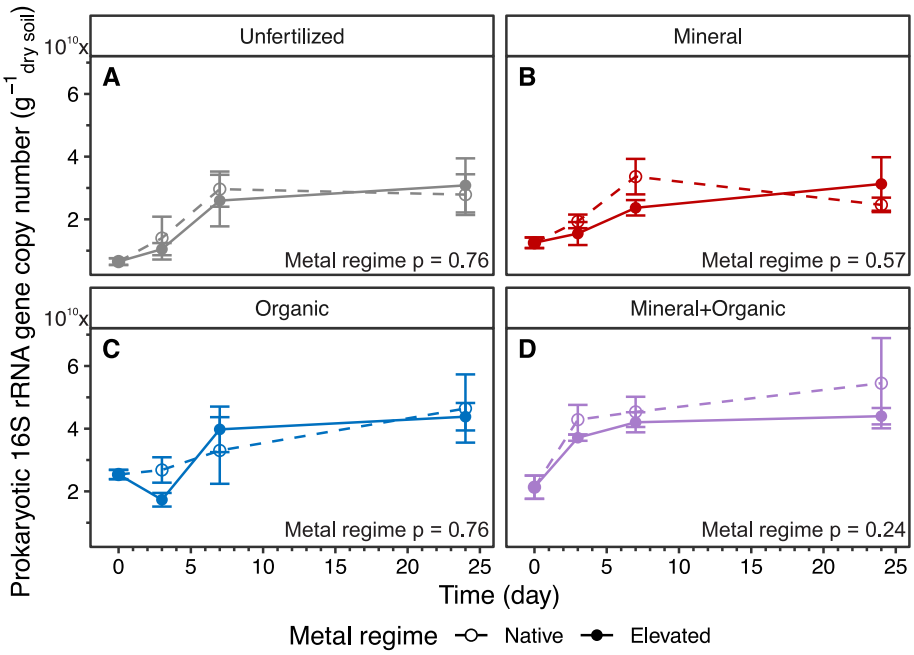

Fig. 3. Bacterial 16S rRNA gene copy numbers in soils with different historical fertilization practices under two metal regimes. Bacterial 16S rRNA gene copy numbers were quantified in agricultural soils fertilized 119 years under four practices: Unfertilized (grey), mineral (red), organic (blue), mineral + organic (purple). Soils either contained native metal contents (empty symbol with dash line) or were exposed to elevated metal levels of +0.2 ppm_w Cd, +33.3 ppm_w Zn, +4.8 ppm_w Pb relative to background (filled symbols with solid line). Three biological replicates, mean values ± standard errors were compared with each other using linear mixed model with repeated measures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

communities in mineral-fertilized and unfertilized soils diverged more than in both organic-fertilized soils.

To target the analysis of bacteria affected by metal amendment, we extracted prokaryotic OTUs that showed significant differences ($p < 0.1$) between native and elevated metal regimes within each fertilization practice from the total bacterial OTU pool, regardless of the direction of change. Most significantly different OTUs due to the metal amendment were found in mineral-fertilized (10), followed by the combined mineral and organic-fertilized (8), unfertilized (5), and organic-fertilized (1) soils (Fig. 4A). Taxonomic classification was performed at the family level and, when possible, at the genus level. All OTUs from unfertilized soils were classified at the family level. In contrast, only seven OTUs in mineral-fertilized and six OTUs in the combination of mineral and organic-fertilized soils were classified at this taxonomic level, with none in organic-fertilized soils (Fig. 4B). Metal amendment affected bacterial community composition most on day 7. Since the strongest shifts occurred at this time point, we examined the direction of change, finding increased genera abundance in mineral-fertilized soil (Fig. 4C) and decreased abundance in other regimes, with the smallest response in organic-fertilized soils. Organic-fertilized soils clustered closest to one another, followed by unfertilized soils, while mineral-fertilized soils were the most divergent from the others.

Linking prokaryotic community changes with geochemical dynamics in a PCA analysis (Fig. 5) revealed that unfertilized and mineral-fertilized soils clustered together but separated for native and elevated metal content, for which the latter correlated to mobile Cd and Zn. In both types of soil prokaryotic diversity correlated with cellular enzyme activity. Organic-fertilized and mineral + organic-fertilized soils clustered together independent of metal content. In these two fertilization regimes, prokaryotic numbers and respiration correlated with soil pH, electrical conductivity, and mobile organic carbon and nitrogen. Prokaryotic lag phase growth rate and mobile Pb barely contributed to biogeochemical properties in any soil.

4. Discussion

4.1. Fate of amended metals in soils under different long-term fertilization

In this study, we investigated the fate of realistic levels of Cd, Zn, and Pb amendments in agricultural soils that had been either not fertilized or fertilized with NPK, manure, or both for 119 years. To approximate the bioavailable fraction of these metals, we targeted the mobile fraction, specifically those extractable with 0.01 M $CaCl_2$ (Kim et al., 2015).

The unfertilized soils became nutrient-depleted over 119 years (Francioli et al., 2016), as indicated by the lowest initial electrical conductivity and concentrations of mobile nutrients (Table S3; Fig. S7). High initial cellular enzymatic activities suggested that prokaryotes actively mined for nutrients, likely degrading minerals and SOM over time, reducing metal-binding sites. Consequently, a portion of the added metals remained in the mobile fraction, making them accessible to soil prokaryotes. Approximately 1.5 % of Cd and 0.9 % of Zn of the total amended metals were mobile, with metal binding strength increasing in the order of Cd < Zn < Pb (calculated from Eq. (1), see methods). This trend reflects the metals' chemical properties, such as the first hydrolysis constant and ionic radius, which influence their stability in soils (Sastre et al., 2007; Usman, 2008).

Mineral fertilization over 119 years introduced inorganic salts (NH_4^+, NO_3^-, K^+, and PO_4^{3-}) into the soil (Francioli et al., 2016), increasing electrical conductivity compared to unfertilized soils. These salts provide readily available N, P, and K but lead to carbon limitation for soil prokaryotes, due to low mobile OC levels (Fig. S7; Hartmann and Six (2023) Prokaryotes responded by decomposing SOM, which is reflected in high initial carbon-cycling enzyme activities, likely degrading organic binding agents that stabilize soil structure (Bronick and Lal, 2005), and reducing metal-binding sites. As a result, metals had limited sites to bind and had to compete with fertilizer-derived NPK cations. The continuous amendment of NH_4^+ also may promoted prokaryotic nitrification (He et al., 2007), as evidenced by the presence of nitrifying bacteria *Nitrosomonadaceae* (Prosser et al., 2014), which could contribute to reduction in soil pH by approximately 0.5 units compared to unfertilized soil over

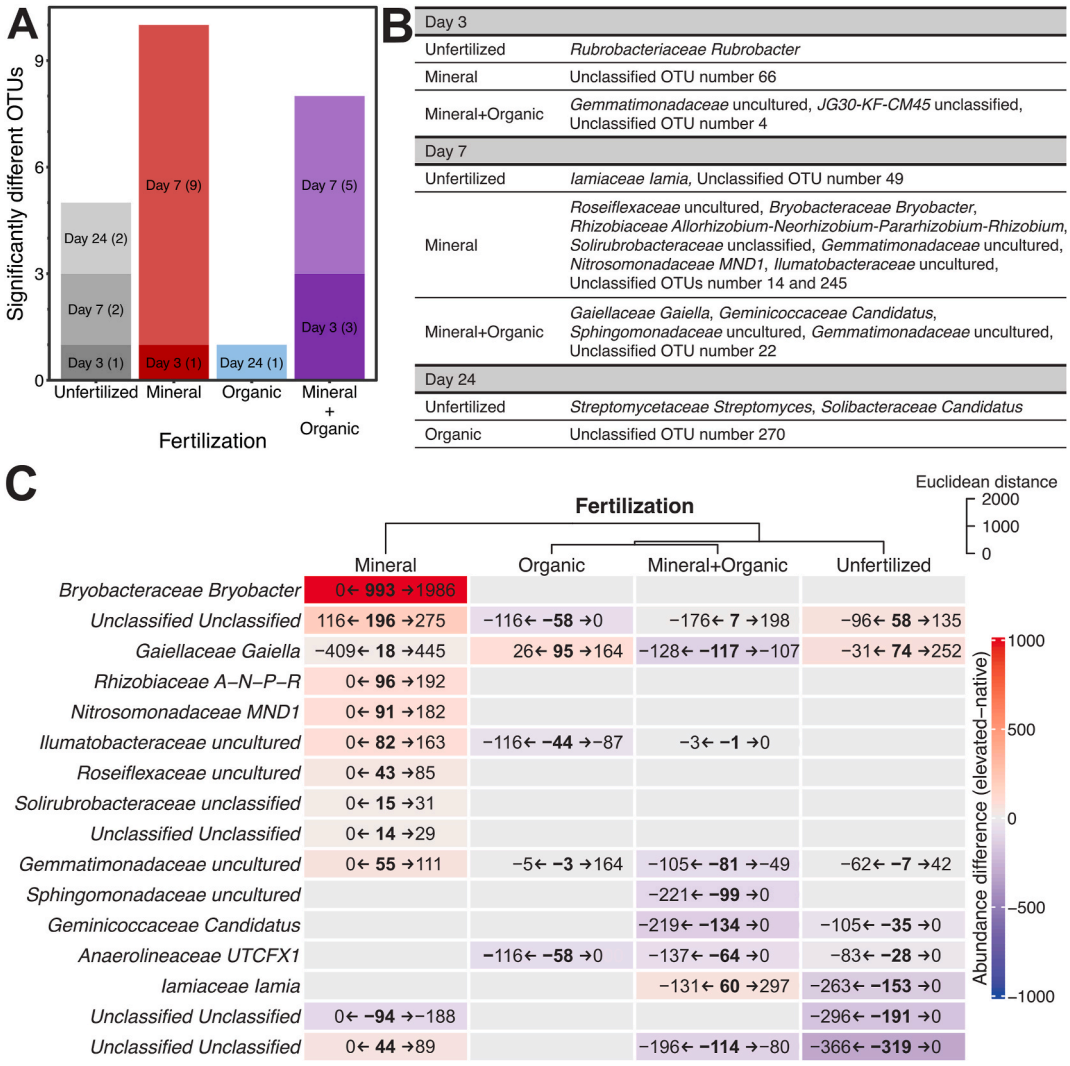

Fig. 4. Bacterial community composition in soils with different historical fertilization practices under two metal regimes. (A) Numbers of statistically different OTUs ($p < 0.1$) between native and elevated (+0.2 ppm_w Cd, +33.3 ppm_w Zn, +4.8 ppm_w Pb) metal regimes were quantified in agricultural soils fertilized for 119 years under four practices: Unfertilized (grey), mineral (red), organic (blue), and mineral + organic (purple) fertilization. The color gradient from darkest to brightest indicates the incubation's progression from days 3, 7, to 21. Values in brackets represent the number of statistically significant OTUs on a specific day. Three biological replicates were compared using a linear model. (B) Taxonomy (classified to Family and Genus levels) of statistically different OTUs between native and elevated metal regimes presented in A. (C) Abundance differences of statistically different OTUs between elevated and native metal soils within each fertilization regime on Day 7. Average difference values (in bold) were used to construct the heatmap per assigned Genus (left of heatmap); the variation between replicates is indicated by the lowest value on the left and the highest on the right of the bold-averaged value. OTUs statistically significant for all fertilization regimes on Day 7 were used to construct the dendrogram (top of the heatmap). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

119 years. This acidification increased surface charge, reduced metal binding due to particle repulsion (Appel and Ma, 2002; Park et al., 2011), and inhibited metal coprecipitation (Palansooriya et al., 2020). As a result, the capacity to immobilize amended metals was decreased in mineral-fertilized soils, leaving the highest proportions of metals remaining in the mobile fraction: approximately 5.7 % for Cd and 4.5 % for Zn.

Organic fertilization with manure increased SOM content by about 30 $\%_w$ compared to unfertilized soils (Francioli et al., 2016). Increased SOM functional groups (e.g., carboxyl, phenol, hydroxyl, and carbonyl) and minerals (e.g., carbonates, phosphates, aluminum and iron oxides, and clays) enhanced metal scavenging through sorption, precipitation, or complexation (Caporale and Violante, 2016; Lair et al., 2007; Park et al., 2011; Sun et al., 2017). Minerals and SOM also form organo-mineral composites, which increased surface area, positive charge, and available adsorption sites, improving metal adsorption capacity (Bao et al., 2022). Manure application buffered soil pH through reversible protonation of organic acids (Naramabuye and Haynes, 2006; Whalen et al., 2000), acid neutralization through carbonates and bicarbonates (Eghball, 1999; Li et al., 2012), maintaining soil pH above 7. At this pH, soil particles acquire a negative surface charge, enhancing positively charged metal binding (Park et al., 2011). The nutrient-rich environment also supported a high density of prokaryotes, evidenced by high initial cell numbers and respiratory activity (Fig. 5), which altered the organic carbon stock composition and increased exposed metal-binding sites. These conditions led to the most effective metal immobilization among all fertilization treatments, leaving only approximately 1.0 % for mobile Cd and 0.3 % for Zn.

The combined use of mineral and organic fertilization further increased soil OC and NPK salt contents (Francioli et al., 2016), resulting in the highest electrical conductivity and mobile nutrient levels among all treatments. The prokaryotic community in the combined mineral and

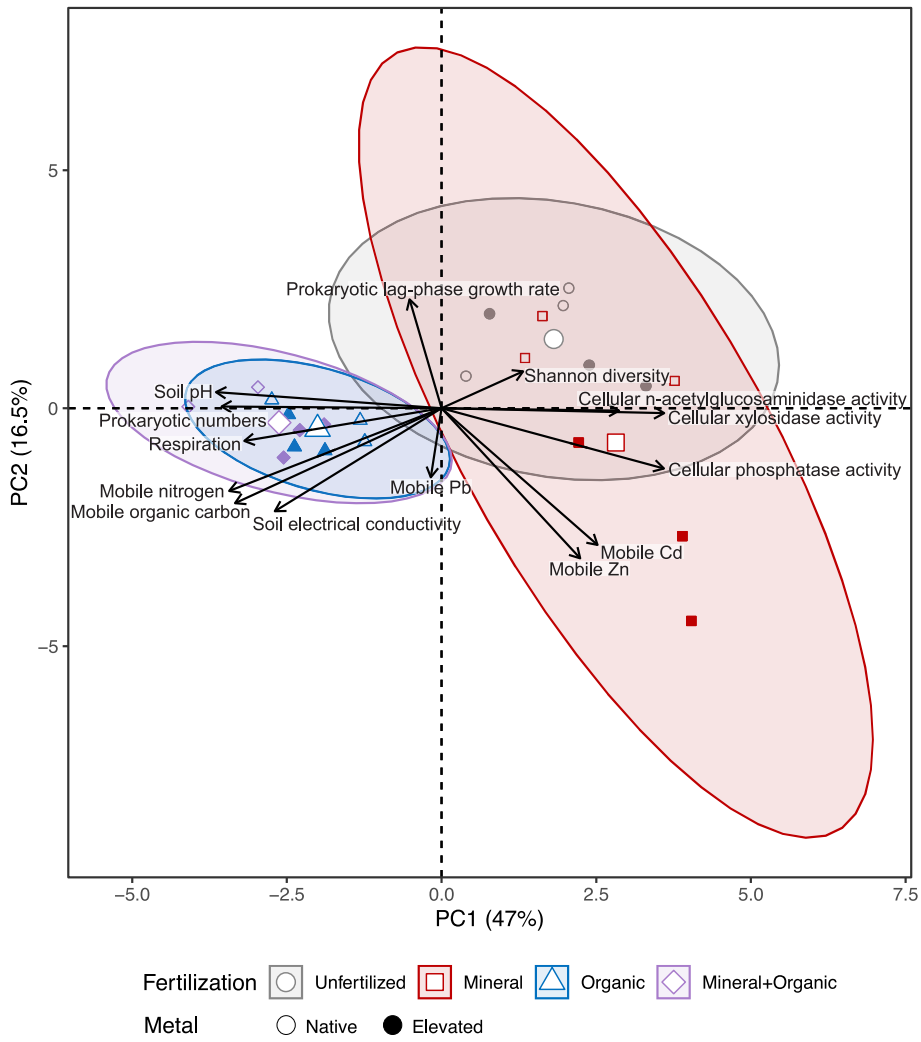

Fig. 5. Principle component analysis biplot with the relationships between soil prokaryotic and geochemical variables in agricultural soils fertilized for 119 years under four practices: Unfertilized (grey circle), mineral (red square), organic (blue triangle), mineral + organic (purple diamond) fertilization. Soils either contained native metal contents (empty symbol) or were exposed to elevated metal levels of +0.2 ppm_w Cd, +33.3 ppm_w Zn, +4.8 ppm_w Pb relative to background values (filled symbol). Points represent individual samples; larger white symbols indicate group centroids. Ellipses represent 95 % confidence intervals around treatment group centroids. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

organic-fertilized soils resembled that in organic-fertilized soils more than mineral-only soils (Fig. S8). Manure amendment contributed to longer NPK retention, as indicated by slightly increasing electrical conductivity compared to soils with only organic fertilization. Ammonia amendment likely promoted ammonia oxidation similarly to mineral-fertilized soil. However, the acidification was less pronounced due to the buffering capacity of manure-derived SOM, compared to solely mineral-fertilized soils, resulting in a soil pH remaining above 7. The combined fertilization boosted prokaryotic activity compared to solely organic-fertilized soil, indicated by the highest CO_2 production, rapid prokaryotic growth, and low cellular enzymatic activities. Despite accelerated prokaryotic metabolism, the high OC content allowed prokaryotes to thrive while maintaining sufficient metal-binding sites. This resulted in similar metal immobilization as in solely organic-fertilized soils, with approximately 1.0 % for Cd, 0.5 % for Zn, and 0 % for Pb of the total amended metals remaining mobile.

4.2. *Effect of long-term fertilization on prokaryotic resistance and resilience to metal amendment*

The amendment of low, realistic amounts of metals primarily affected the prokaryotic community within the first 7 days, indicating that the amended metals caused only mild stress across all fertilization treatments. However, the response of the prokaryotic communities varied depending on the fertilization regime, which correlated with the level of metal bioavailability. Overall, the impact on the prokaryotic community followed this pattern: organic < organic + mineral < unfertilized < mineral.

In unfertilized soils, the increased metal bioavailability likely forced prokaryotes to activate protective mechanisms, such as enhanced metal export and metal-complexing carbon exudation, which required additional energy (Azarbad et al., 2016). This energy allocation towards extracellular hydrolytic enzyme production reduced prokaryotic growth, evidenced by a slight decline in cell numbers. Notably, five taxa were significantly affected by different metal regimes throughout the experiment. Among them, *Rubrobacteriaceae Rubrobacter* and *Streptomycetaceae Streptomyces* known to thrive in high metal environments (Du et al., 2023; Khudur et al., 2018), increased in relative abundance. *Streptomyces*, in particular, is known for its widespread metal protection mechanisms (Álvarez et al., 2013). Conversely, *Lamiaceae Iamia* decreased in relative abundance under elevated metal conditions by day 7. Although relatively novel to soil environments and not well-characterized for metal resistance, *L. lamia* is recognized for nitrate reduction (Kurahashi et al., 2009) and as a potential

plant-growth-promoting bacterium (Panke-Buisse et al., 2015). Its decline could negatively impact soil health and nutrient cycling.

Mineral fertilization reduced the soil's capacity to store nutrients and metals, prompting prokaryotes to mine for nutrients, resulting in initial cellular enzymatic activities similar to those in unfertilized soil. Due to reduced metal-binding sites, prokaryotes in mineral-fertilized soil faced higher exposure to mobile Cd and Zn. This reduction led to increased activity of protective mechanisms and possible cell repair processes, requiring additional energy and nutrients. This increased activity was reflected in nearly doubled enzymatic activities under elevated metal conditions compared to the native metal regime. The focus on metal defense diverted resources away from growth, resulting in the slowest prokaryotic growth among the soils. Additionally, the mineral-fertilized soil exhibited the most significant community shift upon metal amendment, with the largest number of significantly different OTUs, particularly on day 7. Prokaryotes that are both metal-tolerant and involved in nitrogen and phosphorus cycling, such as *Roseiflexaceae* uncultured (Luo et al., 2024; Wang et al., 2022), *Bryobacteraceae Bryobacter* (Camargo et al., 2023; He et al., 2022), *Rhizobiaceae Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (Bellabarba et al., 2019; Díez-Méndez and Menéndez, 2021), *Solirubrobacteraceae* unclassified (Camargo et al., 2023; Xiang et al., 2024), *Gemmatimonadaceae* uncultured (Hu et al., 2021; Wang et al., 2024), *Nitrosomonadaceae MND1* (Chun et al., 2021; Prosser et al., 2014), *Ilumatobacteraceae* uncultured (Wang et al., 2024; Zárate et al., 2021) increased in relative abundance under metal stress, indicating an intensified effort to acquire nutrients while coping with metal toxicity. Overall, the prokaryotic community in mineral-fertilized soil was the most affected by metal amendments among all tested soils (Fig. 5). This could be partially explained by the fact that this community had the highest exposure to amended metals. However, as indicated by initial $CaCl_2$-extractable metal levels (Fig. S3), this soil already faced elevated Cd concentrations prior to amendments, likely due to long-term phosphate fertilizer use, which often contains Cd impurities (Nziguheba and Smolders, 2008). Contrary to the expectation that prolonged exposure fosters acclimatization or tolerance, the pronounced response suggests that extended metal inputs do not necessarily confer resistance but may instead reveal the potential vulnerability of such systems to further metal stress.

Organic fertilization with manure enriched the soil with nutrients, which facilitated prokaryotic growth and metal immobilization. This dual benefit reduced the stress from both nutrient scarcity and metal toxicity. Consequently, soil prokaryotes did not need to increase energy investment in nutrient acquisition or metal defense. Enzymatic activities remained constant across both metal regimes, alongside high CO_2 production (Fig. S6) and stable prokaryotic growth (Fig. 5). This favorable environment allowed the prokaryotic community to develop similarly under both metal regimes without shifting toward metal-resistant or nutrient-acquiring taxa, with minimal shifts in composition between elevated and native metal conditions.

In soil treated with a combination of mineral and organic fertilizers, high levels of nutrients and metal-binding sites minimized the exposure of prokaryotes to metals, resulting in minimal disruption to biogeochemical processes (Fig. 5). The prokaryotic community was similar to that in solely organic-fertilized soil, with relatively minor impacts from metal amendment. However, unlike in the solely organic-fertilized soil, metal amendment in the combined fertilization led to a slight alteration of the prokaryotic community, with a notable increase in significantly different OTUs between native and elevated metal regimes, particularly on day 7. Interestingly, some taxa that increased in solely mineral-fertilized soil under metal stress decreased in the combined fertilization regime. For instance, the relative abundance of *Gaiellaceae Gaiella* and *Sphingomonadaceae Uncultured*, both known degraders of organic aromatic compounds (Waigi et al., 2017; Zhao et al., 2021), decreased. Similarly, taxa involved in nitrogen and phosphorous cycling, such as denitrifying *Gemmatimonadaceae* (Wang et al., 2024) and phosphorus-mobilizing *Geminicoccaceae Candidatus* (Proença et al., 2018) also declined under elevated metal conditions. Interestingly, while *Gemmatimonadaceae* increased relative abundance in solely mineral-fertilized soils under amended metal, their abundance decreased in the combined mineral and organic-fertilized soils, suggesting that metal amendment drove distinct selection patterns in different fertilization regimes. The decline in taxa involved in nutrient cycling and complex organic compound degradation suggests that metal amendment made previously adsorbed NPK cations from mineral fertilizers more available to prokaryotes, reducing the need for specialized nutrient cyclers. This shift likely resulted in a decline in these specialized prokaryotes, allowing more opportunistic r-strategists to dominate under the new conditions.

This study has two main limitations. First, using air-dried soil for incubation may have influenced microbial community structure and activity, and thus affected comparability to in-situ dynamics. Using air-dried soil for incubation is a common practice in soil microbiology to ensure treatment consistency and uniform starting conditions (Haney et al., 2004; Schroeder et al., 2021). While microbial functions can recover after rehydration, allowing meaningful comparisons (Edwards et al., 2024; Wang et al., 2021), fresh soil incubations could provide further insights into microbial responses under more in situ-like conditions. Future studies directly comparing air-dried and fresh soils would help clarify the extent to which pre-treatment affects microbial dynamics and metal-induced effects. Second, it focused on low-dose metal concentrations, reflecting typical agricultural practices in uncontaminated soils, but microbial responses to higher metal pollution may be different. Higher metal concentrations could result from lower-quality fertilizers or pre-existing contamination (e.g., post-industrial sites) and investigating these conditions is essential for understanding microbial tolerance limits and defense mechanisms.

4.3. Environmental implications

In summary, fertilization practices significantly impacted metal immobilization in soil. Long-term mineral fertilization reduced soil health, increased metal availability to prokaryotes and slowed down recovery compared to unfertilized soils. In response to metal stress, these prokaryotes enhanced nutrient acquisition, potentially further reducing soil fertility. Organic fertilization improved soil conditions by enhancing metal immobilization and supporting prokaryotic growth. Combining mineral and organic fertilization is proposed as a strategy to optimize crop yields while maintaining soil health (Reganold and Wachter, 2016). Here, it offered the benefits of both approaches: preserved fertility and limited metal mobilization. However, upon metal amendment, dual fertilization imbalanced C, N, and P cycles compared to solely organic-fertilized soils, which may impact long-term fertility.

CRediT authorship contribution statement

Aleksandra Pieńkowska: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jenin Fleischmann:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Sören Drabesch:** Writing – review & editing, Methodology, Formal analysis. **Ines Merbach:** Writing – review & editing, Methodology. **Gennuo Wang:** Writing – review & editing, Formal analysis, Data curation. **Ulisses Rocha:** Writing – review & editing, Validation, Supervision, Formal analysis. **Thomas Reitz:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **E. Marie Muehe:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2025.126747.

Data availability

Data will be made available on request.

References

Alloway, B.J., 2013. Sources of Heavy Metals and Metalloids in Soil. Springer, Dordrecht, The Netherlands.
Altermann, M., Rinklebe, J., Merbach, I., Körschens, M., Langer, U., Hofmann, B., 2005. Chernozem—Soil of the year 2005. J. Plant Nutr. Soil Sci. 168, 725–740.
Álvarez, A., Catalano, S.A., Amoroso, M.J., 2013. Heavy metal resistant strains are widespread along streptomyces phylogeny. Mol. Phylogenet. Evol. 66, 1083–1088.
Appel, C., Ma, L., 2002. Concentration, pH, and surface charge effects on cadmium and lead sorption in three tropical soils. J. Environ. Qual. 31, 581–589.
Atafar, Z., Mesdaghinia, A., Nouri, J., Homaee, M., Yunesian, M., Ahmadimoghaddam, M., Mahvi, A.H., 2010. Effect of fertilizer application on soil heavy metal concentration. Environ. Monit. Assess. 160, 83–89.
Azarbad, H., Van Gestel, C.A., Niklińska, M., Laskowski, R., Röling, W.F., Van Straalen, N.M., 2016. Resilience of soil microbial communities to metals and additional stressors: DNA-based approaches for assessing "stress-on-stress" responses. Int. J. Mol. Sci. 17, 933.
Bates, D., Mächler, M., Bolker, B., Walker, S., 2014. Fitting linear mixed-effects models using lme4. arXiv preprint arXiv:1406.5823.
Bao, Y., Bolan, N.S., Lai, J., Wang, Y., Jin, X., Kirkham, M., Wu, X., Fang, Z., Zhang, Y., Wang, H., 2022. Interactions between organic matter and Fe (hydr) oxides and their influences on immobilization and remobilization of metal (loid) s: a review. Crit. Rev. Environ. Sci. Technol. 52, 4016–4037.
Bellabarba, A., Fagorzi, C., diCenzo, G.C., Pini, F., Viti, C., Checcucci, A., 2019. Deciphering the symbiotic plant microbiome: translating the most recent discoveries on rhizobia for the improvement of agricultural practices in metal-contaminated and high saline lands. Agronomy 9, 529.
Berti, A., Dalla Marta, A., Mazzoncini, M., Tei, F., 2016. An overview on long-term agro-ecosystem experiments: present situation and future potential. Eur. J. Agron. 77, 236–241.
Blair, N., Faulkner, R.D., Till, A.R., Korschens, M., Schulz, E., 2006. Long-term management impacts on soil C, N and physical fertility: part II: bad Lauchstadt static and extreme FYM experiments. Soil Tillage Res. 91, 39–47.
Bronick, C.J., Lal, R., 2005. Soil structure and management: a review. Geoderma 124, 3–22.
Camargo, A.P., de Souza, R.S., Jose, J., Gerhardt, I.R., Dante, R.A., Mukherjee, S., Huntemann, M., Kyrpides, N.C., Carazzolle, M.F., Arruda, P., 2023. Plant microbiomes harbor potential to promote nutrient turnover in impoverished substrates of a Brazilian biodiversity hotspot. ISME J. 17, 354–370.
Caporale, A.G., Violante, A., 2016. Chemical processes affecting the mobility of heavy metals and metalloids in soil environments. Curr. Pollut. Rep. 2, 15–27.
Chun, S.-J., Kim, Y.-J., Cui, Y., Nam, K.-H., 2021. Ecological network analysis reveals distinctive microbial modules associated with heavy metal contamination of abandoned mine soils in Korea. Environ. Pollut. 289, 117851.
Diacono, M., Montemurro, F., 2011. Long-term effects of organic amendments on soil fertility. Sustain. Agricult. 2, 761–786.
Díez-Méndez, A., Menéndez, E., 2021. Rhizobium presence and functions in microbiomes of non-leguminous plants. Symb. Soil Microorgan. Biol. Appl. 241–266.
Du, L., Zhong, S., Luo, K., Yang, S., Xia, J., Chen, Q., 2023. Effect of metal pollution on the distribution and co-occurrence pattern of bacterial, archaeal and fungal communities throughout the soil profiles. Chemosphere 315, 137692.
Edwards, J.D., Love, S.J., Phillips, R.P., Fei, S., Domke, G., Parker, J.D., McCormick, M., LaRue, E.A., Schweitzer, J.A., Bailey, J.K., 2024. Long-and short-term soil storage methods other than freezing can be useful for DNA-based microbial community analysis. Soil Biol. Biochem. 191, 109329.
Eghball, B., 1999. Liming effects of beef cattle feedlot manure or compost. Commun. Soil Sci. Plant Anal. 30, 2563–2570.
Erisman, J.W., Galloway, J.N., Seitzinger, S., Bleeker, A., Dise, N.B., Petrescu, A.R., Leach, A.M., de Vries, W., 2013. Consequences of human modification of the global nitrogen cycle. Phil. Trans. Biol. Sci. 368, 20130116.
European Environment Agency, 2022. Nutrients and heavy metals in European agriculture: current and critical inputs in relation to air. Soil Water Qual.
Fang, X., Wang, J., Chen, H., Christl, I., Wang, P., Kretzschmar, R., Zhao, F.-J., 2021. Two-year and multi-site field trials to evaluate soil amendments for controlling cadmium accumulation in rice grain. Environ. Pollut. 289, 117918.
FAO & UNEP, 2021. Global Assessment of Soil Pollution: Report. Rome, Italy.
Francioli, D., Schulz, E., Lentendu, G., Wubet, T., Buscot, F., Reitz, T., 2016. Mineral vs. organic amendments: microbial community structure, activity and abundance of agriculturally relevant microbes are driven by long-term fertilization strategies. Front. Microbiol. 7, 1446.
German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol. Biochem. 43, 1387–1397.
Grosse, M., Hierold, W., Ahlborn, M.C., Piepho, H.-P., Helming, K., 2020. Long-term field experiments in Germany: classification and spatial representation. Soils 6, 579–596.
Gu, Z., Eils, R., Schlesner, M., 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics 32, 2847–2849.
Haney, R., Franzluebbers, A., Porter, E., Hons, F., Zuberer, D., 2004. Soil carbon and nitrogen mineralization: influence of drying temperature. Soil Sci. Soc. Am. J. 68, 489–492.
Hartmann, M., Six, J., 2023. Soil structure and microbiome functions in agroecosystems. Nat. Rev. Earth Environ. 4, 4–18.
He, J.z., Shen, J.p., Zhang, L.m., Zhu, Y.g., Zheng, Y.m., Xu, M.g., Di, H., 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environ. Microbiol. 9, 2364–2374.
He, L., Wang, B., Cui, H., Yang, S., Wang, Y., Feng, Y., Sun, X., Feng, Y., 2022. Clay-hydrochar composites return to cadmium contaminated paddy soil: reduced Cd accumulation in rice seed and affected soil microbiome. Sci. Total Environ. 835, 155542.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J.: J. Math. Method. Biosci. 50, 346–363.
Houba, V., Temminghoff, E., Gaikhorst, G., Van Vark, W., 2000. Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. Commun. Soil Sci. Plant Anal. 31, 1299–1396.
Hu, X., Wang, J., Lv, Y., Liu, X., Zhong, J., Cui, X., Zhang, M., Ma, D., Yan, X., Zhu, X., 2021. Effects of heavy metals/metalloids and soil properties on microbial communities in farmland in the vicinity of a metals smelter. Front. Microbiol. 12, 707786.
Igiri, B.E., Okoduwa, S.I., Idoko, G.O., Akabuogu, E.P., Adeyi, A.O., Ejiogu, I.K., 2018. Toxicity and bioremediation of heavy metals contaminated ecosystem from tannery wastewater: a review. J. Toxicol. 2018.
Kassambara, A., Mundt, F., 2020. Factoextra: Extract and visualize the results of multivariate data analyses. R Package Version 1.0.7. https://CRAN.R-project.org/package=factoextra.
Khudur, L.S., Gleeson, D.B., Ryan, M.H., Shahsavari, E., Haleyur, N., Nugegoda, D., Ball, A.S., 2018. Implications of co-contamination with aged heavy metals and total petroleum hydrocarbons on natural attenuation and ecotoxicity in Australian soils. Environ. Pollut. 243, 94–102.
Kim, R.-Y., Yoon, J.-K., Kim, T.-S., Yang, J.E., Owens, G., Kim, K.-R., 2015. Bioavailability of heavy metals in soils: definitions and practical implementation—a critical review. Environ. Geochem. Health 37, 1041–1061.
Kurahashi, M., Fukunaga, Y., Sakiyama, Y., Harayama, S., Yokota, A., 2009. Iamia majanohamensis gen. nov., sp. Nov., an actinobacterium isolated from sea cucumber Holothuria edulis, and proposal of Iamiaceae fam. nov. Int. J. Syst. Evol. Microbiol. 59, 869–873.
Lair, G., Gerzabek, M., Haberhauer, G., 2007. Sorption of heavy metals on organic and inorganic soil constituents. Environ. Chem. Lett. 5, 23–27.
Latare, A., Kumar, O., Singh, S., Gupta, A., 2014. Direct and residual effect of sewage sludge on yield, heavy metals content and soil fertility under rice–wheat system. Ecol. Eng. 69, 17–24.
Lehmann, J., Bossio, D.A., Kögel-Knabner, I., Rillig, M.C., 2020. The concept and future prospects of soil health. Nat. Rev. Earth Environ. 1, 544–553.
Lemire, J.A., Harrison, J.J., Turner, R.J., 2013. Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat. Rev. Microbiol. 11, 371–384.
Li, R., Khafipour, E., Krause, D.O., Entz, M.H., de Kievit, T.R., Fernando, W.D., 2012. Pyrosequencing reveals the influence of organic and conventional farming systems on bacterial communities. PLoS One 7, e51897.
Li, X., Meng, D., Li, J., Yin, H., Liu, H., Liu, X., Cheng, C., Xiao, Y., Liu, Z., Yan, M., 2017. Response of soil microbial communities and microbial interactions to long-term heavy metal contamination. Environ. Pollut. 231, 908–917.
Liu, B., Sträuber, H., Saraiva, J., Harms, H., Silva, S.G., Kasmanas, J.C., Kleinsteuber, S., Nunes da Rocha, U., 2022. Machine learning-assisted identification of bioindicators predicts medium-chain carboxylate production performance of an anaerobic mixed culture. Microbiome 10, 48.
Lopes, C., Herva, M., Franco-Uría, A., Roca, E., 2011. Inventory of heavy metal content in organic waste applied as fertilizer in agriculture: evaluating the risk of transfer into the food chain. Environ. Sci. Pollut. Control Ser. 18, 918–939.

Lueders, T., Manefield, M., Friedrich, M.W., 2004. Enhanced sensitivity of DNA-and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. Environ. Microbiol. 6, 73–78.
Luo, G., Li, L., Friman, V.-P., Guo, J., Guo, S., Shen, Q., Ling, N., 2018. Organic amendments increase crop yields by improving microbe-mediated soil functioning of agroecosystems: a meta-analysis. Soil Biol. Biochem. 124, 105–115.
Luo, X.-F., Liu, M.-Y., Tian, Z.-X., Xiao, Y., Zeng, P., Han, Z.-Y., Zhou, H., Gu, J.-F., Liao, B.-H., 2024. Physiological tolerance of black locust (Robinia pseudoacacia L.) and changes of rhizospheric bacterial communities in response to Cd and Pb in the contaminated soil. Environ. Sci. Pollut. Control Ser. 31, 2987–3003.
McDevitt, C.A., Ogunniyi, A.D., Valkov, E., Lawrence, M.C., Kobe, B., McEwan, A.G., Paton, J.C., 2011. A molecular mechanism for bacterial susceptibility to zinc. PLoS Pathog. 7, e1002357.
Melaku, S., Dams, R., Moens, L., 2005. Determination of trace elements in agricultural soil samples by inductively coupled plasma-mass spectrometry: microwave acid digestion versus aqua regia extraction. Anal. Chim. Acta 543, 117–123.
Merbach, I., Schulz, E., 2013. Long-term fertilization effects on crop yields, soil fertility and sustainability in the static fertilization experiment Bad Lauchstädt under climatic conditions 2001–2010. Arch. Agron Soil Sci. 59, 1041–1057.
Naramabuye, F., Haynes, R.J., 2006. Effect of organic amendments on soil pH and Al solubility and use of laboratory indices to predict their liming effect. Soil Sci. 171, 754–763.
Nziguheba, G., Smolders, E., 2008. Inputs of trace elements in agricultural soils via phosphate fertilizers in European countries. Sci. Total Environ. 390, 53–57.
Palansooriya, K.N., Shaheen, S.M., Chen, S.S., Tsang, D.C., Hashimoto, Y., Hou, D., Bolan, N.S., Rinklebe, J., Ok, Y.S., 2020. Soil amendments for immobilization of potentially toxic elements in contaminated soils: a critical review. Environ. Int. 134, 105046.
Panke-Buisse, K., Poole, A.C., Goodrich, J.K., Ley, R.E., Kao-Kniffin, J., 2015. Selection on soil microbiomes reveals reproducible impacts on plant function. ISME J. 9, 980–989.
Park, J.H., Lamb, D., Paneerselvam, P., Choppala, G., Bolan, N., Chung, J.-W., 2011. Role of organic amendments on enhanced bioremediation of heavy metal (loid) contaminated soils. J. Hazard Mater. 185, 549–574.
Proença, D.N., Whitman, W.B., Varghese, N., Shapiro, N., Woyke, T., Kyrpides, N.C., Morais, P.V., 2018. Arboriscoccus pini gen. nov., sp. Nov., an endophyte from a pine tree of the class Alphaproteobacteria, emended description of Geminicoccus roseus, and proposal of Geminicoccaceae fam. nov. Syst. Appl. Microbiol. 41, 94–100.
Prosser, J.I., Head, I.M., Stein, L.Y., 2014. The Family Nitrosomonadaceae, the Prokaryotes: Alphaproteobacteria and Betaproteobacteria. Springer, Berlin/Heidelberg, pp. 901–918.
R Core Team, 2021. R: a Language and Environment for Statistical Computing.
Rasmussen, P.E., Goulding, K.W., Brown, J.R., Grace, P.R., Janzen, H.H., Korschens, M., 1998. Long-term agroecosystem experiments: assessing agricultural sustainability and global change. Science 282, 893–896.
Reganold, J.P., Wachter, J.M., 2016. Organic agriculture in the twenty-first century. Nat. Plants 2, 1–8.
Rodríguez-Eugenio, N., McLaughlin, M., Pennock, D., 2018. Soil Pollution: a Hidden Reality. FAO, Rome, Italy.
RStudio Team, 2022. Rstudio: Integrated Development Environment for R.
Russell, V. Lenth, 2021. Emmeans: Estimated Marginal Means, Aka Least-Squares Means.
Sahab, S., Suhani, I., Srivastava, V., Chauhan, P.S., Singh, R.P., Prasad, V., 2021. Potential risk assessment of soil salinity to agroecosystem sustainability: current status and management strategies. Sci. Total Environ. 764, 144164.
Sastre, J., Rauret, G., Vidal, M., 2007. Sorption–desorption tests to assess the risk derived from metal contamination in mineral and organic soils. Environ. Int. 33, 246–256.
Schroeder, J., Kammann, L., Helfrich, M., Tebbe, C.C., Poeplau, C., 2021. Impact of common sample pre-treatments on key soil microbial properties. Soil Biol. Biochem. 160, 108321.
Shade, A., Peter, H., Allison, S.D., Baho, D.L., Berga, M., Bürgmann, H., Huber, D.H., Langenheder, S., Lennon, J.T., Martiny, J.B., 2012. Fundamentals of microbial community resistance and resilience. Front. Microbiol. 3, 417.
Stoate, C., Baldi, A., Beja, P., Boatman, N., Herzon, I., Van Doorn, A., De Snoo, G., Rakosy, L., Ramwell, C., 2009. Ecological impacts of early 21st century agricultural change in Europe–a review. J. Environ. Manag. 91, 22–46.
Sun, F., Polizzotto, M.L., Guan, D., Wu, J., Shen, Q., Ran, W., Wang, B., Yu, G., 2017. Exploring the interactions and binding sites between Cd and functional groups in soil using two-dimensional correlation spectroscopy and synchrotron radiation based spectromicroscopies. J. Hazard Mater. 326, 18–25.
Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal toxicity and the environment. Molecular, clinical and environmental toxicology: volume 3. Environ. Toxicol. 133–164.
The James Hutton Institute, 2024. https://www.microresp.com/.
Usman, A.R.A., 2008. The relative adsorption selectivities of Pb, Cu, Zn, Cd and Ni by soils developed on shale in New Valley, Egypt. Geoderma 144, 334–343.
Waigi, M.G., Sun, K., Gao, Y., 2017. Sphingomonads in microbe-assisted phytoremediation: tackling soil pollution. Trends Biotechnol. 35, 883–899.
Wang, F., Che, R., Deng, Y., Wu, Y., Tang, L., Xu, Z., Wang, W., Liu, H., Cui, X., 2021. Air-drying and long time preservation of soil do not significantly impact microbial community composition and structure. Soil Biol. Biochem. 157, 108238.
Wang, J.L., Liu, K.L., Zhao, X.Q., Gao, G.-F., Wu, Y.H., Shen, R.F., 2022. Microbial keystone taxa drive crop productivity through shifting aboveground-belowground mineral element flows. Sci. Total Environ. 811, 152342.
Wang, X., Chen, T., Ge, Y., Jia, Y., 2008. Studies on land application of sewage sludge and its limiting factors. J. Hazard Mater. 160, 554–558.
Wang, Y., Cai, J., Chen, X., Guo, B., Liu, J., Qiu, G., Li, H., 2024. The connection between the antibiotic resistome and nitrogen-cycling microorganisms in paddy soil is enhanced by application of chemical and plant-derived organic fertilizers. Environ. Res. 243, 117880.
Whalen, J.K., Chang, C., Clayton, G.W., Carefoot, J.P., 2000. Cattle manure amendments can increase the pH of acid soils. Soil Sci. Soc. Am. J. 64, 962–966.
Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L.D.A., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., 2019. Welcome to the tidyverse. J. Open Source Softw. 4, 1686.
Xiang, Y., Lan, J., Dong, Y., Zhou, M., Hou, H., Huang, B.-T., 2024. Pollution control performance of solidified nickel-cobalt tailings on site: bioavailability of heavy metals and microbial response. J. Hazard Mater. 471, 134295.
Zárate, A., Dorador, C., Valdés, J., Molina, V., Icaza, G., Pacheco, A.S., Castillo, A., 2021. Benthic microbial diversity trends in response to heavy metals in an oxygen-deficient eutrophic bay of the Humboldt current system offshore the Atacama Desert. Environ. Pollut. 286, 117281.
Zhao, Y., Duan, F.-A., Cui, Z., Hong, J., Ni, S.-Q., 2021. Insights into the vertical distribution of the microbiota in steel plant soils with potentially toxic elements and PAHs contamination after 60 years operation: abundance, structure, co-occurrence network and functionality. Sci. Total Environ. 786, 147338.