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# Effects of digestate soil amendment on the fate of trace metals and on the soil microbial community ${}^{\star}$

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# ABSTRACT

This study evaluated, over a 28-day soil column experiment, (i) the fate of trace metals (Zn, Cu, Pb) in a loamy sand soil after amendment with a non-source-separated municipal solid waste (OFMSW) digestate; (ii) the impact of the OFMSW digestate on soil microbial community; and (iii) the effects of two pharmaceuticals (metformin and lamotrigine) on trace metal fate and microbial community dynamics. Three conditions were tested: natural digestate, digestate spiked with metformin, and digestate spiked with lamotrigine. Soil samples were collected over time to measure trace metal concentrations and fractionation and characterize the soil prokaryotic community using Illumina next generation sequencing technology.

Results showed Pb mobility factor (MF) increased over time by 182% in amended soil and 126% in the other soil layers, while Zn MF increased by 85% and decreased by 36%, respectively. Total metal concentrations were, nevertheless, low, pointing out that the impact of these metals on the soil in this study should be low. Cu MF remained constant (ca. 1.75%). Digestate amendment increased soil microbial diversity, with Shannon Index rising from 4.9 to 5.6, and shifted its composition over time, promoting a more diverse community. Initially dominated by Firmicutes, it stabilized by day 14 with Proteobacteria and Bacteroidota becoming dominant. Pharmaceuticals at environmentally relevant concentrations did not significantly affect metal behaviour or prokaryotic community structure.

These findings suggest digestate may immobilize contaminants, making it a promising resource for soil improvement practices. However, preliminary treatment and monitoring are crucial for its safe application within circular bioeconomy strategies.

# 1. Introduction

Anaerobic digestion is a robust waste treatment process that treats organic waste producing biogas, used as a renewable energy source, and digestate (Fermoso et al., 2019; Kaur et al., 2020). In the last decade, thanks to the change in soil amendment and fertilizer regulations (European Parliament and Council, 2019), the use of digestate as soil amendment gained a lot of attention. However, only few studies have shown non-source-separated municipal solid waste (MSW) digestate to be a promising resource for marginal land restoration (Fang et al., 2017; Gu and Bai, 2018). Digestate was shown to have promising agronomic properties, improving soil structure, and increasing water retention capacity, potentially favouring greater biomass yield and soil biodiversity (Coelho et al., 2020; Tampio et al., 2016). Digestates, originating from animal waste, manure and sewage sludge, were found to have high microbial abundance, with methanogenic species and microbial species

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of agronomical and environmental importance being detected in high and low abundance, respectively (Coelho et al., 2021, 2020). Moreover, some studies found digestate to stimulate the decomposer microbial species and favour microbial activity in soils, especially in the presence of plants (Caracciolo et al., 2015; Walsh et al., 2012). However, other studies found digestate to have had only a temporary impact on the soil microbial community's diversity, suggesting a low agronomical potential in terms of community diversity enhancement, maintaining the native soil microbial species (Coelho et al., 2020). Furthermore, compared to other biosolid amendments, non-source-separated MSW digestate reduced the risk of trace metal leaching due to a higher amount of mineralized organic matter and a lower quantity of dissolved organic carbon, favouring trace metal adsorption and immobilization onto the organic matter and mineral constituents of the soil (Fang et al., 2017; Shaheen et al., 2013). However, high content of trace metals (European Parliament and European Council, 2013) and of contaminants of emerging concern (European Commission and Directorate-General for European CommissionDirectorate-General for Environment, 2022a), like pharmaceutical and personal care products, can be a risk for the ecosystem and human health, as they can be toxic and/or give origin to toxic secondary compounds, and they can transfer through the soil system and accumulate in the flora and fauna (Dragicevic et al., 2018; Teglia et al., 2011). Despite the moderately low risk of MSW digestates, there is still a possibility of transport and redistribution of contaminants inherently present in the digestate within the soil (Teglia et al., 2011), making it essential to better understand how the fate of contaminants and the soil ecosystem are affected by MSW digestate application.

Given the current governmental push towards the use of digestate, originating amongst others from non-source-separated municipal biowaste (referred to as digestate from now on), as soil amendment, and the scarcity of studies on the topic (Baldasso et al., 2023; Bonet-Garcia et al., 2023; Carabassa et al., 2020; Tampio et al., 2016), further investigation is needed. Moreover, to the authors' knowledge very few studies have focused on the effects that this type of digestate, originating from non-source-separated municipal biowaste, specifically its solid fraction, can have on the soil microbial community. In fact, this type of digestate is characterized by an increased amount of anthropogenic inert materials and higher concentrations of organic and inorganic contaminants, which can negatively affect the soil environment. While previous research showed that digestate originating from animal waste, manure, and sewage sludge can enhance agronomic properties and sometimes promote microbial diversity and favour beneficial microbial activity, the impacts of the solid fraction of the digestate under study are largely unexplored. The present study focused on the fate of contaminants, trace metals and pharmaceutical compounds deriving from the digestate, and potential effects on the soil microbial community. Specifically, this study aimed to evaluate the bioavailability and distribution of Zn, Cu and Pb in a poor-quality soil after amendment with a digestate contaminated with these metals and with two pharmaceutical compounds, classified as contaminants of emerging concern, namely metformin and lamotrigine, and to evaluate the changes in the microbial community after amendment application. The study aimed to assess whether trace metals found in the digestate may migrate through the amended soil reaching the deeper soil layers after soil amendment. Moreover, the presence of the pharmaceutical compounds may impact the fate of the trace metals, potentially leading to the formation of either more or less immobile complexes. These contaminants, together with the changes in soil properties, could affect the soil microbial community, either promoting or hindering its development. Zn, Cu and Pb were selected because they were found in highest concentrations in the digestate selected for this study and they are metals that are easily taken up and stored by plants with a varying degree of toxicity (Bonet-Garcia et al., 2023). The two pharmaceutical compounds, metformin and lamotrigine, were selected as they are two of the most commonly used drugs for treating diabetes and epilepsy, respectively. These compounds are frequently detected in biosolids, digestates and soils (Briones and

Sarmah, 2019; Karpov et al., 2021; Paz et al., 2016), showing different adsorption behaviours (Baldasso et al., 2024). Overall, this work contributes to deepen the understanding of (i) trace metals' fate in soil after digestate application and (ii) digestate's effects on the soil microbial community, both in the absence or presence of pharmaceutical compounds.

# 2. Materials and methods

#### 2.1. Materials

The chemical products utilized for the study were ethanol puriss. p.a. > 98% (603-002-00-5, Honeywell), analytical grade hydrogen peroxide >30% w/v (H/1800/15, Fischer Scientific), nitric acid puriss. p.a. 65% (84380-M, Sigma-Aldrich), acetic acid glacial 100% p.a. (100063, Merck), hydroxylamine hydrochloride ACS reagent 98.0% (255580, Sigma-Aldrich), ammonium acetate ACS reagent grade >97% (238074, Sigma-Aldrich), metformin hydrochloride (PHR1084, Sigma-Aldrich) and lamotrigine (PHR1392, Sigma-Aldrich), all being of the highest available purity. Standard stock metallic element solutions were purchased from Sigma-Aldrich. Deionized water with conductivity <0.1 mS cm<sup>-1</sup> was used to prepare solutions and make dilutions. All common plastic and glass utensils were placed in a 20% (v/v) nitric acid bath and carefully washed with deionized water before use.

# 2.2. Solid matrices characterization

The soil was sampled from a construction site in Ermesinde, Porto (PT), homogenized and sieved to <2 mm. The soil classification was obtained through the Portuguese national soil database INFOSOLO (Ramos et al., 2017), which classified the soil as a Cambisol deriving from metamorphic Schist, with a fine loamy sand texture. The digestate was sampled at the end of the digestate solid-liquid separation line of Tratolixo non-source-separated biowaste treatment unit in the MSW treatment plant (Mafra, Portugal). The solid matrices were characterized for their main physical and chemical properties (Supporting Information, Table S1) following the methodology reported in Baldasso et al. (2023). The soil and digestate were combined in a proportion of 14 to 1 (dry weight) and thoroughly mixed to form a homogeneous mixture of amended soil. The soil, digestate and amended soil were also characterized for total trace metal concentration and fractionation following the methodology described in section 2.4.

#### 2.3. Soil mesocosm column experiment

The soil mesocosm column experiment was conducted utilising transparent acrylic plastic columns with 6 lateral regularly spaced-out sampling ports and a base port connected to a glass bottle for the collection of possible soil leachate (Supporting Information, Fig. S1). Soil columns were assembled by layering a first bottom layer of inert gravel (2 cm) for drainage, a mid-layer of soil (37 cm), manually packing at 5 cm strata at a time (Lewis and Sjöstrom, 2010), and a final layer of soil amended with digestate (15 cm), to simulate digestate application on marginal land soil. The soil columns were wrapped in tinfoil, to block sunlight, and placed in an outdoor greenhouse with an average temperature of 25  $^\circ\text{C}.$  The soil columns were irrigated with 200 mL of deionized water once a week to ensure an average water content of 80 % of soil water holding capacity. The experiment had a duration of 28 days, as in a preliminary study with similar conditions, that lasted for 90 days, a stabilization in metal mobility after 21 days was observed (Baldasso et al., 2023). Three experimental conditions were defined, each conducted in triplicates: soil amended with digestate (E-Dig); soil amended with digestate spiked with metformin (E-Met) and soil amended with digestate spiked with lamotrigine (E-Lmt). Since these pharmaceutical compounds were not expected to be present in the selected digestate, they were added in the amended soil to allow a comparison

between natural digestate-amended soil and digestate-amended soil contaminated by pharmaceutical compounds. For this reason, E-Met and E-Lmt experimental conditions were prepared by spiking the digestate with 20 mg/L metformin or lamotrigine aqueous solution, to reach a contaminant concentration of around 2000  $\mu$ g/kg (dw) and thoroughly mixing it. Then, this spiked digestate was used to prepare the digestate-amended soil, which was put on the top of the soil column immediately after preparation. The concentration of pharmaceutical compounds was selected based on that detected in biosolids, digestates and soils reported in the literature (Briones and Sarmah, 2019; Karpov et al., 2021; Paz et al., 2016).

Sampling of the soil columns was carried out on days 1, 14 and 28 by collecting soil samples from the column's 5 side ports (the 6th port was above the soil) with caution to preserve the distribution of the soil layers. Samples were stored at -20 °C until processing. During the 28 days of experiment no soil leachate was obtained from the bottom ports. The solid samples were analysed for Zn, Cu and Pb total concentration and fractionation, and for soil prokaryotic community diversity and composition. The sampling days were chosen according to the findings of previous soil mesocosm experiment (Baldasso et al., 2023) that showed no significant changes after day 21. Additional samples were collected at the end of the experiment to measure the pH of the system using the ISO method for Soil quality (NF ISO10390).

### 2.4. Analytical determinations

The samples' total metals concentrations were determined by atomic absorption spectroscopy (AAS) after an acidic high-pressure microwave digestion as in Baldasso et al. (2023). The digestion was carried out with nitric acid to obtain the environmentally available, thus potentially bioavailable and mobile, metal concentration. The analysis was carried out in triplicate for samples of initial soil, digestate and amended soil, and for all single replicate samples of each soil column. Blank samples were also prepared and analysed following the same procedure to ensure no contamination occurred during the execution. Details on AAS method precision and analytical parameters can be found in Supporting Information (Table S2).

Metal fractionation followed the BCR sequential extraction method as in Baldasso et al. (2023) to evaluate (i) the soluble and exchangeable fraction, (ii) the reducible iron/manganese oxides fraction, (iii) the oxidizable organic matter and sulphide fraction and (iv) the residual metal fractions. Fractionation results were reported as percentage of total trace metal concentration. The residual fraction was obtained by subtracting the other defined fractions from the total trace metal concentration obtained through acidic microwave digestion previously mentioned. Blank samples were prepared and analysed following the same procedure, confirming no contamination. Metal fractionation was carried out in triplicate for samples of initial soil, digestate and amended soil, and for all single replicate samples of each soil column.

### 2.5. Data analysis

Total metal concentrations and metal fractionations were assessed as average of three replicates with corresponding standard deviation. A statistical analysis was performed with SPSS Statistics software (version 28.0.1.0) to evaluate (i) significant differences along soil depth and (ii) significant effects of time and experimental conditions on total concentration and fractionation results. Firstly, non-parametric Kruskal-Wallis test followed by a non-parametric pairwise comparison test was performed to evaluate if and which metal concentrations differed significantly by sampling depth. Secondly, after subdividing the data into two distinct groups (amended layer and soil layers) and verifying the assumptions of normality and homoscedasticity, a two-way ANOVA (or MANOVA) followed by a one-way ANOVA (or MANOVA) and pairwise comparison with post-hoc Tukey HSD test were performed to evaluate if time and experimental conditions had significant interaction and main effects on metal concentration (and fractionation) and which samples differed significantly between each other. The metal concentrations (and fractions) were set as dependent variables and sampling depth, time and experimental conditions were set as independent factor variables. Statistical significance level was set to 0.05.

#### 2.6. Soil prokaryotic community

Prokaryotic community analysis was conducted in triplicate for initial soil, digestate and amended soil samples (day 0), and on one replicate for each soil column samples originating from the amended soil layer (port 1) and the mid soil layer (port 4) collected on days 1, 14 and 28. The samples were collected during the soil mesocosm experiment (section 2.3), placed in sterile sampling bags and stored at -20 °C. The prokaryotic community analysis followed three steps: DNA extraction, next generation sequencing (NGS) with Illumina MiSeq technology for 16S rRNA amplicons, and bioinformatic data analysis.

# 2.6.1. Environmental DNA extraction and 16S rRNA amplicon sequencing

Environmental DNA was extracted from all solid samples (0.25 g) using the PowerSoil Pro® DNA Isolation Toolkit (MO BIO Laboratories, Inc.). The extracted environmental DNA was quantified by fluorometry using Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were prepared and sent to Genoinseq (Cantanhede, Portugal) for amplification and sequencing.

Amplification was carried out using the specific primer pair, forward 515F-Y, 5′- GTGYCAGCMGCCGCGGTAA -3'; and reverse 806rB, 5′-GGACTACNVGGGTWTCTAAT -3' (Caporaso et al., 2011), targeting the V4 hypervariable region of 16S rRNA gene. At Genoinseq laboratories, amplification products were purified and normalized using Sequalprep<sup>™</sup> 96-well plate kit (Thermo Fisher Scientific, Waltham, USA) (Comeau et al., 2017). Then, pair-end sequencing was conducted in the Illumina MiSeq® sequencer with the V3 chemistry (Illumina Inc., San Diego, CA, USA).

#### 2.6.2. Bioinformatic analysis

Raw fastq reads obtained from Illumina MiSeq® sequencing were quality-filtered by Genoinseq (Schmieder and Edwards, 2011; Schubert et al., 2016) using default parameters. The bioinformatic analysis consisted of an upstream and downstream workflow, all performed using RStudio (version 2023.03.1 + 446). The upstream analysis used raw fastq reads and followed the DADA2 pipeline (package version 1.26) with default settings, providing an Amplicon Sequence Variance (ASV) table as output. ASVs representative sequences were then classified against the SILVA database (v.138.1) (Quast et al., 2013) for taxonomic classification. The downstream analysis investigated the diversity and the distribution of the prokaryotic community in and among the samples. The main packages used were phyloseq (version 1.42.0), vegan (version 2.6-4) and ggplot2 (version 3.4.2). Samples' alpha-diversity was evaluated through Shannon, inverse Simpson and Chao indexes coupled with a non-parametric ANOVA and pairwise Wilcoxon rank sum test. Samples' beta-diversity was assessed through non-metric multidimensional scaling (NMDS) coupled with a PERMANOVA, and the samples' taxonomic profiles were visually assessed using bar-plots.

#### 3. Results and discussion

To give a concise presentation of the data, most detailed figures supporting the results discussed hereafter are provided in the Supporting Information, while key observations are highlighted in the main text and supported by discussion.

# 3.1. Trace metal analysis

# 3.1.1. Total trace metal concentration

Zn, Cu and Pb were the trace metals selected for the experiment

because they were the elements that had concentrations in the digestate higher than those in the soil (Supporting Information, Table S3), enabling to perform the experiment without spiking the amended soil with metals and reproduce a real-world scenario of digestate application on land. The total concentrations of trace metals found in the digestate were all within the metal limits imposed by EU regulation (300 mg/kgdw for Zn, 200 mg/kgdw for Cu and 100 mg/kgdw for Pb) on soil improvers and growing media (European Commission and Directorate-General for European CommissionDirectorate-General for Environment, 2022b), suggesting that digestate from non-source-separated household biowaste can be a potential resource for land application and should not be officially classified solely based on its feed material, particularly when considering metal content (Beggio et al., 2019).

Zn, Cu and Pb total concentration along the soil column profile for day 1, day 14 and day 28 are reported in Fig. S2. The concentrations of Zn, Cu and Pb on day 1 in the digestate amended soil layer (port 1) were found to be similar to those of the initial digestate amendment being 96, 44 and 28  $\mu$ g/g, respectively; while their concentrations in the other soil column layers (port 2, 3, 4 and 5) were found to be similar to those of the initial soil being 26, 33 and 25  $\mu$ g/g, respectively (Table S3). Furthermore, Zn, Cu and Pb concentrations in the digestate amended soil layer were significantly higher than their respective concentrations in the other soil column layers for all experimental conditions (presence/ absence pharmaceuticals) and sampling days (p-values <0.05). In fact, Zn, Cu and Pb concentrations in the digestate amended layers ranged between 72 and 108, 28–41, and 24–37  $\mu$ g/g, while in the other soil layers the concentrations ranged between 16 and 22, 28–36, and 18–29  $\mu$ g/g, respectively.

Considering the first soil column layers, neither experimental conditions nor time had a significant effect on trace metals' total concentrations (p-values >0.05). Additionally, Zn, Cu and Pb concentrations did not vary significantly (p-value >0.05) amongst the other soil column layers (port 2, 3, 4 and 5). Moreover, neither sampling time nor experimental conditions exhibited a significant effect on Zn and Cu concentrations (p-value >0.05). In general, Pb concentrations of day 1 (23–43  $\mu$ g/g) were significantly higher (p-value <0.05) than those of day 14 and day 28 (18–25  $\mu$ g/g). The statistical analyses results are reported in Supporting Information.

Overall, these results indicated no significant downward movement of trace metals from the top amended soil layer to the deeper soil layers during the 28-day experiment. Other studies have shown also that other types of digestate had lower risk of metal leaching compared to sewage sludge and mineral fertilizers, even during intense precipitation events (Dragicevic et al., 2018; Gu and Bai, 2018). Results can be explained by (i) the alkaline pH of the soil column system (average 8.6 at the end of the experiment) which increases the soil's total negative surface charge favouring the retention of metal cations (Shaheen et al., 2013) and (ii) the experimental set-up which could have led to low soil leaching, key factor in colloidal particle transport (Adriano, 2001; Teglia et al., 2011). Furthermore, the presence of metformin and lamotrigine did not have any statistically significant effect on trace metals' total concentration along the soil profile through time, which agrees with the findings of Baldasso et al. (2023) who studied the condition of digestate contaminated with metformin. Their study observed a slight trace metal migration from the top amended soil layer to the underlaying soil layer at the third experimental week (day 21) which was not observed in the current study. This difference could be attributed to random formation of preferential flow paths causing uneven water distribution and to an unforeseen alteration in the integrity of the soil layers due to the sampling process.

# 3.1.2. Trace metal fractionation

Zn fractionation results (Fig. S3) showed that the amended soil (port 1) had significantly higher (p-values <0.05) exchangeable fraction (20–54 %), higher reducible fraction (31–48 %) and lower oxidizable-residual fraction (2–46 %) than the other soil layers (4.5–14 %, 4–11

%, and 79-91 %, respectively). The exchangeable fraction in the amended soil increased, while the oxidizable-residual fraction decreased, from day 1 (20-32 % and 20-45 %, respectively) to day 28 (41-54 % and 13-2 %, respectively). Zn fractionation in the other soil layers was unaffected by experimental conditions (presence/absence of pharmaceutical) or time (p-value >0.05). Overall, these results show that Zn in the amended soil changed form over time, increasing its bioavailability and potential mobility, consistent with studies on biowaste and sewage sludge amendments (Gu and Bai, 2018; Zheljazkov and Warman, 2004). This contrasts with previous studies on Zn aging in soils (Sayen et al., 2009; Sayen and Guillon, 2014, 2010), which reported a decrease in exchangeable forms as Zn redistributed to more strongly bound fractions. These findings highlight the distinct temporal behaviour of Zn in natural soils versus in digestate-amended soils. The increase in Zn bioavailability over time can be attributed to its high affinity toward Fe and Mn oxides, a process favoured under alkaline pH conditions, and its tendency to form complexes with labile organic molecules (Bradl, 2004), which can be more easily available or break with time due to organic degradation. This behaviour may also explain the increase in exchangeable Zn for E-Met and E-Lmt, as Zn might be in competition with the pharmaceutical compounds for soil adsorption sites (Baldasso et al., 2024).

Cu fractionation results (Fig. S4) showed that the amended soil had a higher reducible fraction (4.3-16 %) and a lower oxidizable-residual fraction (82-94 %) than the other soil layers (1.6-3.7 % and 93-97 %, respectively; p-value <0.05). This agrees with past studies finding Cu availability and mobility to be linked to soil's organic matter content (Gu and Bai, 2018). Cu tends to form very stable complexes with organic matter especially in alkaline environments (Zheljazkov and Warman, 2004) and has great affinity for silicate mineral groups, all of which lower Cu availability and mobility. The loamy sand soil used had 3.4 %dw organic matter content of and high silicate minerals content, explaining the predominance of Cu in the oxidizable-residual fraction. Moreover, these fractions in the soil layers were not affected by time contrarily to those in the amended soil. Cu reducible fraction increased from day 1(4.3-7.3 %) to day 14 (14-16 %) at the expense of Cu oxidizable-residual fraction. This can be caused by organic matter mineralization, leading to the release of Cu metallic cations that can readily react with available metal oxides (Adriano, 2001). Moreover, the presence of metformin and lamotrigine did not affect Cu fractionation along the soil profile most likely due to the synergistic adsorption behaviour observed between Cu and the two pharmaceutical compounds (Baldasso et al., 2024).

Pb fractionation (Fig. S5) showed that the amended soil had a higher reducible fraction (31-92 %) and a lower oxidizable-residual fraction (3.8-64 %) than the other soil layers (3.0-20 % and 77-96 %, respectively; p-value <0.05). Moreover, Pb distribution was not significantly affected by the presence of pharmaceuticals, most likely due to Pb's greater affinity toward Fe/Al/Mn oxides, organic matter, silicate minerals and its possible precipitation at alkaline pH (Baldasso et al., 2024). In the amended soil layer, the exchangeable and reducible fractions increased from day 1(0.73 % and 49 %) to day 28 (2.7 % and 73 %, respectively). While the oxidizable-residual fraction decreased from day 1 (50 %) to day 28 (25 %). This contrasts the findings reported in Baldasso et al. (2023), who found that Pb distribution in the amended soil layer was constant over time with Pb found predominantly in the oxidizable-residual fraction. Similarly, Pb fractions in the other soil layers were also affected by time (p-value <0.05). The exchangeable and reducible fractions increased from day 1 (1.0 and 5.0 %) to day 28 (2.6 and 12 %, respectively). While the oxidizable-residual fraction decreased from day 1 (94 %) to day 28 (85 %). It is known that Pb behaviour in soil is affected by different mechanisms, predominantly adsorption onto specific soil and clay minerals characterized by Mn, Fe and Al oxides for which Pb exhibits the strongest affinity (Bradl, 2004). The soil used in the experiment presented high contents of quartz and kaolinite, with minor contents of calcite and muscovite, providing

different reactive solid surfaces, especially Al-oxides. Pb adsorption onto Al-oxides is known to be characterised by two phases: a first quick reaction on free surface site and a second reaction governed by diffusion in the mineral particles and lower site affinity (Bradl, 2004). This mechanism coupled with organic matter mineralization can explain the increase in Pb reducible fraction through time, with the simultaneous reduction of oxidizable-residual fraction. (Adriano, 2001).

#### 3.1.3. Trace metal mobility factor

The trace metal mobility factor (MF) was calculated for all soil column layers through time to better understand the potential mobility of trace metals along the soil profile. The MF was calculated using relative contents of the bioavailable metal fractions following equation (1) (Kabala and Singh, 2001).

$$MF = \frac{Exchangeable}{(Exchangeable + Reducible + Oxidizable \& Residual)} *100$$
(1)

The MF results are reported in Fig. 1, with detailed data provided in Table S4. Zn MFs were consistently higher compared to Cu and Pb across all experimental conditions. Zn MFs decreased trend with depth and increased with time in all experimental conditions. In the digestate-amended soil, Zn MF increased from 20 to 32 % on day 1–41–54 % on day 28, while in the other layers it decreased slightly from 7.4 to 12 % on day 1–4.5–8.3 % on day 28. Cu MF remained relatively stable, ranging from 1.2 to 2.3 % in digestate-amended soil and 1.3–2.2 % in the other soil layers, with no clear trend over time. On the other hand, Pb MFs increased with time across all the soil column layers. In the digestate-amended layer, it increased from 0.7 to 0.8 % on day 1 to 0.9–3.4 % on day 28, while in the other layers it increased from 0.6 to 1.7 % on day 1–1.4–3.8 % on day 28.

Overall, the digestate amendment appears to have increased the trace metals' potential mobility in the amended soil over time compared to the deeper soil layers, enhancing their bioavailability. Zn was found to be the most mobile element, posing greater environmental risk, followed by Cu and then Pb, generally agreeing with past studies (Kabata-Pendias and Pendias, 2001; Ogundiran and Osibanjo, 2009).

However, the low increase of total metal concentrations and of exchangeable fractions in the deeper soil layers due to digestate amendment suggests that the mobility of these metals may not represent a significant risk. Over a longer period, these trace metals could potentially migrate to deeper soil layers. Further long-term experiments (e.g, 1 year or more), with molecular studies, are needed to fully understand the aging process of these trace metals in digestate-amended soils, as change in the soil's physical and chemical properties over time (e.g., pH, redox conditions, content of OM, complexation with organic molecules, microbial activity) can significantly influence metal mobility and bioavailability. For instance, an acidic environment increases the solubility of many trace metals and may lead to dissolution of metal-containing minerals, which increases their concentration in the soil solution, thereby contributing to their mobility and availability (Kabata-Pendias and Pendias, 2001). Additionally, the tested concentrations of metformin and lamotrigine did not significantly affect the fate and distribution of these trace metals within the soil system. However, higher pharmaceutical concentrations or the presence of other organic contaminants could alter the behaviour of the trace metals, warranting further investigation into potential cumulative effects and their impact on environmental risk.

#### 3.2. Soil microbial community

Initial soil (collected on day 0) and soil samples taken below the digestate amended soil layer (port 2 and 4) presented very low amounts of eDNA, confirming that the soil used was poor in terms of living organisms (Table S5). This high difference in eDNA between the amended and non-amended soil demonstrated a key role of the digestate amendment in increasing the microbial abundance in the selected soil. The low amount of eDNA obtained in the non-amended soil prevented an effective amplification of the extracted DNA, compromising the prokaryotic community analysis for these samples. Therefore, the results reported below focus only on the prokaryotic community profile of the initial digestate and the digestate amended soil over time.

A total of 2,947,832 raw sequences were obtained from Illumina



Fig. 1. Trace metal mobility factor (MF) of Zn, Cu and Pb for the digestate-amended soil (line with full circle) and the natural soil layers (line with empty circle), for all experimental conditions tested. Digestate (E-Dig) - light blue, digestate spiked with metformin (E-Met) – yellow, and digestate spiked with lamotrigine (E-Lmt) – dark blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

MiSeq sequencing, ranging from 35,181 to 211,092 sequences per sample, with an average of 89,328 sequences. A total of 1,926,007 cleaned sequences were obtained at the end of the upstream workflow, ranging from 23,182 to 141,030 sequences per sample with an average of 58,363 sequences, corresponding to 65 % of the average input (Supporting Information, Table S6).

### 3.2.1. Prokaryotic community richness and diversity

Rarefaction analysis, based on ASVs, showed that sequencing reached saturation for all samples, confirming good coverage of the community diversity (Fig. S6).

The prokaryotic communities' richness and alpha-diversity results (Figs. S7–A, Table S7 and Table S8) revealed that the abundance of ASVs per sample matched the Chao index, indicating similar species richness across all samples. Their close correspondence suggests good representation of the rare biosphere, indicating that additional sampling would have unlikely revealed other unique species. However, it is important to consider that the Chao index provides an estimate, and further studies could provide additional insights into the rare species dynamics giving additional information on overall community structure. Moreover, neither experimental condition nor time significantly affected species richness (p-value >0.05). Shannon and Inverse Simpson indices showed an increase in the community's alpha-diversity over time (p-value <0.05), indicating growing community diversity.

The beta-diversity (Figs. S7–B and Table S9) indicates a clear prokaryotic community separation, significantly affected by time (p-value <0.05). Samples collected at the beginning of the experiment (day 0) and on day 1 formed one cluster, while samples from day 14 and day 28 formed another. These findings show that digestate amendment clearly increased the microbial abundance of the soil. Other studies have shown other types of digestate also stimulated prokaryotic growth and community abundance in soil (Caracciolo et al., 2015; Walsh et al., 2012).

The microbial community's structure in general increased diversity over time. This may indicate that part of the microorganisms introduced by the digestate (e.g., anaerobic microorganisms) were gradually replaced by other microorganisms more suited to the new environmental conditions (e.g., aerobic microorganisms). Previous studies observed that part of the dominant prokaryotes from digestate are not able to permanently colonize the soil (Coelho et al., 2020; Johansen et al., 2013; Pathan et al., 2021). Also, no clear effect of the added pharmaceuticals on the prokaryotic community structure or diversity was observed, despite the acknowledged potential of microorganic contaminants to influence the soil microbial communities (Alexandrino et al., 2017; Bôto et al., 2023; Duarte et al., 2019). This could be attributed to various factors, such as the pharmaceuticals' concentration, the physico-chemical properties of the digestate-amended soil (such as pH, mineral content and organic matter content), pharmaceuticals adsorption behaviour (Baldasso et al., 2024), and potential interactions with trace metals that alter their bioavailability (Fernandes et al., 2022). Moreover, microorganisms originating from the digestate might have high adaptability and contaminant degradation potential due to their continuous exposure to a multitude of contaminants (Rahman et al., 2021).

# 3.2.2. Prokaryotic community taxonomic composition

On average 99.7 % of ASVs belonged to bacteria domain, while <0.15 % to archaea domain and <0.13 % to unclassified species (Supporting Information, Table S10).

For the archaea domain, the most abundant archaeal groups were the Methanobacteriaceae and Methanomicrobiaceae family followed by those of Nitrososphaeraceae and Methanomethylophilaceae. These families have been detected in different digestates and digestate amended grasslands and peatlands (Cadillo-Quiroz et al., 2014; Coelho et al., 2020; Roopnarain et al., 2023). The abundance of these archaea groups (Table S11), calculated based on the total archaea abundance, varied between the digestate and the amended soil samples. The most abundant

genera of the Methanobacteriaceae and Methanomicrobiaceae families were *Methanobacterium* and *Methanoculleus*, respectively. These organisms live in anaerobic conditions and can potentially increase the soil's methane emission through hydrogenotrophic methanogenesis pathways, specifically in soils favouring anaerobic condition (e.g., peatlands) (Cadillo-Quiroz et al., 2014), however this was not the case for the current study which utilized a loamy sand soil.

Considering the bacteria domain, the samples' taxonomic composition was evaluated by comparing the relative abundance of reads on phylum and genus basis. Results at phylum level showed that the initial digestate, initial amended soil and samples collected on day 1 had similar bacterial community composition with the main phyla being Firmicutes, Proteobacteria, Bacteroidota, Caldatribacteriota and Actinobacteriota (Fig. 2-A). For the samples collected on day 14 and day 28 for all experimental conditions, a similar bacterial composition was observed with the main phyla being Proteobacteria, Bacteroidota, Firmicutes, Actinobacteriota, Planctomycetota, Deinococcota, Patescibacteria and Desulfobacterota. Over time a clear decrease in the phyla Firmicutes and Caldatribacteriota was observed. On the contrary, a clear increase in Proteobacteria was observed, while other phyla like Planctomycetota, Deinococcota, Patescibacterota increased their relative abundance to values on average above 2 %.

The bacterial communities' composition in digestate are known to be intrinsically dependent on the operational conditions of the anaerobic digestion process, the feed material and the inoculum used (Coelho et al., 2021). Other studies also observed that Firmicutes, Bacteroidota and Proteobacteria were the dominating and the most metabolically active phyla in fresh digestate/compost. Moreover, a growth of Actinobacteria, Planctomycetota and Deinococcota with organic amendment aging has been reported (Coelho et al., 2020; Roopnarain et al., 2023; Zhao et al., 2022). Firmicutes and Bacteroidota (fermenting bacteria) are known to have a fundamental role in organic matter mineralization (Zhang et al., 2022). Firmicutes are more stress-resistant and capable of using recalcitrant C sources, while Bacteroidota are more environment-sensitive and mainly use labile organic matter sources (Naylor et al., 2022; Zhang et al., 2022; Zhao et al., 2022). These characteristics, together with a change from anaerobic to aerobic conditions, can explain the decrease in Firmicutes and the increase in Bacteroidota in amended soil samples. Moreover, Proteobacteria and Actinobacteria are known to have key roles in soil carbon, nitrogen, and sulphur cycling, the same as Planctomycetota, Patescibacteria and Desulfobacterota that have been positively correlated with soil C/N ratio (Zhao et al., 2022). All these phyla that presented a tendency to increase their relative abundance after a period of soil stabilization (around 14 days), have already been described as characteristic phyla of soil bacteria communities (Naylor et al., 2022).

This variation in bacterial community composition was also found at the genus level (Fig. S8). Most of the genera found have been detected also in other organic amendments and amended soils (Coelho et al., 2020). The initial digestate, the initial amended soil and samples of day 1 had similar taxonomic pattern of the top 10 genera (Fig. 2-B), which was different from those in day 14 and day 28 samples, which were similar to one another. Furthermore, the phyla of the top 10 genera confirmed the prokaryotic community composition found at the phylum level, with Fastidiosipila and Tepidimicrobium of the Firmicutes phylum being the dominant genera of initial and day 1 samples, and Pusillimonas, Thiopseudomonas, Aquamicrobium and Pseudomonas of the Proteobacteria phylum being the dominant genera of day 14 and day 28 samples. Fastidiosipila and Tepidimicrobium are known to be characteristic genera of the meso/thermophilic AD process, with the first being important for the conversion of complex organic molecules to volatile fatty acids and carbon dioxide and the second being important in the hydrogenotrophic pathways of methanogenesis (Fermoso et al., 2019; Roopnarain et al., 2023). Their functions highlight their AD origins and explain why they were found in great abundance in the digestate samples. Moreover, these genera could lead to potential soil greenhouse gas emissions (carbon



Fig. 2. (A) Taxonomic profiles at phylum level (phyla  $\geq$ 2% relative abundance) and (B) taxonomic profiles of the top 10 genera of the bacterial communities of initial digestate, initial amended soil and samples taken from the first soil column layers through time (day 1, day 14 and day 28) for the different experimental conditions (without (D) or with spiking of lamotrigine (D-LMT) or metformin (D-MET).

dioxide and methane) when found in favourable anoxic conditions, especially after heavy precipitation events with low soil drainage (Naylor et al., 2022). *Pusillimonas, Thiopseudomonas, Aquamicrobium* and *Pseudomonas* are all gram-negative bacteria that have been detected in diverse soil and engineered environments (wastewater treatment plants, anaerobic digestors, biofilters, etc). Amongst these genera, *Pseudomonas* is known to be a plant-growth-promoting genus (Pathan et al., 2021), while *Aquamicrobium* and *Pusillimonas* are known to have pollutant-degrading capacities specifically towards hydrocarbons and petroleum derived compounds (Wang et al., 2021). Additionally, *Rhodopirellula* (Žure et al., 2017) and *Actinomyces* (Nazari et al., 2022) can contribute to the global carbon and nitrogen cycles, and several other processes, *Sporosarcina* (Janarthine and Eganathan, 2012) and

Leucobacter (Zhu et al., 2022) are plant growth promoting species, Marinobacter (Raddadi et al., 2017) and Taibaiella (Kim et al., 2016) can have a role in biogeochemical cycling of organics and metals, and Paenacalcigenes (Mitzscherling et al., 2022) is proposed as a potential plastic-polymer degrader. In addition, Paenibacillus, Iamia and Streptococcus are considered important metal biosorbents that have been applied successfully in contaminated soils; they have been found in the amended soil samples suggesting they can contribute to the immobilization of metals originating from the digestate, reducing their threat towards the soil environment (Rana et al., 2021). The observed variations in bacterial composition, particularly with the increase of the above-mentioned genera, likely played a role in modulating the mobility and bioavailability of trace metals in the digestate amended soil (Giller et al., 1998; Gadd, 2010; Violante et al., 2010). These genera could have impacted the state of trace metals in several ways, such as through the release of certain metabolites and organic acids, which could interact with trace metals via complexation or chelation reactions altering the metals' mobility (Kim et al., 2016). Additionally, their ability to degrade complex organic compounds could release trace metals into the soil solution, increasing their solubility and bioavailability and thus promoting their biosorption (Wang et al., 2021). In fact, these genera are known to act as biosorbents and bioaccumulate trace metals, they can also induce metal precipitation ultimately enhancing their immobilization in the soil matrix (Raddadi et al., 2017; Rana et al., 2021).

# 4. Conclusions

The present work showed that Cu, Pb and Zn, originating from nonsource-separated biowaste digestate, remained in the top amended soil layer during the experimental time. These metals in the soil layers were primarily found in the oxidizable-residual fraction over time, suggesting strong bonds with soil organic matter and mineral groups decreasing their mobility and preventing their redistribution among the other fractions. Contrarily, the metals in the top amended soil layer were found also in the exchangeable and the reducible fractions making them more bioavailable and potentially more mobile. However, the quantity of metals in these fractions was low diminishing the potential environmental impact of the amendment. Furthermore, Zn was the metal with greatest mobility potential followed by Cu and Pb. Zn and Pb mobility potential increased over time in the amended soil layer indicating a greater risk for metal leaching with aging. This is in contrast with nonamended soils where Zn mobility decreased with aging. So further investigation is needed to understand how digestate aging affects metal mobility and fate in soils. Additionally, digestate amendment led to an increase in microbial abundance in soil and a stabilization of the prokaryotic community after 14 days, that could favour plant growth, organic contaminant degradation and trace metal immobilization. The presence of metformin and lamotrigine, at the tested levels, had no influence on metal behaviour and soil prokaryotic community structure. Overall, non-source-separated biowaste digestate proves to be a valuable resource for soil remediation given its metal sorption and immobilization capacity and its positive effect on soil microbial abundance. When coupled with other strategies, as plant cover, it can effectively remediate marginal land, boosting local bioeconomies and empowering communities.

#### CRediT authorship contribution statement

Veronica Baldasso: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Maria Paola Tomasino: Writing – review & editing, Methodology. Stéphanie Sayen: Writing – review & editing, Conceptualization. Emmanuel Guillon: Writing – review & editing, Conceptualization. Luigi Frunzo: Writing – review & editing, Funding acquisition, Conceptualization. Carlos A.R. Gomes: Writing – review & editing, Supervision, Conceptualization. Maria João Alves: Resources. Ricardo Castro: Resources. Ana Paula Mucha: Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. C. Marisa R. Almeida: Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

#### Associated content

The supporting information for this chapter is reported in Supporting Information. This content includes detailed data (in table and figure format) supporting the *Results and discussion* section in the main text, in addition it includes details on soil, digestate and amended soil characterization, the total metal concentration and fractionation results of initial solid matrices, details of statistical analysis performed and details on the soil microbial community characterisation.

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# Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maria Joao Alves reports a relationship with Tratolixo - Tratamento de Resíduos Sólidos, E.I.M., S.A that includes: employment. Ricardo Castro reports a relationship with Tratolixo - Tratamento de Resíduos Sólidos, E.I.M., S.A that includes: employment. If there are other authors, they 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.2025.125961.

### Data availability

Data will be made available on request.

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