

Phytoremediation of contaminated soils aiming metal recovery – including microorganisms’ role and bioaugmentation possibilities

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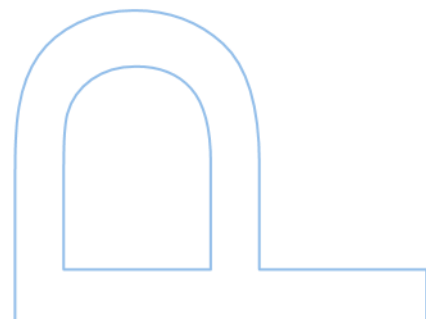
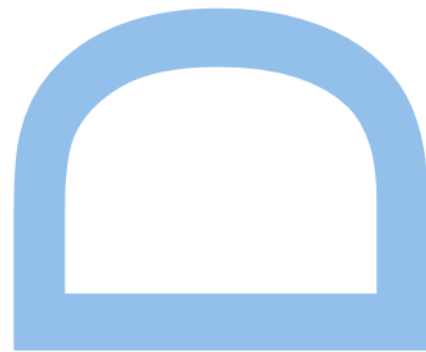
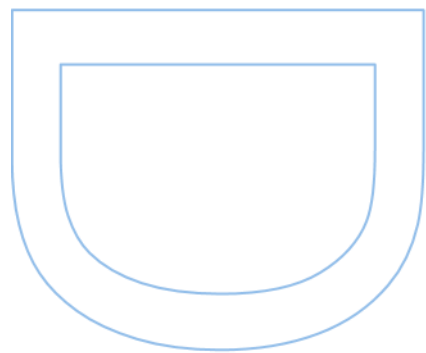
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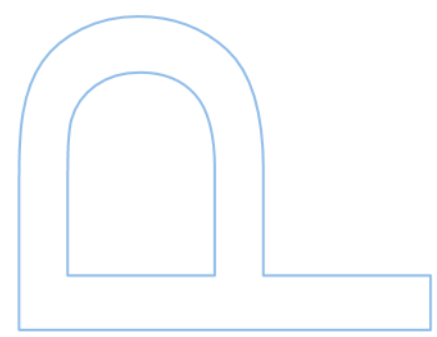
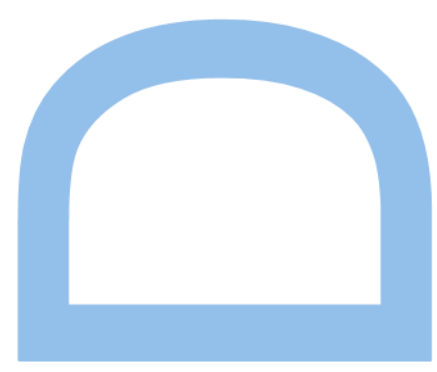
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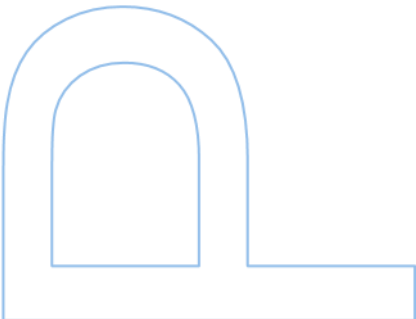
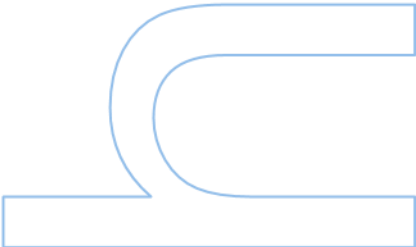
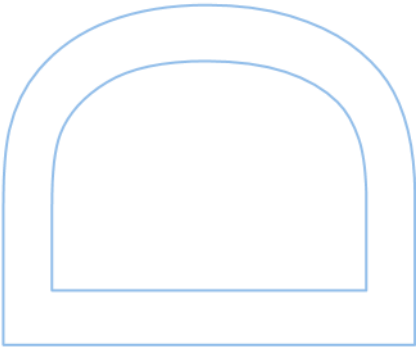


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Metal-Micro exploitation



Dedicated to my loving family and friends

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Resumo

A degradação do solo ameaça a segurança alimentar global, a saúde dos ecossistemas e o desenvolvimento sustentável. Simultaneamente, o crescimento populacional intensifica o consumo de recursos, a geração de resíduos e a procura por energia limpa. A digestão anaeróbica emerge como uma estratégia crucial para a produção sustentável de energia e a estabilização de resíduos. O seu subproduto, o digerido, revela potencial como condicionador de solo, no entanto, persistem incertezas ambientais, especialmente em relação aos metais vestigiais (TMs). Estratégias de fitogestão utilizando plantas bioenergéticas são soluções ecologicamente responsáveis que podem ser utilizadas para destoxificar, reduzir a mobilidade ou extrair TMs dos solos após a adição de digerido. Ao mesmo tempo, as plantas e os microrganismos associados podem beneficiar das propriedades fertilizantes do digerido e melhorar ainda mais a qualidade do solo. Em última análise, a biomassa vegetal pode ser reaproveitada como uma valiosa matéria-prima no processo de digestão anaeróbica. A presente tese investiga a influência do digerido num solo marginal, avaliando o potencial de fitogestão de duas espécies de plantas bioenergéticas, *Panicum virgatum* e *Pennisetum alopecuroides*, para TMs. Cada capítulo aborda aspetos cruciais, incluindo a mobilidade e biodisponibilidade de metais, a exploração das comunidades microbianas do solo e a avaliação do potencial de produção de biometano a partir da biomassa das plantas utilizadas. Ao abordar de forma abrangente essas questões, a tese visa contribuir para a compreensão do impacto do digerido no solo marginal e apresentar uma valiosa orientação para otimizar a fitogestão de TMs.

Os principais resultados destacam a eficácia do digerido de RSU (Resíduos Sólidos Urbanos) na melhoria da matéria orgânica do solo (OM) e no teor de nutrientes, promovendo o crescimento tanto de *P. virgatum* quanto de *P. alopecuroides*. Ao mesmo tempo, essas plantas mitigaram a migração de TMs presentes no digerido através do perfil do solo. As medições feitas na solução do solo e através de Gradientes Difusivos em Filmes Finos (DGT) surgem como as técnicas adequadas para avaliar a biodisponibilidade de TMs. Os resultados indicam que a OM pode influenciar significativamente o comportamento dos TMs em solos suplementados com digerido geridos por plantas bioenergéticas. Além disso, as plantas bioenergéticas cultivadas na

presença de digerido demonstram uma produção aumentada de biometano em comparação com aquelas cultivadas sem suplementação de digerido. Este trabalho de investigação constitui uma base sólida na procura de abordagens sustentáveis e ecológicas para a gestão do solo e a produção de energia renovável, com potenciais aplicações para mitigar problemas ambientais associados à utilização de digerido.

Palavras-chave: restauração de solos marginais, digerido, metais vestigiais, espécies de plantas bioenergéticas, fitogestão, produção de bioenergia.

Abstract

Soil degradation endangers global food security, ecosystem health, and sustainable development. Simultaneously, population growth intensifies resource consumption, waste generation, and the demand for clean energy. Anaerobic digestion emerges as a pivotal strategy for sustainable energy production and waste stabilization. Its by-product, digestate, exhibits potential as a soil conditioner, yet environmental uncertainties persist, notably regarding trace metals (TMs). Phytomanagement strategies using bioenergy crops are ecologically responsible solutions that can be used to detoxify, reduce mobility or extract TMs from soils after digestate amendment. At the same time, the plants and associated microorganisms can benefit from fertilizing properties of digestate and further enhance the soil quality. Ultimately, plant biomass can be repurposed as a valuable feedstock in the anaerobic digestion process. The thesis investigates the influence of digestate on marginal soil, evaluating the phytomanagement potential of two dedicated non-food bioenergy crops, *Panicum virgatum* and *Pennisetum alopecuroides*, for TMs. Each chapter addresses crucial aspects, encompassing metal mobility and bioavailability, exploration of soil microbial communities, and the assessment of potential biomethane production from the biomass of plants employed. By comprehensively addressing these questions, the thesis aims to contribute to the understanding of digestate impact on marginal soil and present a valuable roadmap for optimizing the phytomanagement of TMs with dedicated bioenergy crops.

The main findings underscore the effectiveness of digestate in enhancing soil organic matter (OM) and nutrient content, fostering the growth of both *P. virgatum* and *P. alopecuroides*. Concurrently, these plants mitigate the migration of digestate-borne TMs through the soil profile. Soil solution and Diffusive Gradients in Thin Films (DGT) measurements emerge as the optimal techniques for evaluating TM bioavailability. The results indicate that OM could significantly influence TM behavior in digestate-amended soils managed by bioenergy crops. Furthermore, bioenergy crops grown in presence of digestate demonstrate heightened biomethane production compared to those grown without digestate supplementation. This research serves as a solid foundation in our pursuit of sustainable and eco-friendly approaches to soil management and renewable energy production, with potential applications for mitigating environmental concerns associated with digestate usage.

Key words: restoration of marginal soils, digestate, trace metals, bioenergy crops, phytomanagement, bioenergy production.

Résumé

La dégradation des sols menace la sécurité alimentaire mondiale, la santé des écosystèmes et le développement durable. La croissance démographique intensifie simultanément la consommation de ressources, la génération de déchets et la demande en énergie propre. La digestion anaérobie émerge comme une stratégie cruciale pour la production d'énergie durable et la stabilisation des déchets. Son sous-produit, le digestat, présente un potentiel en tant qu'améliorant de sol, mais des incertitudes persistent sur le plan environnemental, notamment en ce qui concerne les métaux traces (MTs). Les stratégies de phytomanagement utilisant des cultures énergétiques sont des solutions écologiquement responsables qui peuvent être employées pour détoxifier, réduire la mobilité ou extraire les MT des sols après l'amendement au digestat. Dans le même temps, les plantes et les microorganismes associés peuvent bénéficier des propriétés fertilisantes du digestat et améliorer davantage la qualité du sol. En fin de compte, la biomasse végétale peut être réutilisée en tant que matière première précieuse dans le processus de digestion anaérobie. La thèse explore l'influence du digestat sur les sols marginaux, évaluant le potentiel de phytomanagement de deux cultures bioénergétiques dédiées, *Panicum virgatum* et *Pennisetum alopecuroides*, pour les MTs. Chaque chapitre aborde des aspects capitaux, englobant la mobilité et la bio disponibilité des MTs, l'exploration des communautés microbiennes du sol, et l'évaluation du potentiel de production de méthane à partir de la biomasse des plantes utilisées. En abordant de manière approfondie ces questions, la thèse vise à contribuer à la compréhension de l'impact du digestat sur les sols marginaux et à présenter une feuille de route précieuse pour optimiser le phytomanagement des TM avec des cultures bioénergétiques dédiées.

Les principales conclusions soulignent l'efficacité du digestat pour améliorer la matière organique du sol (OM) et le contenu en nutriments, favorisant ainsi la croissance à la fois de *P. virgatum* et de *P. alopecuroides*. Parallèlement, ces plantes atténuent la migration des MTs transportés par le digestat à travers le profil du sol. Les mesures de la solution du sol et les mesures de Gradient de Diffusion en Couche Mince (DGT) émergent comme les techniques optimales pour évaluer la biodisponibilité des Ms. Les résultats indiquent que la OM pourrait influencer significativement le comportement des MTs dans les sols amendés par le digestat et gérés par des cultures énergétiques. De plus, les cultures à vocation énergétiques cultivées en présence de digestat montrent une production de méthane accrue par rapport à celles cultivées sans supplémentation en digestat. Cette recherche constitue une base solide dans notre objectif d'approches

durables et respectueuses de l'environnement en matière de gestion des sols et de production d'énergie renouvelable, avec des applications potentielles pour atténuer les préoccupations environnementales liées à l'utilisation du digestat.

Mots-clés : restauration des sols marginaux, digestat, métaux traces, cultures bioénergétiques, phytomanagement, production de bioénergie.

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List of Abbreviations

AAS	Atomic absorption spectrometry
AD	Anaerobic digestion
Al	Aluminium
As	Arsenic
B	Cumulative specific methane production
Bmax	Ultimate methane production
BCF	Bio-concentration factor
BCR	EU community bureau of reference
Ca	Calcium
Cd	Cadmium
CEC	Cation exchange capacity
CH ₄	Methane
Cl	Chlorine
cmolC Kg ⁻¹	Centimoles charge per kilogram (units of cation exchange capacity)
Co	Cobalt
CO ₂	Carbon dioxide
Cr	Chromium
Cu	Copper
DGT	Diffusive gradients in thin films technique
DOC	Dissolved organic carbon
DNA	Deoxyribonucleic acid

DOM	Dissolved organic matter
dw	Dry weight
EEM	Excitation emission matrix
Eh	REDOX potential
EPS	Extracellular polysaccharide substances
ETAAS	Atomic absorption spectrometry with Electrothermal atomization
EU	European union
FAAS	Atomic absorption spectrometry with Flame atomization
FAO	Food and agriculture organization of the United Nations
FCUP	Faculty of Sciences of the University of Porto
fe	Elution factor
Fe	Iron
FTIR	Fourier-transform infrared spectroscopy
H	Hydrogen
h	Hour(s)
HCl	Hydrochloric acid
HF	Hydrofluoric acid
HIX	Humification index
HNO ₃	Nitric acid
HRT	Hydraulic retention time
HSO ₄	Sulfuric acid
ICP-MS	Inductively coupled plasma mass spectroscopy
ISO	International organization for standardization
ITS	Internal transcribed spacer
ITPS	Intergovernmental technical panel on soils
IUPAC	International union of pure and applied chemistry
K	Specific rate constant or apparent kinetic constant
K	Potassium
KCl	Potassium chloride
Ksv	Stern–Volmer constant
LOD	Limit of detection
LOI	Loss on ignition (%)
M	Molar mass
mg·Kg ⁻¹	Milligrams per kilogram

mg·L ⁻¹	Milligrams per liter
Mg	Magnesium
mm	Millimeter
mM	Millimolar
Mn	Manganese
MSW	Municipal solid waste
N	Nitrogen
Na	Sodium
NaCl	Sodium chloride
NaH ₂ PO ₄	Monosodium phosphate
NaOH	Caustic soda
Na ₃ PO ₄	Trisodium phosphate
Ni	Nickel
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
OM	Organic matter
P	Phosphorus
PCR	Polymerase chain reaction
	Phosphorus pentoxide
P ₂ O ₅	
Pb	Lead
PO ₄ ³⁻	Phosphate
pH	-log ₁₀ (Hydronium ion activity)
PVC	Polyvinylchloride
rRNA	Ribosomal ribonucleic acid
S	Sulphur
SOC	Soil organic carbon
SO ₄ ²⁻	Sulphate
SSAD	Sewage sludge anaerobic digestate
TOC	Total organic carbon
TM	Trace metal
UP	University of Porto
EPA	United States Environmental Protection Agency
VFAs	Volatile fatty acids
WHO	World health organization

XRD	X-ray diffraction (analysis of crystal structures)
XRF	X-ray fluorescence (spectrometry)
Zn	Zinc
$\mu\text{g}\cdot\text{g}^{-1}$	Microgram per gram (ppm)

1. Chapter 1: Introduction

1.1 Understanding global soil status and issues with soil quality

The soil, often overlooked and underestimated, is a finite and vital resource that plays a crucial role in sustaining biodiversity, clean water, agricultural productivity, and environmental resilience. Soil is facing unprecedented challenges worldwide mainly due to anthropogenic activities, climate change, and unsustainable land management practices.

The human population is predicted to reach 9.7 billion in 2050 and to increase further to 10.9 billion by the end of the century (D. Gu et al., 2021). Estimates of global food demand based on these population forecasts and on expected dietary shifts indicate that food production in 2050 will need to increase by 40-70 percent compared to 2010 (ITPS, 2015). With this estimation, agricultural soils will have to maintain and improve their productivity to ensure food security (Molotoks et al., 2018). Most of this growth will occur in low-income countries, many of which have infertile soils and low levels of agricultural productivity. Furthermore, under the increasing pressures of a changing climate, healthy soils will be needed to build resilience to floods and drought and help carbon sequestration. In the case of the European Union, 60-70% of soils are considered unhealthy (EU, 2021), prompting a mission to ensure that at least 75% of soils in each EU Member State are healthy or significantly improved by 2030 to support ecosystem services.

Some of the most significant threats to soil function at the global scale are soil erosion, loss of soil organic carbon (SOC), and nutrient imbalance. Careful soil management can increase the food supply and provide a valuable lever for climate regulation and a pathway for safeguarding ecosystem services. The Intergovernmental Technical Panel on Soils (ITPS) believes that (i) stabilizing or increasing global SOC through locally appropriate practices and (ii) rehabilitating marginal and degraded lands, are two of the main strategies to follow that will help address decreasing soil quality, food insecurity and avoid further environmental and land degradation (ITPS, 2015). As outlined by Lal (2009), a key strategy for enhancing SOC levels involves the reintegration of biosolids onto land and fostering biomass growth (Figure 1). Therefore, research endeavors should concentrate on optimizing these practices to maximize their efficacy and environmental safety in improving soil health and productivity. By prioritizing these approaches, we can establish a foundation for sustainable land management practices

and alleviate the detrimental impacts of soil degradation on global food security and environmental sustainability.

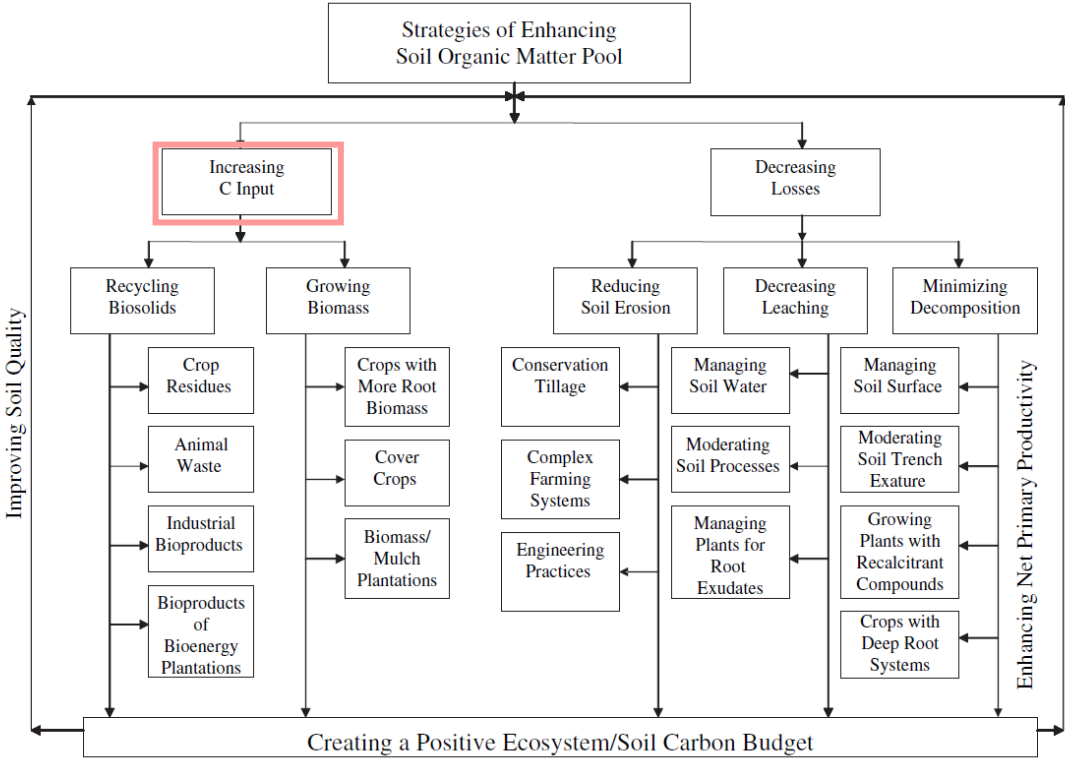


Figure 1. Strategies of improving and maintaining the soil organic matter pool. Reprinted from Lal (2009).

1.2 Digestate for marginal soil reclamation

1.2.A Origin of digestate: Anaerobic digestion

The anaerobic digestion (AD) process consists in the degradation and stabilization of organic materials (such as industrial and municipal wastewaters, sludge, solid wastes, manures, agricultural residues, and energy crops) (Figure 2) under anaerobic conditions by a microbial consortium. This process comprises four stages: hydrolysis, acidogenesis, acetogenesis/dehydrogenation, and methanation. In the hydrolysis stage, large compounds like carbohydrates, fats, and proteins are degraded into simpler components by hydrolytic-fermentative bacteria, leading to the production of sugars, amino acids, and fatty acids. Through acidogenesis these compounds are converted into volatile fatty acids by fermentative bacteria. Hydrogen-producing acetogenic bacteria degrade volatile fatty acids into acetate, hydrogen, and carbon dioxide (CO₂) during the acetogenesis phase. Methane-forming archaea (methanogens) produce methane (CH₄) and carbon dioxide (CO₂) in the final methanation phase. The degradation occurs in digesters optimized for microbial activity, considering factors like mixing, temperature, and pH. Digesters are classified according to the feeding pattern (i.e. batch, continuous, semi-continuous) (De Vrieze et al., 2013; Hossain et al., 2022; Wu et al., 2024), the number of stages (single stage or two stages, i.e. acidogenic and methanogenic) (Cremonez et al., 2021; Ward et al., 2008) and the temperature of the process (psychrophilic, <30 °C, mesophilic, 30–40 °C, or thermophilic, 50–60 °C conditions) (Nie et al., 2021), and the fluid-dynamic (i.e. plug flow, completely mixed, hybrid) (Hurtado et al., 2015; Sadino-Riquelme et al., 2018; P. Wei et al., 2023), which in turn depend on feedstock concentration and condition (wet or dry conditions). The resulting biogas, composed mainly of CH₄ (55–75%) and CO₂ (25–45%) (and small amounts of water vapor, traces of H₂S, NH₃ and H₂, and possibly other contaminants like siloxanes), is considered a clean biofuel suitable for transportation or injection into the gas grid after purification. Alternatively, it can be converted into heat and electricity using a combined heat and power system, the primary exploitation method in European agricultural AD plants.

The byproduct, digestate, consists of microbial biomass and undigested material, usually separated into liquid and solid fractions. The liquid fraction is rich in nitrogen (N) and potassium (K), while the solid fraction contains residual fibers and phosphorus. The use of digestate as fertilizer or soil improver is of interest due to economic and environmental benefits, offering an alternative to commercial fertilizers and contributing to nutrient recycling (Lamolinara et al., 2022; Monlau et al., 2015; Wang & Lee, 2021).

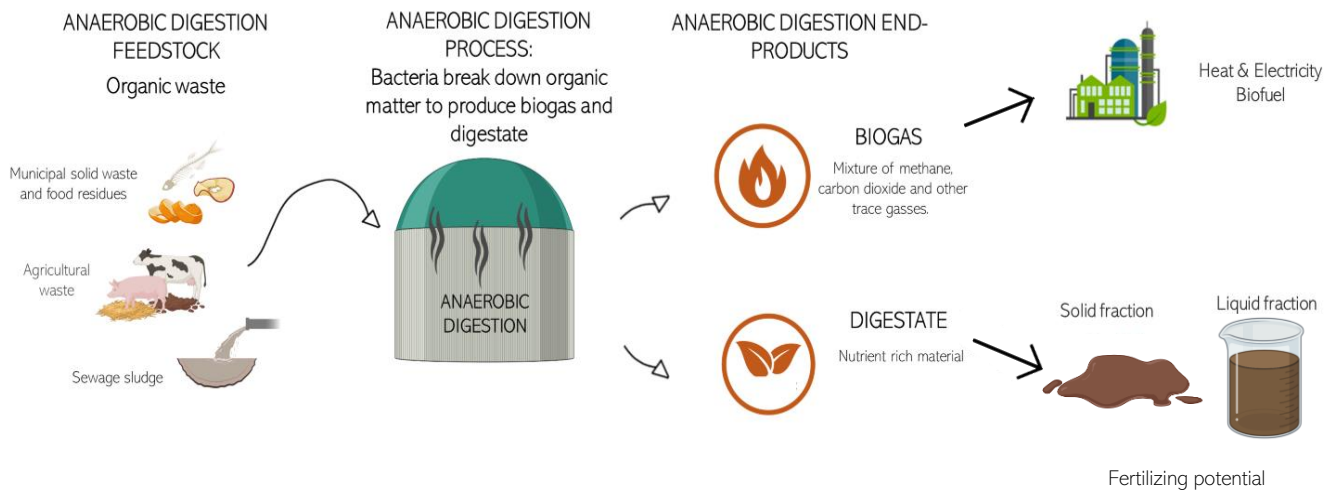


Figure 2. Infographic of the anaerobic digestion process and valuable byproducts. Figure created with Biorender.com License agreement number: GI254UKAL8.

1.2.A.1 Global status of anaerobic digestion

Due to the above-mentioned unprecedented population growth forecasts, it is expected that by 2050 the world will generate 3.4 billion tons of waste per year (Kaza et al., 2018). The management of this large volume of waste is a serious problem for environmental sustainability. In addition, a larger population is leading the planet towards further global challenges such as continuous uptrend of natural resource consumption and energy insecurity due to global dependence on fossil energy. Along with all significant environmental costs that this implies. Global energy consumption is estimated to rise to 739 quadrillion Btu in 2040 (Ahmad & Zhang, 2020; Capuano, 2018). AD has proven to be one of the most economical and effective technologies capable of addressing not only the increasing demand for improved waste treatment practices, but also immediate requirements for resources recovery and cleaner energy production (Atelge et al., 2020). Therefore, global status of AD is progressing rapidly. In recent years, there has been a strong development of AD units worldwide, particularly in countries like China, Germany, USA, Italy, UK, and France (Akhiar et al., 2020). These countries are leading in the biogas sector due to their long establishment, intensive research, and government incentives for renewable energy and waste management solutions. In the specific case of the European Union (EU) there were about 18,774 biogas plants in 2020 with more than 10 GW of installed electric capacity. According to the EBA Statistical Report 2021 (EBA, 2021) biogas and biomethane production amounted to 191 TWh in 2020 (18.0 bcm of energy) in Europe. Germany has the highest biogas production in Europe, with over 10,000 operational biogas plants (Liebetrau et al., 2019), followed by Italy with 1995

operating plants in 2016 (EBA, 2021; Pirelli et al., 2021), France (837), United Kingdom (715 plants) and Switzerland (634) (EBA, 2021). Most biogas plants in Germany are fed with substrates from agriculture (Liebetrau et al., 2019). Asian countries have shown increasing interest in biogas technology, and with ongoing research, the biogas sector in Asia is expected to grow in the coming years. India has a significant potential for biogas expansion, with an estimated biogas potential of 310 to 655 billion m³/year by 2040 (Mittal et al., 2019; Singh & Kalamdhad, 2023). China is a major biogas producer, with approximately 26.5 million plants in 2007. Biogas production in China mainly originates from sewage treatment, but there are also over 40 million domestic small biogas plants (L. Deng et al., 2017; Y. Deng et al., 2014; Song et al., 2014). The United States currently has over 2,400 operational biogas production sites, with over 15,000 new sites identified for development, across all states, including farms, water recovery facilities, food waste digesters, and landfill gas projects. The new biogas systems could generate 103 trillion KWh of electricity annually, significantly reducing greenhouse gas emissions (EPA, 2021). In the case of Australia, there were an estimated 242 digesters operating in 2017 (Carlu et al., 2019). Most of these plants are landfill and wastewater plants. Waste-based biogas production in Australia holds significant potential, estimated to become a £2.24 billion per year industry (WBA, 2018). With a large livestock and human population generating considerable biosolids and food waste, the potential biogas output is around 7.5 million m³. In South America, the adoption of AD has been growing in certain regions, especially in Brazil. In 2020, Brazil, produced about 1.83 billion Nm³ of biogas, with 37 units being under construction (Mühl & de Oliveira, 2022). Overall, approximately 79% of the AD plants in Brazil are used in the agricultural and livestock sector, although this sector only produces 11% of the total biogas produced in the country. The industrial sector only operates 78 CE plants (12%) but accounts for 16% of the total biogas production. There are only 57 waste treatment plants in the country (9% of the AD plants), producing 73% of the biogas in Brazil. Although African countries are still in the early stages of developing AD, there is growing interest, development, and implementation of this technology for renewable energy production and waste management solutions. National biogas programs have been implemented in several African countries, including Kenya, Uganda, Ethiopia, Tanzania, Rwanda, Cameroon, Burkina Faso, and Benin (Roopnarain & Adeleke, 2017). In addition, household biogas digester implementation is increasingly being promoted in rural areas of Asia, Africa, and South America (for example in countries such as China, India, Nepal, Vietnam, Cambodia, Bangladesh, Bhutan, Kenya, Rwanda, Bolivia, Colombia, and Peru) (Garfí et al., 2016).

With the increasing popularity of AD as a sustainable waste management and energy production solution, there is a concurrent surge in the generation of vast quantities of digestate. The escalating production of digestate requires careful and appropriate management strategies to address environmental, agricultural, and logistical challenges. Without effective management, the improper disposal or utilization of digestate could lead to adverse environmental impacts, such as nutrient runoff and soil and groundwater contamination. Hence, the development and implementation of robust management practices are imperative to harness the benefits of AD while mitigating potential risks, ensuring the fully sustainable integration of this technology into the circular economy scheme.

1.2.B Digestate composition and fertilizing properties

In general, digestate holds considerable potential as a nutrient-rich fertilizer. However, its composition and fertilizer value are highly influenced by factors such as the feedstock composition, inoculum source, AD operating conditions (e.g., pH, temperature, organic loading rate, hydraulic retention time), AD configuration (e.g., presence of post-fermenters), and the application of pretreatment on biomass fed to AD (Coelho et al., 2018; Monlau et al., 2015).

Digestate typically exhibits slightly alkaline pH values (>7.5), primarily resulting from the degradation of volatile fatty acids (VFAs), ammonia (NH₃) production during the process, and the addition of strong bases or carbonates to regulate pH and buffer capacity (Ward et al., 2008; Tambone et al., 2010). The organic matter (OM), total organic carbon (TOC), and ash content in digestate are highly variable. Labile organic components break down easily during AD, leading to a digestate enriched with recalcitrant organic compounds (Fernández-Domínguez et al., 2021; Insam et al., 2015; Nkoa, 2014; Tambone et al., 2009). Efficiency of OM conversion through mesophilic (35 °C) or thermophilic (55 °C) AD typically ranges from 13 % to 65 %, depending on substrate type, anaerobic reactor parameters (e.g., organic loading rate, hydraulic retention time), and feedstock pretreatment. Even after pretreatment, around 35 % of organic matter may remain in the digestate. The ammonium (N-NH₄⁺) content in digestate is linked to the initial total kjeldahl nitrogen (TKN) content in the feedstock. During AD, TKN transforms into soluble inorganic nitrogen, mainly N-NH₄⁺ and NH₃, with their balance dependent on temperature and pH. Higher pH and temperature result in a higher fraction of free ammonia. Digestates from highly degradable feedstocks (i.e. poultry and pig manure) are characterized by elevated N-NH₄⁺/TKN ratios and low C/N ratios, while lignocellulosic feedstocks that are low in N (i.e. sorghum, maize silage) lead to a low N-NH₄⁺/TKN ratio

in digestate. Digestates also contain other macro-nutrients (P, K, S), with increased availability due to the breakdown of easily degradable carbohydrates and conservation of lipids and recalcitrant lignin (Tambone et al., 2010). Also trace elements and micronutrients (S, Fe, Co, Ca, Mg, Se, Ni), including trace metals (TMs), are present. These TMs may originate from the initial feedstock or may be introduced as supplements to enhance digester performance (as described later). Recycling of OM and nutrients from digestate back to the soil is considered the most sustainable utilization of digestate (Peng & Pivato, 2019). Land application of digestate may be an effective strategy to improve marginal soil quality. Digestate may alter soil properties in different ways; directly through the intrinsic properties of the digestate, or indirectly, by changing soil physical, chemical and/or biological properties. Digestate has been proved to reduce soil's bulk density, boost SOC, increase saturated hydraulic conductivity, raise soil capacity of water retention, improve aggregate stability, influence positively on the soil microbial growth and biodiversity, correction of soil's acidity (Johansen et al., 2013; Lamolinara et al., 2022; Möller & Müller, 2012; Monlau et al., 2015; Nkoa, 2014; Panuccio et al., 2021; Slepiciene et al., 2020). Despite all its advantages as a soil improvement agent, there are many properties of the digestate that may limit its use, such as presence of organic contaminants, emerging contaminants, pathogens, and TMs.

1.2.C Trace metals in digestate

TMs present in digestate may originate in feedstocks or can be introduced into digesters to enhance biogas production yield and rate (Fermoso et al., 2015; Moestedt et al., 2016; Molaey et al., 2018). The total concentrations of TMs are consistently higher in digestates compared to feedstocks, with concentrations increasing during AD due to microbial mineralization and the loss of volatile solids (Ciavatta et al. 1993). TMs are distributed between liquid and solid fractions, but they predominantly accumulate in the solid phase along with sulfides/phosphates and residual organic fractions (Fermoso et al. 2015). However, some evidence suggests that AD enhances the complexation of TMs with organic ligands, consequently reducing the mobility of TMs in the digestate (Lavado et al. 2005; Marcato et al. 2009). TMs encompass elements crucial for regular metabolic processes, referred to as micronutrients (e.g., Fe, Mn, Cu, Zn, Mo), which can become harmful in excessive amounts, and elements like As, Hg, Pb, or Cd, which even at low concentrations can be very noxious to animals while having a lesser impact on plant growth and development. The order of metals based on their toxicity to living organisms

is as follows: Hg > Cu > Zn > Ni > Pb > Cd > Cr > Sn > Fe > Mn > Al (Tchounwou et al., 2012).

1.3 Behavior of trace metals in digestate-amended soils

When applying digestate as a bio-fertilizer, excessive concentrations of digestate-borne TMs can be transferred to soils, in fact, several legislative restrictions exist on the use of digestates as soil amendments (EPA, 2021; Foster & Prasad, 2021; Malhotra et al., 2022; Nkoa, 2014; Saveyn & Eder, 2014; Stürmer et al., 2020; van Midden et al., 2023). Moreover, as above-mentioned, digestate can induce physical and chemical changes in soil that may result in alterations of TMs speciation, bioavailability, and mobility, such as shifts in pH, redox potential (Eh), cation exchange capacity (CEC), a raise of soil organic carbon (SOC), dissolved organic carbon (DOC), and humic substances, an enhancement of microbial activity, etc. Increases in metal bioavailability and mobility represent a potential environmental hazard, given the risk of toxicity to living organisms, potential bioaccumulation in the food chain, leaching and contamination of groundwater sources, ecological disruption, etc.

1.3.A The concept of speciation, bioavailability, and mobility of trace metals

The mere presence of an element in the environment does not necessarily imply that the element has an impact on living organisms. Firstly, the elements must be in a “bioavailable” form (Almeida et al., 2019). There is no universally accepted definition for bioavailability, and it is usually a non-quantitative concept. Despite the uncertainty associated with the term, there is a general consensus that considers an element as bioavailable if it is in a chemical form that living organisms (such as plants, animals, and microorganisms) can absorb readily and if, once absorbed, it affects the life cycle of the organisms (WHO, 2007). The International Standards Organization (ISO) defines bioavailability in the guidelines of 17402 (ISO, 2008) as “the degree to which chemicals present in the soil may be taken up or metabolized by human or ecological receptors or are available for interaction with biological systems”.

Understanding the speciation of metals is crucial for assessing their bioavailability, mobility, fate, and potential toxicity of metals in soils. Therefore, to prevent an overestimation of the risks associated with metals in environmental compartments, it is crucial to consider the concentration of element species. Element species refer to the diverse physical and chemical forms in which an element is found in the environment. The distribution of an element among these various forms is termed its speciation, as

described by Templeton et al. (2000). Metal speciation will be defined by (i) the oxidation state of the metal, (ii) associations and complexes to solids and dissolved species (surface complexes, metal-ligand bonds, surface precipitates), and (iii) the molecular geometry and coordination environment of the metal (Brown et al., 1999). All of the above components can be interrelated and often difficult to separate. Moreover, they all have chemical, biological, and physical considerations and for this reason metal speciation is truly a multidisciplinary endeavor.

Within the soil system, metal ions are present in three essential phases: the solid phase, the solution phase, and the gaseous phase, with the former two being particularly crucial. The bioavailability of metals can be estimated by the re-supply of the metal to the mobile pool (soil solution) from more stable phases (metals in and associated with solid species). The association of elements with the solid phase can be either (i) reversible, involving physical and electrochemical sorption processes where complexation with organic or inorganic ligands in soils plays an essential role, or (ii) irreversible (chemisorption), where elements become part of mineral lattices (considered to be the most immobile fraction of metals in soil) (Adriano, 2013; Adriano & Adriano, 2001; Alloway, 1995; Kabata-Pendias, 1993). In the solid part of the soils, the most immobile fraction of TMs occurs in the minerals' structure, whereas the most mobile fraction corresponds to TMs bound on the surface of minerals and organic matter of the soil. In the soil solution, various species are present, including free hydrated metal ions and complexes with organic ligands (such as amino-, carboxyl-, and phenolic groups) and inorganic ligands (such as carbonate, chloride, hydroxide, nitrate, and sulfate), as well as colloids and pseudocolloids (Alloway, 1995).

The Figure 3 represents an overview of TMs interactions with soil compartments.

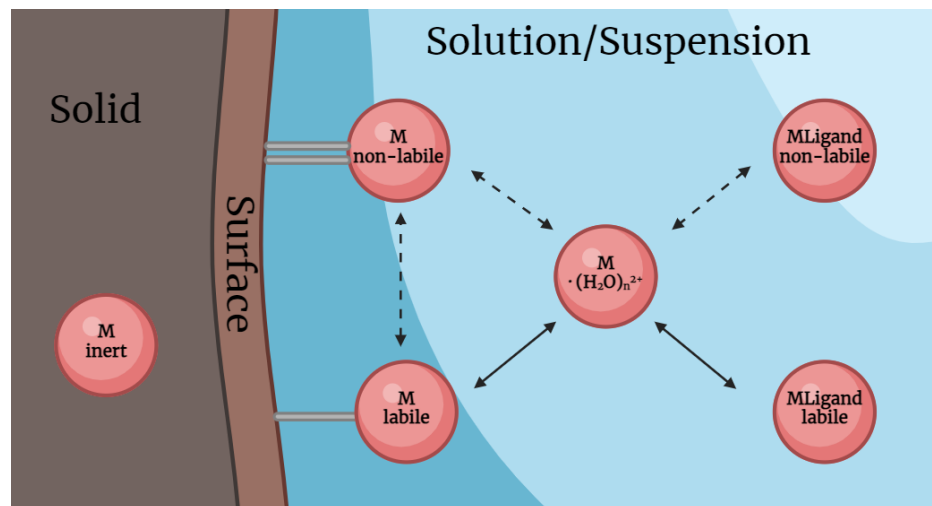


Figure 3. Schematic representation of metal equilibria in soils. Adapted from Alloway (1995). Broken arrows represent kinetically constrained reactions. Reversible arrows represent instantaneous reactions. **Minert** refers to metals that are essentially inert, existing in a state that only reacts to alterations in solution conditions over extended periods, potentially through processes like mineral weathering, organic decomposition, or shifts in redox state. Examples include: (i) metal ions enclosed within the structure of primary (or secondary) minerals due to isomorphous substitution, only released through mineral weathering; (ii) resilient primary (e.g., sulfide) or secondary (e.g., phosphate, carbonate) metal compounds that gradually undergo solubilization via slow oxidation reactions or dissolution over prolonged durations; (iii) recalcitrant organic compounds containing metals; (iv) enduring anthropogenic contaminants like metal fragments and vitrified particles. Non-labile metal, denoted as **Mnon-lab**, refers to metal that is not held reversibly, demonstrating sorption hysteresis due to its presence in forms that are kinetically constrained. This type of metal responds slowly to changing conditions over days to months. Desorption of non-labile metal can occur without alterations in redox status or the decomposition of the sorbing substrate. It may be found within soil solids, in solution/suspension linked to ligands (ML) like humic/fulvic acids or contained within micron and sub-micron-sized particulates (Lombi et al., 2003). Examples include dehydrated metal ions diffused into mineral structures or occluded through co-precipitation reactions, surface-adsorbed metals held strongly on mineral or organic surfaces, internal constituents of metal compounds in solubility equilibrium with the soil solution, and non-reversibly held metal complexes in solution and sub-micron particulates

in suspension. **Mlabile** represents labile metal ions capable of reversible responses (adsorption or desorption) to changes in solution equilibrium, exhibiting no adsorption hysteresis. Labile metals can exist in various chemical forms, including soluble ligand complexes (ML; inorganic and organic). Examples include exchangeably held hydrated metal ions in clay interlayers and the diffuse double layers of humus acids, specifically-adsorbed metal ions on clay edges, Fe/Mn/Al hydrous oxide surfaces, and humus, metal ions on the surface of metal compounds in solubility equilibrium with the soil solution, and reversibly held metal complexes in solution, including most inorganic species and a portion of the metal bound to dissolved humic and fulvic acid. $M \cdot (H_2O)_n^{2+}$ means free hydrated metal ions, often considered the immediately bioavailable form, though evidence suggests that complexes in solution (MLlabile and possibly MLnon-labile) also contribute to apparent bioavailability. All forms in the soil solution ($M \cdot (H_2O)_n^{2+}$, MLlabile, MLnon-labile) are subject to transportation in drainage water. Figure created with Biorender.com license number: BP269BGG23.

1.3.B Factors and processes governing metal speciation and mobility

The reactions governing metal speciation in soils, and consequently metal fate and mobility, primarily involve: (i) sorption (adsorption and absorption)/desorption processes, (ii) dissolution/precipitation (or coprecipitation), (iii) complexation to inorganic and organic ligands, and (iv) reduction/oxidation reactions (Alloway, 1995; Li et al., 2022; Reeder et al., 2006; Strawn, 2021; Yan et al., 2022). These processes are in turn driven by soil properties (such as pH, Eh potential, presence of organic and inorganic ligands, presence of clay minerals, microbial biomass etc.). Therefore, physical, and chemical changes induced by digestate amendment can have a significant impact on metal speciation.

The boundaries between the above-mentioned mechanisms are rather diffuse and they may eventually be described in a more unified manner. In this context, an attempt was made to delineate them in distinct sections.

1.3.B.1 Sorption

Surface complexation and surface precipitation represent two primary adsorption processes in soils. The former involves the adsorption reaction between an aqueous chemical species and a functional group on the soil surface, while the latter pertains to the three-dimensional accumulation of an adsorbate (e.g., metal ion) onto a soil surface. Functional groups on the soil surface can either be inorganic or organic. Inorganic

functional groups are found at the surfaces of secondary minerals including hydrous oxides, oxides and oxyhydroxides of Fe and Mn; hydrous oxides of Al and Si, and clay minerals such as kaolinite and smectites (Manceau et al., 2002). Carboxyl, carbonyl, and phenolic are examples of organic functional groups that occur on the surfaces of humic and fulvic acids, and non-humic substances in soils. Surface complexes are formed when aqueous metal ions interact with surface functional groups through either physical (electrostatic attraction) or chemical (covalent or ionic bonds) interactions, forming outer-sphere or inner-sphere complexes, respectively. The pH and metal ion concentration in the solution play pivotal roles in surface complexation reactions (X. Gu & Evans, 2008; Shi et al., 2021). Nevertheless, additional metal mechanisms, including ion exchange, chelation, electrostatic interaction, or microprecipitation, can be involved in the metal adsorption process, either synergistically or independently, comprising both weaker and stronger bonds (Alloway, 1995; Adriano, 2001; Kabata-Pendias & Pendias, 2001). Additionally, metal ions can be adsorbed on living and dead/inactive cells through metabolism-independent processes (Vijayaraghavan & Yun, 2008), such as interactions with hydroxyl, carboxyl, amino, phosphoryl and sulfhydryl functional groups on cell surfaces. Desorption involves the liberation of adsorbed ions into the soil solution. Metals, whether attached to soil solid particles specifically or nonspecifically, can transition into a soluble state, particularly under altered conditions, such as shifts in pH. However, it has been observed that desorption processes are not fully efficient, meaning that only a portion of the sorbed metals becomes soluble (Bakes et al., 1995).

The overall strength of metal retention in soils reflects several factors, including: (i) residual properties originating from the metal source material; (ii) the inherent affinity of metal ions for soil adsorption surfaces and soluble soil ligands; (iii) the array of adsorption surfaces within soils; (iv) the more “variable” soil properties, such as pH, redox potential (Eh), cation exchange capacity, water content, temperature, biological activity, and salt concentration; (vi) the duration of contact between soil and metal (Alloway, 1995; Kabata-Pendias & Pendias, 2001).

Bioaccumulation represents another sorption mechanism, denoting the assimilation of metals by living cells (microorganisms and plant roots) through metabolism-dependent or independent transport processes (DalCorso et al., 2013; Ledin, 2000; Priya et al., 2022). Soil microorganisms can also mediate ion exchange reactions that affect the speciation and metals, making them bioavailable for uptake (Figure 4). Upon entering living cells, metals may undergo binding, precipitation, accumulation, sequestration

within specific intracellular organelles, or translocation to specific structures, depending on the organism and the element in question.

1.3.B.2 Precipitation

Metal ions create relatively insoluble precipitates with oxyanions (OH^- , CO_3^{2-} , PO_4^{3-} , and SO_4^{2-}) both in the soil solution and on solid soil particles (Alloway, 1995). At low surface sorption densities, metal ions form complexes (outer and/or inner sphere) on mineral surfaces. However, with increasing surface loading, metal ions transition into three-dimensional sorption complexes called surface precipitates. Surface precipitation involves various mechanisms, including (i) the reprecipitation of a dissolved mineral surface and an adsorbed metal ion forming a coprecipitate, (ii) modification of the solubility product at the mineral-water interface (surface-induced precipitation), and (iii) nucleation of surface sites leading to homogeneous precipitates when monolayer surface coverage is exceeded. Coprecipitation relies on the dissolution rate of a mineral surface. Aqueous metal ions also form precipitates with ligands in the soil solution. This occurs at pH 7 and above, where oxyanions (SO_4^{2-} , CO_3^{2-} , PO_4^{3-} , OH^- , AsO_4^{3-} and Cl^-) dominate the soil solution. Metal-ligand complexes may either remain in solution, precipitate, or get adsorbed onto mineral surfaces as ternary complexes. Microbial cells can also play a role in the precipitation of metals in soils by releasing organic acids into the extracellular medium, leading to the formation of insoluble metal compounds, a concept known as bioprecipitation (Figure 4).

1.3.B.3 Complexation

As above-mentioned, metals can form complexes with both inorganic and organic ligands. Complexes with organic matter are of special importance. Organic matter in soil encompasses a wide range of both non-humic and humic substances, including (i) organic matter bound to soil minerals, (ii) dissolved organic matter (DOM) in the soil solution, and (iii) particulate organic matter (Baldock & Nelson, 2000; Kallenbach et al., 2016; Schmidt et al., 2011). Metal can form complexes with carboxyl, hydroxyl, thiol, amine, and other active, functional groups on organic matter surface, which have high affinity for metal ions (Huang et al., 2018). Overall, these complexation reactions of metals-organic matter will have an influence on metal mobility, speciation and bioavailability (Ashworth & Alloway, 2008; Harter & Naidu, 1995). They can either increase metal mobility through the formation of mobile DOM-metal complexes or reduce metal mobility through the formation of complexes that are adsorbed and stabilized by soil surfaces, as previously described, thereby reducing the bioavailability of metals in soils to a certain extent (Clemente et al., 2006; Kulikowska et al., 2015).

1.3.B.4 Reduction/oxidation reactions

Metal(loid)s can exist in multiple oxidation states, with redox reactions governing their transformation, mobility, and toxicity. Abiotic redox processes occur on various surfaces including humic substances, Fe(III)- and Mn-oxides, sulfides, and ferrous species, controlling the reactivity and transformation of metal(loid)s in soils. Mn oxides, acting as an "electron pump," uniquely catalyze a wide range of redox reactions (Borch et al., 2010), including the oxidation of metal cations like Pb, Co, Cu, and Ni. The toxicity of metal(loid)s varies with their oxidation state; for instance, arsenite is more toxic and bioavailable than arsenate. Manganese oxides play a crucial role in oxidizing As(III) to As(V), affecting arsenic's mobility and toxicity. Similar oxidation processes occur for other heavy metals like Co, Pb, Ni, and Cu (Sun & Doner, 1996). Reduction reactions, facilitated by organic material, sulfides, and ferrous species, can release As into solution under reducing conditions. As an additional example, chromate, highly toxic and carcinogenic, contrasts with nontoxic Cr(III) essential for animal nutrition. Reduction reactions converting Cr(VI) to Cr(III) are significant, especially given Cr(VI)'s mobility in soils. Cr(VI) reduction by soil organic matter leads to stable Cr(III)-organic complexes (Borch et al., 2010).

Many microorganisms possess the capacity to either reduce or oxidize metal ions as part of their metabolic processes, utilizing metals as electron donors or acceptors (Burgin et al., 2011). Through such mechanisms, microorganisms contribute significantly to the removal of heavy metals from polluted soils by reducing them to a lower redox state, as demonstrated by studies (Lovley, 1993; Jing et al., 2007) (Figure 4).

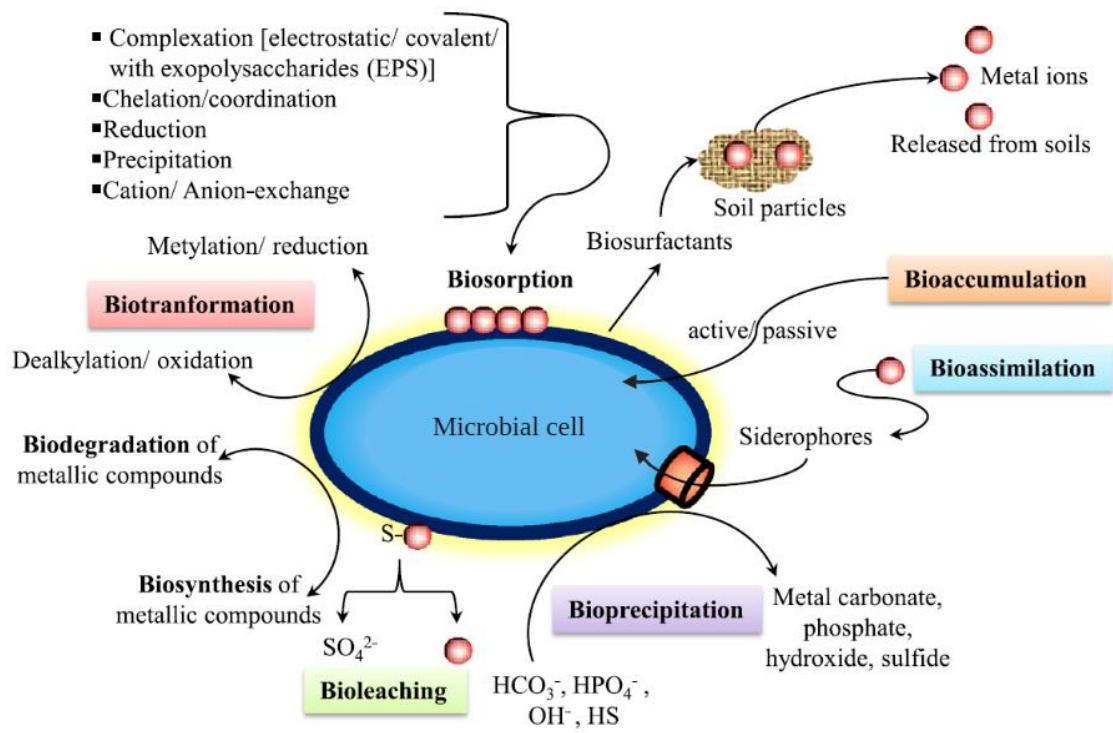


Figure 4. Illustration of different types of microbial interactions with TMs in soils. Adapted from Ahemad (2019).

1.4 Phytomanagement of trace metals in digestate-amended soils

To ensure the environmental safety of land application of digestate, it is essential to prevent the occurrence of excessive concentrations of bioavailable and mobile TMs resulting from this practice. The challenge associated with TMs presence in soils is their non degradability through any biological or physical processes, resulting in their persistent presence in soil for long periods of time, thereby posing a prolonged environmental hazard (Adriano 2001; (Kabata-Pendias, 2011; Kabata-Pendias & Pendias, 2001; Suman et al., 2018).

Phytomanagement is an *in-situ*, cost-effective and ecologically responsible strategy that uses the potential of plants and their associated microorganisms to (i) eliminate excessive concentration of TMs from soil and/or (ii) lower their bioavailability in soil and potential for leaching (Awa & Hadibarata, 2020; Bhat et al., 2022; Tangahu et al., 2011; Z. Wei et al., 2021). Additionally, the presence of plants offers further benefits by

fostering a healthier soil environment. This includes the promotion of nutrient cycling, increased SOC storage (as above mentioned, Figure 1), erosion prevention, and enhancement of soil structure. Therefore, further improving the quality of the digestate-amended soil. As above mentioned, there are essential TMs such as Co, Cu, Fe, Mn, and Zn that play an important role in physiological and biochemical processes of plants, such as chlorophyll biosynthesis, photosynthesis, DNA synthesis, protein modifications, redox reactions, sugar metabolism, and nitrogen fixation. While other TMs, such as Cd, Hg, Pb, U, V and W, are not needed for any physiological and biochemical functions and can have toxic effects on plants, even at low concentrations. However, both groups are noxious to plants at high concentrations.

Plants employ different mechanisms to cope with toxic concentrations of TMs, with the main strategies being phytostabilization and phytoextraction. Phytostabilization involves the absorption of TMs on the roots or precipitation of TMs within the rhizosphere, limiting their mobility and bioavailability in the soil. In this process, the plant alters soil chemistry (via root exudation and/or alteration of soil pH, organic matter, redox potential, etc.) to facilitate the absorption and/or precipitation processes of TMs in the soil (N. S. Bolan et al., 2011). Phytoextraction involves the absorption of TMs from the soil by the roots and their accumulation in aboveground biomass, such as shoots and leaves (Lasat, 2002). Phytoextraction is of special interest due to the possibility of TMs recovery, with hyperaccumulator species being desirable for this purpose (Chaney et al., 2007; Cristaldi et al., 2017). When exposed to elevated concentrations of TMs, hyperaccumulator plants can accumulate them in their above-ground parts without displaying phytotoxicity symptoms (Van der Ent et al., 2013). Crucial physiological steps of TMs detoxification in hyperaccumulators include boosted TMs uptake from the rhizosphere, reduced TMs sequestration in root vacuoles, intensified TMs loading into the xylem for transport to shoots, and stimulated TMs influx across the leaf cell plasma membrane and sequestration in the leaf vacuole (Suman et al., 2018).

The effectiveness of phytoremediation processes in metal-contaminated soil is improved by microbial activities in root and rhizosphere soils through two complementary mechanisms: (i) direct facilitation of phytoremediation, wherein plant-associated microbes boost metal translocation (facilitating phytoextraction) or decrease the mobility/availability of metal contaminants in the rhizosphere (promoting phytostabilization); and (ii) indirect support for phytoremediation, which involves plant growth-promoting microorganisms (PGPM), such as plant growth-promoting bacteria (PGPB), rhizobia, and arbuscular mycorrhizal fungi (AMF). These microorganisms

exhibit plant growth-promoting (PGP) traits, including the synthesis of indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophores, N fixation, and the solubilization of phosphorus (P) and potassium (K). This enhances plant nutrient uptake and biomass production, thereby aiding in the removal or containment of pollutants (Y. Ma et al., 2011; Manoj et al., 2020; Raklami et al., 2022; Sharma, 2021; Wenzel, 2009).

Figure 5 shows a graphic representation of the phytomanagement processes.

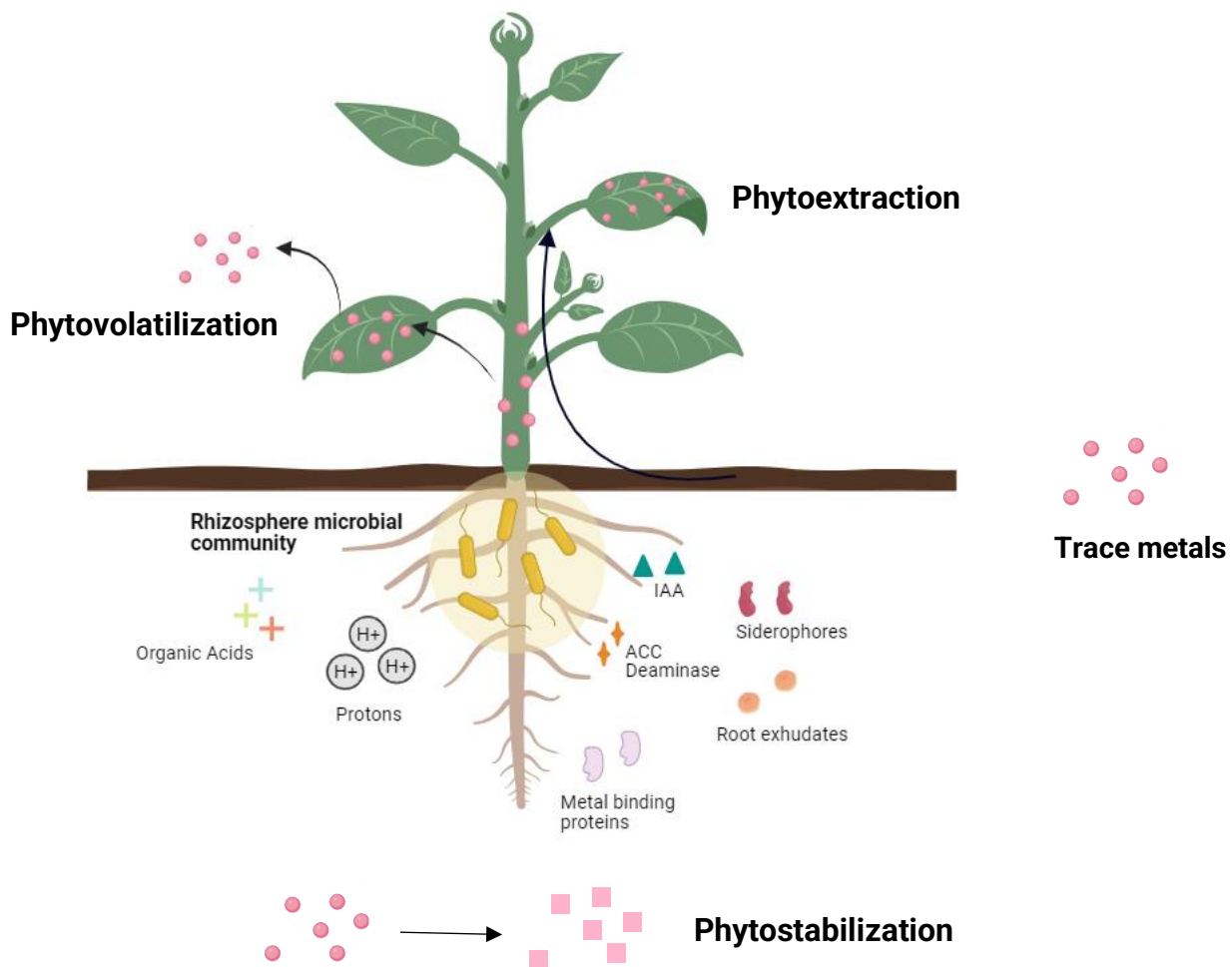


Figure 5. Graphic representation of trace metals phytoremediation processes. Figure created with Biorender.com; license number: WZ269BGOCX.

1.4.A Specific case of bioenergy crops for phytomanagement of digestate amended soil

Non-invasive nonfood dedicated bioenergy crops that can grow on marginal and contaminated lands and provide high yields of reliable and sustainable biomass with low production costs are gaining increasing attention. Among these, perennial grasses (such as switchgrass, elephant grass, red canary grass, miscanthus, fountain grass etc.) are regarded with particular importance. Many of dedicated energy crop plants offer the dual benefits of phytomanagement and bioenergy production, as they are classified as TMs hyperaccumulators (Jha et al., 2017; Pandey et al., 2016; Pandey & Singh, 2020; Van Ginneken et al., 2007). Their nonedible nature made up primarily by hemicellulose, cellulose, and lignin makes their raw material a very attractive feedstock to produce biofuels or biobased products. As lignocellulosic feedstock, perennial grasses are expected to play an important role in achieving long-term goals for energy policy in cutting CO₂ emissions and contributing to the maintenance of energy supplies (Iordan et al., 2023; W. Liu et al., 2023; Monti et al., 2012; Paustian et al., 2000). Also, by growing them on marginal land, they do not conflict with food production, and reduces the pressure to produce energy crops on land that would otherwise be used to produce food crops, avoiding higher prices and food insecurity. Furthermore, perennial grasses can provide benefits in terms of soil structure and stability (e.g., reduced soil loss, erosion, and runoff), soil quality (e.g., increase in soil fertility, organic matter, and nutrient retention), and biodiversity (e.g., cover for native wildlife), thus potentially enhancing marginal soil health. Switchgrass (*Panicum virgatum*) and fountain grass (*Pennisetum alopecuroides*) have been identified as promising species for TM phytoremediation and bioenergy production. Previous studies have reported the capacity of *P. virgatum* to phytoremediate Cd, Cr, Zn, and Pb from contaminated soils (Chen et al., 2012; Guo et al., 2019), whereas *P. alopecuroides* has been recently identified as an effective Cr phytoremediator for the first time (Jia et al., 2022). Both plant species are potential bioenergy feedstocks (Fairley, 2011; McLaughlin & Adams Kszos, 2005; Tripathi et al., 2016; M. Zhang et al., 2016).

Utilizing bioenergy crops for the phytomanagement of marginal soils amended with digestate offers multiple benefits by ensuring environmental safety, generating biomass for bioenergy production, and further enhancing the overall quality of the digestate-amended marginal soil. This process not only rehabilitates the soil but also makes it potentially viable for agriculture, ensuring future food production if the need arises.

1.4.A.1 Valorizing bioenergy crops after phytomanagement of digestate-amended soil

Originally designed solely for soil cleanup, phytoremediation now encompasses the valorization of harvested phytobiomass, once considered undesirable waste. In recent years, the utilization of phytobiomass for bioenergy production has made phytotechnologies highly competitive in remediation practices. The shift towards sustainability and the circular economy emphasizes the importance of utilizing all available resources sustainably, aligning with the principles of the bioeconomy (Scarlat et al., 2015). Biochemical conversion methods like composting reduce organic matter content and concentrate TMs-enriched fractions (Singh & Kalamdhad, 2012; Yang et al., 2019). Additionally there are cases where the TM concentration in the final compost is below regulatory thresholds, thus becoming suitable fertilizers. Phytomining recovers valuable TMs from plant biomass, closing the metal life cycle sustainably (Wang et al., 2020). Thermochemical processes offer efficient biomass energy conversion, producing valuable products like biochar, biogas, or biofuels while concentrating metals predictably. Biomass energy conversion through AD emerges as a promising cost-effective option for post-phytoremediation plant biomass management (Lee et al., 2017). Profits from energy production can significantly reduce remediation costs, potentially making phytoextraction a profitable activity.

In the context of this study, the TMs potentially recovered from the digestate-amended soil by the bioenergy crops can be re-introduced in the AD process, fostering the closure of the material loop. However, the AD of biomass containing TMs requires careful consideration due to the significant influence TMs can exert on anaerobic bacterial activities, as previously mentioned. TMs can exhibit stimulatory, inhibitory, or even toxic effects on AD processes depending on their concentrations (Oleszkiewicz & Sharma, 1990). Therefore, further research is needed to better understand and address the implications of TMs-containing post-remediation plant biomass in AD processes.

1.5 Aims

The objective of this thesis was to investigate the potential of *Panicum virgatum* and *Pennisetum alopecuroides*, along with their associated microorganisms, in mitigating TMs from a marginal soil amended with digestate through phytomanagement techniques. Additionally, the research aimed to explore the feasibility of utilizing the harvested biomass for biogas production. The study focused on two distinct types of digestates: municipal solid waste (MSW) digestate and sewage sludge anaerobic digestate (SSAD). Through these investigations, the thesis sought to contribute to our

understanding of soil reclamation through sustainable digestate management practices and renewable energy production.

1.5.A Specific aims

The specific aims were: (i) Investigate the impact of MSW digestate amendment on the quality of marginal soil; (ii) Assess the fate and fractionation of TMs throughout the soil profile in MSW digestate-amended soil undergoing phytomanagement by *P. virgatum* and *P. alopecuroides*; (iii) Study the effects of *P. virgatum* or *P. alopecuroides* in shaping the evolution of microbial communities in the topsoil over time following MSW digestate amendment. Additionally, analyse the microbial communities in different plant root compartments in the presence and absence of MSW digestate; (iv) Evaluate different methods for determining the bioavailability of TMs in SSAD-amended soil phytomanaged by *P. virgatum*; (v) Understand the influence of organic matter nature and reactivity on the behaviour of TMs in SSAD-amended soil phytomanaged by *P. virgatum*; and (vi) Explore the biogas production potential of post-phytomanagement biomass from *P. virgatum* and *P. alopecuroides*.

1.6 Thesis outline

This thesis comprises 7 chapters. Chapter 1 serves as the introduction, providing a comprehensive overview of the topic.

Chapter 2 delves into three key areas: (i) evaluating the impact of MSW digestate application on the health of a marginal soil, (ii) examining the influence of plants on the mobility of MSW digestate-borne TMs (Zn, Cu, Cr, Pb, Ni, Mn, and Fe) throughout the soil profile, including total metal levels and fractionation in various soil layers, and (iii) assessing plant growth performance and the uptake capacity of TMs (Pb, Zn, Cu).

Chapter 3 investigated the evolution of prokaryotic and fungal communities in MSW digestate-amended soil managed by *P. virgatum* or *P. alopecuroides*. This chapter also analysed the root microbiome of these plants in the presence or absence of MSW digestate, utilizing 16S and ITS amplicon sequencing technique.

In Chapter 4 the focus shifted to understanding TMs (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, Pb and As) mobility and bioavailability in SSAD-amended soil in the presence or absence of *P. virgatum*, through a multi-approach assessment. This was assessed through a combination of diverse methods, namely, soil solution analysis, sequential extraction, diffusive gradients in thin films (DGT) and accumulation into *P. virgatum* tissues.

Chapter 5 explored the nature of the "bound" fraction of organic matter in a SSAD amended soil phytomanaged by *P. virgatum* using colorimetric methods and emission matrix (EEM) methodology. Additionally, the chapter investigated the reactivity of Cu(II) (as an example of a TM) bound to organic matter through EEM coupled with fluorescence titration quenching.

Chapter 6 investigated the potential for biogas production from post-phytomanagement biomass of *P. virgatum* and *P. alopecuroides* through biochemical methane potential (BMP) tests.

Chapter 7 provides a synthesis of the key findings and insights derived from the preceding chapters, along with offering future research perspectives.

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2. Chapter 2: Metal mobility in an anaerobic-digestate amended soil: the role of two bioenergy crops and their metal phytoremediation potential.

This chapter is based on the publication:

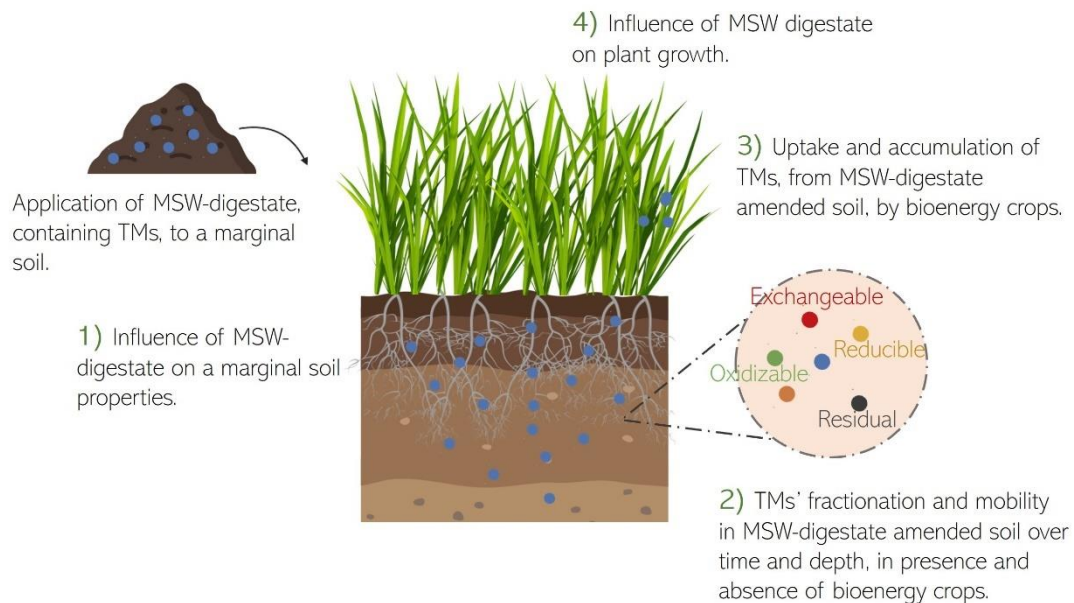
Neus Bonet-Garcia, Veronica Baldasso, Valentin Robin, Carlos R Gomes, Gilles Guibaud, Maria João Alves, Ricardo Castro, Ana Paula Mucha, C. Marisa R. Almeida, "Metal mobility in an anaerobic-digestate amended soil: the role of two bioenergy crop plants and their metal phytoremediation potential", 2023, Frontiers in Environmental Science, section Toxicology, Pollution and the Environment, 11, (21 pages) doi: 10.3389/fenvs.2023.1267463

Abstract

Panicum virgatum and *Pennisetum alopecuroides*, two non-food bioenergy crops were evaluated for their capacity to phyto-manage trace metals (Pb, Zn, Ni, Fe, Mn, Co, Cr, and Cu) from municipal solid waste digestate after its application to a marginal soil. For that, 90-day vertical soil column mesocosm (columns with 0.6 x 0.2 m) experiments were carried out to assess (i) the impact of digestate application on a marginal soil's health, (ii) plant effect on digestate-borne trace metals' mobility along the soil profile (measuring total metal levels and fractionation in different soil layers by atomic absorption spectroscopy, and (iii) plant growth performance and trace metals (Pb, Zn, Cu) uptake capacity. Results showed that trace metals were mostly confined in the 0-0.2 m soil horizon over the course of the experimental period, migrating from the digestate amended soil layer (0-0.1 m) to the layer underneath (0.1-0.2 m) within the first 21 days and remaining stable afterwards. No evidence of trace metals' mobility to deeper soil layers was detected. Trace metals' migration was reduced when *P. virgatum* and *P. alopecuroides* were present, suggesting a phytoremediation (phytostabilization) effect. For both plant species, no trace metals accumulation in roots was observed (bioconcentration factor, BCF<1) although both plants showed potential for Zn translocation to aboveground tissues (translocation factor, TF>1). The growth of both plants was positively affected by municipal solid waste digestate amendment, which also

improved soil quality (increased concentration of total organic carbon and available phosphorus, as well as cation exchange capacity and water holding capacity).

2.1 Graphical abstract



2.2 Introduction

The world's population is expected to increase by nearly 2 billion people in the next 30 years (UN 2022). This will lead to a consumption of vast amounts of resources, production of billions of tons of waste and wastewater (Kaza et al., 2018), and an unprecedented demand for clean and affordable energy. At the same time, demographic expansion, rising expectations of living standards, and scarcity of natural resources have made soil degradation a major issue now, posing a serious threat to human wellbeing (FAO, 2019).

As mentioned in Chapter 1 General Introduction, anaerobic digestion (AD) has proven to be one of the most economical and effective technologies capable of addressing not only the increasing demand for improved waste treatment practices but also immediate requirements for resource recovery and cleaner energy production (Atelge et al., 2020). This technology is a promising alternative, having the ability of turning a variety of organic waste products, such as organic fraction from municipal solid waste (MSW) into two potentially useful end products: renewable bioenergy in the form of biogas and digested

solids, hereafter referred to as “digestate.” However, because of its intrinsic benefits, AD is widely applied in Europe (Di Maria et al., 2016) and large amounts of digestate are being produced. Recycling and valorization of digestate through proper digestate management practices is needed. Digestate is an organic matter and nutrient-rich material that can be applied to soils as fertilizer or soil improver (Albuquerque et al., 2012; ITPS, 2015; M. E. Lee et al., 2021; Monlau et al., 2015; W. Wang and Lee, 2021), which can mitigate the overreliance on environmentally unsustainable chemical fertilizers (Nkoa, 2014). These chemical fertilizers require a high raw material and energy input for their production, and their application can lead to nitrification and loss of soil carbon at higher application rates (Nkoa, 2014; Cheong et al., 2020). In addition, a fraction of the carbon contained in organic amendments, such as digestate, can be sequestered and stabilized in soil. So, land application of digestate could help minimize greenhouse gas emission into the atmosphere (Paustian et al., 2016). Nonetheless, digestate application is not entirely harmless, as it can contain variable concentrations of harmful chemical pollutants, such as trace metals (TMs), and/or pathogenic microorganisms, especially if it originates from non-separated municipal solid waste. TMs, despite occurring at low concentrations ($<1,000 \text{ mg kg}^{-1}$), can have a significant biological effect, either as essential nutrients or as environmental contaminants (Robinson et al., 2009). These TMs may originate from the feedstocks used in AD but can also be added in digesters for optimizing the biogas production yield and rate (Fermoso et al., 2015; Moestedt et al., 2015; Molaey et al., 2018). TMs' total concentrations are always higher in the digestate than in the feedstock, being distributed between the digestate's liquid and solid fractions, but mostly accumulate in the digestate solid fraction together with sulfide and phosphate ions and residual organic fraction (Fermoso et al., 2015). TMs include both elements essential for normal metabolic processes, called micronutrients (e.g., Fe, Mn, Cu, Zn, and Mo), which can become extremely toxic at high concentrations, and elements such as As, Hg, Pb, or Cd, which when present at low concentrations are very noxious to humans and animals, while affecting plant growth and development to a lesser extent. Hence, one of the main obstacles in applying MSW digestate on soils is related with the possibility of introducing excessive amounts of TMs into the soil ecosystem (Jacobs, 1981; Adriano and Adriano, 2001; Kabata-Pendias and Pendias, 2001). In fact, there are strict legislative norms regarding the maximum amounts of TMs that could be incorporated into arable soil (Mininni et al., 2015; European Commission, 2019). Consequently, for the MSW digestate to be safely classified as a usable “product” rather than a hazardous “waste,” strategies to remove excessive TM concentrations are needed.

The remediation of matrices contaminated with TMs is a challenge because of their non-degradability (Kabata-Pendias and Pendias, 2001; Kabata-Pendias, 2011). Conventional remediation options commonly involve excavation, physicochemical treatments (such as stabilization, soil washing, and chemical reduction/oxidation), and off-site disposal to “secured” landfills. Such remedial options are generally expensive, may produce adverse effects on ecosystems, and often require appropriate methods for waste disposal (Liu et al., 2018). Phytoremediation is widely viewed as a green, cost-effective, and ecologically responsible alternative to the environmentally destructive physical–chemical remediation methods currently practiced. Phytoremediation can be an in situ option to remove excessive TM concentrations and lower TM bioavailability in soil amended with MSW digestate (Tangahu et al., 2011; Awa and Hadibarata, 2020). Phytoremediation of TM-contaminated matrices is based on immobilization of TMs in the rhizosphere soil and roots (phytostabilization) and on the mobilization, uptake, and transfer of TMs into the aboveground plant organs (phytoextraction) (Wei et al., 2021; Bhat et al., 2022). The phytoremediation efficiency depends on multiple factors such as TM speciation and bioavailability, solid matrix properties, and plant species (Terry and Banuelos, 2000; Sayen et al., 2019; Qin et al., 2022). Moreover, plants’ presence can further increase the organic matter content in the soil. This organic matter can form water-soluble and/or water-insoluble TM complexes (Zeng et al., 2011). Through the formation of these complexes, organic matter can dissolve, mobilize, and transport TMs in soils and/or accumulate them in certain soil horizons. This can contribute to a reduction of TM mobility toward other reservoirs, namely, living organisms or groundwater. Among phytoremediation mechanisms, phytoextraction is of special interest due to the possibility of TM recovery. The phytoextraction potential of any plant species is primarily driven by two essential key factors: 1) shoot biomass and 2) total TM concentration accumulated in shoot biomass (Hernández-Allica et al., 2008). Hence, a crucial aspect in phytoremediation trials is the choice of the most appropriate plant species to the conditions and matrices to be remediated. As mentioned in Chapter 1 General Introduction, energy crops could be a suitable option. In fact, these plants are typically grown because of their high-rate biomass production, which can later be used to produce biofuels or combusted to generate heat or electricity. Most of the dedicated energy crops, besides offering the dual benefits of phytoremediation and bioenergy production, easily adapt to unfavourable conditions and are TM accumulators, sequestering exceptionally high amounts of the absorbed TMs into their biomass (Tripathi et al., 2016). These plants could be used for direct TM uptake from soils amended with MSW digestate, while profiting from the fertilizing properties of the digestate itself (Antonkiewicz et al., 2017;

M.-S. Lee et al., 2021; Mucha et al., 2019; Seleiman et al., 2012). Eventually, plants' biomass could afterward be used as a feedstock to re-introduce the TMs in the AD process (Garuti et al., 2018), thus allowing it to close the material loop. This is still a scarcely explored subject, and more research to understand the phytoremediation mechanisms in these plants under such conditions is needed. Furthermore, this would allow further expansion of the quadruple task of waste management, soil reclamation, resource recovery, and bioenergy production.

When applying in situ TM phytoremediation techniques to the MSW digestate-amended soil, it is essential to follow changes in the TM behavior in the presence of plants. To our knowledge, there is a lack of studies focusing on the effects of MSW digestate contaminated with TMs on the soil, namely, on TM mobility through the soil profile and on the plants' influence on their behavior. Therefore, the present work aimed to evaluate the potential of two dedicated perennial bioenergy crops for the phytomanagement of MSW digestate, contaminated with TMs, after its application to a poor soil, the plants' influence on TM mobility, and how the soil and plants could benefit from the amendment properties of MSW digestate. The selected plant species, switchgrass (*Panicum virgatum*) and fountain grass (*Pennisetum alopecuroides*), have been identified as promising species for TM phytoremediation and bioenergy production. Previous studies have reported the capacity of *P. virgatum* to phytoremediate Cd, Cr, Zn, and Pb from contaminated soils (Chen et al., 2012; Guo et al., 2019), whereas *P. alopecuroides* has been recently identified as an effective Cr phytoremediator for the first time (Jia et al., 2022). Both plant species are potential bioenergy feedstocks (McLaughlin and Adams Kszos, 2005; Fairley, 2011; Tripathi et al., 2016; Zhang et al., 2016).

We hypothesize that the MSW digestate will improve a marginal soil's health and that any TM contamination from the MSW digestate will be tackled by plants through phytoremediation processes, preventing soil contamination and giving a new value to the TM-contaminated MSW digestate. For that, a series of 90-day vertical soil column mesocosm (0.6 × 0.2 m) experiments were performed to assess the effects of the application of the MSW digestate on the quality of a marginal soil and evaluate phytoremediation processes of *P. virgatum* and *P. alopecuroides* over time in terms of 1) plant effects on TM mobility along the soil profile (through the assessment of total metal concentration and BCR sequential extraction at different soil layers), 2) plant growth performance, and 3) plants' TM uptake capacity.

2.3 Materials and methods

2.3.A Soil, digestate and plants

Soil samples were collected from the vicinity of a construction field in Ermesinde in the municipality of Valongo, North Portugal (41°12'25.7" N, 8°32'26.7" W). The soil was identified through the European Soil Database (Hiederer, 2013). Data were complemented with INFOSOLO, the Portuguese online database for soil profile data (Ramos et al., 2017), following the procedure described by Baldasso et al. (2023). The soil was classified as an Entisol deriving from schist, a medium-grade metamorphic rock, with a soil texture ranging from loamy to clayey. This soil was selected as a good candidate for soil reclamation as it displays poor agronomic qualities (low OM, available nutrients, and water holding capacity (WHC) (Table S1 in Appendix I)) and low TM content (see Results), ideal to follow the mobility of TMs initially present in the contaminated digestate.

The solid fraction of MSW digestate used in these experiments was collected from the mesophilic full-scale anaerobic digestion plant of Tratolixo, a Portuguese company located in the municipality of Mafra, Portugal, that treats the non-source separated organic fraction of MSW at the end of the solid–liquid separation process, specifically after centrifugation. The physical and chemical characterization of the soil, MSW digestate, and MSW digestate-amended soil is included in (Table S1, Appendix I).

Two energy crops, *P. virgatum* and *P. alopecuroides*, were selected for this study. Plants were selected according to pre-established criteria, considering the following standards: metal (Cr, Mn, Fe, Co, Cd, Ni, Cu, Zn, and Pb) uptake capacity; ability to produce a large volume of biomass; high energy potential; ability to be grown in marginal soils; non-edible; non-invasive behavior and of low-cost maintenance (Chen et al., 2012; Jia et al., 2022; McLaughlin and Adams Kszos, 2005; Shrestha et al., 2019; M. Zhang et al., 2016). One-month-old plants were obtained from a greenhouse located in Vigo, Galicia, Spain, where they were germinated and grown in a commercial substrate at 22°C ± 2°C.

2.3.A.1 Initial samples characterization

Elemental composition (Ca, K, Mg, and P) analysis was performed using XRF on the ground sample using ED-XRF SPECTRO XEPOS, XEP05 (SPECTRO Analytical Instruments, AMETEK, France). The carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) content in soil was determined using a 2400 CHNS Organic Elemental Analyzer 100 V (PerkinElmer, MA, USA). Total carbon (TC) content was also obtained through the CHNS analysis. Dry weight (DW) and water content (WC) were measured according to

the oven drying method (ISO: 2720, 1973). The volatile dry weight and volatile organic matter content were determined using the loss on ignition (LOI) approach (Nelson and Sommers, 1996). The cation exchange capacity (CEC) was determined using the cobalt hexamine method in triplicate for each sample (Ciesielski et al., 1997). The pH value was obtained following the normalized ISO 10390:2005 method, in a 1:5 soil/H₂O solution. The water holding capacity was determined in accordance with the modified funnel method provided by Bernard (1963). The particle density was measured using the pycnometer method according to Blake and Hartge (1986). The wet bulk density was determined following the volumetric cylinder standard method (Blake and Hartge, 1986). Porosity was calculated from particle and bulk densities as described in Flint and Flint (2002). X-ray diffraction (XRD) patterns of the initial soil were recorded. An analysis was conducted using a Bruker D8 ADVANCE diffractometer (Bruker, MA, USA) at the Cu-K α wavelength ($\lambda_{K\alpha}$ = 0.1541 nm), between 2° and 60° 2 θ , with a 0.02° 2 θ step and a counting time of 1 s/step and rotating sample holder (15 rpm). Prior to XRD, the sample was gently ground using a non-amorphized ball mill in zirconium oxide (Pulverisette 23, Fritsch). The obtained XRD patterns were processed using EVA software (Bruker). Fourier-transform infrared spectroscopy (FTIR)-ATR (attenuated total reflectance) measurements were performed using an FTIR spectrometer (PerkinElmer, MA, USA) operating in the attenuated total reflectance mode in the middle infrared (MIR) region (4,000–600 cm⁻¹) at a 2 cm⁻¹ resolution for 15 scans.

2.3.B Experimental design

Experiments were performed in vertical soil columns. Three treatment conditions, each with two replicates, were assembled: T0 (soil + MSW digestate), T1 (soil + MSW digestate + *P. virgatum*), and T2 (soil + MSW digestate + *P. alopecuroides*) (Figure 6). One control column for each plant species, with the soil but no MSW digestate, was also assembled to evaluate both the plant growth and plant natural TM content in the absence of the MSW digestate. A total of eight columns were assembled.

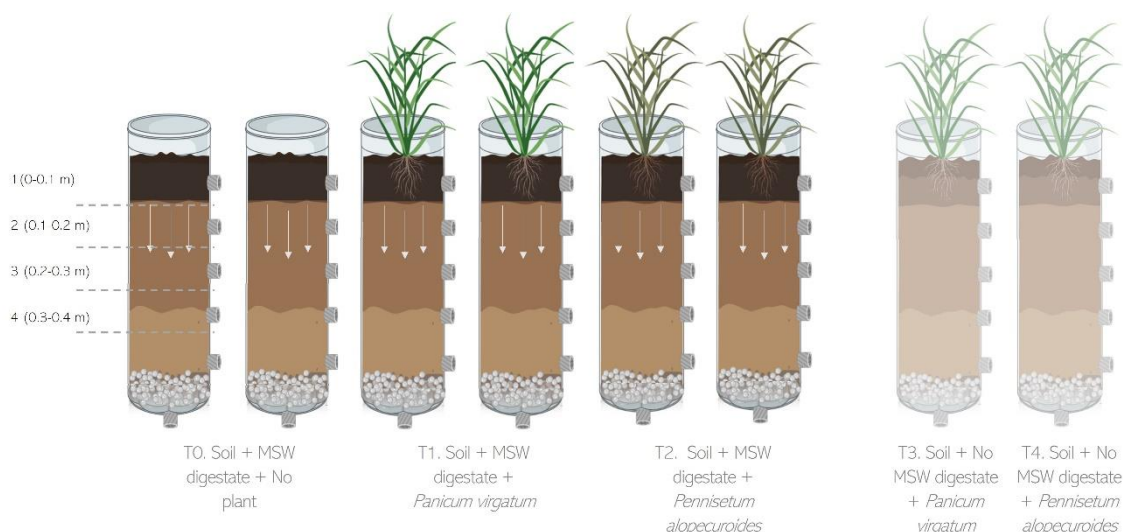


Figure 6. Schematic of the phytoremediation experiments in soil column mesocosms. T3 and T4 are control columns with no digestate amendment. Figure created using BioRender.com. License agreement number: VT254U6HT3.

Soil columns consisted of rigid PVC columns (0.2 m diameter × 0.6 m height) with five sampling ports including a hole at the bottom, with a tap, for leachate collection, whenever needed (Figure 6). The lower 0.02 m of each column was filled with inert pebbles (to avoid soil loss and allow water drainage), followed by addition of the homogenized marginal land soil (9.5 L), previously sieved to <2 mm. To avoid positive preferential flows through the soil, the columns were filled without air gaps, enabling an equal level of homogeneity. Finally, the soil was slightly compacted. A thoroughly manually mixed MSW digestate–soil mixture (proportion 1:1 v/v and 2 L each, to simulate digestate incorporation in the agriculture soil) was placed at the top of the uncontaminated soil in a 0.15-m layer, which is approximately equal to the depth of digestate incorporation on agricultural fields (0.1–0.2 m) (Albuquerque et al., 2012). Next, the soil present in the columns was saturated by adding a total of 5 L of deionized H₂O. During the experiment (90 days), the soil columns were regularly irrigated to maintain 80% of the water holding capacity, which required adding 200 mL of deionized H₂O weekly. The columns were fully covered with aluminum foil to avoid sunlight interferences. After 1 week of acclimation (day 7), ten 1-month-old seedlings of *P. virgatum* and *P. alopecuroides*, with similar sizes, were transplanted into the respective column and irrigated with deionized water according to plant requirement, always maintaining a selected WHC. The plants were grown for 83 days, with the total time of the experiment being 90 days. The experiments were carried out in a semi-open

environment, inside of the building of the Faculty of Sciences of the University of Porto, Portugal, with a natural day/night regime and at room temperature ($21 \pm 1^\circ\text{C}$).

Soil samples from T0, T1, and T2 columns were collected on days 0, 7, 21, 35, and 90 at the following depths: 0–0.1 m; 0.1–0.2 m; 0.2–0.3 m; and 0.3–0.4 m using the sampling ports (at each sampling time, one sample at each depth per duplicate columns) (Figure 6). Soil-column leachates were also collected (when available). All the samples were stored at 4°C in polyethylene tubes for subsequent analyses of total metal contents and metal fractionation. At the end of the experiment, plants from T1, T2 (three plant replicates per duplicate column from each treatment), T3, and T4 (three plant replicates per single column from each treatment) were harvested to assess plant growth and metal content in plant tissues and to estimate the plant metal uptake.

2.3.C Metal analysis for total content and fractionation

The metal (Zn, Cu, Pb, Cr, Ni, Mn, Co, Cd, and Fe) content was determined in initial samples (soil, MSW digestate, and MSW digestate-amended soil, each in triplicates), in the soil layer samples collected over time (in duplicates considering the two columns for each treatment), in leachates (when available), and in different plant tissues, with the plants being collected at the end of the experimental period. For each plant tissue, triplicate samples from each column were prepared and analyzed, after homogenizing the total amount of plant tissue from the respective column. All solid samples mentioned previously were initially dried (at room temperature until constant weight). Leachates were acidified with HNO_3 (at 1%) and stored at 4°C until analysis.

TM levels were measured by atomic absorption spectroscopy (AAS), either with flame (PerkinElmer, AAnalyst 200) or electrothermal atomization (PerkinElmer, PinAAcle 900Z, coupled to an AS 900 autosampler), depending on metal levels, after high-pressure digestion in a microwave apparatus (ETHOS 1, Milestone Inc. (Shelton, CT, USA)) following the US EPA 3052 protocol with concentrated HNO_3 (65%) and H_2O_2 (30%), except the leachate samples that were analyzed directly. To quantify TMs in different plant structures, ca. 0.5 g of plant tissues was weighed in a microwave Teflon vial and 1 mL of HNO_3 (65%) and 5 mL of H_2O_2 (30%) were added. The same procedure was used for the MSW digestate and samples collected from the 0–0.1 m layer over time from MSW-digestate-amended columns (T0, T1, and T2) with ca. 0.5 g of each sample being placed in microwave Teflon vials. For the initial soil sample and samples from 0.1–0.4 m layers from the MSW-digestate-amended columns (T0, T1, and T2), only 5 mL of concentrated HNO_3 for ca. 0.25 g of the sample was used. The total running time for the

microwave digestion was 25 min: 5 min at 250 W, 5 min at 400 W and 5 min at 500 W, and 10 min at 0 W. The vessels were then allowed to cool at room temperature, and the samples were transferred to 50-mL tubes with the addition of deionized H₂O (up to 20 mL). The solutions were stored at 4°C until AAS analysis. The afore mentioned procedures were previously validated in the laboratory (Almeida et al., 2004). Metal quantification was carried out by external calibration with aqueous metal standard solutions. For that, working metal standard solutions were prepared by appropriate dilutions of stock standard solutions with deionized water. Triplicate samples were run to ensure the precision of quantitative results.

Metal fractionation in initial samples (soil, MSW digestate, and MSW digestate-amended soil, each in triplicate) and soil layer samples collected over time from soil columns (in duplicates considering the two columns for each treatment) was estimated by sequential extraction according to the BCR procedure described by Rauret et al. (1999), including minor modifications, as previously reported (Almeida et al., 2004). Three fractions were prepared: 1) exchangeable and bound to carbonates (more bioavailable); 2) reducible and bound to Fe and Mn (hydro) oxides; 3) oxidizable and bound to the organic material and sulfide plus the residual (the less bioavailable). For this purpose, the samples (ca. 0.5 g) were extracted successively with 20 mL of a 0.11 M CH₃COOH solution (exchangeable fraction) and 20 mL of a 0.5 M NH₂OH.HCl solution (fraction bound to Fe and Mn oxyhydroxides). The extractions were carried out using an end-over-end shaker (Unitronic Reciprocating Shaking Bath, JP Selecta) at room temperature. TM concentrations in these solutions were measured by AAS, as described previously. The metal in the third fraction was calculated based on the difference between the total metal content and the sum of the metal content in the two extraction solutions mentioned previously. For the initial soil, digestate, and digestate-amended soil samples, fractionation was carried out for Fe, Mn, Zn, Cu, Pb, and Cr. For the samples collected during the soil column experiment, fractionation analysis was carried out only for Zn, Cu, Pb, and Cr.

All the material used in sample preparation was washed with deionized water, placed in a nitric acid solution (20%, v/v) overnight, and washed thoroughly again with deionized water.

2.3.D Plant biomass yield and morphological traits

Three young leaves (approximately 7 cm in length) from each column were selected at the beginning of the phytoremediation experiment (day 7) and their height was measured

over time (day 21, 35, and 90) to assess the growth dynamics until the end of the experiment, an average was calculated for each treatment and for each time point. At the end of the experiment plants were harvested separately from each column and homogenized, three replicates of each plant tissue (leaves and roots) were taken separately from each column homogenized samples. Both roots and leaves were dried at room temperature ($25 \pm 2^\circ\text{C}$), until a constant weight was reached, to obtain dry weight value, an average for each treatment was calculated.

2.3.E Metal accumulation in plants

To evaluate metal accumulation in plants, the bioaccumulation coefficient factor (BCF) and translocation factor (TF) were calculated with concentrations determined in soils and plants at the end of the experiment. The BCF is the ratio of the metal concentration in the root to that in the soil, and the TF is the ratio of the metal concentration in the shoot to that in the root. The BCF and TF were calculated with the formula mentioned as follows, previously reported by Yoon et al. (2006):

$$\text{BCF} = C_{\text{root}}/C_{\text{soil}}$$

$$\text{TF} = C_{\text{shoot}}/C_{\text{root}}$$

Here, C_{root} is the metal concentration in the plant root (mg kg^{-1}), C_{soil} is the metal concentration in the soil (mg kg^{-1}), and C_{shoot} is the metal concentration in the plant shoot (mg kg^{-1}). C_{soil} was calculated as the average of the metal concentration in the two initial soil layers, the 0–0.1 m layer (the MSW digestate-amended soil) and the 0.1–0.2 m layer (the soil layer beneath the MSW digestate-amended soil), as at the end of the experiment (90 days), the plant roots were in contact with both soil layers.

2.3.F Statistical analysis

The samples' mean, standard deviation, and maximum and minimum values were analyzed using Microsoft Excel 2019. Statistical tests were performed using SPSS Statistics software (version 26.0). The Shapiro–Wilk test was used to test if the distribution of the datasets deviates from a comparable normal distribution. The null hypothesis was rejected if p value < 0.05 . Significant differences among soil column samples collected over time and depth were evaluated through a parametric one-way analysis of variance (ANOVA). The same procedure was followed to evaluate significant differences between metal concentrations in different plant tissues. Two-way ANOVA was used to evaluate the differences in the total metal and metal fraction concentrations between different conditions. The dependent variable was metal concentration in all

cases. The variables were defined as continuous. Significant differences ($p < 0.05$) were detected by a Tukey's Honestly Significant Difference (HSD) post hoc test. Principal component analysis (PCA) and Pearson's correlation were used to identify the potential interrelation among total TM concentrations, and with depth, pH, and presence/absence of the plants, in T0, T1, and T2 columns. Pearson's correlation was tested at 99% confidence level. For the PCA, data were log-transformed and eigenvalues >1 were extracted through a varimax rotation. The analyses were performed using SPSS Statistics (version 26.0) and RStudio (version 4.3.0).

2.4 Results

2.4.A Initial soil, MSW digestate and MSW digestate amended soil characterization

Soil, MSW digestate, and MSW digestate-amended soil (1:1 v/v soil + MSW digestate) were initially characterized. The results are presented in Appendix I (Figure S2, Figure S3, and Table S1). X-ray diffraction and attenuated total reflection–Fourier-transform infrared spectroscopy analysis of the soil–solid phase revealed a clay mineral composition of mainly quartz, associated to phyllosilicates such as kaolinite and muscovite (Figure S2, Appendix I). Traces of carbonates ($p < 0.5\%$) were inferred from CHNS analysis before and after acid treatment, and the results are in accordance with weak carbonate band observed on ATR spectra. The loss on ignition of soil was $2.4 \pm 0.1\%$ (Table S1, Appendix I), hence considered a poor clayey soil (Brady et al., 2008). This was expected as this was a marginal soil originating from a construction site. The application of MSW digestate, which had an LOI of $22.0 \pm 0.8\%$, substantially increased ($p < 0.05$) the soil LOI to $6.5 \pm 0.1\%$. This is in accordance with what has been previously reported regarding biosolids', such as anaerobic digestate or sewage sludge, ability to increase the soil organic matter content (Gerzabek et al., 2001; Parat et al., 2005; Carabassa et al., 2020). The application of MSW digestate to the soil also increased the soil organic carbon (SOC), the major component of soil organic matter content, from $0.20 \pm 0.05\%$ to $4.4 \pm 0.7\%$. Similarly, Tambone et al. (2009) reported a high concentration of organic carbon in the organic municipal solid waste (OFMSW) digestate.

Initial soil pH agrees with the soil mineralogical composition, that is, slightly basic, due to traces of carbonates (Table S1, Appendix I). Addition of MSW digestate slightly enhanced the soil pH, from 7.9 ± 0.2 to 8.4 ± 0.1 ($p < 0.05$). This could be explained by the alkaline nature of MSW digestate (pH 8.8 ± 0.4). Furthermore, there could be an increase of negatively charged functional groups in the soil, coming from the MSW digestate-borne organic matter, such as carboxyl ($-\text{COOH}$), phenolic ($-\text{OH}$), and amino

(-NH₂) groups (Adusei-Gyamfi et al., 2019). There was also a significant increase ($p < 0.05$) in the cation exchange capacity in the soil amended with MSW digestate (from 4.4 ± 0.2 to 15.2 ± 2 cmol (+)/kg) (Table S1), probably induced by the higher amount of negatively charged sites, as previously mentioned. Overall, this would increase the capacity of the soil to hold positively charged ions. The increase of the CEC in organic component-amended soil has also been previously reported (Panuccio et al., 2021). The CEC of MSW digestate was 40 ± 3 cmol (+)/kg, which is similar to the values found in literature (20.3%–53.4%) (Teglia et al., 2011a; 2011b). It is worth mentioning that the increase of the CEC of soils is of particular interest in the context of soil improvement, for example, an increased CEC has been proven to reduce the entry of nutrient loads into groundwater systems (Bargmann et al., 2014). The application of MSW digestate also increased the levels of available P (P₂O₅) in the soil from $0.1\% \pm 0.02\%$ to $0.78\% \pm 0.03\%$ (Table S1, Appendix I), similarly to what was observed by Bachmann et al. (2014), Hupfauf et al. (2016), and Tan et al. (2021). These studies showed that the application of the digestate increased the content of plant-available P in the soil to the same extent as highly soluble mineral P fertilizer and undigested dairy slurry, so the digestate could be a suitable substitute for inorganic fertilizers. Ultimately, these changes can promote plant growth and yield. The contents of total nitrogen (TN) in soil also increased after MSW digestate application. These results were consistent with the previous findings, showing that organic matter, N, and P in the digestate improved both plant biomass and chemical properties of the soil (Garg et al., 2005; Arthurson, 2009; Głowacka et al., 2020). Soil calcium (CaO) and magnesium (MgO) also increased ($p < 0.05$) with MSW digestate amendment, from $1.3\% \pm 0.6\%$ to $8.0\% \pm 0.4\%$ and from $0.30 \pm 0.08\%$ to $2.6\% \pm 0.9\%$, respectively (Table S1, Appendix I). High values have been found in digestate-amended soils (Doyeni et al., 2021). CaO and MgO are important adsorbents of TMs in soils. They are binders that can react with TM salts, and hence induce TM precipitation (i.e., insoluble complex compounds or hydroxides) due to their alkaline nature (Spence and Shi, 2004).

Both the bulk and particle density were lower in the soil amended with MSW digestate, decreasing from 2.2 ± 0.3 g·mL⁻¹ and 2.54 ± 0.01 g·mL⁻¹ to 1.3 ± 0.2 g·mL⁻¹ ($p < 0.05$) and 1.8 ± 0.9 g·mL⁻¹ ($p > 0.05$), respectively (Table S1, Appendix I). Organic matter found in the digestate can make clay soils less dense and heavy (Pagliai et al., 1981; Pagliai and Antisari, 1993). A field experiment by Garg et al. (2005) showed that the amendment of soils with liquid digestate from agricultural waste reduced the bulk density and increased the saturated hydraulic conductivity and moisture retention capacity of soils.

These findings corroborate the results on the amending properties of typical anaerobic digestate, and they suggest that MSW digestate could be considered an effective organic amendment material. These changes in soil characteristics could influence the TM content, distribution, bioavailability, and, ultimately, toxicity (Adriano and Adriano, 2001; Kabata-Pendias and Pendias, 2001) and influence plants' development and of soil microbial communities. Nevertheless, it is worth noting that these results were obtained after the recent amendment of the soil, and long-term experiments should be conducted to determine the MSW-digestate effects in the soil over an extended timeframe.

2.4.B Total and fractionation metal content in the soil, MSW digestate, and MSW digestate-amended soil

The total concentrations of TMs in the soil, MSW digestate, and MSW digestate-amended soil are given in Figure 7. Cd was below the limit of quantification (LOQ) ($0.5 \mu\text{g}\cdot\text{g}^{-1}$) in all samples; therefore, this metal was not considered for further analysis. Cr and Ni concentrations in the MSW digestate were below the limit values stated in Portuguese (Decree Law No 73/2011, 2011) and European ((EU) 2019/1,009 (European Commission, 2019)) regulations for Class III (soil for plant crops not intended for human and animal food), whereas Pb, Zn, and Cu exceeded values stipulated in the Portuguese regulation (Table S2, Appendix I). No limit values for Mn and Fe were found in the legislations. Overall, MSW digestate amendment markedly increased ($p < 0.05$) the concentrations of Pb, Zn, Ni, Mn, and Cu in the soil. The increase of total TM concentrations in the topsoil after the application of biosolid fertilizers has been extensively reported (Koutroubas et al., 2014; Latare et al., 2014; Lloret et al., 2016; Marguí et al., 2016; Dragicevic et al., 2018). However, for Fe, an opposite trend was observed as the Fe content was much higher in the soil than in MSW digestate-amended soil ($p < 0.05$). No significant differences ($p > 0.05$) were observed in concentrations of Cr and Co between the soil and the MSW digestate-amended soil, although Cr concentration tends to be higher in MSW digestate.

A modified BCR sequential extraction protocol (Rauret et al., 1999) was carried out to evaluate TM availability changes upon soil amendment with the MSW digestate for Fe, Mn, Cu, Zn, Pb, and Cr (Figure 8). For Cd, Co, and Ni, no metal fractionation was carried out, as their levels in the initial matrices were low. The sequential extraction method identifies TMs in the MSW digestate-amended soil which could be potentially mobilized in the short and long term and ultimately become an environmental hazard or TMs that could be more bioavailable to plant and microbial communities, either potentiating plant uptake or increasing metal toxicity. In the present study, chemical fractionation of TMs in

solid matrices consisted in three fractions (exchangeable, reducible, and oxidizable + residual). Preliminary tests indicated that the percentages in the oxidizable fraction were very low (results not shown), so it was decided to join this fraction to the residual one. Overall, MSW digestate amendment increased the potential mobility and availability of Fe and Mn ($p < 0.05$) (exchangeable fraction) in the soil, while no significant ($p > 0.05$) influence was found in the Pb, Cu, and Cr fraction distribution. In the initial soil, Fe seemed to be mainly present in the residual and oxidizable fraction (least mobile forms). Application of MSW digestate significantly influenced Fe distribution, increasing exchangeable and reducible fractions ($p < 0.05$), and thus its potential availability. Ali et al. (2015) reported that the application of biofertilizers led to a drop in soil pH, which resulted in an increase of available Fe. However, in this study, MSW digestate induced a slight increase in the soil pH, which is generally linked to an increase in the cationic metal retention in soil surfaces via sorption, inner-sphere surface complexation, and/or precipitation and multinuclear type reactions (McBride, 1994). Therefore, the increase in the available forms, in this case, could be caused by 1) trace organic acids present in the MSW digestate or 2) recent TM soil enrichment, which was not yet allowed to become sequestered and adsorbed by soil constituents. Similarly, the application of MSW digestate increased ($p < 0.05$) the exchangeable fraction of Mn, while it decreased the reducible fraction in the soil. Considering the recent application of the MSW digestate into the soil, this result could be attributed to MSW digestate containing Mn in exchangeable form and the subsequent release into the soil after amendment. Similar results were reported by Karimi et al. (2020), who studied Mn availability in the biochar-amended soil. The decrease of the reducible fraction and an increase in the residual + oxidizable fraction could be explained by the transformation of Mn into a more stable fraction through the formation of insoluble complexes with organic matter functional groups (Dhaliwal et al., 2019; Li et al., 2021). Upon addition of MSW digestate to the soil, the Zn concentration in the reducible fraction increased ($p < 0.05$), whereas that in the exchangeable fraction dropped. However, the total amount of oxidizable + residual fractions was not significantly affected by MSW digestate. This could be linked to the presence of Mn in the MSW digestate. Hydrous Mn oxides, along with organic matter, can decrease the reactivity of metals in the soil through adsorption (Jing et al., 2023). Similarly, Qiao et al. (2003) reported an increase of Zn concentration in the reducible fraction of paddy soil after biosolid application, as well as Bose et al. (2008), who found similar results on an industrial waste-amended soil. However, other case studies showed an increase in the proportion of Zn bound to the acid-extractable fraction after biosolid amendments (Planquart et al., 1999; Qiao et al., 2003; Yang et al., 2018; Wydro et al.,

2021). Both in the initial soil and MSW digestate-amended soil, the largest portion of Pb, Cr, and Cu was found distributed among the oxidizable + residual fractions (bound to organic matter and sulfides and the remaining non-silicate-bound TMs). It is considered that TMs are dominantly present in the residual fraction, as preliminary results showed low TM amounts in the oxidizable fraction. This indicates that these TMs are less readily bioavailable to plants.

MSW digestate amendment in the soil seemed to increase the bioavailability of Fe and Mn, whereas it maintained low bioavailability for Pb, Cu, and Cr. For Zn, on the other hand, the MSW digestate slightly decreased its bioavailability. Based on the comparison with previously published data, the results also seem to indicate that the origin, type, and properties of the digestate, as well as field conditions, will differently influence TM bioavailability. However, the results in this section reflect the effect of a recent application of MSW digestate to the soil; the following sections will describe and discuss the longer-term effects.

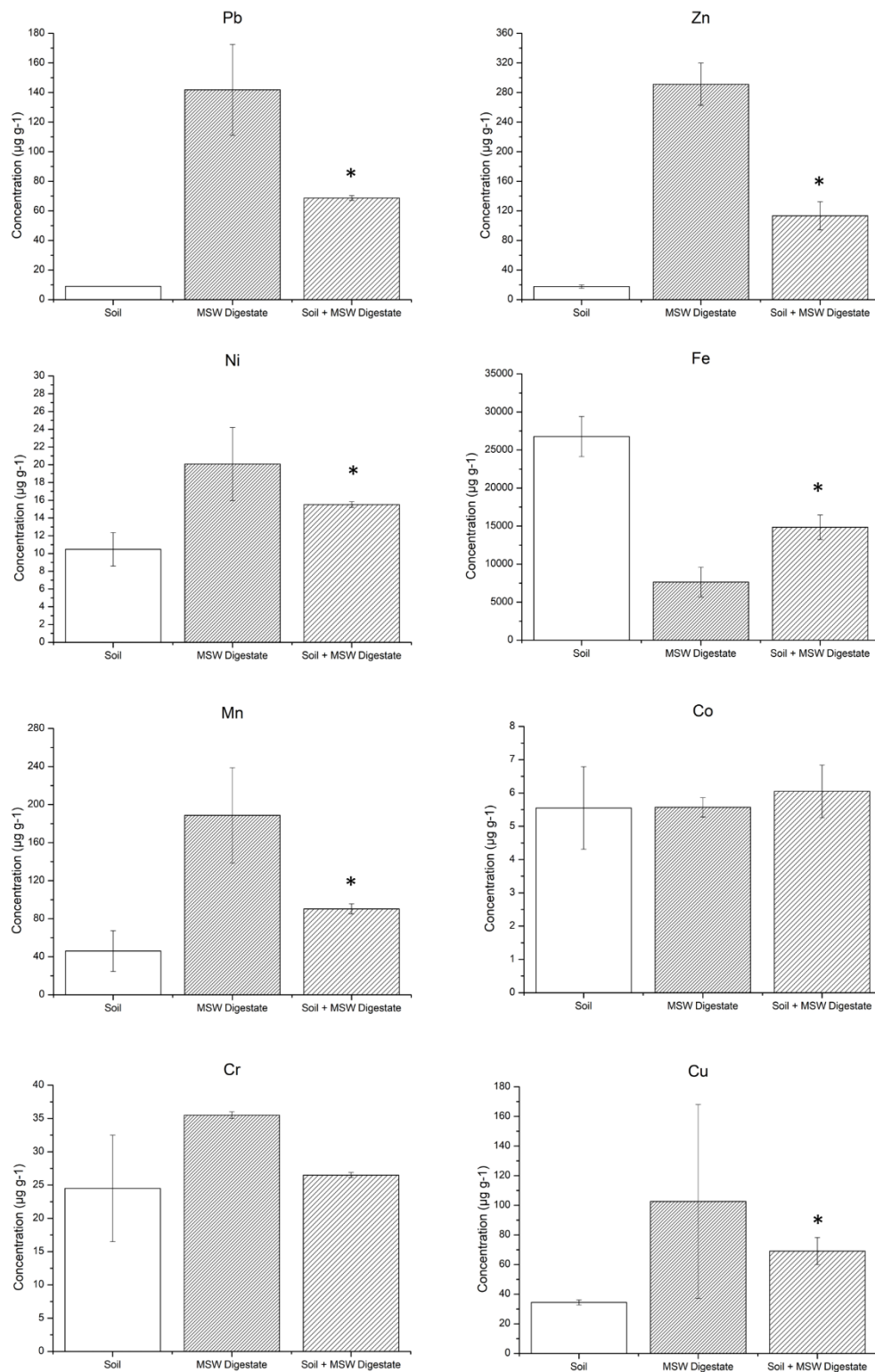


Figure 7. Total metal (Pb, Zn, Ni, Fe, Mn, Co, Cr, and Cu) concentration ($\mu\text{g g}^{-1}$ dw) (+SD) of the initial samples (soil, MSW digestate, and MSW digestate-amended soil).

Error bars indicate the standard error of the mean of three measurements ($n = 3$). * Values in the MSW digestate-amended soil are significantly different than that in the unamended soil at the 0.05 level of significance assessed by Tukey's multiple comparison test.

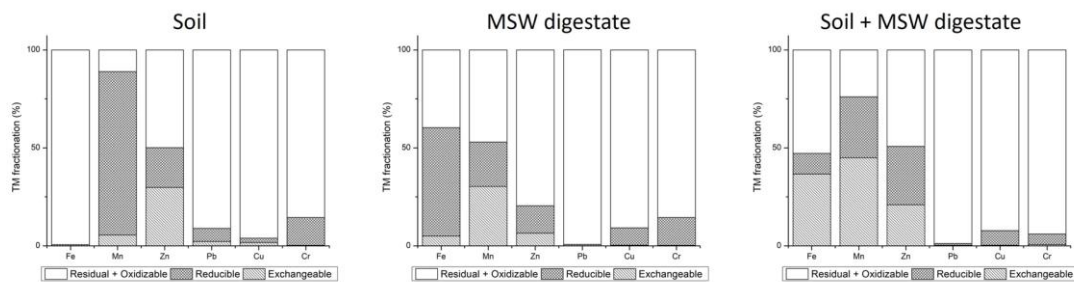


Figure 8. Percentage of selected metals (Fe, Mn, Zn, Pb, Cu, and Cr) in different fractions (exchangeable, reducible, and residual + oxidizable) in the initial samples (soil, MSW digestate, and MSW digestate-amended soil). Exchangeable (acid-soluble and bound to carbonates); reducible (bound to Fe and Mn oxides); residual + oxidizable (bound to the soil matrix and organic matter). Error bars are not illustrated in the figure; the standard error of the mean of three measurements ($n = 3$) are indicated in Table S4, Appendix I.

2.4.C Total metal concentration along the soil profile through time

Understanding the migration and fractionation of TMs in the soil environment after MSW digestate amendment is of great significance for implementing relevant risk-control strategies (Latosińska et al., 2021). The total content of Zn, Cu, Cr, Pb, Mn, Ni, and Fe in the different layers of the soil column after 7, 21, 35, and 90 days of MSW digestate application is shown in Figure 9. Levels of Cd and Co were not determined due to their low concentrations, which would prevent observing significant differences over time.

Overall, in a short-term period (90 days), relatively little downward movement of TMs occurred. TMs (Cr, Mn, Ni, Cu, Zn, and Pb) tended to mostly remain in the upper layer (0–0.1 m), with the one with MSW digestate-amended soil, moving slowly from the surface to the second layer, within the top 0.2 m of the soil profile. After the stabilization period, at day 7 before plant transplantation, concentrations for all TMs were significantly higher ($p < 0.05$) in the 0–0.1 m soil layer (MSW digestate-amended soil) when compared

to deeper layers and decreased from the top to bottom, except for Fe, which already presented a higher concentration in the initial soil ($p < 0.05$). In soil columns with no plant (T0, Figure 9), as a general trend, at day 21, significant increases ($p < 0.05$) of Zn, Cu, Pb, Cr, Ni, and Mn concentrations were observed in the 0.1–0.2 m layer, the layer below the MSW-amended soil, after which TM concentration did not vary significantly ($p > 0.05$). These results suggest the migration of Zn, Cu, Pb, Cr, Ni, and Mn from the MSW digestate-amended soil to the soil layer beneath (0.1–0.2 m) during the first 21 days after MSW digestate application, which then remained relatively stable until the end of the experimental period (day 90), with no statistically significant differences ($p > 0.05$) among days 21, 35, and 90. No evidence of TM movement deep into the soil profile was detected since the TM concentration in the depths below 0.2 m essentially remained unchanged for all treatments during the whole experimental period. Similar results have been previously reported (Harris and Urie, 1986; Farrell et al., 2010) showing that TM levels in the soil after bio-amendment applications remained essentially constant in the top 0.1 m of soil, while it progressively declined in the sub-surface layers after a longer period. Similarly, a recent study conducted by Piśkuła and Stępień (2021) showed that the addition of increasing amounts of organic matter in the soil reduced the leaching of TMs deep into the soil profile (especially on light soils that are highly sandy). However, an opposite trend was recorded here for Fe, that presented an upward trend over depth (lower concentration was observed in upper layers) and non-significant differences ($p > 0.05$) over time, except for a big decline observed at the 0.1–0.2 m layer between day 7 and day 21, which after that point remained stable. This could be explained by the fact that the Fe concentration did not originate from the MSW digestate, as observed in the initial samples' characterization, which showed that MSW digestate amendment induced a decrease in the soil's Fe concentration.

The observed Zn, Cd, Pb, Cr, Ni, and Cd behavior in T0 could be explained by the known affinity of TMs for different fractions of soil organic matter (Fujikawa et al., 2000; Fujikawa and Fukui, 2001; Milne et al., 2003; Hartland et al., 2012; Šípková et al., 2013) and larger levels of organic matter in top layers induced by the MSW digestate amendment. Within organic matter, several distinct functional groups (such as hydroxyl, carbonyl, carboxyl, carbohydrate, and phenol) exhibit a strong capacity for forming complex compounds with metals, including insoluble complexes which can ultimately reduce metal mobility and transport (Tang et al., 2014; Borggaard et al., 2019). An additional piece of evidence is the increase of negatively charged sites in the soil (CEC) upon MSW amendment, previously explained (Table S1, Appendix I), which was likely because of the increase of

organic matter, indicating the ability of the upper layer of soil to hold positively charged ions (such as TM cations) (Sumner and Miller, 1996). Another possible explanation is the observed increase in the soil pH upon MSW digestate amendment (from 7.9 to 8.4, Table S1, Appendix I), a value that was maintained until the end of the experiment (Table S3, Appendix I). The maintained higher pH over time could be explained by various processes, with emphasis on the release of OH^- ions due to the decarboxylation of organic anions during the mineralization of organic carbon and the consumption of H^+ ions through the protonation of organic molecules (Shi et al., 2019; Yan et al., 1996). Many researchers have observed a significant reduction in the leaching of TMs under an increase in soil pH value (Fulekar and Dave, 1991; Wang et al., 2020). This slightly basic pH can induce some TM precipitation under the form of hydroxides and/or carbonates (Ford and Sparks, 2001). Furthermore, this pH value is favorable for the sorption of TMs onto clay minerals, present in the studied soil (Harter, 1983; Lair et al., 2007). Finally, it has been observed in the first section that MSW digestate amendment increased CaO and MgO concentrations in the soil (Table S1, Appendix I), which are considered to be major adsorbents of TMs in soils due to their alkaline nature (Spence and Shi, 2004), which could also explain the high retention of TMs in the upper layers. A similar trend of TM migration was found in soil columns with *P. virgatum* and *P. alopecuroides* (treatments T1 and T2), with the TM content also remaining mainly confined in the 0–0.2-m layers over the course of the experiment. However, TM concentrations in the 0.1–0.2 m-layer on days 21, 35, and 90 were significantly ($p < 0.05$) lower when compared to those in soil columns without plants (T0), indicating a potential protective effect of the studied plants, preventing metal mobility through the soil profile. A clear decline in TM concentration over depth can be observed, especially for Zn, Cu, and Pb. As previously mentioned, the TM distribution at depths below 0.2 m remained constant during the experiment for all treatments. Similarly, *P. virgatum* has been widely reported to reduce Zn concentrations in soil (Aderholt et al., 2017; B.-C; Chen et al., 2012; Kacprzak et al., 2014; Masters et al., 2016), as well as Cr (Li et al., 2011), which was not observed in the current study. Fewer studies focus on *P. alopecuroides* (Chen et al., 2020). However, other species of the same genus have shown the TM uptake capacity, reducing TM concentrations in soils, similar to what was found in this study. For example, Lin et al. (2020) used *Pennisetum hybridum* to treat municipal sewage sludge. In the study of He et al. (2021), digestate application was used to improve the Cd phytoremediation potential of *Pennisetum hybridum*.

Small but not significant differences ($p > 0.05$) were observed between columns with different plant species, except for the Zn concentration in the 0.1–0.2 m layer at day 90, which was lower ($p < 0.05$) in the presence of *P. virgatum* than with *P. alopecuroides*. The reduced migration of TMs observed when plants were present could be explained by the activity in the rhizosphere. Both plants and rhizosphere microorganisms (bacteria, archaea, and fungi) can contribute to lowering TM mobility (Plekhanova et al., 2022). They both can release chelating compounds outside cells, for example, secondary metabolites such as biosurfactants, TM-binding proteins (metallothionein), metallophores, and low molecular weight organic acids and iron-chelating compounds (siderophores) (Tao et al., 2004; Lambers et al., 2009; Seshadri et al., 2015; Fresno et al., 2017; Barra Caracciolo and Terenzi, 2021), which can induce sorption and/or precipitation processes and decrease the mobility and accessibility of TMs significantly. A recent study from Grybos et al. (2022) showed the potential of microorganisms to release chelating agents for Fe and Pb. Furthermore, these compounds can enhance the plant's and rhizosphere microorganisms' metabolically active uptake of TMs (McGrath et al., 2001). In fact, the Zn and Cu concentration decrease could be explained by plant uptake due to their essential nature for plants, as they were involved in many enzymatic processes necessary, for example, for proper photosynthesis, metabolism of carbohydrates and proteins, oxidation processes, and synthesis of DNA, RNA, and chlorophyll. It is important to note that in both the absence and presence of plants, Pb, Zn, Ni, Fe, Mn, Cr, and Cu concentrations in column leachates collected over time were below the detection limit ($250 \mu\text{g}\cdot\text{L}^{-1}$, $17 \mu\text{g}\cdot\text{L}^{-1}$, $125 \mu\text{g}\cdot\text{L}^{-1}$, $174 \mu\text{g}\cdot\text{L}^{-1}$, $66 \mu\text{g}\cdot\text{L}^{-1}$, $0.75 \mu\text{g}\cdot\text{L}^{-1}$, and $100 \mu\text{g}\cdot\text{L}^{-1}$, respectively), indicating no significant leaching of TMs from the soil column.

To better visualize the obtained findings, correlation coefficients were calculated (Pearson's at the 0.05 significance level) and principal component analysis was performed to determine the relationships between total concentrations of TMs in the soil profile and among the pH, depth, and presence/absence of plants in soil columns T0, T1, and T2 (Table S5 and Figure S3, Appendix I). As previously observed, Pb, Cr, Zn, Ni, Mn, and Cu were negatively correlated with depth ($p < 0.05$), whereas the opposite was observed for Fe ($p < 0.05$). Concentrations of Pb, Cr, Zn, Ni, Mn, and Cu over time and depth were positively correlated with each other ($p < 0.05$), while they were negatively correlated with Fe ($p < 0.05$). The positively correlated metal concentrations with each other could indicate a synergy among the leaching of these metals through the soil profile (Gräfe et al., 2004; Kumpiene et al., 2008). Regarding plant presence, Mn,

Zn, Ni, and Cu total concentrations in the soil profile were negatively correlated with the presence of both *P. alopecuroides* and *P. virgatum*, while Pb was negatively correlated with *P. virgatum*, and Cr was negatively correlated with *P. alopecuroides*, although these correlations were not significant ($p > 0.05$).

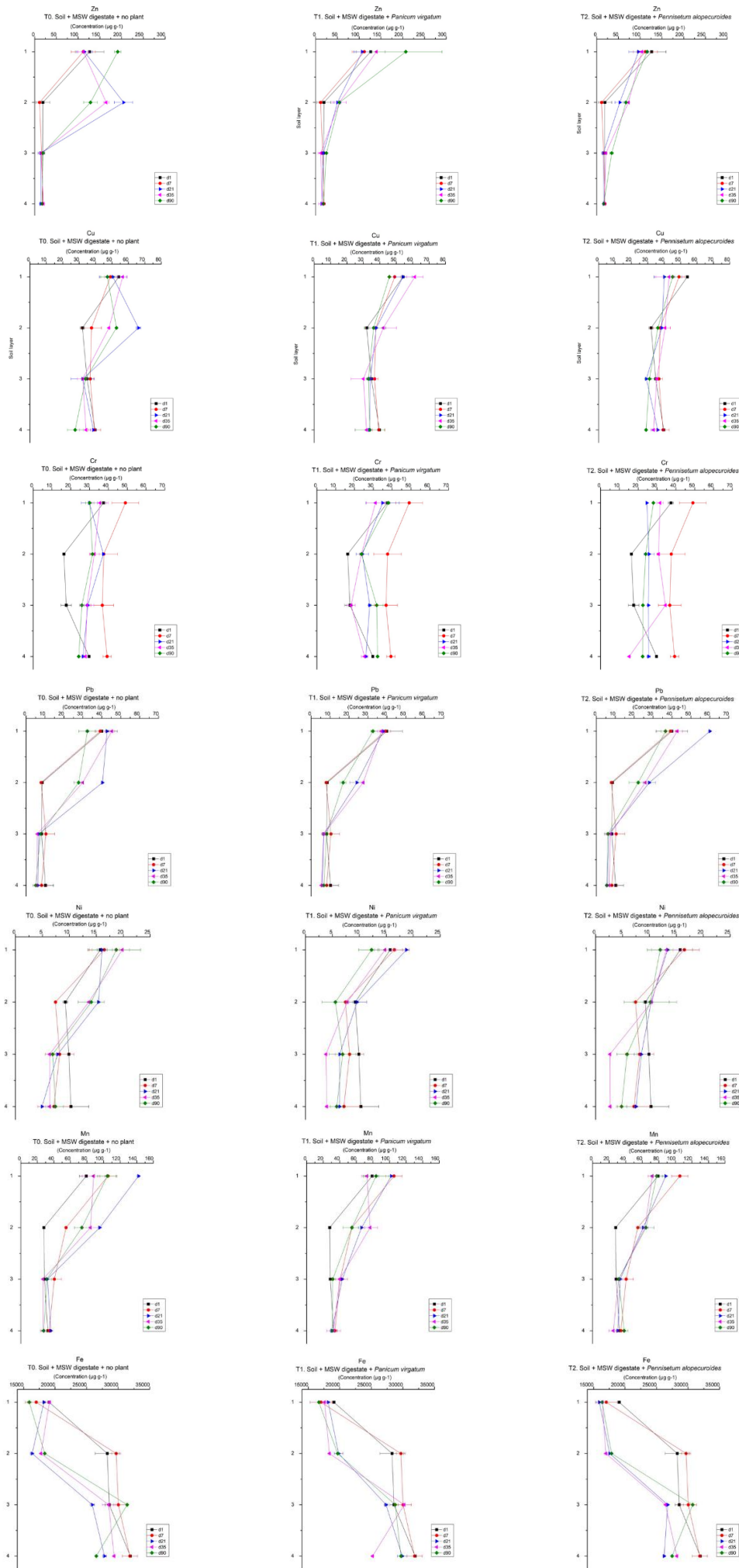


Figure 9. Total metal (Zn, Cu, Cr, Pb, Ni, Mn, and Fe) concentration ($\mu\text{g}\cdot\text{g}^{-1}$ dw) in soil solid phase along soil depth; layers (1, 0–0.1 m; 2, 0.1–0.2 m; 3, 0.2–0.3 m; 4, 0.3–0.4 m) in soil columns with and without plants over time (days 1, 7, 21, 35, and 90). Plants were transplanted into the respective columns at day 7. Error bars indicate the standard error of the mean of the three measurements per each duplicated column ($n = 2 \times 3$).

2.4.D Metal fractionation as a function of time, depth, and presence of plants.

To evaluate the evolution of the potential availability of TMs in the soil amended with MSW digestate over time and depth, and possible plant presence effects, metal fractionation was determined in samples collected in soil columns after the stabilization period (7 days) and at the end of the experimental period (90 days) for Cu, Cr, Pb, and Zn. The TMs of concern could cause ecotoxicological effects in the soil ecosystem upon amendment (Figure 10).

At day 7, Cu, Cr, and Pb were found mostly in the oxidizable + residual fraction in all soil layers, indicating that TMs were in their less mobile state, as previously observed for the initial samples. This was followed by the reducible fraction (TMs bound to oxides which are unstable under reducing conditions), while the exchangeable fraction (bound to the soil by weak adsorption in the particles, therefore the most “mobile” fraction) was very low. Zn was found to be the most bioavailable TM; in the top layer (0–0.1 m), approximately 25% of the total concentration of Zn corresponded to the exchangeable fraction, whereas 47% corresponded to the reducible fraction. According to the literature, the mobility of the selected TMs in soils decreases in the following order: $\text{Zn} > \text{Cu} > \text{Pb}$ (Finzgar et al., 2007). Regarding soil columns without plants (treatment T0), in general, at the end of the experimental period, Zn, Cu, Cr, and Pb were mostly found in the oxidizable and residual fractions. As previously detailed, these TMs are probably non-mobile, not available, being bound to stable, high-molecular weight substances (Kazi et al., 2005; Van Poucke et al., 2020a; Van Poucke et al., 2020b), being probably sequestered by organic matter in the soil matrix. Overall, the reducible fraction of TMs in the first soil layer (0–0.1 m) decreased over time when compared to that on day 7, increasing in the second soil layer (0.1 m–0.2 m), accordingly to the migration of TMs observed for the total TM concentration. Layers below 0.2 m remained practically unchanged. Concerning Zn, both exchangeable and reducible fractions decreased in the 0–0.1 m layer, while both fractions increased in the 0.1–0.2 m layer. However, no significant ($p > 0.05$) changes were observed in TM fractions between day 7 and day 90. Previous studies reported that there is a risk in the application of bio-amendments from

municipal waste or urban sources, such as digestate or sewage sludge, in soil since both quantity and bioavailability of TMs may increase in the soil (Cambier et al., 2019). However, in the present study, a low metal mobility was observed without a significant increase in metal bioavailability. This was even more noticeable when the plants were present. No significant ($p > 0.05$) changes between presence (T1 and T2) and absence of plants (T0) were observed in Cu and Cr fractionation. However, for Pb, while its fractionation in columns with *P. virgatum* (T1) was similar to that found in soil columns without plant (T0), *P. alopecuroides*'s presence (T2) seemed to slightly increase the Pb reducible fraction. These results are in accordance with the reported high solubility of Pb at low pH (Sauvé et al., 1997). For Zn, however, both plants clearly changed Zn fractionation and availability, increasing it in the second soil layer (0.1–0.2 m) ($p < 0.05$). This could be explained by the rhizosphere activities, discussed in the previous section, which can in turn induce a decrease in pH (Table S3, Appendix I). The 0–0.2 m (rhizosphere) layer of both soil columns with the plants (*P. virgatum* and *P. alopecuroides*) showed a one-unit pH decrease compared to the 0.2–0.4 m layer, 7.1 vs. 8.2. This pH decrease could be explained by a root-mediated pH shift, which could be attributed to a combination of mechanisms, including 1) the cation–anion exchange balance; 2) organic acid release; 3) root exudation and respiration, 4) redox-coupled processes; 5) release of H^+ ions by the roots in their immediate vicinity (Seitz et al., 2022). Soil pH decrease can increase TM mobility and availability since it plays a major role in the sorption of metals as it directly controls the solubility of metal hydroxides, as well as of metal carbonates and phosphates (Sauvé et al., 2000; Michaud et al., 2007; Blossfeld et al., 2010; Seshadri et al., 2015; Antoniadis et al., 2017). Soil pH can also affect metal hydrolysis, ion-pair formation, and organic matter solubility.

Nonetheless, despite some changes in metal availability in upper layers, the combined study of total TM concentration and fractionation indicated that the presence of plants reduced metal transport through the soil profile in the short term (90 days).

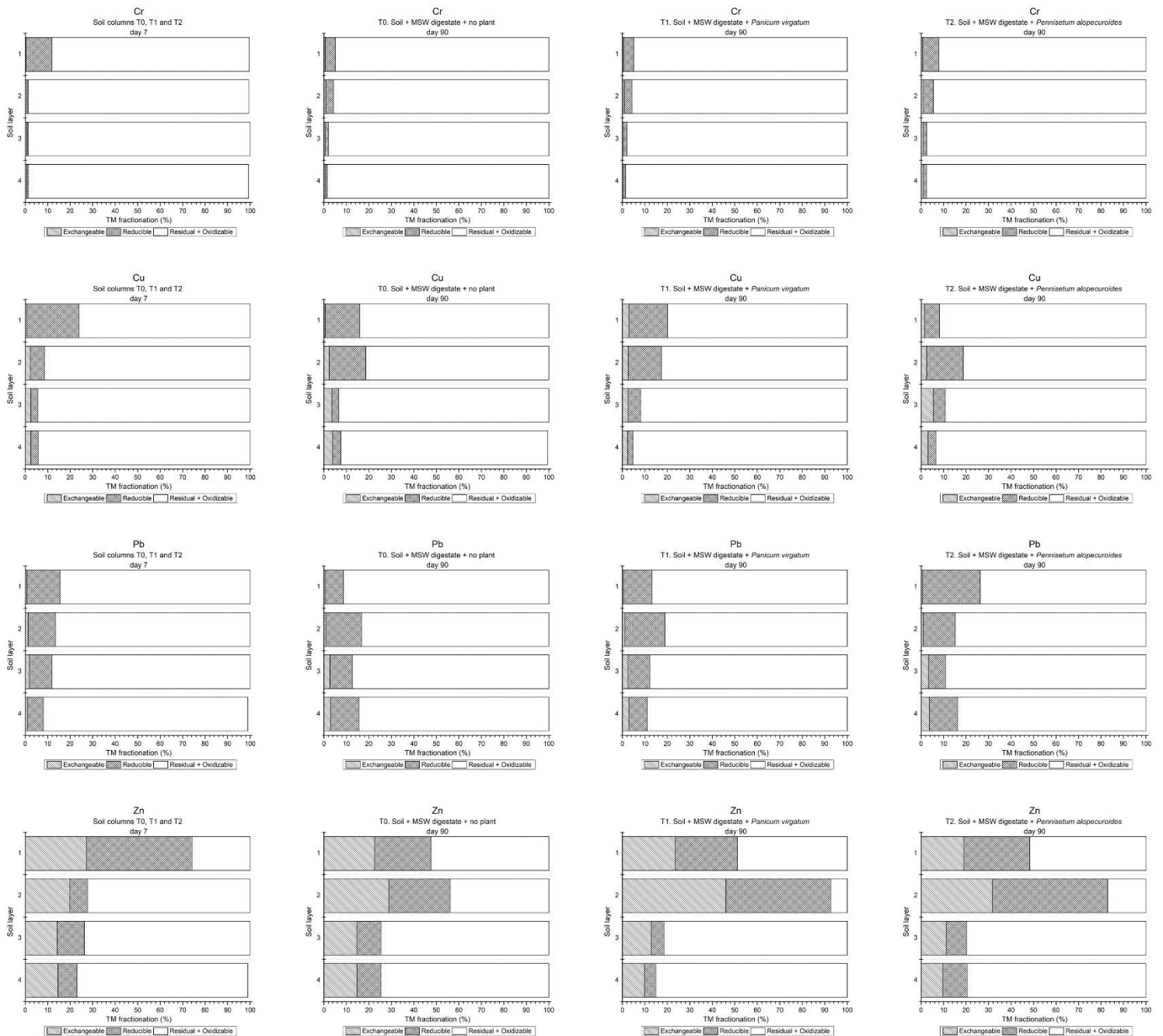


Figure 10. Percentage of metal fractions (Cr, Cu, Pb, and Zn) (exchangeable, reducible, and residual + oxidizable) along soil layers (1, 0–0.1 m; 2, 0.1–0.2 m; 3, 0.2–0.3 m; 4, 0.3–0.4 m) in soil columns after the initial stabilization period (day 7) and the end of the experimental period (day 90). Three measurements per each duplicated column ($n = 2 \times 3$) were performed.

2.4.E Effects of MSW digestate on plant growth

Positive significant effects ($p < 0.05$) of MSW digestate amendment were observed on the growth of both plants (Figure 11). At the end of the experimental period, both plants presented a significantly higher ($p < 0.05$) dry matter biomass in MSW digestate-amended soil conditions when compared to the unamended soil (Figure 11). *P. virgatum* in the MSW digestate-amended soil presented a greater ($p < 0.05$) total biomass than *P. alopecuroides* under the same conditions. Furthermore, plants' height evolution over time was increased with MSW digestate amendment (Figure 11). Digestates can enhance plant growth directly through physiological and nutritional effects. Some substances found in digestates can function as natural plant hormones (auxins and gibberellins) and can improve seed germination, root initiation, and uptake of plant nutrients. Digestates can also serve as sources of biologically available N, P, and K, which can contribute to plant growth (Liang et al., 1996; Tambone et al., 2010). For instance, in this study, it has been shown that MSW digestate application increased P2O5 concentration in soil (Table S1, Appendix I). Indirectly, digestates may affect plant growth through modifications of the physical, chemical, and biological properties of the soil, for example, enhanced soil water holding capacity and CEC and improved soil structure (Stevenson, 1994). In addition, recent studies have identified beneficial microbes in digestates, such as plant growth promoting bacteria (PGPB) (Qi et al., 2017). In fact, it has been demonstrated that the application of different digestates from different sources improved crop yields (Zheng et al., 2019; Jimenez et al., 2020; Jamison et al., 2021; Panuccio et al., 2021), as observed in the current study. Tan et al. (2021) showed that the bioenergy crop hybrid giant Napier grass increased the total yield when digestate was applied. In addition, Walsh et al. (2012) found that grasses amended with liquid digestate gave similar or better yields than those receiving either N or NPK inorganic fertilizers. Alburquerque et al. (2012) found that the addition of the digestate increased the soil microbial biomass and activities, which provided a greater amount of organic carbon to the soil, ultimately causing a positive effect on crop yields. Lopushniak et al. (2021) performed prognostic models of *P. virgatum* L., and according to their results, the use of sewage sludge provided a higher dry biomass yield than unamended soil. Rodgers & Anderson (1989) already demonstrated the benefits of sludge amendment in *P. virgatum* and other grass crop species. Yue et al. (2017) reported how municipal sewage sludge biochar amendment induced an improvement of poor urban soil fertility and turf grass nutrition and growth.

Therefore, the amendment with the MSW digestate was beneficial to plants. Higher plant growth can lead to higher exudation of compounds which can influence TM mobility and availability and could influence the TM behaviors as discussed in the previous sections, contributing for a higher retention of TMs in the layer of the soil amended with the MSW digestate, reducing TM mobility in the layer of the soil beneath it. A higher plant growth and an increase in plant biomass can also lead to a high amount of TMs being removed from contaminated matrices due to a higher metal uptake, which will also reduce the possibility of TM mobility through the soil profile.

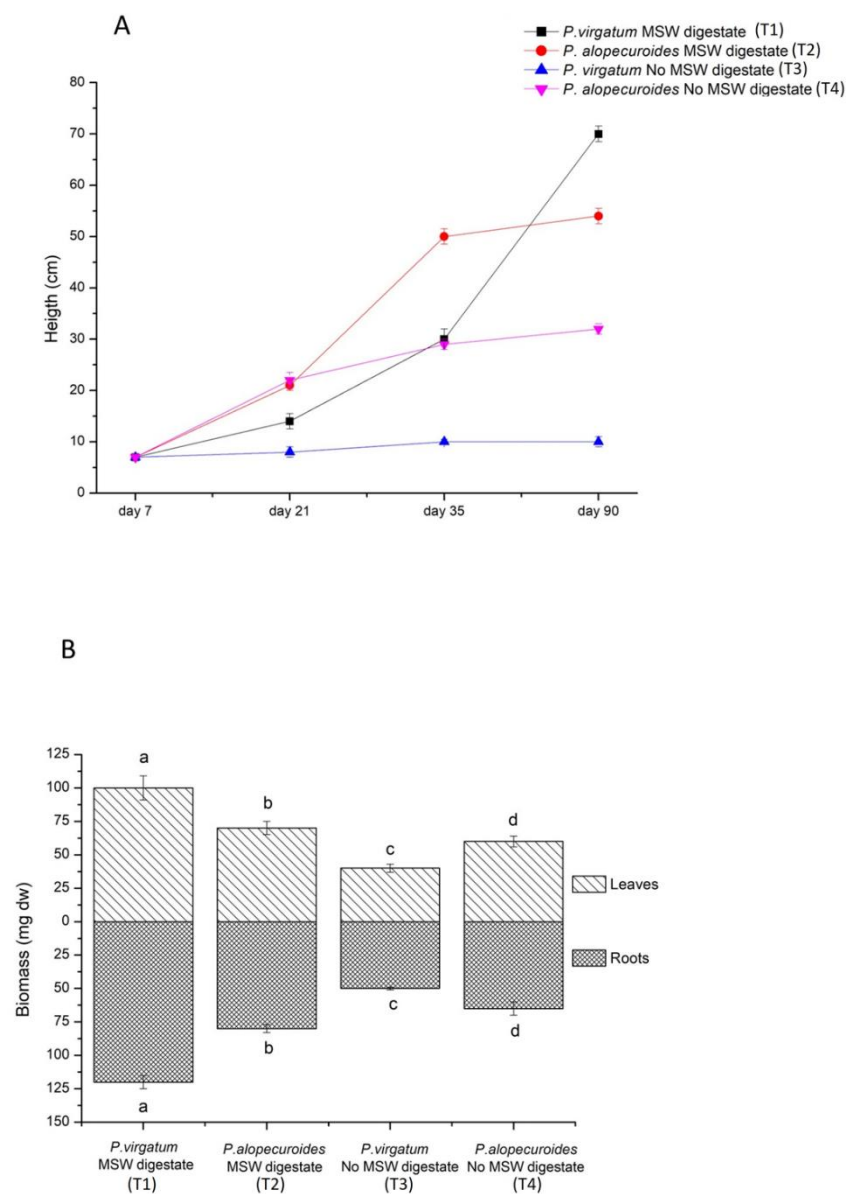


Figure 11. (A) Average height (cm) (+SD) over time of *P. virgatum* and *P. alopecuroides* grown in the presence or absence of MSW digestate. (B) Average dry mass (mg) (+SD)

at the end of the experimental period (day 90) of *P. virgatum* and *P. alopecuroides* grown in the presence or absence of MSW digestate. Error bars indicate the standard error of the mean of three measurements per each duplicated column ($n = 2 \times 3$) in the case of plants from MSW-amended columns (T1 and T2) and three measurement per single column ($n = 1 \times 3$) for plants in the unamended column (T3 and T4). Columns with different letters are significantly different at the 0.05 level of significance by Tukey's multiple comparison test.

2.4.F Plant metal uptake

After harvesting the plants at the end of the experiment, the total concentration of the two essential TMs (Zn and Cu) and one non-essential TM (Pb) in plant tissues was determined to evaluate whether the plants were adopting a phytoextraction strategy.

When analyzing phytoextraction, different factors should be taken into consideration: 1) the plant's potential to accumulate the TM and 2) the plant's ability to transfer the accumulated contaminants from belowground to the aboveground tissues. Therefore, the bioconcentration factor and translocation factor were calculated to evaluate TM uptake, mobilization into the plant tissues, and storage in the shoot parts. The BCF is described as the ability of plants for elemental accumulation from the substrate (Radziemska, 2018), being used to measure the TM accumulation efficiency in plants; BCF values >1 indicate a potential TM hyperaccumulator species (Zhang et al., 2002). The TF explains the ability of a plant to translocate the TM from the roots through the shoots and leaves, mediated by the xylem and/or phloem cells (Rascio and Navari-Izzo, 2011), being used to evaluate plants' capacity for phytoextraction.

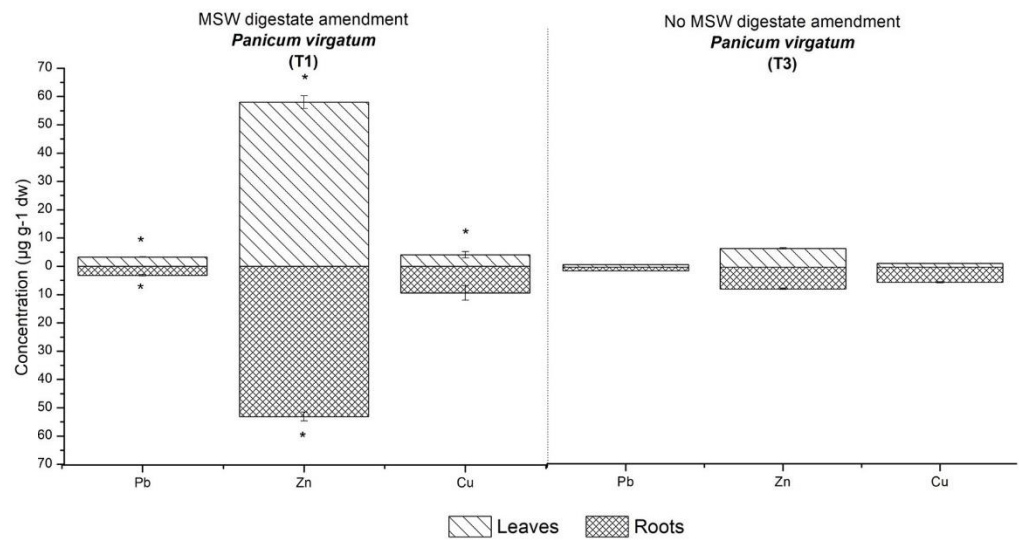
Pb, Zn, and Cu concentrations in plant tissues grown in the MSW digestate-amended soil were significantly higher than in plants grown in the unamended soil ($p < 0.05$) (Figure 12), being the most noticeable for Zn. In general, Zn levels were statistically higher ($p < 0.05$) in *P. virgatum*. In both plant species, the concentration of TMs in the leaves and roots followed the order $\text{Zn} > \text{Cu} > \text{Pb}$; both in the MSW digestate-amended and -unamended soil, however, the differences between Cu and Pb concentrations were not statistically significant. This was in accordance with the metal fractionation results where Zn was found mostly in exchangeable and reducible fractions, considered to be the most bioavailable TM in the MSW digestate-amended soil and so more easily taken up. In addition, according to the total TM concentration, Zn was the most abundant TM.

Both in the unamended and MSW digestate-amended soil, the BCF <1 was observed for both plants for all the TMs. BCF values were slightly higher for *P. virgatum* (Figure 13). Even though Zn and Cu are essential elements for all living organisms (Bowen, 1966), no evidence of bioconcentration was found. In addition, despite Zn being the most available TM, no evidence of hyperaccumulation in plants was found. Regarding TM translocation abilities, for Zn a TF >1 was observed for both plant species. As mentioned previously, a TF value higher than 1 indicates that the TM is stored mainly in the aboveground part of the plant, which is primarily responsible for phytoextraction (Nirola et al., 2015). The observed results suggest a potential for both plants to relocate Zn to the aboveground biomass. The highest accumulation of the contaminants in the aerial part is interesting from the phytoextraction point of view. Once the contaminants are in the aerial part, the biomass harvest will contain the metals removed from the MSW digestate-amended soil. Previous studies have shown that *P. virgatum* can uptake TMs such as Cd, Cr, and Zn from contaminated soils (Cui et al., 2011; Aderholt et al., 2017; Afzal et al., 2017; Guo et al., 2019). Mei et al. (2020) also showed *P. alopecuroides* had good Cu, Pb, Zn, and Cd uptake abilities when exposed to synthetic stormwater in bioretention plants, as also observed in this study, although without the bioconcentration capacity. Differences in TM accumulation between the two plant species could be attributed to different plant uptake capacities; variations in the plant biomass, as *P. virgatum* presented a higher biomass; or the differences in the rhizosphere microbial community structure (not determined), rather than metal availability differences, which were not significant, as discussed previously. On the other hand, differences of the TF between the MSW digestate-amended and unamended soil could be explained by the differences in TM bioavailability and the plant biomass between the different conditions, as well as the differences in rhizosphere microbial diversity. The TM type, sources, physical and chemical behavior, environmental factors (Usman et al., 2020), plant biomass, and rhizosphere microbial diversity (Wood et al., 2016) can all play a role in TM extraction and accumulation by plants.

Therefore, the decrease in Zn, Cu, and Pb in the MSW digestate-amended soil layer and reduced migration into the soil layer beneath it when plants were present could be more closely associated with the physical and chemical stabilization of TMs in the soil through phytostabilization processes rather than direct root metal accumulation by the plants. However, the plants showed a capacity to translocate and accumulate Zn in aboveground tissues. The results suggest that different phytoremediation processes may be occurring, which combined, can help immobilize TMs in the rhizosphere and

plant tissues (Kidd et al., 2009; Alkorta et al., 2010) and protect deeper layers of the soil from TM migration. Further research would help identify the phytoremediation mechanisms adopted by the plants, which in turn would help optimize the process.

A



B

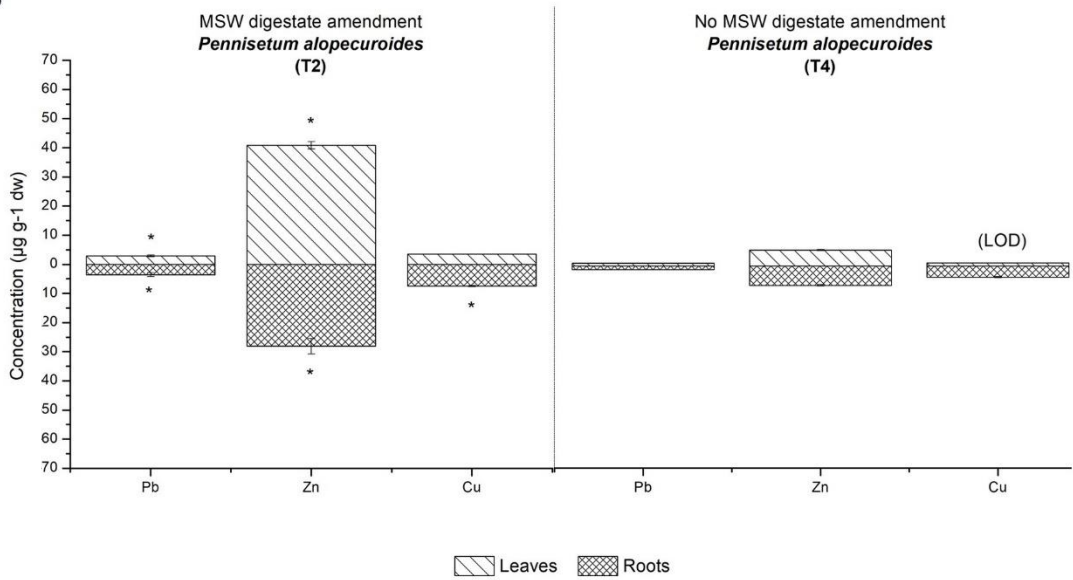


Figure 12. Concentration ($\mu\text{g}\cdot\text{g}^{-1}$) on dry weight basis (+SD) of Pb, Zn, and Cu at the end of the experimental period (day 90) in roots and leaves of *P. virgatum* and *P. alopecuroides* grown in the presence or absence of MSW digestate. (A) *P. virgatum* grown in the presence and absence of MSW digestate, (B) *P. alopecuroides* grown in the presence and absence of MSW digestate. (*) indicates the significant differences of each metal concentration between the presence and absence of the MSW amendment at the 0.05 level of significance by Tukey's multiple comparison test. Error bars indicate the standard error of the mean of the three measurements from two columns ($n = 2 \times 3$) for T1 and T2, and the mean of three measurements from a single column ($n = 1 \times 3$) for T3 and T4. The values were below the limit of detection (LOD) for Cu ($1.5 \mu\text{g}\cdot\text{g}^{-1}$).

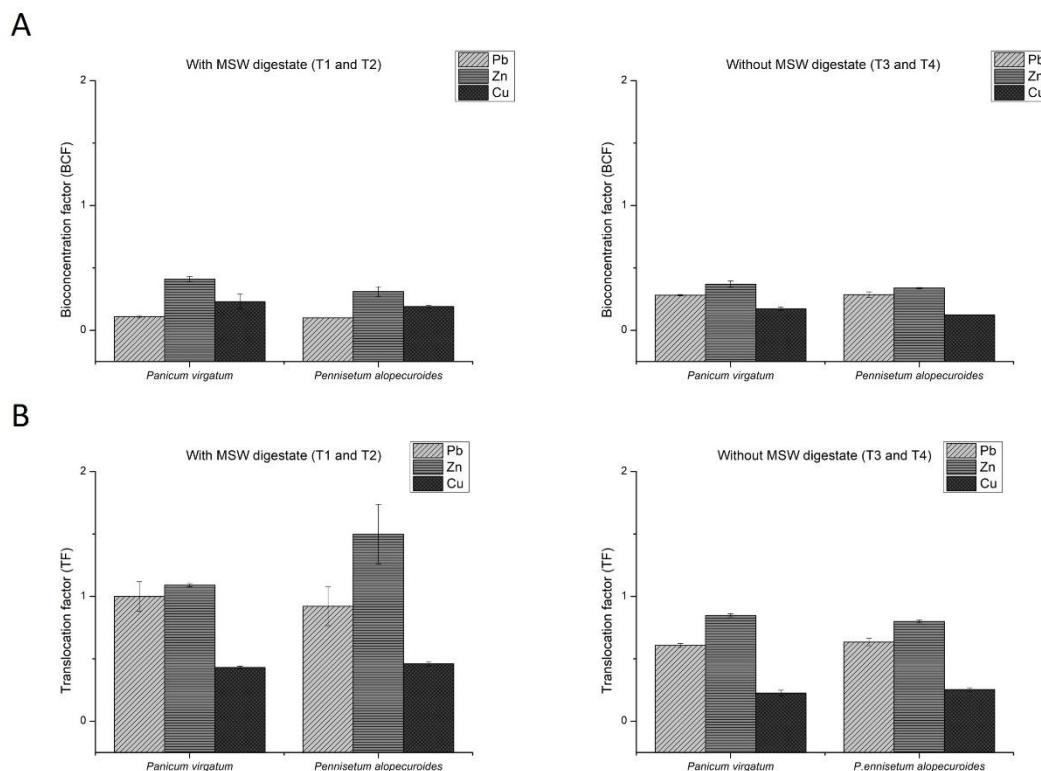


Figure 13. Root bioconcentration factor (BCF) and translocation factor (TF) (+SD) of Pb, Zn, and Cu for *P. virgatum* and *P. alopecuroides* grown in the (A) MSW digestate amended soil and (B) unamended soil, at the end of the experimental period (day 90). Error bars indicate the standard error of the mean of the three measurements from two columns ($n = 2 \times 3$) for T1 and T2 and the mean of three measurements from a single column ($n = 1 \times 3$) for T3 and T4.

2.5 Conclusions

The results showed that MSW digestate application increased the organic matter content and the macro- and micronutrients in the marginal soil, indicating a potentially suitable use in soil reclamation. Furthermore, MSW digestate exhibited a positive impact on the growth rate and biomass yield of *P. virgatum* and *P. alopecuroides*, with the latter showing a slightly less biomass growth.

Overall, total TM concentrations increased in the 0.1–0.2 m soil layer over time, the soil layer beneath the digestate-amended soil, suggesting a downward migration of TMs. Nevertheless, total TM concentrations tended to be confined in the upper layers (0–0.2 m) of the soil profile, indicating no metal migration to the deep soil layers and no evidence of risk, for instance, of groundwater contamination. The highest concentrations in bioavailable metal fractions were found in the first layers, the 0–0.1-m MSW digestate-amended soil and 0.1–0.2 m, especially for Zn. The other TMs, namely, Cu, Cr, Fe, Mn, and Pb, were mostly found in oxidizable–residual fractions in all layers of the soil profile, showing a low availability. *P. virgatum* and *P. alopecuroides* reduced TM migration to the 0.1–0.2 m layer, in particular that of Zn, Cu, and Pb, suggesting a protective effect. Moreover, in general, no significant differences were observed among TM fractionation between the presence and absence of plants. In fact, only an increase in Zn availability in the 0.1–0.2 m layer was observed when *P. virgatum* was present. Within the studied conditions, higher concentrations of TMs were found in both plants' tissues when exposed to MSW digestate-amended soils relatively to unamended soils. However, no bioconcentration capacity was observed for the two plants.

Although the current findings cannot be extended to all soils, for the bio-amendment types and field situations, it is expected that the current research will add to the body of knowledge regarding the potential benefit of the MSW digestate, along with its impact on soil attributes and the use of phytoremediation technologies as an economically green alternative to increase the safety of such practices. Further experiments are needed to analyze the long-term effects and large-scale implementation.

Ultimately, this approach may help achieve the Mission Board's proposal to the European Commission that aims to ensure 75% of healthy soils by 2030 for food, people, nature, and climate (EC, 2020). Furthermore, it may also help address the United Nations Sustainable Development Goals (SDGs) 7 ("Ensure access to affordable, reliable, sustainable and modern energy for all"), 13 ("Climate action"), and 15 ("Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests,

combat desertification, and halt and reverse land degradation and halt biodiversity loss”) (United Nations, 2016).

Authors contribution

NB-G: conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing—original draft. VB: methodology and writing—review and editing. VR: methodology, validation, visualization, and writing—review and editing. CG: resources and writing—review and editing. GG: conceptualization, funding acquisition. MA: resources and writing—review and editing. RC: resources and writing—review and editing. AM: conceptualization, funding acquisition, supervision, and writing—review and editing. MA: conceptualization, funding acquisition, supervision, validation, visualization, and writing—review and editing.

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3. Chapter 3: Response of soil and plant microbiome to digestate amendment and phytomanagement of a marginal soil

This chapter is based on the manuscript prepared for submission as:

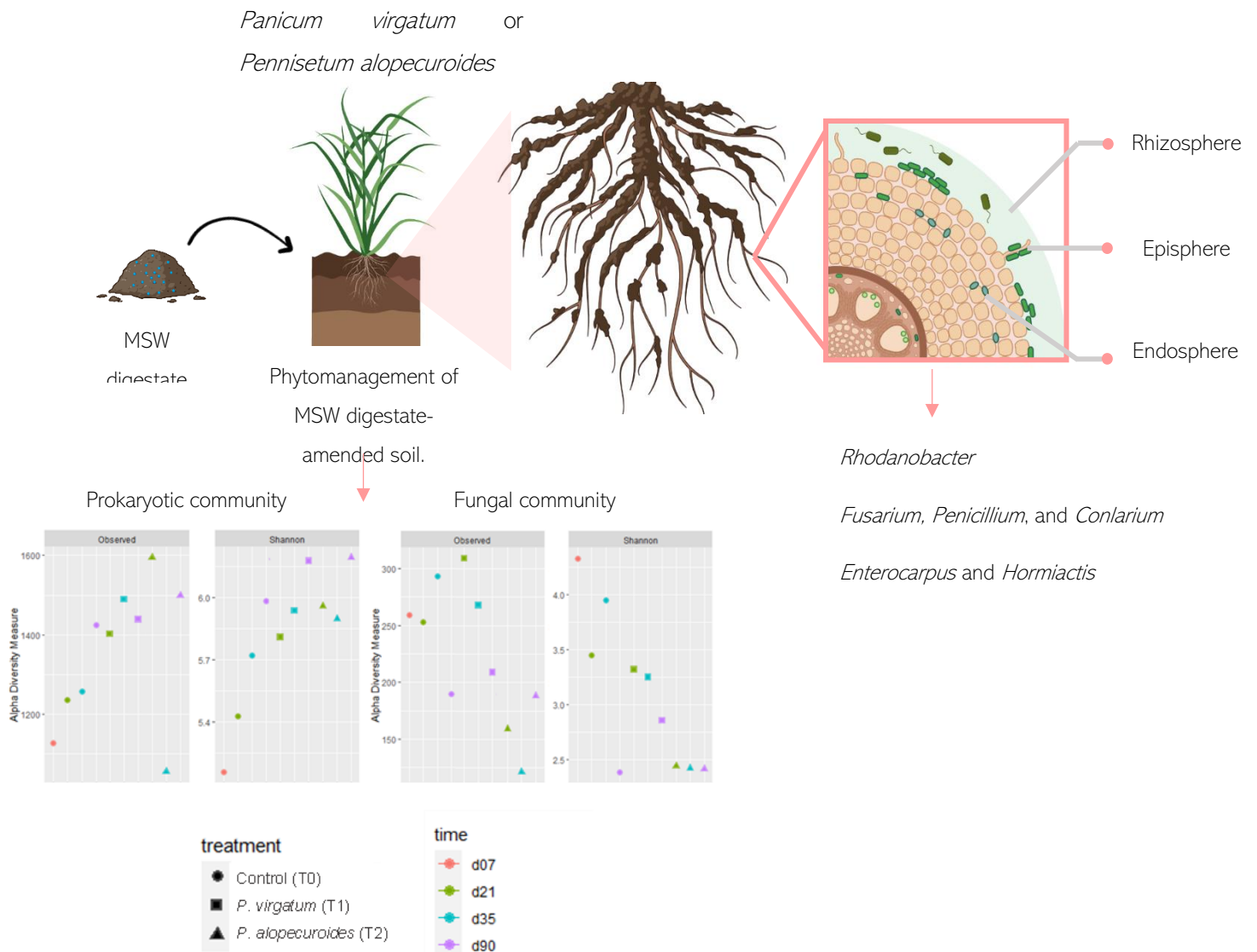
Neus Bonet-Garcia, Joana P. Fernandes, Maria Paola Tomasino, Isabelle Bourven, Gilles Guibaud, C. Marisa R. Almeida and Ana Paula Mucha. Response of soil and plant microbiome to digestate amendment and phytomanagement of a marginal soil

Abstract

This study explores the impact of municipal solid waste (MSW) digestate on soil microbial communities during a 90-day phytoremediation experiment. The research focuses on: (i) the evolution of prokaryotic and fungal communities in topsoil over time after MSW digestate amendment, with and without *Panicum virgatum* or *Pennisetum. alopecuroides*; and (ii) the prokaryotic and fungal communities in different plant root compartments in the presence and absence of MSW digestate. Following 90 days of MSW digestate amendment, topsoil displayed higher prokaryotic diversity, enriched in the presence of plants. Changes in soil properties induced by MSW digestate could be identified as the main factor contributing to the restructuring of microbial communities over time. By day 90, MSW digestate-amended soil demonstrated dominance in the prokaryotic phyla Proteobacteria, Bacteroidota, Firmicutes (Bacteroidota), Actinobacteriota (Actinomycetota), Planctomycetota, Verrucomicrobia, and the fungal phylum Ascomycota. The presence of *P. virgatum* and *P. alopecuroides* intensified the abundance of Planctomycetota, Chloroflexi, Verrucomicrobia, and Acidobacteria. The prokaryotic genera - *Aequorivita*, *Arenibacter*, *Actinomyces*, and *Tissierella* - found in the amended topsoil were absent in plant root compartments. *Rhodanobacter* may constitute a core microbiome for these plants. Predominant fungal genera in the root microbiome - *Fusarium*, *Penicillium*, and *Conlarium*, may contribute to a core microbiome fostering plant growth. *Enterocarpus* and *Hormiactis* identified in amended topsoil were also detected in plant root microbiomes when grown in presence of MSW digestate, suggesting potential plant selection. By elucidating the dynamics of microbial communities in response to MSW digestate amendment and bioenergy crops presence, this study sheds light on the potential of phytomanagement of digestate amended soil as a viable practice. Furthermore, comprehending the microbiome of bioenergy crops

cultivated in MSW digestate-amended soil enriches our knowledge of potentially crucial microorganisms that could aid plants in phytomanagement efforts.

3.1 Graphical abstract



Prokaryotic phyla: Proteobacteria, Bacteroidota, Firmicutes, Actinobacteriota (Actinomycetota), Planctomycetota, Chloroflexi, Verrucomicrobia, Acidobacteria.

Fungal phylum: Ascomycota.

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3.2 Introduction

Soil health is critical for the appropriate functioning of terrestrial ecosystems. Unfortunately, as the global population continues to grow, there is increasing pressure on land resources, leading to a decline in soil quality and health at a global scale, which can induce deleterious impacts on essential ecosystem services (FAO, 2019). On the other hand, global population growth also brings an increasing production of waste derived from anthropogenic activities, especially organic waste as the demand for food rises (Chen et al., 2020). It is essential to adopt sustainable waste management practices that reduce the amount of waste generated and maximize the recovery of resources. As mentioned in Chapter 1, General Introduction, anaerobic digestion (AD) is widely adopted for treating diverse organic wastes with simultaneous production of renewable energy source and a nutrient-rich by-product called digestate, which can be separated into a solid and a liquid fraction. However, AD's increasing popularity carries a growing production of digestate, the management of which has become a challenge (Kaur et al., 2020). Land application of digestate can be regarded as an increasingly attractive strategy to reduce landfill disposal and decline in soil fertility while decreasing the reliance on commercial fertilizers (Lu & Xu, 2021; Monlau et al., 2015; Mucha et al., 2019; Nkoa, 2014; Singh & Agrawal, 2008). In soils, solid digestate can promote the formation of stable aggregates and improve soil aeration, water-holding capacity, soil porosity, cation exchange capacity, and enhance soil organic C storage. Moreover, the use of organic amendments has been linked to both an increase of microbial biomass and changes in microbial community structure and diversity (Badagliacca et al., 2020; Cattin et al., 2021; Cytryn et al., 2011; De la Fuente et al., 2013; Gomez et al., 2006; Li et al., 2013; Marschner et al., 2003; Reardon & Wuest, 2016). These alterations are likely to be connected with changes in the functional diversity of soil microbial communities (Fierer et al., 2012), which could potentially improve soil physicochemical properties, fertility and crop production (Nannipieri et al., 2003; Shu et al., 2022; Torsvik & Øvreås, 2002). One challenge with the field application of digestate lies in its chemical safety (Golovko et al., 2022; Lamolinara et al., 2022), since it may result in the release of digestate-borne contaminants, such as trace metals (TMs), in the environment. TM content in digestate and its potential implications for the use as fertilizer has been a subject of increasing interest during the last decade (Bonetta et al., 2014; Coelho et al.,

2018; Govasmark et al., 2011; Kupper et al., 2014; Párraga-Aguado et al., 2017). TMs pollution could become a problem of great magnitude owing to their toxicity and persistence in soil, and their ability to negatively affect soil health, plant growth, food quality, and animal and human health and as so they should be removed from digestate.

Phytomanagement is a nature-based solution that can be regarded as a sustainable, environment friendly and cost-effective approach for managing TM-containing matrices, using TM-tolerant plants and their associated microorganisms to detoxify, reduce mobility or extract TMs. Therefore, it is a promising management option to attenuate the potential environmental risks associated with land application of digestate and restore the soil fertility and ecosystem functions. At the same time, plants and their associated microorganisms can benefit from the fertilizing properties of digestate. Bioenergy crops have been studied as potential candidates for phytoremediation (Barbosa et al., 2015; Pandey et al., 2016; Prabha et al., 2021; Van Ginneken et al., 2007), because they are fast-growing and have deep root systems and networks, thus having the potential of limiting contaminants dispersal in the environment. These crops can also be harvested and used for biofuel production, providing a dual benefit. In our previous study (Bonet-Garcia et al., 2023, Chapter 2) we integrated the phytomanagement of TMs with the application of digestate amendment to restore a marginal soil. We demonstrated that the application of municipal solid waste digestate (MSW) improved the quality of a marginal soil, albeit leading to increased total concentrations of TMs in the soil. However, the TMs introduced by the digestate exhibited low potential bioavailability. When bioenergy crops *Panicum virgatum* and *Pennisetum alopecuroides* were employed for phytomanaging the MSW digestate-amended soil, there was a notable reduction in the migration of Zn, Pb and Cu through the soil profile over time. These bioenergy crops not only mitigated the transport of TMs but also benefited from the enhanced soil quality resulting from the digestate amendment, reflected as an increase of plant biomass. These findings prompt the question about the microbial communities present during the phytomanagement of MSW digestate-amended soil, potentially playing a direct or indirect role in reducing TMs migration and supporting plant growth.

As so, this study aimed to investigate microbial community changes over time during the phytomanagement of a MSW digestate amended soil. Specific aims included: (i) investigate the evolution of the prokaryotic and fungal community structure over time in topsoil (0-0.1 m) after amendment with MSW digestate, both in the absence or presence of *P. virgatum* or *P. alopecuroides*, and (ii) compare the prokaryotic and fungal community structure in different root compartments (rhizosphere, episphere and

endosphere) of *P. virgatum* and *P. alopecuroides* grown in the presence or absence of MSW digestate at the end of the experiment (day 90). For that, a 90-day phytoremediation experiment was performed in vertical soil column mesocosms where a MSW digestate amended soil layer was placed on the top of the soil columns and the plants were transplanted to each respective soil column. We hypothesize that (i) the MSW digestate amendment will increase the prokaryotic and fungal diversity of soil, (ii) the presence of plants will result in higher prokaryotic and fungal diversity in the amended topsoil over time, and (iii) the root microbiome of plants cultivated with MSW digestate will exhibit greater diversity compared to those grown without MSW digestate.

3.3 Material and methods

3.3.A Initial samples

Soil samples were collected from the vicinity of a construction field the municipality of Valongo, Portugal (41°12'25.7"N 8°32'26.7"W). The solid fraction of MSW digestate used in these experiments was collected from an AD plant treating non-source separated organic fraction of MSW. Soil and MSW digestate physical and chemical characterization are included in (Table S1, Appendix II). Details on samples and characterization are shown in Chapter 2.

As described in Chapter 2, two bioenergy crop plants, *P. virgatum* and *P. alopecuroides*, were selected for this study. Plant selection was performed according to a pre-established criteria considering the following standards: metals (Cr, Mn, Fe, Co, Cd, Ni, Cu, Zn, Pb) uptake capacity; ability to produce a large volume of biomass; high energy potential; ability to be grown in marginal soils; non-edible; non-invasive behaviour and low-cost maintenance (Chen et al., 2012; Jia et al., 2022; McLaughlin & Adams Kszos, 2005; Shrestha et al., 2019; Zhang et al., 2016). One month old plants were obtained from a greenhouse located in Vigo, Galicia, Spain, where they were germinated and grown in commercial substrate at 22 ± 2 °C.

3.3.B Experimental design and sample collection.

Vertical soil column mesocosm experiments were assembled, in which three treatment conditions, with two replicates each, were tested: T0 (Soil + MSW digestate); T1 (Soil + MSW digestate + *P. virgatum*) and T2 (Soil + MSW digestate + *P. alopecuroides*) (Figure 1) (as described in Bonet-Garcia et al. (2023) and Chapter 2). Additionally, control columns for each plant species, with soil but no MSW digestate, were assembled to evaluate the root microbiome of *P. virgatum* and *P. alopecuroides* without MSW digestate amendment. The microcosms were assembled in rigid PVC columns (0.2 m

diameter × 0.6 m height) with 5 sampling ports including a hole at the bottom, with a tap, for leachate collection whenever needed (Figure 14). The lower part of each column (0.02 m) was filled with inert pebble (to avoid soil loss and allow water drainage), followed by addition of homogenized uncontaminated soil (0.3 m) previously sieved to <2 mm. To avoid positive preferential flows through the columns, soil columns were filled without air gaps enabling an equal level of homogeneity, subsequently, the soil was slightly manually compacted and saturated by adding a total of 5 L of deionized water. Afterwards, thoroughly manually mixed MSW digestate-soil mixture (proportion 1:1 v/v, 2 L each, to simulate digestate incorporation in agriculture soil) was placed at the top of uncontaminated soil as a 0.1 m layer, which is approximately the depth of digestate incorporation on agricultural fields (between 0.1 – 0.2 m) (Albuquerque et al., 2012). Experiments were carried out in a semi-open environment, inside of the Faculty of Sciences building of the University of Porto, Portugal, with a natural day/night regime at room temperature (21 ± 1 °C). Columns were fully covered with aluminum foil to avoid sunlight interferences. After 1 week of acclimation (day 7), one-month old seedlings with similar sizes of *P. virgatum* and *P. alopecuroides* were transplanted into the respective columns. The plants were grown for 83 days starting on 26th of April 2021). During the experimental time (90 days), the mesocosms were manually irrigated with 200 mL of deionized H₂O each week, to maintain 80 % of water holding capacity (WHC). Details are also given in Chapter 2.

Soil samples for microbial analysis were collected from (i) the initial unamended soil, initial MSW digestate and initial mixture soil + MSW digestate; (ii) the top layer of T0, T1 and T2 treatments on days 7, 21, 35 and 90. (iii) At the end of the experiment (day 90), roots from *P. virgatum* (in T1 and T3 treatment) and *P. alopecuroides* (in T2 and T4 treatments) were collected for root microbiome characterization.

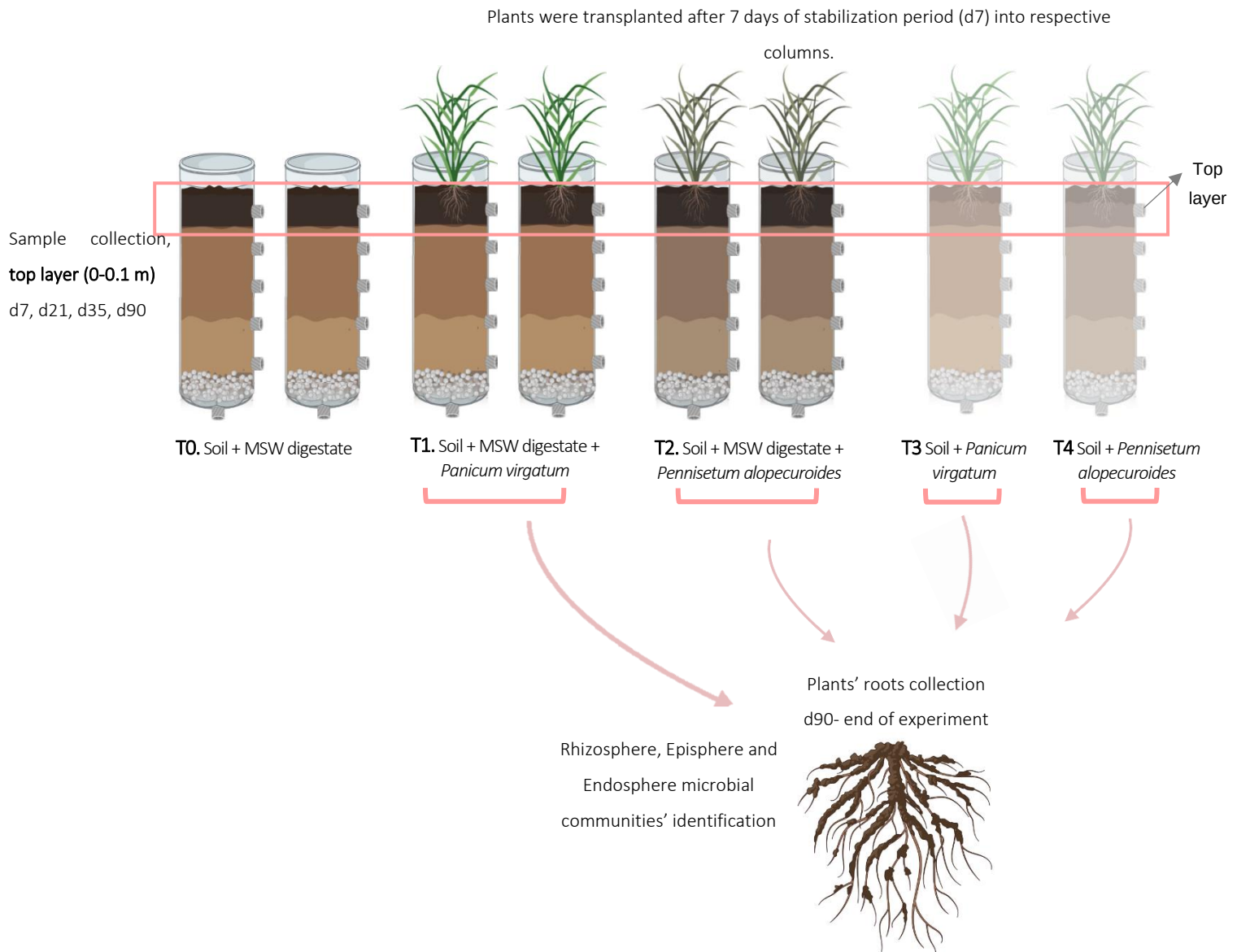


Figure 14. Graphic representation of soil columns mesocosms experimental design and sample collection for microbial community analysis. Figure created with Biorender.com. License agreement number: VT254U6HT3.

3.3.C Plants' microbial community.

At the end of the experimental period (day 90), roots from *P. virgatum* and *P. alopecuroides* and respective rhizosediment were collected and separated as follows: rhizosphere (~1mm thickness around the root), episphere (from roots surface) and endosphere (from surface-sterilized roots). For that, *P. virgatum* and *P. alopecuroides* roots were collected from T1, T2, T3 and T4 treatments, and the rhizosediment was

separated from the root by shaking. In case of T1 and T2, rhizosphere soil of plants from duplicated columns was homogenized. The rhizosphere soil which could not be removed by shaking was collected manually using a spatula. Then, to separate the episphere community, different roots from the plants were washed with 10 mL of sterile physiological solution (0.9 % NaCl) containing 0.01% Tween 80 by vortex-shaking for 2 min. To isolate the endophytic community, several roots were vigorously washed in physiological solution (0.9 % NaCl) for 10 min and surface sterilized in 70 % ethanol for 3 min, 0.9 % sodium hypochlorite for 5 min, 70 % ethanol for 20 min and rinsed four times for in sterile deionized water (4 successive washes, 30 seconds each) before a final washing step in sterile deionized water for 10 min, finally, roots were smashed with sterile mortar and pestle. Root compartment samples were stored at -20 °C until DNA extraction.

3.3.D DNA extraction

DNA was extracted from: (i) initial samples (unamended soil, MSW digestate and MSW-digestate amended soil); (ii) soil samples of the topsoil (0 -0.1 m) layer (the MSW digestate amended soil) from T0, T1 and T2 treatments on days 7, 21, 35 and 90 (soil samples from duplicated columns were homogenized) and (iii) root microbiome (rhizosphere, episphere and endosphere) of *P. virgatum* and *P. alopecuroides* at day 90 in presence (T1 and T2) and absence (T3 and T4) of MSW digestate, using the DNeasy PowerSoil Pro Kit (Qiagen GMBH, Germany). For the initial samples, topsoil samples over time and rhizosphere soil, 0.5 g (wet weight) were used to extract DNA, following the manufacturer's instructions. On the other hand, to extract the endosphere and episphere prokaryotic and fungal community, some modifications to the extraction protocol were performed. In detail, 2/3 zirconia beads were added to the PowerBead Pro Tube containing the plant tissue and the CD1 solution and the tubes were incubated at 55° for 10 min (Jiang et al., 2015). Then the tubes were vortex for 25 min and then, the protocol provided by the supplier was followed. In the end, elution buffer was incubated at 65 °C for 5 min prior to use. The DNA concentration was quantified using the Qubit® dsDNA HS Assay Kit and the Qubit® 3.0 Fluorometer (Invitrogen, Carlsbad, CA). The DNA quality based on the ratios 260/280 and 260/230 nm was assessed with DeNovix DS-11 DS-11-FX spectrophotometer, using 1 µl of DNA sample.

3.3.E Prokaryotic and fungal community characterization.

For prokaryotic community, the 16S rRNA gene fragment (hypervariable V4 region) was amplified using the primers set 515F-Y 5'- GTGYCAGCMGCCGCGGTAA -3', 806rB 5'- GGACTACNVGGGTWTCTAAT -3' (Caporaso et al., 2011; McCormick et al., 2014).

While, for fungal community, the internal transcribed spacer ITS2 was amplified using the primers set ITS3F 5'-CATCGATGAAGAACGCAG-3', ITS4R 5'-TCCTSCGCTTATTGATATGC-3' (Tedersoo et al., 2014). The first PCR conditions for the 16S rRNA involved a 3 min denaturation at 95 °C, followed by 10 cycles of 98 °C for 20 s, 57 °C for 30 s and 72 °C for 30 and a final extension at 72 °C for 5 min. The first PCR conditions for the ITS2 of 25 cycles of 98 °C for 20 s, 47 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min.

In the second PCR reaction, indexes and sequencing adapters were added to both ends of the amplified target region according to manufacturer's recommendations. Negative PCR controls were included for all amplification procedures. Then, PCR products were purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal). Raw reads were extracted from Illumina MiSeq® System in fastq format and quality filtered with PRINSEQ version 0.20.4 (Schmieder & Edwards, 2011) to remove sequencing adapters, reads with less than 150 bases and trim bases with an average quality lower than Q25 in a window of 5 bases (refs). The forward and reverse reads were merged by overlapping paired end reads with AdapterRemoval version 2.1.5 (Schubert et al., 2016) using default parameters.

The sequence analysis of 16S rRNA and ITS2 gene amplicons was carried out using DADA2 pipeline (version 1.12.1) (Callahan et al., 2016). The pipeline was used to filter reads and chimeras, join paired end reads, denoise and dereplicate sequences and providing an Amplicon Sequence Variance (ASV) table as output (Callahan et al., 2017). The demultiplexed and primer-clipped reads were trimmed based on quality and length for prokaryotic 16S V4 sequences (240 and 160 bp for forward and reverse reads, respectively) and for fungal ITS2 sequences (190 and 150 bp for forward and reverse reads respectively, to maintain a high Q score and sequence overlap). Trimmed sequences were dereplicated, de-noise, merged and chimeras were removed. The resulting 16S rRNA gene ASVs were taxonomically classified against SILVA v.132 using the Naive Bayes classifier, (available at <https://www.arb-silva.de/download/archive/qiime>) (Yilmaz et al., 2014). The ITS2 ASVs were taxonomically classified using UNITE v.9 (available at <https://unite.ut.ee/repository.php>) (Nilsson et al., 2019).

3.3.F Downstream data analysis.

The alpha-diversity indices including Observed ASVs and Shannon index (Shannon & Weaver, 1949) were obtained from the ASVs tables using the R package *phyloseq* (v1.27.6). Bray-Curtis dissimilarity coupled with non-metric multidimensional scaling (NMDS) was conducted to indicate the community dissimilarities (Bray & Curtis, 1957) using the R packages *phyloseq* (v1.27.6) (McMurdie & Holmes, 2013) and *vegan* (v2.6–2) (Oksanen et al., 2019). The graphs were made using the R packages *ggplot2* (v. 3.3.6) (Wickham et al., 2016), *scales* (v. 1.1.0) (Wickham et al., 2019a), *dplyr* (v. 1.0.10) (Wickham et al., 2019b), *gridExtra* (v. 2.3) (Auguie & Antonov, 2017), *RColorBrewer* (v 1.0.1) and *cowplot* (v. 1.0.0) (Wilke et al., 2019).

For the community composition analysis, a pre-treatment of transformation to relative abundance was performed for each sample with the R package *phyloseq* (v1.30.0) (McMurdie and Holmes, 2013). For analysis at the phylum level, total taxa were included and taxa that contributed with more than 1 % of the relative abundance for each sample was pooled together. For analysis at the genus level, total taxa were included and taxa that contributed with more than 2% or 5 % of the relative abundance for each sample was pooled together. The taxonomic analyses at both phylum and genus levels were done with R package *phyloseq* (v. 1.30.0) (McMurdie and Holmes, 2013) and the corresponding graphs were made using the R packages *tidyverse* (v. 1.3.0) (Wickham et al., 2019c) and *ggplot2* (v. 3.3.6) (Wickham, 2016).

3.3.G Statistical analysis.

Dissimilarity results were tested by permutational multivariate ANOVA (PERMANOVA) using the Adonis function (*vegan*) for beta group significance (p-value < 0.05). Parameters treatment and time were tested in soil columns samples.

3.4 Results

3.4.A Upstream Sequence Outputs

Total prokaryotic and fungal communities analysis at phylum and genus level of (i) initial samples (soil, MSW digestate and MSW digestate amended soil), (ii) soil samples from top (0-0.10 m) layer of T0, T1 and T2 columns collect over time (days 7, 21, 35 and 90), and (ii) root microbiome (endosphere, episphere and ehizosphere) from *P. virgatum* and *P. alopecuroides* in presence (T1 and T2 columns) or absence (T3 and T4 columns) of MSW digestate at day 90, were performed through amplicon sequencing of v4 hypervariable region in the 16S rRNA gene for prokaryotic community characterization (both bacterial and archaeal community), and Internal transcribed spacer (ITS2) region (located between 5.8S and 28S rRNA genes) for fungal community characterization. Sequencing results were subjected to bioinformatic workflow.

For prokaryotic sequences (16S v4), after filtering out low-quality and short sequence reads, a total of 2,403,087 raw sequences were obtained from 19 samples. The number of prokaryotic sequences varied from 78,284 to 190,159 per sample. These sequences were clustered into ASVs, and the number of ASVs ranged from 953 to 1,477. The ASVs which were taxonomically assigned to eukaryotic, mitochondrial or chloroplast sequences were removed from further analysis. The rarefaction curves of all were generated and normalized to the minimum number of sequences. For fungal sequences (ITS2), after filtering out low-quality and short sequence reads, a total of 1,582,410 raw sequences from 19 samples were obtained. The number of fungal sequences varied from 25,452 to 147,462. These sequences were clustered into ASVs, and the number of ASVs ranged from 321 to 1,240.

3.4.B Prokaryotic and fungal community characterization of initial samples (soil, MSW digestate and MSW digestate amended soil).

3.4.B.1 Alpha diversity

Results of prokaryotic and fungal community characterization of initial samples are reported in Figure 15. No fungal community analysis from initial soil sample was performed due to low amount of DNA obtained after soil samples extraction. Alpha-diversity indices - richness index (Observed ASVs)) and Shannon index- of prokaryotic community increased in MSW digestate amended soil when compared both to the initial unamended soil and the MSW digestate (Figure 15A). Regarding fungal community richness index (Observed ASVs)) and Shannon index were also higher in MSW digestate amended soil when compared to the MSW digestate (Figure 15D).

3.4.B.2 Taxonomic profiles

The phylogenetic diversity of the prokaryotic community in the initial soil was primarily distributed among 3 different bacterial phyla: Proteobacteria (Pseudomonadota) (81 %); Bacteroidota (10 %) and Firmicutes (Bacillota) (6%) (Figure 15B). The Proteobacteria (Pseudomonadota) phylum was partially represented by the genus *Pseudomonas* (39%) while Bacteroidota was mainly represented by the genus *Chitinophaga* (9%). Genera within the Firmicutes (Bacillota) phylum were found at abundances below 2% (Figure 15C).

The phylogenetic diversity of MSW digestate was mainly distributed among 5 bacterial phyla: Firmicutes (Bacillota) (39 %), Bacteroidota (20 %), Cloacimonadota (12%), Thermotoga (8 %) and Halanaerobiaeota (6 %), and 1 fungal phylum: Ascomycota (61%) (Figure 15B and 15E). Firmicutes (Bacillota) was mainly represented by the genera *Tepidimicrobium* (15%) and *Fastidiosipila* (7%). Bacteroidota was characterized by the genera *Flavobacterium* (9%) and *Aequorivita* (2%). Thermotoga was mainly constituted by the genus *Thermotoga* (7%). Genera within the phyla Cloacimonadota and Halanaerobiaeota were found with abundances of less than 2% (Figure 15C). The fungal phylum Ascomycota was predominantly represented by the genera *Geotrichum*, *Aspergillus*, *Penicillium*, *Gibberella*, *Schizothecium*, *Trichoderma* and *Fusarium* (with abundances ranging from 2 - 6%) (Figure 15F).

In general, initial incorporation of MSW digestate into soil stimulated marked shifts in the phylogenetic composition and relative abundance of major bacterial taxa. Although the MSW digestate-amended soil continued to be dominated by the same three bacterial phyla as the unamended soil - Bacteroidota, Firmicutes and Proteobacteria (Pseudomonadota)-, their relative abundances and genera composition were notably different (Figure 15B). The phylum Bacteroidota (which increased by 23% in soil after MSW digestate amendment) was partially represented by the genera *Flavobacterium*, *Gelidibacter*, *Aequorivita* and *Chitinophada*, with abundances ranging 2 - 8%) (Figure 15C), Firmicutes (Bacillota) (which increased by 22% in soil after MSW digestate amendment) was partially represented by the genus *Tepidimicrobium* (6%) (Figure 15C). Proteobacteria (Pseudomonadota) (which decreased by 59% in soil after MSW digestate amendment) was partially composed by the genera *Pseudomonas* (11%) and *Aquamicrobium* (3%) (Figure 15C). The most abundant fungal phyla in MSW digestate amended soil were Ascomycota (55%) (Figure 15E), (which was partially represented by the genera *Geotrichum*, *Aspergillus*, *Penicillium*, *Gibberella*, *Schizothecium*, *Trichoderma* and *Fusarium* with abundances ranging from 2 - 5%) (Figure 15F), and

Chytridiomycota (12%) (Figure 15E), whose genera were found at abundances below 2% (Figure 15F). In addition, the prokaryotic phyla Cloacimonadota, Thermotogota, Campilobacterota, Synergistota, Caldatribacteriota and Halanaerobiaeota, potentially borne from the MSW digestate, had increased to abundances $> 1\%$ in the MSW digestate amended soil when compared to the unamended soil.

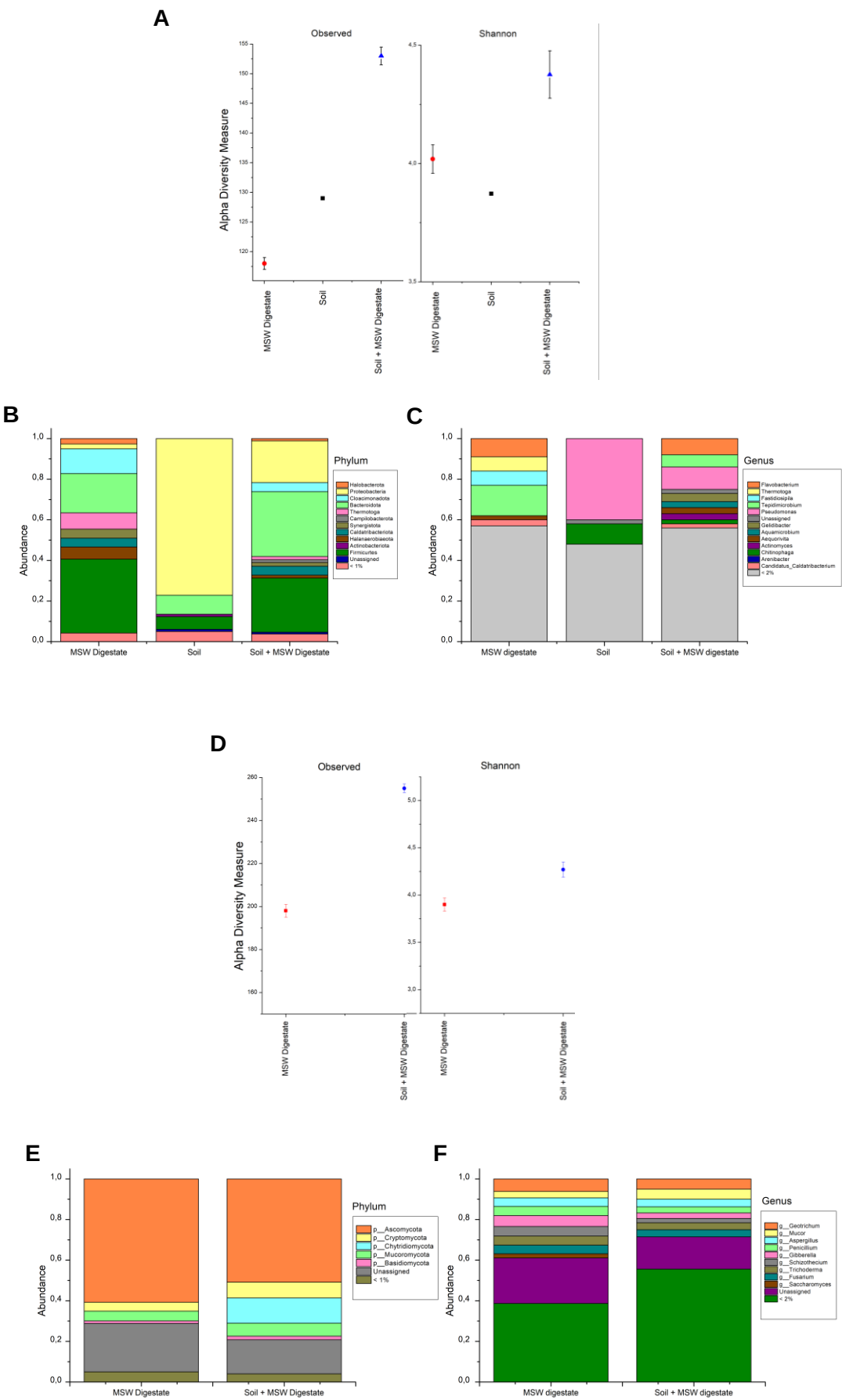


Figure 15. (A) Prokaryotic (16S v4-Bacterial and archaeal) and (D) Fungal (ITS2) community Alpha-diversity indices - richness index (Observed ASVs) and Shannon index- of initial MSW digestate, initial soil and initial MSW digestate amended soil. Error bars indicate standard error of the mean (n = 2). No data was obtained for soil's fungal community due to low concentration of DNA recovered from initial soil samples. (B, C) Composition of Prokaryotic (16S v4) and (E, F) Fungal (ITS2) communities in initial samples, showing the most abundant (B and D) phyla (with relative abundance > 1% of total reads) and (C and F) genera (with relative abundance > 2% of total reads). Minor phyla (< 1%) and genera (< 2%) were pooled together.

3.4.C Prokaryotic and fungal community evolution over time in top amended soil

The results pertaining to the prokaryotic and fungal community analysis in the topsoil, unplanted (T0) or planted with *P. virgatum* (T1) or *P. alopecuroides* (T2), collected on days 7 (the initial day of the phytoremediation experiment), 21, 35, and 90, are presented in Figure 16 and Figure 17.

3.4.C.1 Alpha and beta diversity

Overall, the prokaryotic biodiversity present in the topsoil (0 - 0.1 m) of T0 increased in richness (Observed ASVs) and Shannon index over time. Some fluctuations were observed in presence of plants. In T1, Shannon index increased over time, while richness (Observed ASVs) increased from day 7 to day 35, whereas slightly decreased on day 90. In T2, richness (Observed ASVs) increased from day 7 to day 21, highly decreased between day 21 and day 35, and increased again on day 90. Similarly, Shannon index in T2 slightly decreased between day 35 and day 90. By day 90, richness (Observed ASVs) and Shannon index had considerably increased compared to day 7, specially in presence of plants (T1 and T2) (Figure 16A). In terms of fungal community richness (Observed ASVs), no clear trend over time was discernible for T0. However, a decrease over time was noted for T1, while for T2, there was an initial increase followed by a subsequent decrease. By the end of the experiment (day 90), all treatments (T0, T1, and T2) showed a lower richness compared to day 7. In terms of fungal Shannon index, overall, it can be observed a decrease over time for all treatments. At day 90, similar values were found for T0 and T2, while T1 showed a higher diversity (Figure 16B).

NMDS of the Bray–Curtis dissimilarity analysis revealed a clear distinction of prokaryotic communities between day 7 and day 90 in all treatments (Figure 16C). Minimal differences were observed between days 7, 21, and 35. By day 90, prokaryotic microbial

communities in both T1 and T2 (columns with plants) exhibited significant dissimilarity compared to the prokaryotic communities in T0 (column without plants) at the same time. In terms of the fungal community (Figure 16D), T2 exhibited higher dissimilarity over time, while T0 and T1 demonstrated relatively lower dissimilarity over the same period. By day 90, T1 and T2 showed a considerable difference from T0. The Adonis test identified a significant p-value for the factor of time in both prokaryotic ($p < 0.01$) and fungal ($p < 0.05$) communities, while no significant p-value was not found for the factor of treatment (Table S1, Appendix II).

3.4.C.2 Taxonomic profiles

Results of prokaryotic and fungal taxonomical identification at phylum (> 1% abundant) and genus (> 2% abundant) level are presented in Figure 17. A restructuring of microbial community can be observed over time, being different between the absence of plants (T0) and the presence of plants (T1 and T2). At the beginning of the phytoremediation experiment (Initial, day 7) the prokaryotic community was dominated by Bacteroidota (40%), Proteobacteria (Pseudomonadota) (26%) and Firmicutes (Bacillota) (24%). It can be noticed that phyla Cloacimonadota, Thermotogota, and Halanaerobiaeota identified in the initial amended soil, were not longer present (<1% abundance). By day 90, Proteobacteria (Pseudomonadota) had experienced a slight increase in T0 when compared to day 7 (from 26% to 32%). In T1 and T2, however, the relative abundance of Proteobacteria (Pseudomonadota) was slightly lower, contributing to 22% in both T1 and T2. A noteworthy rise in Proteobacteria (Pseudomonadota) relative abundance was noted in T1 between day 7 and day 35, increasing from 27% to 42%. A decline in the Proteobacteria (Pseudomonadota) genus *Aquamicrobium* - potentially introduced by the MSW digestate amendment – was observed over time, reaching abundances ranging from 3 to less than 2% in all treatments by day 90. Furthermore, in the presence of plants (T1 and T2), the abundance of *Pseudomonas*—a major genus in the unamended soil that had initially shown a decline following MSW digestate amendment— continued to decrease over time. By day 90, its abundance had diminished to less than 2%. By day 90, the phylum Firmicutes (Bacillota), which initially experienced an increase due to MSW digestate amendment, exhibited a decline across all treatments, notably in the presence of plants (T1 and T2). Its relative abundance decreased from 24% on day 7 to 11% in T0, and further to 5% in T1 and 6% in T2 by day 90. Consequently, a reduction in the abundance of Firmicutes (Bacillota) genera, potentially introduced by the MSW digestate amendment, including *Flavobacterium*, *Tepidimicrobium*, *Fastidiosipila*, and *Gallicola*, can be observed over time. On day 7,

their range was from 8% to 4%, while by day 90, it narrowed to $\leq 3\%$ in all treatments. The phylum Bacteroidota also exhibited a decline in all treatments, decreasing from 38% on day 7 to 23% in T0, 19% in T1, and 21% in T2. The Bacteroidota genus *Gelidibacter* (potentially introduced by the MSW digestate amendment) decreased from 3% on day 7 to $< 2\%$ by day 90 in all treatments. However, Bacteroidota genera *Aequovirita* (a genera already observed in MSW digestate and initial MSW digestate amended soil) and *Arenibacter* had increased in presence of *P. virgatum* (T1) from $< 2\%$ on day 7, to 3% by day 90. In presence of *P. alopecuroides*, the Bacteroidota genera *Aequovirita*, *Tissierella* and *Ohtaekwangia* also had increased to 4% and 2%, respectively by day 90. In contrast to the above-mentioned phyla, Actinobacteriota (Actinomycetota) experienced an increase in relative abundance, from 3% on day 7 to 9%, 8%, and 7% in T0, T1, and T2, respectively. In agreement, the Actinobacteriota (Actinomycetota) genus *Actinomyces* increased to 3% and 4% in T0 and T2, respectively by day 90. Relative abundances of Planctomycetota, Chloroflexi and Verrucomicrobiota rose over time in all treatments, especially in presence of plants, from $< 1\%$ on day 7 to 9%, 2% and 3%, respectively, in T0; 16%, 10% and 8%, respectively, in T1; and 15%, 12% and 7%, respectively, in T2 on day 90. The presence of plants also favoured the emergence of the phylum Acidobacteriota by day 90 (3% and 4% in T1 and T2, respectively). This upward trend highlights the impact of plant presence on the enrichment of these microbial groups under the studied conditions. Most of the genera within these phyla were found with abundances $< 2\%$. As an opposite trend, the Planctomycetota genus *Rhodopirellula*, which was 5% abundant on day 7, decreased over time in presence of plants (T1 and T2) until reaching abundances below 2 %.

Regarding fungal community, the most abundant phyla at the beginning of the experiment (day 7) were Ascomycota (65%), Basidiomycota (11%), and Mucoromycota (10%). It is noteworthy that Chytridiomycota was a dominant phylum in the initial MSW digestate-amended soil, constituting 12% of the relative abundance (Figure 17), however, it was no longer observed by day 7. Overall, among the fungi, Ascomycota was the dominant phylum in all the treatments and times. However, a restructuration of the genera within Ascomycota was found over time. The genera *Penicillium* and *Geotrichum* experienced a decrease over time (from 22% and 10%, respectively on day 7, to abundances ranging from 6% to $< 2\%$ by day 90 in T0, T1 and T2). On the other hand, the genera *Enterocarpus* and *Homiactis*, which were $< 2\%$ abundant on day 7, increased to 38% and 29%, respectively in T0; 25% and 32%, respectively, in T1; and 31% and 30%, respectively in T2 by day 90. The genera *Candida* and *Iodophanus* seemed to

surge in presence of *P. alopecuroides* (T2) between day 21 and day 35, however they both were < 2% by day 90. By day 90, *Scedosporium* was observed in presence of both *P. virgatum* and *P. alopecuroides*. The phyla Basidiomycota and Mucoromycota experienced a decrease over time. At the end of the experimental period (day 90), both Basidiomycota and Mucoromycota had a relative abundance of <1% in T0 and T1. In the case of T2, Mucoromycota was <1% in T2 while Basidiomycota was 2%. A pronounced decrease of Basidiomycota and Muscoromycota from day 7 to day 21 was found in T1, from 11% and 10%, respectively, at day 7 to <1% at day 21. Consequently, a decrease of the Muscoromycota genus *Mucor* was observed (from 8% on day 7 to < 2% by day 90).

In summary, the results suggest that the microbial genera initially introduced by the MSW digestate amendment become depleted over time, prompting a reorganization of the microbial community. This restructuring is likely influenced by the physical and chemical changes induced by the amendment. These findings highlight the dynamic impact of plant presence on the enrichment and evolution of microbial groups during the phytoremediation process.

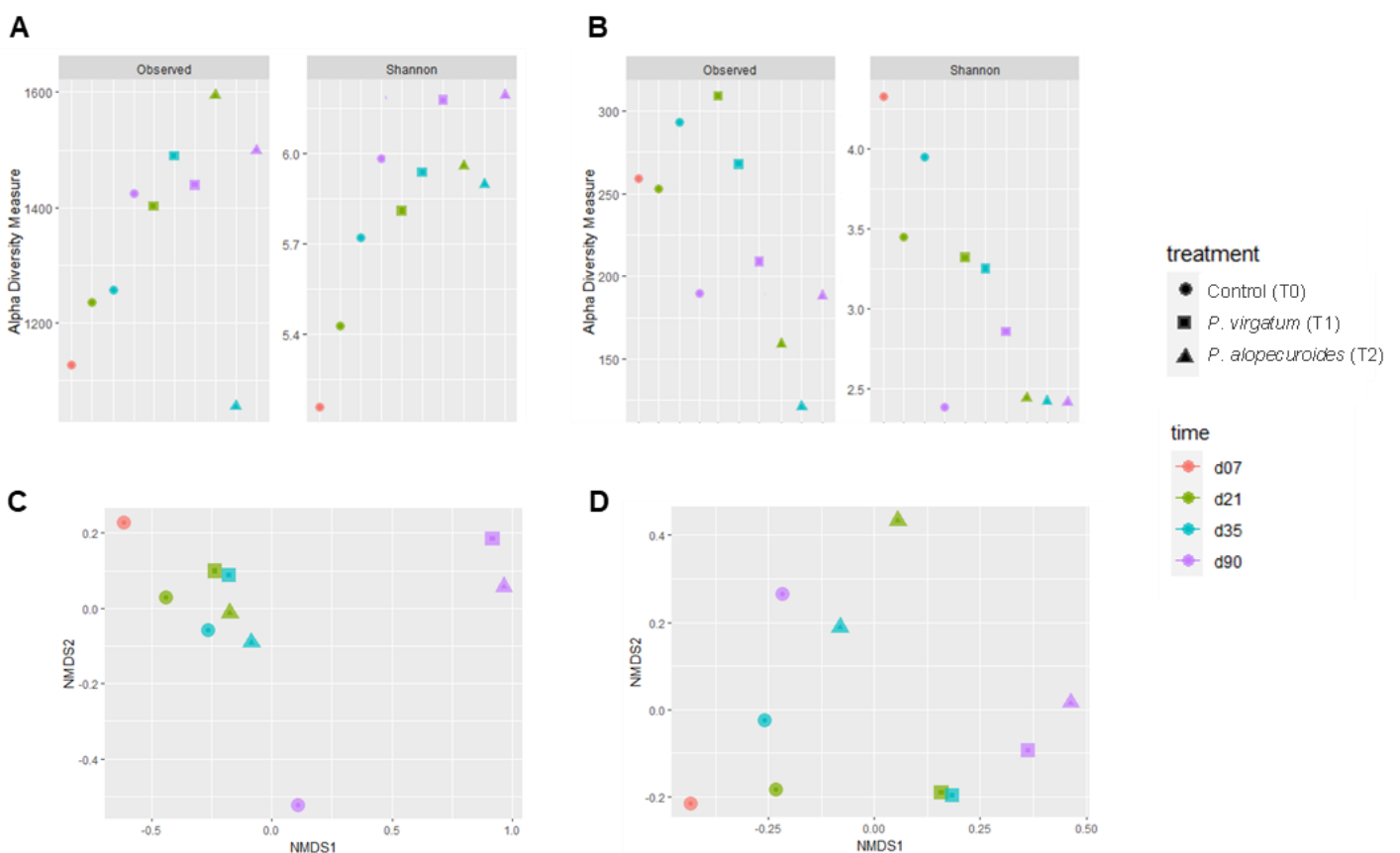
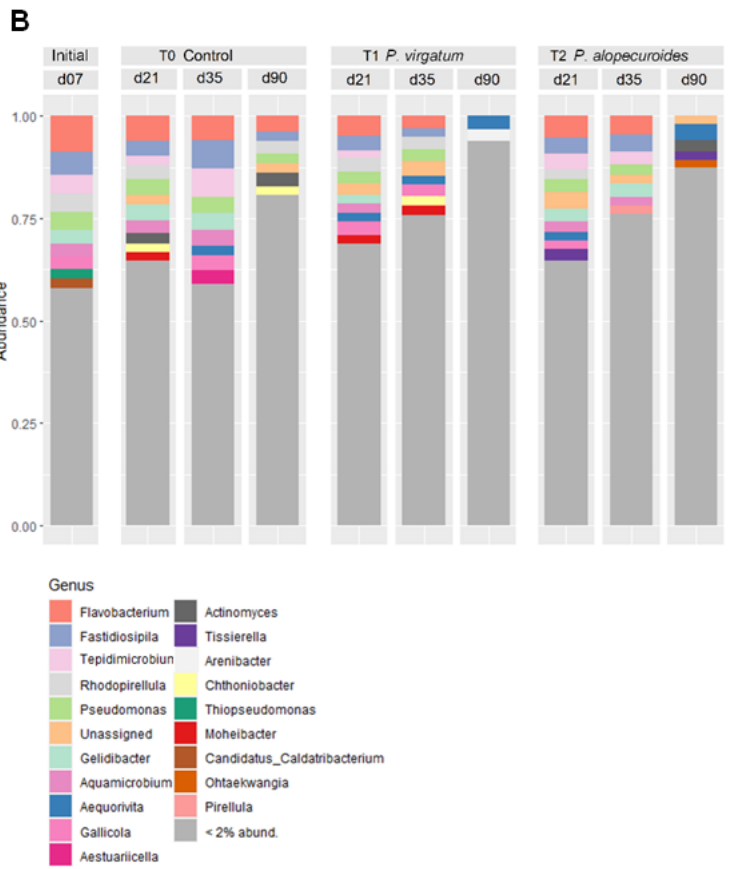
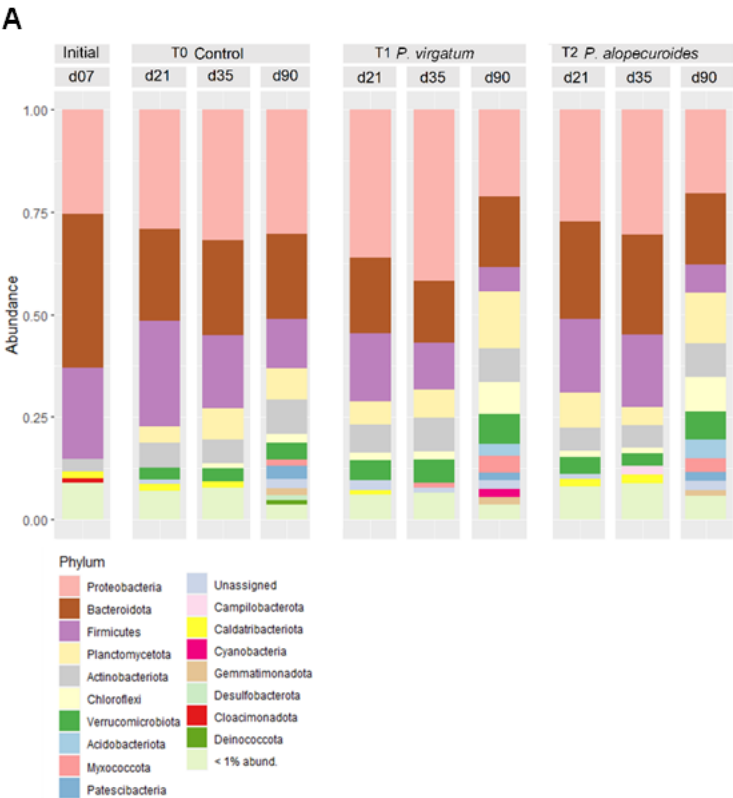


Figure 16. Alpha-diversity indices - richness index (Observed ASVs) and Shannon index for the (A) Prokaryotic and (B) Fungal community of 0-0.1 m soil samples from T0, T1 and T2 over time, calculated based on ASV (Amplicon Sequences Variant). (C) Prokaryotic (16S v4) and (D) fungal (ITS2) community beta diversity of 0-0.1 m soil samples from T0, T1 and T2 over time. Non-metric multidimensional scaling (NMDS) comparison based on Bray–Curtis dissimilarity index. Samples from topsoil (0- 0.1 m) layer of T0, T1 and T2 over time. Different colours indicate different days (red: d7; green: d21; blue: d35; purple: d90). Different shapes indicate different treatments (circle: control T0; square: column with *P. virgatum* T1; triangle: column with *P. alopecuroides* T2).



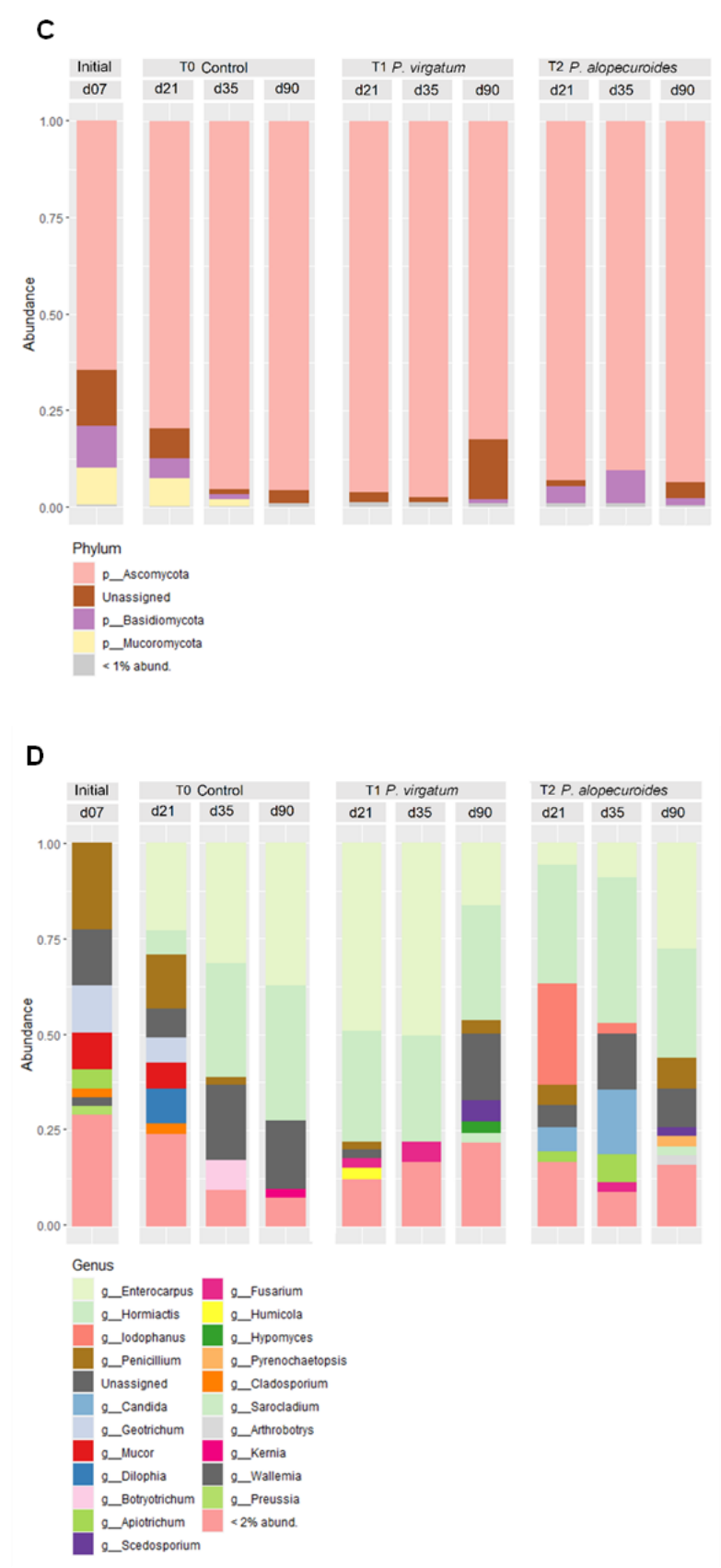


Figure 17 Relative abundance of prokaryotic (16S v4) sequences at (A) phylum and (B) and fungal (ITS2) sequences at (C) phylum and (D) genus level in topsoil (0-0.10 m) layer of soil columns T0, T1 and T2 over time (d7, 21, 35 and 90). Minor phyla (< 1% of total reads) and minor genera (< 2 % of total reads) were pooled together.

3.4.D *P. virgatum* and *P. alopecuroides* root microbiome at the end of the experimental period (day 90).

Results of the characterization of the prokaryotic and fungal community in *P. virgatum* and *P. alopecuroides* roots, grown with or without MSW digestate, are presented in Figure 18, Figure 19, and Figure 20.

3.4.D.1 Alpha and beta diversity

In terms of the prokaryotic community in *P. virgatum* roots, the richness (Observed ASVs) and Shannon index of the episphere and rhizosphere were higher in the absence of MSW digestate (T3). Whereas for the endosphere, the richness and diversity were higher when *P. virgatum* was grown in soil amended with MSW digestate (T1). In T1, the episphere exhibited the lowest richness, while the rhizosphere had the highest richness. In T3, the episphere showed the highest richness, followed by the rhizosphere, and the endosphere with the lowest value. Both in absence and presence of MSW digestate, Shannon index in *P. virgatum* increased from endosphere to rhizosphere (Figure 18A). Regarding the fungal community, richness (observed ASVs) of the rhizosphere was higher in the absence of MSW digestate (T3), while the episphere and the endosphere had similar richness values between presence (T1) and absence (T3) of MSW digestate. Shannon index of the rhizosphere and the endosphere was higher in the presence of MSW digestate amendment (T1), while the opposite was found for the episphere (a big difference can be observed between presence (T1) and absence (T3) of MSW digestate). In T1, the richness increased from the endosphere to the rhizosphere, while the diversity presented its highest value in the rhizosphere, followed by the episphere and the endosphere (Figure 18B). Concerning the prokaryotic community in *P. alopecuroides* roots, the richness of the endosphere and the rhizosphere was higher in the absence of MSW digestate (T4). Whilst the richness of the episphere was higher when *P. alopecuroides* was grown in soil amended with MSW digestate (T2). In T2, the episphere had the highest richness, while the rhizosphere exhibited the lowest value. Contrarily, in T4, the episphere had the lowest richness indices, while the rhizosphere had the highest. In the case of diversity, the rhizosphere and episphere showed higher

values in T2, while the endosphere's diversity was similar both in T2 and T4. In T4, Shannon index increases from endosphere to rhizosphere, while in T2, Shannon index in the episphere was higher than in the rhizosphere (Figure 18A). In terms of fungal community, the rhizosphere and the episphere richness was higher in absence of MSW digestate (T4), while richness in the endosphere was higher in presence of MSW digestate (T2). Shannon index in the rhizosphere was similar between T2 and T4, while diversity in the episphere was higher in T4, and diversity in the endosphere was greater in T2. In *P. alopecuroides* grown with MSW digestate, richness and diversity increased from the endosphere to the rhizosphere. The Shannon index of the rhizosphere was similar between *P. virgatum* and *P. alopecuroides* in all treatments (Figure 18B).

Beta diversity analysis of the prokaryotic community showed that rhizosphere, episphere and endosphere of *P. virgatum* grown in soil amended with MSW digestate were clearly different from those of *P. virgatum* grown in absence of MSW digestate (Figure 18C). However, this difference was not as notable in the case of *P. alopecuroides*. Beta diversity of the fungal community showed that the endosphere was markedly separated from the episphere and rhizosphere in *P. virgatum* and *P. alopecuroides* grown with and without MSW digestate (Figure 18D). The endosphere of *P. virgatum* was very different when grown with and without MSW digestate, while this difference was less pronounced in case of *P. alopecuroides*. The Adonis test did not yield a statistically significant p-value for the factors "plant," "MSW digestate presence," or "root compartment" (Table S2, Appendix II).

3.4.D.2 Taxonomic profiles

Taxonomic profiles of the prokaryotic and fungal community in *P. virgatum* and *P. alopecuroides* roots, grown with or without MSW digestate, are illustrated in Figure 19 and Figure 20.

A. Endosphere:

Prokaryotic community

The endosphere of both *P. virgatum* and *P. alopecuroides* grown in absence and presence of MSW digestate was dominated by Proteobacteria (Pseudomonadota) (with a relative abundance ranging from 47% to 62%) and Bacteroidota (ranging from 11% to 18%). In the endosphere of both plants, Planctomycetota and Actinobacteriota (Actinomycetota) had a higher abundance in presence of MSW digestate (17% and 11%, respectively, in *P. virgatum* and 9% and 6%, respectively, in *P. alopecuroides*) when compared to the absence of MSW digestate (9% and 3%, respectively in *P. virgatum*

and 3% and 4%, respectively in *P. alopecuroides*). These results also indicate a higher presence of Planctomycetota and Actinobacteriota (Actinomycetota) in *P. virgatum*. Verrucomicrobia was present in all treatments (3-5%). Myxococcota was present in *P. virgatum* both in presence (5%) and absence (4%) of MSW digestate, while Firmicutes (Bacillota) was only found in presence of MSW digestate (5%). On the other hand, *P. alopecuroides* showed presence of Acidobacteria both in presence and absence of MSW digestate (6% and 2%, respectively).

Higher differences were observed at genus level, especially in *P. virgatum* between absence and presence of MSW digestate. While *Caulobacter*, *Ohtaekwangia*, *Pseudomonas* and *Flavobacterium* were present in the absence of MSW digestate (12%, 8%, 3% and 3%, respectively), they were $\leq 1\%$ in the presence of MSW digestate. To the detriment of them, *Rhodanobacter*, *Paenibacillus*, *Devosia*, *Sorangium*, *Micromonospora* and *Planctomicrobium* became more abundant in the presence of MSW digestate (all ranging from 4- 7% of relative abundance). On the other hand, *Chitinophaga* and *Pir4_lineage* were found both in absence of MSW digestate (ranging from 2 – 5%).

In the case of *P. alopecuroides*, less differences were observed between the absence and presence of MSW digestate. *Rhodanobacter*, *Burkholderia-Caballeronia-Paraburkholderia* and *Flavobacterium* were present in both absence of MSW digestate (7%, 5% and 5%, respectively) and presence of MSW digestate (9%, 5% and 3%, respectively). *Granulicella*, *Arachidicoccus* and *Dongia* were found in the absence of MSW digestate, accounting for 5%, 4% and 3%, respectively, whereas they were all $<1\%$ in the presence of MSW digestate. On the other hand, *Devosia* and *Pir4_lineage* genera were only observed in the presence of MSW digestate with a relative abundance of 6% and 3%, respectively, while in the absence of MSW digestate they both were $<1\%$.

The percentage of shared ASVs between the endosphere grown with and without MSW digestate was notably low for both plants (Figure S1), accounting for only 6% in the case of *P. virgatum* and 13% for *P. alopecuroides*. The endosphere of *P. virgatum* exhibited 51% and 46% of unique ASVs in both presence and absence of MSW digestate. In the case of *P. alopecuroides*, 41% of unique ASVs was observed in the presence of MSW digestate, while 46% in the absence of MSW digestate.

Fungal community

The endosphere of both *P. virgatum* and *P. alopecuroides* grown in absence and presence of MSW digestate was dominated by the phylum Ascomycota ($> 90\%$). Larger

differences were observed at genus level. In *P. virgatum*, *Podospora*, *Schizothecium* and *Poaceascaoma* were the dominant genera in the absence of MSW digestate (44%, 24% and 12%, respectively), while in the presence of MSW digestate, they were all <1%. In detriment of them, *Fusarium*, *Conlarium*, *Homiactis* and *Enterocarpus* became dominant in the presence of MSW digestate (18%; 24%, 18% and 16%, respectively), whereas in the absence of MSW digestate they were $\leq 1\%$.

In the case of *P. alopecuroides*, *Conlarium*, *Fusarium* and *Microdochium* were more abundant in the absence of MSW digestate (24%, 27 % and 5%, respectively) when compared to the presence of MSW digestate (10%, 6% and 2% respectively). Moreover, *Sarocladium*, a genus that was not found in the endosphere of *P. virgatum*, was present in the endosphere of *P. alopecuroides* both in absence of MSW digestate (22%) and presence of MSW digestate (18%). In addition, *Phialocephala*, *Pyrenochaetopsis*, *Geomyces*, *Pseudorhizophila* and *Enterocarpus* genera showed greater relative abundances in the presence of MSW digestate (6%, 8%, 18%, 88% and 5%, respectively) compared to the absence of MSW digestate, where they all had a relative abundance $\leq 2\%$.

As in the case of the prokaryotic community, the percentage of shared ASVs between the endosphere grown with and without MSW digestate (Figure S1, Appendix II) was low for both plants, accounting for 7% in the case of *P. virgatum* and 18% for *P. alopecuroides*. The endosphere of *P. virgatum* showed 49% and 46% of unique ASVs in presence and absence of MSW digestate, respectively. In the case of *P. alopecuroides*, 52% of unique ASVs was observed in the presence of MSW digestate, while 30% in the absence of MSW digestate.

B. Episphere:

Prokaryotic community

The episphere of *P. virgatum* and *P. alopecuroides* both in absence and presence of MSW digestate, was dominated by Proteobacteria (Pseudomonadota) (ranging from 30-45%). Bacteroidota had a higher relative abundance in *P. virgatum* (21% and 24% in presence and absence of MSW digestate, respectively) than in *P. alopecuroides* (8% and 4% in presence and absence of MSW digestate, respectively). On the other hand, Actinobacteriota (Actinomycetota) had a higher abundance in *P. alopecuroides* (29% and 16% in presence and absence of MSW digestate, respectively) than in *P. virgatum* (11% and 5% in presence and absence of MSW digestate, respectively). These results also indicate that Actinobacteriota (Actinomycetota) in the episphere of both plants was

positively influenced by the presence of MSW digestate. Also, in the case of *P. alopecuroides*, the abundance of Actinobacteriota (Actinomycetota) was higher in episphere than in endosphere.

Planctomycetota, Verrucomicrobiota, and Myxococcota were present in all treatments (ranging from 3-8%). Moreover, Patescibacteria, a phylum that was $\leq 1\%$ abundant in the endosphere was found in all treatments (ranging from 3-7%). In the case of *P. virgatum*, Myxococcota seemed to be negatively influenced by the presence of MSW digestate (from 7% without MSW digesta to 3% with MSW digestate) while Firmicutes (Bacillota) positively influenced (from $< 1\%$ without MSW digestate to 7% with MSW digestate). In the case of *P. alopecuroides* Acidobacteria seemed to be negatively affected by the presence of MSW digestate.

Larger differences were observed at genus level. Both in *P. alopecuroides* and *P. virgatum* there is a higher presence of *Rhodanobacter* in presence of MSW digestate (12% and 5% respectively) compared to the absence of MSW digestate ($\leq 2\%$). In case of *P. alopecuroides*, *Paenbacillus* became more abundant in presence of MSW digestate (9%) when compared to the absence of MSW digestate ($< 1\%$). In case of *P. virgatum*, *Sorgarium* and *Planctomicrobium*, which in the endosphere presented higher abundances in the presence of MSW digestate, in the episphere showed higher abundances in the absence of MSW digestate (3%) when compared to the presence of MSW digestate ($< 2\%$). *Flavobacterium* was observed in *P. virgatum* both in presence and absence of MSW, being more abundant in presence of MSW digestate (11% and 3%, respectively).

The observed differences were reflected in the Venn diagram (Figure S2, Appendix II). In *P. virgatum* and *P. alopecuroides*, the percentage of ASVs shared between the presence and absence of MSW digestate was 6% and 20%, respectively. The episphere of *P. virgatum* and *P. alopecuroides*, grown in MSW digestate showed 31% and 49% of unique ASVs, respectively. While 63% and 31% of unique ASVs, respectively when they were grown without MSW digestate.

Fungal community

At phylum level, the fungal community was dominated by Ascomycota ($\geq 80\%$). Larger differences were observed at genus level. In the absence of MSW digestate, the episphere of *P. virgatum* was dominated by *Fusarium*, *Myrmecridium* and *Poaceascoma* (18%, 35% and 6%, respectively). While in presence of MSW digestate, *Fusarium* (60%) and *Enterocarpus* (20%) became the most abundant genera in detriment of the other

genera. In the case of *P. alopecuroides*, the MSW digestate induced a decrease of *Myrmecridium*, *Fusarium* and *Apiotrichum* abundances, which were 10%, 15% and 11%, respectively, abundant in the absence of MSW digestate and became < 2%, 4% and <2%, respectively, abundant in the presence of MSW digestate. On the other hand, *Penicilium*, *Sarocladium*, *Enterocarpus* and *Oidiodendron*, which were 12%, 8% and <2%, respectively, in the absence of MSW digestate, increased to 35%, 18%, 8% and 9%, respectively, in the presence of MSW digestate.

The percentage of shared ASVs between the episphere grown with and without MSW digestate (Figure S2, Appendix II) was 14% in the case of *P. virgatum* and 23% for *P. alopecuroides*. The episphere of *P. virgatum* had 47% and 40% of unique fungal ASVs in presence and absence of MSW digestate, respectively. In the case of *P. alopecuroides*, the episphere in absence of MSW digestate showed almost twice as “unique” ASVs as in presence of MSW digestate (52% and 26%, respectively)

C. Rhizosphere

Prokaryotic community

In the rhizosphere of *P. virgatum* and *P. alopecuroides* both in presence and absence of MSW digestate the most abundant phyla were Proteobacteria (Pseudomonadota) (29-39%), Actinobacteriota (Actinomycetota) (9-14%), Bacteroidota (5-8%), Planctomycetota (6-9 %), Acidobacteriota (8-16%), Verrucomicrobiota (4-8%) and Patescibacteria (6-10%). In the case of *P. virgatum*, in the absence of MSW digestate, Crearchaeota also presented a relatively high abundance (8%).

At genus level, in the case of *P. alopecuroides*, *Rhodanobacter*, *Caulobacter*, *Devosia*, *Burkholderia*-*Caballeronia*-*Paraburkholderia*, *Sorgarium*, *Flavobacterium* and *Pir4_lineage* were more abundant in presence of MSW digestate (ranging from 7%- 2%, respectively) when compared to the absence of MSW digestate (ranging from 2% - <2%). In the case of *P. virgatum*, *Rhodanobacter* had similar abundances between the presence and absence of MSW digestate (8% in both cases). In the presence of MSW digestate *Paenbacillus*, *Devosia* and *Chitinophaga* became > 2% abundant.

In *P. virgatum* and *P. alopecuroides*, the percentage of ASVs shared between the presence and absence of MSW digestate (Figure S3, Appendix II) was 9% and 16%, respectively. The rhizosphere of *P. virgatum* and *P. alopecuroides* grown without MSW digestate had higher percentage of unique ASVs (56% and 57%, respectively) than when grown in presence of MSW digestate (34% and 28%, respectively).

Fungal community

Ascomycota was the most abundant phylum in all conditions ($\geq 88\%$). At genus level, in the case of *P. alopecuroides*, *Fusarium*, *Penicilium* and *Conlarium* had similar abundances in absence (9%, 13% and 9%, respectively) and presence of MSW digestate (8%, 11% and 9%, respectively). The presence of MSW digestate negatively influenced the genus *Trichoderma*, which was 11% abundant in the absence of MSW digestate and became $<5\%$ abundant in presence of MSW digestate. On the other hand, *Pyrenochaetopsis*, *Gaeumannomyces* and *Flavocillium* became $> 5\%$ in presence of MSW digestate. In the case of *P. virgatum*, *Fusarium* and *Penicilium* showed similar abundances in absence (12% and 5%, respectively) and presence (15% and 7%, respectively) of MSW digestate. MSW digestate induced a higher abundance of *Conlarium*, which became 18% abundant and *Enterocarpus* and *Aspergillus* which became $> 5\%$ abundant.

In *P. virgatum* and *P. alopecuroides*, the percentage of ASVs shared between the presence and absence of MSW digestate (Figure S3, Appendix II) was 23% and 34%, respectively. The rhizosphere of *P. virgatum* and *P. alopecuroides* grown without MSW digestate had a higher percentage of unique ASVs (46% and 54%, respectively) than when grown in absence of MSW digestate (31% and 21%, respectively).

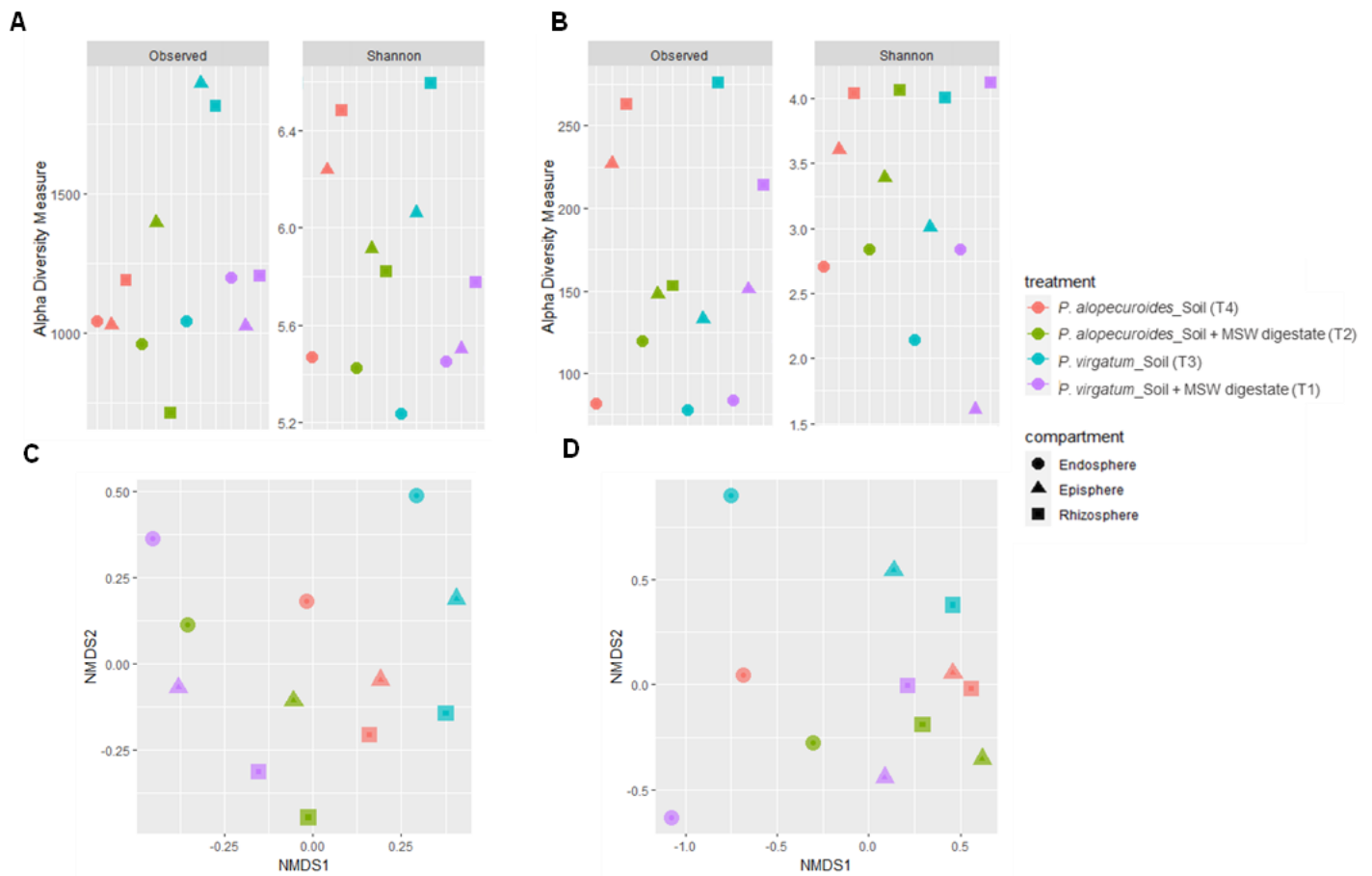


Figure 18. Alpha-diversity indices - richness index (Observed ASVs) and Shannon index for the (A) prokaryotic and (B) fungal community calculated based on ASV (Amplicon Sequences Variant). Non-metric multidimensional scaling (NMDS) comparison of the (C) prokaryotic and (D) fungal communities based on Bray–Curtis dissimilarity index. Samples from *P. virgatum* and *P. alopecuroides* root microbiome at the end of the experimental period (day 90). Different colours indicate the different treatments (red: *P. alopecuroides* in soil without MSW digestate (T4); green: *P. alopecuroides* in soil + MSW digestate (T2); blue: *P. virgatum* in soil without MSW digestate (T3); purple: *P. virgatum* in soil + MSW digestate (T1)). Different shapes indicate different plant root compartments (circle: Endosphere; triangle: Episphere; square: Rhizosphere).

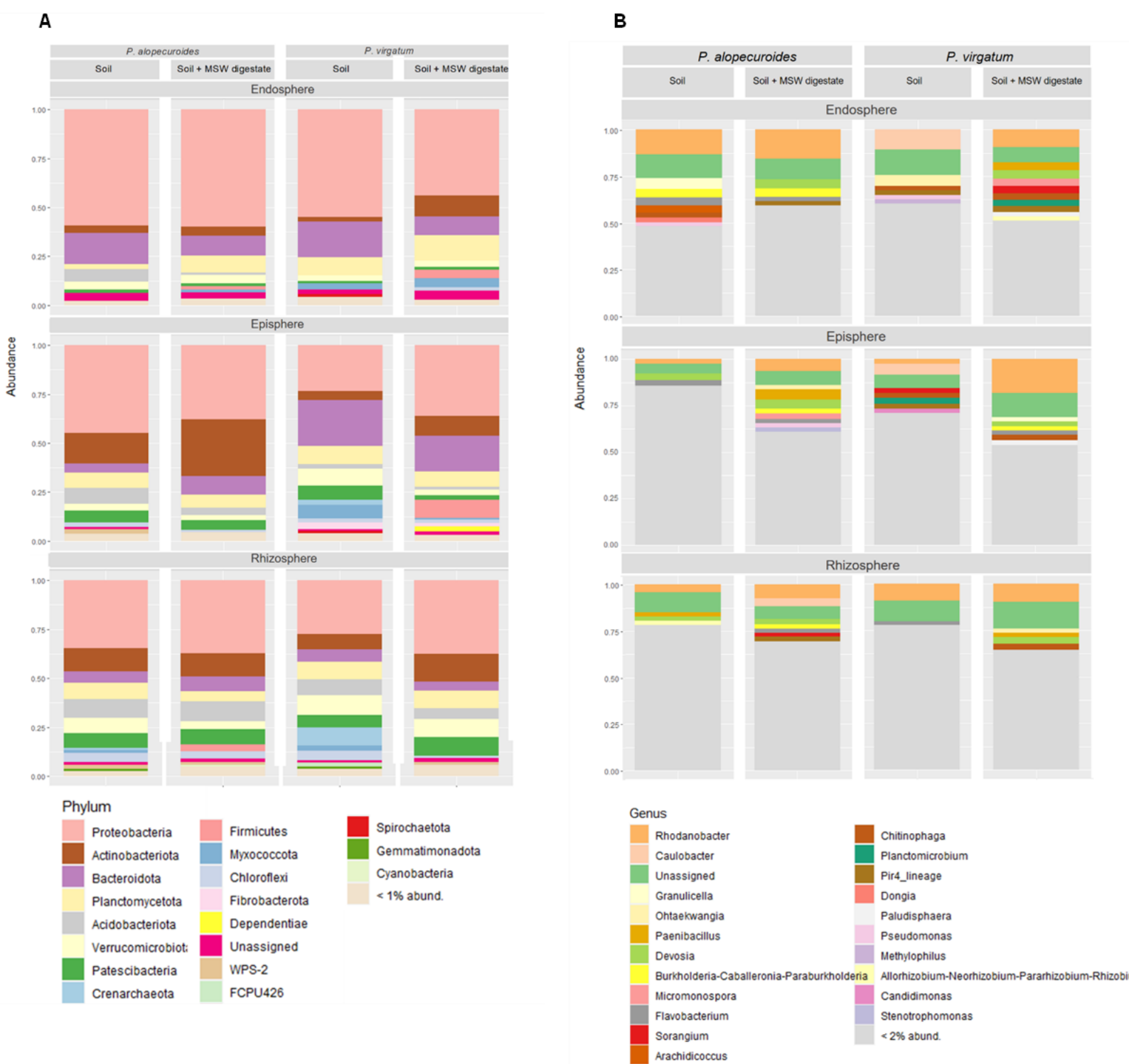


Figure 19. Relative abundance sequences of prokaryotic community at (A) phylum and (B) genus level in *P. virgatum* and *P. alopecuroides* root microbiome at the end of the experimental period (day 90). Minor phyla (< 1% of total reads) and genera (< 2% of total reads) were pooled together.

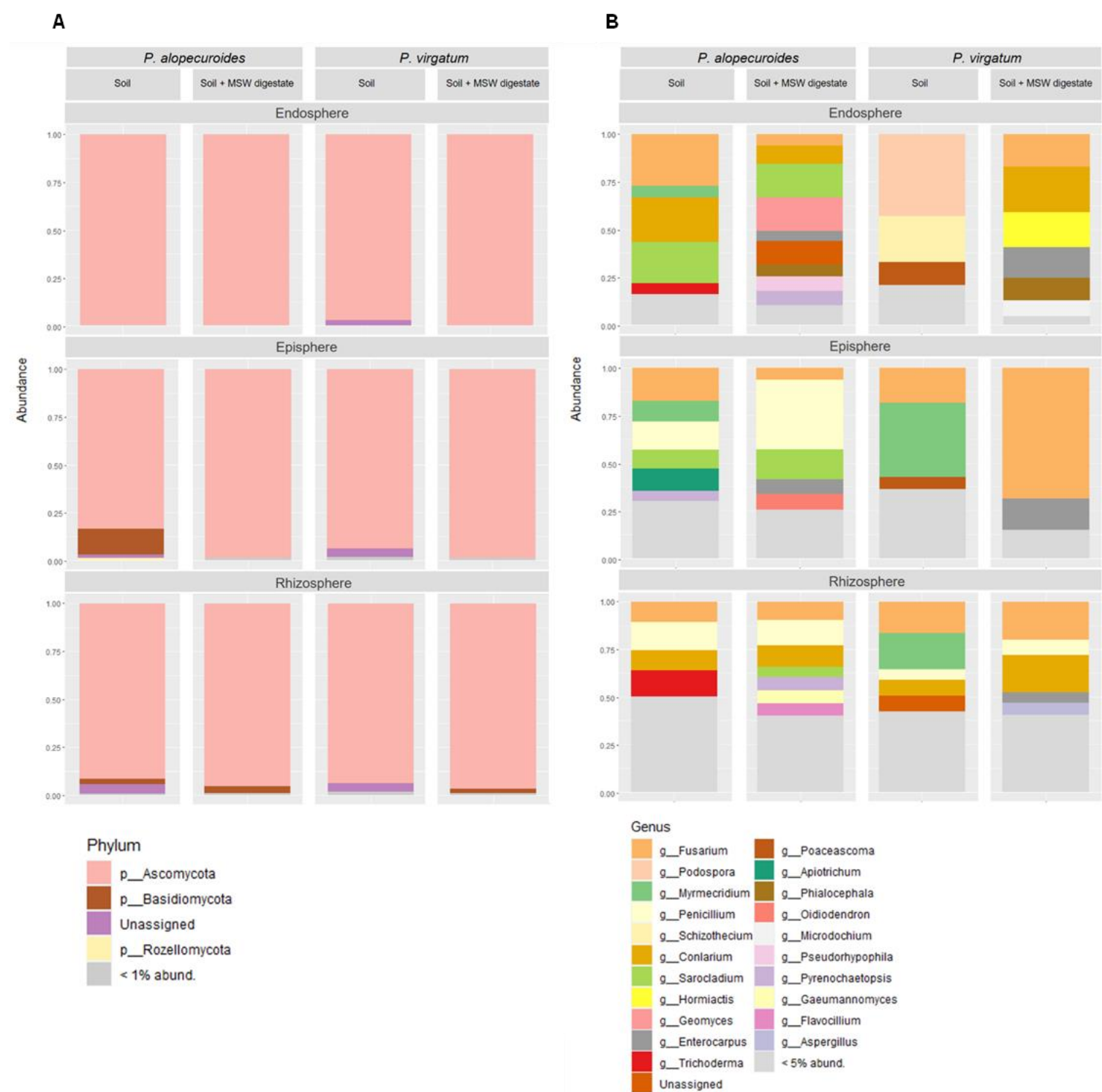


Figure 20. Relative abundance sequences of fungal community at (A) phylum and (B) genus level in *P. virgatum* and *P. alopecuroides* root microbiome at the end of the experimental period (day 90). Minor phyla (< 1% of total reads) and genera (< 5% of total reads) were pooled together.

3.5 Discussion

3.5.A Initial effects of MSW digestate application to soil

The most relevant prokaryotic phyla detected in MSW digestate included Firmicutes (Bacillota), Bacteroidota, Cloacimonadota, and Thermotoga. Previous studies have observed these phyla in anaerobic digestion systems (Centurion et al., 2018; Hassa et al., 2018; Johnson & Hug, 2022; Nelson et al., 2011; Supaphol et al., 2011; Tyagi et al., 2021; Wei et al., 2020; Yi et al., 2014). Similarly, the identified major genera such as *Tepidimicrobium*, *Thermotoga*, *Flavobacterium* and *Fastidiosipila* have been reported in anaerobic digestion units as well (Cazaudehore et al., 2023; S. Chen et al., 2019; Z. Chen et al., 2023; Gong et al., 2019; Tseng et al., 2020; Wu et al., 2016). Concerning fungal phyla in MSW digestate, Ascomycota, Mucoromycota, and Cryptomycota have been identified, and their presence has been documented in anaerobic digestion systems (Matsubayashi et al., 2017; Qin et al., 2021; Yang et al., 2022; Young et al., 2018). Similarly, major genera such as *Geotrichum*, *Aspergillus*, *Penicillium*, *Gibberella*, *Trichoderma*, and *Fusarium* (Borja et al., 1995; Mustafa et al., 2016; W. Sun et al., 2015; Uwineza et al., 2021; Yang et al., 2022; Zanellati et al., 2021) have been previously reported in anaerobic digestion systems. It is noteworthy that certain species within the *Fusarium* genus have been recognized as plant pathogens (Berasategui et al., 2023; Rampersad, 2020). In our preceding study (Bonet-Garcia et al., 2023, Chapter 2), the application of MSW digestate showed no detrimental effects on plants; instead, there was an observed increase in biomass. Nevertheless, it is important to acknowledge that results from these studies should be considered when devising strategies for monitoring digestate land application.

The initial MSW digestate incorporation to soil induced a higher richness and diversity of the prokaryotic community in the marginal soil, which initially presented a poor diversity and richness, mainly dominated by the phylum Proteobacteria. Several authors highlighted an increase of soil microbial biomass (Badagliacca et al., 2020; Cattin et al., 2021; De la Fuente et al., 2013; Garcia-Sánchez et al., 2015; Muscolo et al., 2017; Panuccio et al., 2021; Ren et al., 2020; Walsh et al., 2012) and taxonomic diversity (Diacono et al., 2010) after digestate application, whereas others observed only minor or no changes (Barduca et al., 2021; Johansen et al., 2013; Podmirseg et al., 2019).

As the characterization of the initial MSW digestate-amended soil was conducted immediately after mixing the soil with the MSW digestate, the surge in microbial richness and diversity in the initial mixture was likely induced by the recent incorporation of

microbial communities from the MSW digestate into the soil. This includes the identified prokaryotic genera *Flavobacterium*, *Gelidibacter*, *Aequorivita*, *Tepidimicrobium*, *Aquamicrobium*, and the fungal genera *Geotrichum*, *Aspergillus*, *Penicillium*, *Gibberella*, *Schizothecium*, *Trichoderma*, and *Fusarium*.

Nonetheless, these findings illustrate the immediate influence of MSW digestate following its initial incorporation into the soil. The enduring effects over time are described in the subsequent section.

3.5.B Evolution of prokaryotic and fungal communities over time in phytomanaged MSW digestate amended soil

The temporal dynamics of prokaryotic and fungal communities in topsoil suggest that the effects of MSW digestate application develop gradually over time. For the prokaryotic community, higher richness (Observed ASVs index) and higher Shannon index were observed in the topsoil by day 90. As reported by Alburquerque et al. (2012), application of digestate to soil has been demonstrated to provide a supply of readily available nutrients and ultimately had a favourable impact on microbial community. In our previous study (Bonet-Garcia et al., 2023, Chapter 2) we found that MSW digestate increased ($p < 0.05$) levels of total organic carbon, total nitrogen and available P in soil, which could ultimately boost the prokaryotic community (Drenovsky et al., 2004; Li et al., 2013; Philippot et al., 2023; Zhou et al., 2017). Literature reports a diverse range of results regarding the impact of digestate on soil's prokaryotic community, Garbini et al. (2022) found that diversity slightly increased after 61 days in a soil amended with cattle manure digestate, however no significant changes were reported for richness. Similarly, Urra et al., (2020) found that the application of organic amendments led to a higher soil prokaryotic diversity, compared to mineral fertilization, however that did not result in an enhanced microbial activity and biomass. On the other hand, Luo et al. (2023) found that the digestate-amended soil had no increase in bacterial biomass but had a higher bacterial diversity on day 90 compared with day 0. Different results obtained in literature could be explained by variability in physiochemical properties of different kinds of digestate and soils. Concerning the fungal community, a gradual decline of richness and diversity was observed over time, that may be attributed to the initial spike in diversity on day 7, caused by the recent introduction of fungal communities from the MSW digestate, which gradually declined over time. Conversely, Sun et al. (2016) found that the addition of organic materials increased soil fungal richness, over a long period of time. On the other hand, Luo et al. (2023) observed fungal diversity decreased over, with a fungal

biomass increased over time. The opposite trend observed in the dynamics of prokaryotic and fungal communities over time may be attributed to the distinct ecological strategies employed by bacteria and fungi. Bacteria are often linked to an r-selected strategy, characterized by rapid growth and resource utilization for carbon and nutrients. On the contrary, fungi are associated with a K-selected strategy (Blagodatskaya & Kuzyakov, 2008; Fierer et al., 2009; Fontaine et al., 2003).

In comparison with the control, the presence of *P. virgatum* and *P. alopecuroides* induced an increase in diversity of prokaryotic community over time. While, for the fungal community only *P. virgatum* seemed to have an effect in increasing the Shannon index. Plant roots release a substantial amount of organic carbon into the soil, mostly in form of carbohydrates, amino acids, and organic acids (Hütsch et al., 2002). These compounds fuel microbial activity and shape their community structure in both the rhizosphere and bulk soil (Koranda et al., 2011; Paterson et al., 2007), emphasising the importance of plant roots for maintaining soil microbial functions. stimulating microorganisms with beneficial traits that are needed for both plant growth and health (Chaparro et al., 2014). This further increase in biodiversity could lead to an overall increase resource utilization, functionality, productivity, resiliency, etc. (Bell et al., 2005; Stockdale et al., 2013).

When looking at the prokaryotic and fungal communities' composition, it can be observed that the communities potentially originated from the MSW digestate (such as the prokaryotic genera *Aquamicrobium*, *Flavobacterium*, *Tepidimicrobium*, *Fastidiosipila* and *Gelidibacter* and the fungal genera *Penicillium*, *Geotrichum* and *Mucor* decreased over time, leading to a restructuration of the microbial community. The dominance of the phyla Proteobacteria (Pseudomonadota), Bacteroidota, Firmicutes (Bacillota), Actinobacteriota (Actinomycetota) and Planctomycetota have been previously reported in bio-based fertilizer and digestate amended soils (Luo et al., 2023; Obayomi et al., 2023; Pathan et al., 2021; Pezzolla et al., 2015a; Suproniene et al., 2022). Moreover, the increase of Planctomycetota, Actinobacteriota (Actinomycetota) and Verrucomicrobia over time could be associated to their known role in degrading organic matter and participation in biogeochemical cycling processes (Buckley et al., 2006; Fausto et al., 2018; Fuerst, 2017; Jenkins et al., 2010; Piazza et al., 2019; Sapp et al., 2015). The presence of *P. virgatum* and *P. alopecuroides* lead to a further increase in the phyla Planctomycetota, Chloroflexi, Verrucomicrobia and Acidobacteria. Members of these phyla have been reported to have play pivotal ecological roles in plant-soil ecosystems, including modulation of biogeochemical cycles and influencing plant growth

(Fuerst, 2017; Huber et al., 2016; Kielak et al., 2016; Krzmarzick et al., 2012; Nunes da Rocha et al., 2013). Certain genera occurred in the presence of *P. virgatum* and/or *P. alopecuroides*, including *Aequorivita*, known to play a pivotal role in plant rhizosphere dynamics (Pereira et al., 2021). Similarly, the abundance of *Arenibacter*, associated with carbon and nitrogen metabolism (Sun et al., 2023), rose in presence of *P. virgatum*. Additionally, *Actinomyces*, recognized for its beneficial plant growth-promoting traits (Jog et al., 2016), and *Tissierella*, previously linked to tryptophan metabolism—specifically Indole-3-Acetic Acid (IAA) biosynthesis, a hormone crucial for plant development (Peixoto et al., 2016), occurred in the presence of *P. alopecuroides*. Collectively, these findings highlight their potential contributions to the promotion of plant health.

Janssen (2006) performed a large study of microbial community diversity in healthy soil samples, where the predominant phyla were Proteobacteria (Pseudomonadota) (range 10 - 77%), Acidobacteria (range 5 - 46%) and Actinobacteriota (Actinomycetota) (range 0 – 34%), followed by Verrucomicrobia (range 0 – 21 %), Bacteroidota (range 0 - 18%) , Chloroflexi (range 0 - 16%), Planctomycetes (range 0 - 8%), and Gemmatimonadota (range 0 - 4%) . This healthy soil average microbial community structure composition can be considered as a reference point for comparing microbial community structures in soil samples. Overall, the prokaryotic community in the phytomanaged MSW-digestate amended soil of the current study more closely resembled the reported average prokaryotic community of healthy soil when compared to the initial unamended soil, indicating an improvement in soil health.

Regarding fungal community, a dominance of Ascomycota over time was observed in the MSW digestate amended topsoil both in presence and absence of plants. Similarly, Pezzolla et al. (2015) demonstrated that Ascomycota became predominant 90 days after digestate amendment. Ascomycota are the main decomposers of plant residues in the early successional stages of several soil ecosystems (Klaubauf et al., 2010), and members of Ascomycota are involved in numerous soil functions. These fungi may form symbiotic relationships with mycorrhizae or endophytes that promote plant growth (Bastías et al., 2020; Bonito et al., 2014; Hardoim et al., 2015; Lindow & Brandl, 2003; Toju et al., 2013). At the genus level, a significant rise over time was observed in *Enterocarpus*, a genus previously documented in fertilized soil (Wang et al., 2023, p. 202), and *Homiactis*, a genus previously identified in cultivated soil (Elmholt & Kjøller, 1989).

3.5.C Differences in the endosphere, episphere and rhizosphere of *P. virgatum* and *P. alopecuroides* in the presence and absence of MSW digestate

Despite a positive significant effect of MSW digestate amendment was observed on the growth of both plants (Bonet-Garcia et al., 2023, Chapter 2), it was not possible to observe a discernible advantage in terms of prokaryotic and fungal community diversity in root compartments. On the contrary, results revealed that certain root compartments of both *P. virgatum* and *P. alopecuroides* exhibited higher prokaryotic and fungal diversity in the absence of MSW digestate. This could be explained by the process of root formation, which involves the root colonization by a subgroup of microorganisms from the bulk soil and the subsequent colonization of the episphere by the rhizosphere (Compant et al., 2021). The introduction of a diverse microbial community through MSW digestate could potentially trigger a competitive interaction between the native root microbiome and the exogenous communities. This competition, absent in the unamended marginal soil due to its lower richness and diversity, might account for the observed disparities in the root microbiome of plants cultivated in the presence or absence of MSW digestate.

Overall, the prokaryotic phyla Proteobacteria, Bacteroidota, Actinobacteriota (Actinomycetota) and Planctomycetota and the fungal phylum Ascomycota phylum dominated in all compartments. Acidobacteria, Verrucomicrobia and Patescibacteria were also present in rhizosphere and episphere. Similar taxonomical profiles to those here obtained have been reported in previous studies, where it was shown that most notable root communities are members of the Proteobacteria (Pseudomonadota), Planctomycetota, Bacteroidota, Actinobacteriota (Actinomycetota), Acidobacteria and Ascomycota phyla (Bodenhausen et al., 2013; Bulgarelli et al., 2012; Coleman-Derr et al., 2016; Fierer et al., 2009; Kolton et al., 2017; Peiffer et al., 2013; X. Sun & Guo, 2012; Uroz et al., 2010; Zarraonaindia et al., 2015). A study performed by Mao et al. (2014) found that the rhizosphere of the *P. virgatum* was dominated by Proteobacteria (Pseudomonadota) and Acidobacteria.

At genus level, *Rhodanobacter* demonstrated a notable presence in the root microbiome of both *P. virgatum* and *P. alopecuroides*, both in presence and absence of MSW digestate. This observation suggests that *Rhodanobacter* may constitute a core microbiome component for these plants, indicative of its potential significance for host fitness (Shade & Handelsman, 2012; Vandenkoornhuyse et al., 2015). Moreover,

Rhodanobacter has been documented to exhibit plant growth-promoting effects, further emphasizing its potential role in supporting plant development (Thijs et al., 2014). However, *Aequorivita*, *Arenibacter*, *Actinomyces* and *Tissierella*, observed in the amended topsoil in presence of *P. virgatum* and/or *P. alopecuroides* (above-mentioned) were not observed in plants root compartments, or were present with abundances < 2%.

Similarly, members of the Ascomycota phylum, identified in various plant compartments in both the presence and absence of MSW digestate, particularly the predominant genera *Fusarium*, *Penicillium*, and *Conlarium*, have been reported to possess plant growth-promoting (PGP) traits (Dastogeer et al., 2017; Dastogeer & Wylie, 2017). This suggests the potential inclusion of these genera in the core microbiome of the plants, underscoring their likely contribution to promoting plant growth and overall soil health. Furthermore, *Enterocarpus* and *Hormiactis*, identified in the amended topsoil (as mentioned earlier), were also detected in the root microbiome of the plants, implying a potential selection by the plants.

3.6 Conclusions

After 90 days of MSW digestate amendment, the topsoil exhibited heightened prokaryotic diversity, further enriched in the presence of plants. The restructuring of microbial communities over time could be attributed to changes in soil properties and adaptation of the microbial communities originated from the MSW digestate to the environmental conditions (eg. aerobic conditions). By day 90, MSW digestate-amended soil demonstrated dominance in Proteobacteria, Bacteroidota, Firmicutes, Actinobacteriota, Planctomycetota, Verrucomicrobia, and Ascomycota. The presence of *P. virgatum* and *P. alopecuroides* intensified the abundance of Planctomycetota, Chloroflexi, Verrucomicrobia, and Acidobacteria.

Prokaryotic genera *Aequorivita*, *Arenibacter*, *Actinomyces*, and *Tissierella*, observed in the amended topsoil, were absent in plant root compartments. *Rhodanobacter*, found in the root microbiome with or without MSW digestate, may constitute a core microbiome for these plants. The predominant fungal genera in the root microbiome, *Fusarium*, *Penicillium*, and *Conlarium*, known for their plant growth-promoting traits, may contribute to the core microbiome, fostering plant growth. *Enterocarpus* and *Hormiactis*, identified in the amended topsoil, were also detected in plant root microbiomes, suggesting potential plant selection.

For future research, it is crucial to conduct long-term studies to comprehend the sustained effects of phytomanagement of digestate-amended soil over time. Additionally,

investigating cumulative effects resulting from repeated digestate applications over an extended period is essential. Field trials, maintaining unaltered variables, are vital for comprehensive insights into microbial community performance and the sustainability of anaerobic digestate as an alternative to mineral fertilizers. Moreover, ensuring that phytomanagement of digestate-amended soils promotes a healthy microbial community underscores its potential as a sustainable option for farmers.

Authors contribution

NB-G: conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing—original draft. JF: investigation, methodology, validation, visualization, and writing—review and editing. MT: validation, visualization, and writing—review and editing. IB: writing—review and editing. GG: conceptualization, funding acquisition. MA: conceptualization, funding acquisition, supervision, validation, visualization, and writing—review and editing. AM: conceptualization, funding acquisition, supervision, validation, visualization, and writing—review and editing.

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4. Chapter 4: Trace metal mobility in soil-water-plant system with municipal sewage sludge digestate amendment. Experimental assessment of methodological approaches

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Neus Bonet-Garcia, Valentin Robin, Isabelle Bourven, Rémy Buzier, François Bordas, Ana P. Mucha, C. Marisa R. Almeida, Gilles Guibaud. Trace metal mobility in soil-water-plant system with municipal sewage sludge digestate amendment. Experimental assessment of methodological approaches.

Abstract

The impact of sewage sludge anaerobic digestate (SSAD) application as a land amendment has to be thoroughly understood as it could result in the accumulation or changes in the mobility of potentially toxic trace metals (TM) into soils. Phytomanagement is a green strategy that can help to ensure the safety of this practice. In this study, a 45-day greenhouse vertical soil column mesocosm experiment was conducted to understand how to measure the mobility and actual bioavailability of TMs in digestate-amended soil in presence/absence of plants and what parameters may be influencing TMs behaviour. The originality of the study comes from the multi-approach assessment of the mobile fraction of TMs. TMs concentrations in soil solution were monitored over time and depth, along with physical-chemical parameters, while final TMs potential bioavailability was assessed through a combination of diverse methods (soil solution analysis, sequential extraction, diffusive gradients in thin films (DGT) and accumulation into plant tissues). The research demonstrates a positive association between the application of SSAD and the levels of As, Mn, Fe, Co, Ni, and Cu in the soil solution, increasing them. The amendment resulted in an elevated concentration of dissolved organic carbon (DOC) and anions and cations as well, influencing the behavior of TMs in the soil solution over time and depth. In the presence of *P. virgatum*, Mn and Fe concentrations in the soil solution were further increased, potentially linked to the increased DOC and a reduction in both redox potential (Eh) and pH. A comprehensive evaluation of TMs lability, including DGT analysis, soil solution concentration, and

sequential extraction at the end of the experiment, highlighted Mn, Fe, Co, Al, and Zn as the most readily available TMs, correlating them with elevated concentrations in *P. virgatum* tissues. This study emphasizes the significance of considering TM DGT fluxes and TM soil solution concentrations for estimating the pool of labile TMs in a soil system.

4.1 Introduction

The world's population increase is leading to a consumption of vast amounts of resources, production of billions of tons of waste and wastewater (Kaza et al., 2018), and an unprecedented demand for clean and affordable energy. At the same time, demographic expansion, rising expectations of living standards, and scarcity of natural resources have made soil degradation a current major issue, posing a serious threat to human well-being (FAO, 2019).

Anaerobic digestion (AD), as indicated in Chapter 1, is one of the most-used strategies to give respond to the demand for both sustainable energies and waste stabilization methods (Akhiar et al., 2020; Vasco-Correa et al., 2018). The fertilization benefits of digestates are well documented, as indicated in previous chapters. Therefore, digestate can be used as a soil improver and thereby address the declining soil quality, reducing the use of chemical fertilizers and optimizing management costs by promoting a circular economy (Koszel & Lorencowicz, 2015; Kowalczyk-Juśko et al., 2023; Panuccio et al., 2019; Peng & Pivato, 2019). However, digestates can contain trace metals (TMs) (among other contaminants such as organic contaminants and pathogens), which are added externally to the process or originate from the AD feedstock (Ezebuiri & Körner, 2017; Garuti et al., 2018). Among TMs, both essential and non-essential groups can be found. When applying digestate as a bio-fertilizer, excessive concentrations of digestate-borne TMs can be transferred to soils. To prevent this issue, several legislative restrictions exist on the use of digestates as soil amendments (Di Maria et al., 2020; Foster & Prasad, 2021; Lamolinara et al., 2022; Malhotra et al., 2022; Saveyn & Eder, 2014; Stürmer et al., 2020). Moreover, digestate can induce physical and chemical changes in soil, that may result in alterations in TMs speciation and mobility, such as shifts in pH, redox potential (Eh), cation exchange capacity (CEC), soil organic carbon (SOC), enhancement of microbial activity, etc. The challenge associated with TMs presence in soils is their non degradability through any biological or physical processes, resulting in their persistent presence in soil for long periods of time, thereby posing a prolonged environmental hazard (Adriano & Adriano, 2001; Kabata-Pendias, 2011; Kabata-Pendias & Pendias, 2001). Phytomanagement is an *in-situ*, cost-effective and ecologically responsible strategy that uses the potential of plants to eliminate excessive

concentration of TMs from soil (phytoextraction, phytovolatilization) or lower their bioavailability (phytostabilization) (Awa & Hadibarata, 2020; Bhat et al., 2022; Tangahu et al., 2011; Z. Wei et al., 2021).

In a previous study using a 90-day soil-column mesocosm experiment (Bonet-Garcia et al., 2023, Chapter 2), we demonstrated that applying municipal solid digestate to the topsoil led to increased total concentrations of Pb, Zn, Ni, Mn, Fe and Cu in soil, however digestate-borne TMs showed low potential bioavailability. TMs were mostly confined in the 0-0.2 m soil horizon over the course of the experimental period, migrating in low amounts from the digestate-amended soil layer (0-0.1 m) to the layer underneath (0.1-0.2 m) within the first 21 days and remaining stable afterwards. Notably, when the bioenergy crops *P. virgatum* and *P. alopecuroides* were used to phytomanage the digestate-amended soil, the migration of TMs was further reduced. These bioenergy crops not only mitigated the transport of TMs but also benefited from the improved soil quality resulting from the digestate amendment. Prior research has also reported the increase of total TMs concentrations in topsoil after the application of biosolids fertilizers (Dragicevic et al., 2018; Koutroubas et al., 2014; Latare et al., 2014; Lloret et al., 2016; Marguí et al., 2016). Fewer research focuses on TMs bioavailability in digestate-amended soil, showing varying results that depend on factors such as the origin, type and properties of digestate, as well as soil conditions. While even less studies were found pertaining to the role of *P. virgatum* or *P. alopecuroides* in the phytomanagement of digestate-amended soils (Bonet-Garcia et al., 2023, Chapter 2). The obtained findings in our previous study and those found in literature raised the question of how to measure the mobility and actual bioavailability of TMs in digestate-amended soil in presence/absence of plants, not often measured directly and not always discussed, or only hypothesized, due to lack of full dataset.

TMs potential bioavailability in digestate-amended soils is mostly analyzed through sequential extraction fractionation approaches, which simplify metal speciation into discrete fractions and assume they are at or near equilibrium, which may not accurately represent the complexity of metal interactions with various components in the environment. While these methods can provide insight into potential metal availability, they do not directly measure bioavailability, which depends on a complex interplay of chemical, physical, and biological factors. The determination of the concentration of TMs in the soil solution phase is an attractive approach for all ecological considerations since free ion in soil solution is generally the most common phytoavailable form of TMs. Also, TMs dissolved in soil solution constitute the mobile portion and pose a risk to

groundwater systems. However, measurements of TMs in soil solution may fail to account for the ability of the soil to sustain the solution concentration following depletion. The *in-situ* determination of labile TM fraction can be achieved by the application passive sampling through diffusive gradients in thin film (DGT), which is based on the diffusion of the targeted TM through a diffusive layer (porous gel) followed by an irreversible sorption onto a layer of binding phase. It is a dynamic technique (i.e., it perturbs the equilibrium system and induces resupply) and its measurements in soils depend on labile (i.e., species that can be easily dissociated and resupplied) concentrations in the solution and their resupply from the solid phase. Such behavior can be considered as mimicking the uptake of TMs by plants, and thus TM flux and concentration determination by DGT could be more effective in estimating TMs bioavailability compared with total analysis, chemical extractions, and soil solution analysis (Sun et al., 2014). Several studies concluded that DGT measurements were correlated to the bioavailability of TMs, such as Zn or Cu (Agbenin & Welp, 2012; Almendros et al., 2022; Cornu & Denaix, 2006; Qasim et al., 2016; Sun et al., 2014; Tandy et al., 2011; Xie et al., 2021).

Hence, a multi-approach assessment of the mobile fraction of TMs was adopted in this study (Figure 21), aiming to evaluate the mobility and actual bioavailability of TMs in digestate-amended soil in presence/absence of plants. For that, a greenhouse mesocosm experiment lasting 45 days was carried out using vertical soil columns. In this experiment, sewage sludge anaerobic digestate (SSAD) was applied to marginal soil and phytomanaged with *P. virgatum*. Several methods were applied to address the mention aim: (i) monitoring chemical parameters and TM concentrations in the soil solution phase over time and depth, and (ii) applying and comparing different measures of bioavailability, including TMs BCR fractionation, DGT fluxes, concentrations in the soil solution, and TMs uptake by *P. virgatum*, at the end of the experimental period (day 45). In light of the diverse set of data collected, we hypothesized that the integration of multiple indicators will provide a comprehensive understanding of the dynamic processes governing TM mobility, bioavailability and TM plant uptake in the studied system. This holistic approach can also help decide on the most effective strategy to assess TM bioavailability and guide future research and management practices.

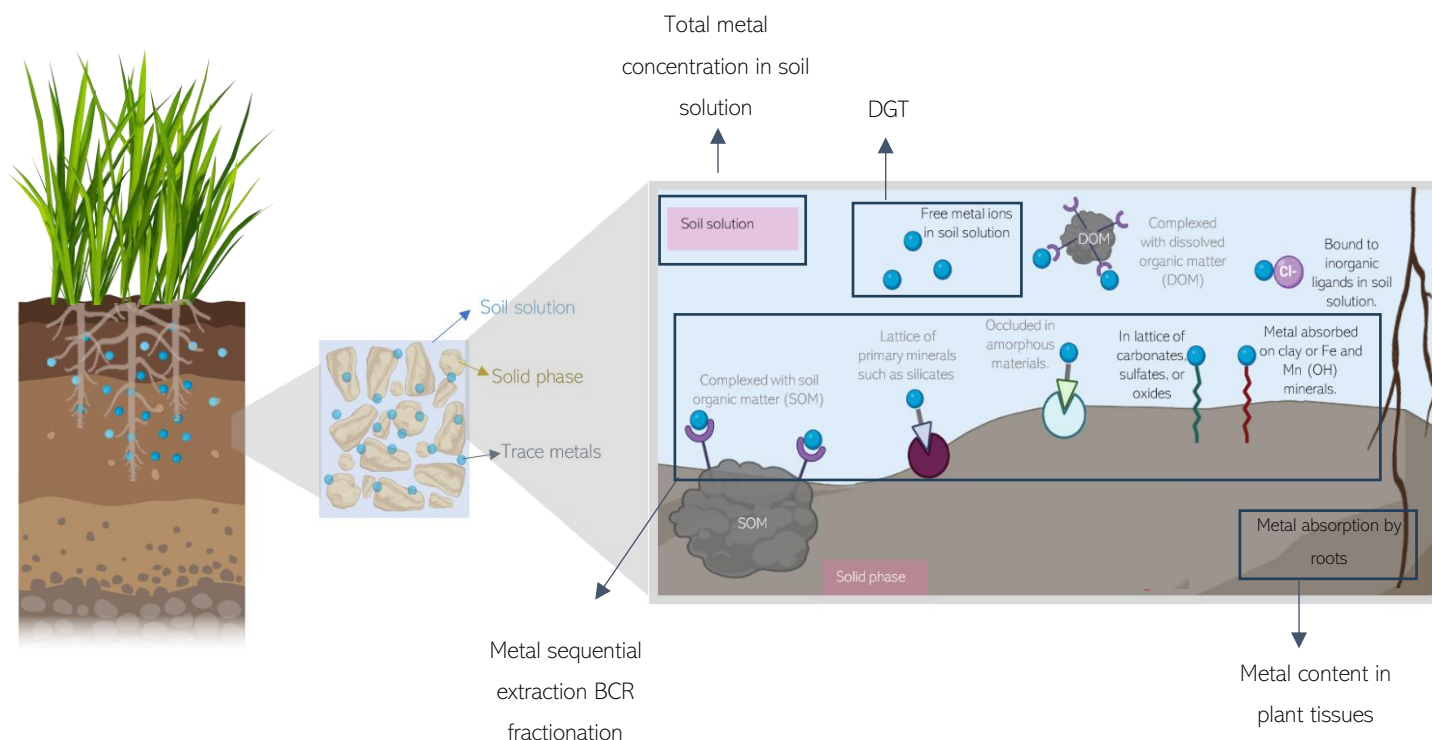


Figure 21. Illustration of the multiapproach assessment adopted in the present study. Figure created with Biorender.com. License agreement number MZ26A4MEDB.

4.2 Material and methods

4.2.A Soil and Sewage sludge anaerobic digestate (SSAD)

Soil samples were collected from the vicinity of a construction field in Ermesinde in the municipality of Valongo, North Portugal (41°12'25.7"N 8°32'26.7"W). Soil was identified and classified as an Entisol, previously described in Bonet-Garcia et al. (2023). Soil was air dried and sieved through <2 mm according to the ISO 11464 standard method before storage and use in columns experiments. The main physical-chemical parameters of soil after 9 months of storage were: pH value of 7.80 ± 0.05 , CEC of $9.3 \pm 0.1 \text{ cmol}(+)\cdot\text{kg}^{-1}$ and SOC of 0.2%.

Sewage sludge anaerobic digestate (SSAD) was collected from a municipal wastewater treatment plant located in Limoges (France). The digestate was derived from activated sludge treated by a mesophilic anaerobic digestion process. The sample was collected in a polypropylene (PP) bucket up to maximum capacity and closed with a lid to limit sample exposure to open air. Once in the laboratory, the sample was stored at 4 °C for less than 24 h before starting the experiment.

Detailed parameters of both SSAD, soil and SSAD amended soil are presented in Appendix III (Table S1 and Figure S1).

4.2.B Soil column mesocosm experiments

Vertical soil column mesocosm experiments were performed. Three treatment conditions were evaluated in separated columns: C1 (unamended soil), C2 (soil + SSAD) and C3 (soil + SSAD + *P. virgatum*) (Figure 22).

4.2.B.1 Column set up

Mesocosms consisted of rigid and opaque PVC cylindrical columns (0.11 m diameter × 0.3 m height) with 3 sampling ports (representing 3 different soil layers: top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) separated by 0.1 m each, and 1 at the bottom for leachate collection. The lower 0.02 m of each column were filled with inert pebble (to avoid soil loss and allow water drainage), followed by the addition of homogenized uncontaminated soil (0.2 m), previously sieved to <2 mm. To avoid positive preferential flows through the columns, soil columns were filled without air gaps enabling an equal level of homogeneity. Finally, soil was slightly compacted. SSAD and soil (proportion 1:3 v/v, final volume 2 L) were thoroughly mixed for 1 h using an orbital 2D shaker. The mixture of SSAD + soil was placed in C2 and C3 at the top of the uncontaminated soil, forming the 0-0.1 m topsoil layer, which is approximately equal to the depth of digestate incorporation on agricultural fields (0.1 – 0.2 m) (Alburquerque et al., 2012). In column C1 the 0-0.1 m topsoil layer was unamended soil. Prior to the experiment, soil present in the soil column mesocosms was saturated by adding a total of 3 L of ultrapure water (Milli-Q system: resistivity 18.2 MΩ·cm⁻¹). During the experiment (45 days), the soil column mesocosms were irrigated after each sampling in order to maintain 100 % of the water holder capacity (WHC), as a fixed reference has to be chosen to enhance reproducibility and standardization. Experiments were carried out for 45 days (including a 14-day stabilization period, after which 7 specimens of *P. virgatum* were transplanted to C3) in a greenhouse, with a natural day/night regime (during the months of September-November) at a temperature of 20 ± 4 °C. Before transplant, plants were grown in commercial substrate for 4 months.

A parallel small column containing unamended soil and 7 specimens of *P. virgatum* was set under the exact same conditions to serve as a control for the plant.

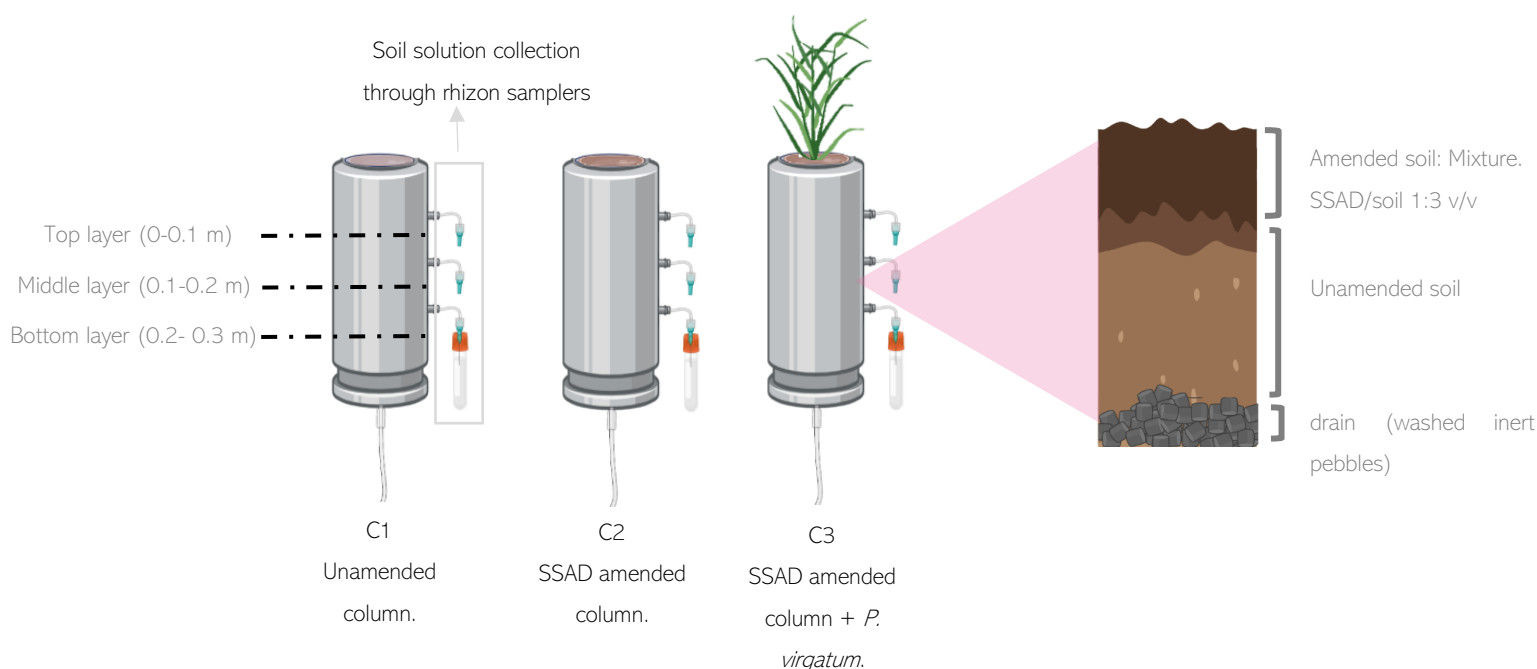


Figure 22. Schematic representation of vertical soil column mesocosm experiments. Figure created with Biorender.com. License agreement number EF26A4MNWI.

4.2.C Sample collection and characterization

4.2.C.1 Soil solution

Soil solution samples were collected using rhizon samplers (mean pore volume size 0.15 μm ; Rhizosphere Research Products, Netherlands) every 48 hours at the following depths: top layer (0-0.1 m), middle layer (0.1-0.2 m), and bottom layer (0.2-0.3 m) (Figure 22). Leachate samples were also collected every 48 hours from the drainage at the bottom of the column. Collected solutions were filtered with a syringe filter in cellulose acetate (pore diameter 0.2 μm) (Minisart) and diluted with ultrapure water (Milli-Q system: resistivity 18.2 $\text{M}\Omega\cdot\text{cm}^{-1}$). TMs concentration, pH, Eh, concentration of anions and cations, dissolved organic carbon (DOC) concentration, and concentrations of proteins, humic substances, sugars and uronic acids, were measured in soil solution. pH and Eh were measured immediately after collection. Samples for DOC and anions and cations analysis were stored at $-20\text{ }^{\circ}\text{C}$ until analysis, whereas those for TMs measurements were acidified (2% HNO_3 (v/v)) (HNO_3 69%, Suprapur) and stored at $4\text{ }^{\circ}\text{C}$. The pH and Eh measurements were conducted in triplicate, whereas for the concentration of TMs, DOC, and anions and cations, only a single measurement was

performed due to the limited quantity of sample, except at the end of the experiment when TMs concentration in soil solution were measured in triplicate.

4.2.C.2 Solid phase

Solid samples were collected at the beginning (initial soil and initial SSAD amended soil) and end of the experiment (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers of C1, C2 and C3). Samples were stored in polyethylene tubes at 4 °C until analysis. TMs total concentration and fractionation, pH, cation exchange capacity (CEC), soil organic carbon (SOC) concentration and concentration of proteins, humic substances, sugars and uronic acids were measured in all solid samples. In initial samples carbon, hydrogen, nitrogen, and sulfur (CHNS) analysis was also performed. All measurements in solid samples were conducted in triplicate.

4.2.C.3 Plant samples

Plant samples were collected at the beginning and end of the experiment from C3 and the control unamended pot, and subsequently stored at -20 °C. Prior to analysis, the samples were dried at room temperature (20 ± 1 °C). The above-ground components (leaves) and below-ground components (roots) were meticulously separated. The total content of TMs was then measured in both plant roots and leaves, with all measurements performed in triplicate.

4.2.C.4 DGT

DGT technique was applied to all layers of C1, C2 and C3 at the end of the experiment (day 45). Good laboratory practices were optimized to limit the risks of contamination, reusable materials were washed using 10% (V/V) HNO₃ bath for 24 h, an additional washing step was included 24 h before DGT assembling day. Materials, gels, reagents and DGT devices were always handled with clean gloves.

DGT preparation

Chelex binding gel strips, with a thickness of 0.04 cm, were provided by the manufacturer (DGT Research Ltd, Lancaster, UK). Polyacrylamide diffusive gels (15% acrylamide and 0.3% cross linker) were prepared according to Zhang et al. (1998), with a thickness of 0.077 cm. The DGT devices were assembled using plastic holders (3.14 cm² exposure area, provided by DGT Research Ltd.) enclosing a Chelex binding gel, a polyacrylamide diffusive gel and a cellulose nitrate membrane (0.4 µm pore diameter, 0.012 cm thickness).

DGT deployment

DGT was deployed in (i) initial SSAD- amended soil (after the 14 days stabilization period) and (ii) Top, middle and bottom layers of C1, C2 and C3 (Figure 22) at the end of the experimental period (day 45). Soil samples were at 100% WHC prior to DGT deployment. DGT devices (in triplicate) were carefully placed on the soil paste with gentle pressure to ensure complete contact between the filter membrane of the device and the soil and kept at 20 ± 1 °C for 24 h. Upon retrieval, DGT devices were washed with ultrapure water (Milli-Q, Millipore), to remove soil particles, and then disassembled. The DGTs were dismantled for binding gel recovery. Chelex binding gels were immediately eluted for 24 h with 2 mL of $1 \text{ mol} \cdot \text{L}^{-1} \text{ HNO}_3$. Eluates were stored at 4 °C until analysis.

DGT blanks were used to control the potential contamination of the samplers during the different steps related to the DGT samplers' usage, i.e., manufacturing, assembly/disassembly, and analysis. A total of 6 DGT blanks were prepared (3 for each DGT deployment day).

Labile elements flux calculations

The mass, M , of analyte accumulated on the binding layer, was calculated according to the following equation:

$$M = \frac{C_e \times V_e}{f_e}$$

Where C_e ($\mu\text{g L}^{-1}$) is the analyte mass concentration in the eluate, V_e (L) is the eluent volume and f_e is the analyte elution factor (0.8).

Mass of analyte accumulated in the binding layer was used to calculate the mean flux from the soil through the DGT device for the deployment time using the following equation:

$$F = M/t$$

Where M (μg) is the mass of analyte accumulated in the binding layer and t (s) is the deployment time.

4.2.C.5 Analysis

pH and Eh in soil solution were measured in triplicate immediately after collection. pH was measured using a pre calibrated pH micro-electrode (Orion™ PerpHecT™ ROSS™, Thermo Fisher Scientific, Waltham, MA, USA). Eh potential was measured using a platinum electrode combined with an Ag/AgCl (KCl saturated) probe (WTW Electrode

sentix ORP). The Eh reported later (mV) is relative to the standard hydrogen electrode and was corrected according to temperature and pH (Bohn 1971). Anions and cations concentrations were analyzed by ionic chromatography (IC) using a Dionex, DX120 chromatograph, equipped with a Dionex IonPac CS12A column with limits of quantification (LOQs) of 0.45, 0.075, 0.075, 0.075, 0.25, 0.1, 0.3, 0.25, 0.25, and 0.15 mg L⁻¹ for NO₃⁻, NO₂⁻, SO₄²⁻, PO₄³⁻, Cl⁻, Na⁺, NH₄⁺, Ca²⁺, Mg²⁺, and K⁺, respectively. Measurements were performed in single replicates.

Water content and dry weight of solid samples were determined in triplicate by oven drying the samples at 105 °C to a constant weight. All the subsequent analyses were calculated according to these dry weight measurements. Subsequently, the loss on ignition (LOI%) was measured by heating the previously dried samples at 550 °C for 2 h (Nelson & Sommers, 1996). LOI values were used to estimate the volatile dry weight. The solid samples' pH was determined in triplicate from the supernatant that was obtained after shaking the samples with ultrapure water in a 1:5 ratio for 2 h on an orbital device (80 rpm), according to the NF ISO 10390:1994 protocol (Ciesielski et al., 1997)(AFNOR, 2004). The assessment of effective CEC was performed in triplicate using the hexamine cobalt trichloride solution standard method (ISO 23470:2007) (Ciesielski et al., 1997). CHNS content in soil was determined in triplicate using a 2400 CHNS Organic Elemental Analyzer 100V (PerkinElmer, MA, USA). The solid organic matter (SOM) extraction procedure was based on cation exchange using a cation exchange resin (CER). The amount of CER required was calculated according to volatile solid (VS) content in samples (Frølund et al., 1996; H. Liu & Fang, 2002; Redmile-Gordon et al., 2014). 70 g of CER (Sigma Aldrich, DOWEX Marathon, sodium form, 20-50 mesh; Sigma Aldrich, Midland, MI) per gram of VS were added to 40 mL of buffer solution (2 mM Na₃PO₄ + 4 mM NaH₂PO₄ + 9 mM NaCl + 1 mM KCl adjusted to pH 7 with HCl 2 M) in 50 mL polypropylene centrifuge tubes, capped, and cooled to 4 °C over night. Next, dry soil samples were transferred to the CER-containing 50 mL tubes. Agitated at 600 tr/min for 1 h at 4°C in an orbital shaker and centrifuged twice at 20000 g for 20 min at 4°C. Supernatants were collected, transferred to new 50 mL polypropylene centrifuge tubes, and stored at -20 °C until analysis. Concentrations of organic carbon in SOM and dissolved organic matter in soil solution (DOM) were quantified by a Shimadzu® TOC-L TOC (Japan) with a LOQ of 0.5 mC·L⁻¹. Measurements were performed in triplicate for SOC and in single replicates for DOC. Humic-like substances, proteins, sugars and uronic acids content were measured in SOM and DOM. Measurements for SOM were performed in triplicate while

measurements for DOM were performed in single replicates. Humic-like substances and proteins concentrations were obtained using the colorimetric method described by Frølund et al., 1995. For sugars and uronic acids, the protocols established by DuBois et al. (1956) and Blumenkrantz and Asboe Hansen (1973) were followed.

The total TMs content from solid samples and plant tissues mentioned above was obtained following an adapted protocol of US EPA 3052: 0.5 g of dry sample (in triplicate) were digested with 5 mL of H₂O₂ (30%), 4 mL of HCl (37%) and 4 mL of HNO₃ (69%) for 10 min at 180 °C by microwave heating (Multiwave Go). Sequential extractions were carried out on all samples (in triplicate) by using a modified BCR procedure (Rauret et al., 1999). Briefly, 0.5 g of sample was successively mixed with each solution (F1, F2, and F3). After every step, the supernatant was obtained by centrifugation (3000g, 20 min), filtered (0.2 µm) and acidified with concentrated HNO₃. Between each fraction, pellets were washed with 20 mL of ultrapure water (Milli-Q system: resistivity 18.2 MΩ·cm⁻¹). F1 corresponds to the acid-soluble/exchangeable fraction and it was extracted using acetic acid (CH₃COOH) (0.11 mol·L⁻¹). The second (F2), the reducible fraction, is related to metal/lroids generally bound to Fe and Mn oxyhydroxides and it was extracted with hydroxylammonium chloride (hydroxylamine hydrochloride) (0.5 mol·L⁻¹). The oxidizable fraction (F3) corresponds to metals associated to organic matter or in sulfide form and it was obtained after a digestion with hydrogen peroxide (300 mg g⁻¹, 8.8 mol L⁻¹) and an extraction with ammonium acetate (1.0 mol L⁻¹). The extractions were carried out in an end-over-end shaker at room temperature. The residual fraction (F4) was calculated as the difference between the total metal content and the sum of the metal content in the three extraction solutions mentioned above.

TMs measurements from soil solution, solid phase, sequential extraction and DGT gels were performed using an Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) device (Agilent Technology 7700x ICP-MS, Santa Clara, CA, USA and using 45Sc and 115In as internal standards). All the samples were adjusted to 2% (V/V) HNO₃ by dilution before the analysis. The LOQs were 0.03 µgCr·L⁻¹, 0.07 µgMn·L⁻¹, 1 µgFe·L⁻¹, 0.03 µgNi·L⁻¹, 0.04 µgCu·L⁻¹, 0.04 µgPb·L⁻¹, 0.04 µgAs·L⁻¹ and 0.03 µgCd·L⁻¹. LOQs for DGT were determined according to the IUPAC recommendations (IUPAC Compendium on Analytical Nomenclature, online version available at https://media.iupac.org/publications/analytical_compendium/) using the mean + 10 standard deviations (SD) of the blanks (2% acidified ultrapure water). Measurements in solid phase, sequential extraction and DGT gels were performed in triplicate, while measurements in soil solution were performed in single replicates.

4.2.C.6 Plant metal uptake

To evaluate the TMs accumulation in *P. virgatum* under the studied conditions, bioaccumulation coefficient factor (BCF) and translocation factor (TF) were calculated using the concentrations determined in top layer (0-0.1 m) of C3 (SSAD-amended column) and *P. virgatum* tissues at the end of the experiment (day 45). BCF and TF were calculated in triplicate using the formula mentioned below, previously reported by Yoon et al. (2006).

$$BCF = C_{\text{root}} / C_{\text{soil}}$$

$$TF = C_{\text{leaves}} / C_{\text{root}}$$

where, C_{root} is the concentration of metal in plant root ($\text{mg} \cdot \text{kg}^{-1}$), C_{soil} the concentration of metal in topsoil ($\text{mg} \cdot \text{kg}^{-1}$) and C_{leaves} the concentration of metal in plant leaves ($\text{mg} \cdot \text{kg}^{-1}$).

4.2.D Statistical analysis

Sample mean, standard deviation, maximum and minimum values were analyzed by Microsoft Excel 2019. Uncertainty bars in graphs represent the standard deviation. Statistical tests were performed using R (version 4.3.0, 2023). Shapiro-Will test was used to test whether the distribution of data sets deviate from a comparable normal distribution, null hypothesis was rejected if $p < 0.05$. Significance of the differences among and between C1, C2 and C3 samples over time and depth were evaluated through a parametric one-way analysis of variance (ANOVA). Significant differences ($p < 0.05$) were detected by a Tukey's Honestly Significant Difference (HSD) post hoc test. Pearson's correlation was used to identify potential correlations (i) between TMs concentration and chemical parameters in soil solution over time and depth (ii) between different measures of bioavailability on day 45 and (iii) between the bioavailability measures in the top layer of C3 and C1 and the TMs concentration in plants tissues (leaves and roots) harvested from C3 and the control unamended pot at day 45 using R-packages *corr* (version 0.4.4) and *ggcorrplot* (version 0.1.4.1).

4.3 Results

4.3.A Evolution of soil solution properties over time (from day 0 to day 45)

4.3.A.1 Chemical parameters

The results of pH, Eh and DOC measurements in soil solution over time across the top (0-0.1 m), middle (0.1-0.2 m), bottom (0.2-0.3 m) layers, as well as in the leachate of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + plant) are presented in Figure 23. DOM composition (fractionation of proteins, humic substances, sugars and uronic acids) and concentrations of anions and cations concentrations are shown in Appendix III (Figure S2 and Figure S3).

4.3.A.1.1 Addition of SSAD to soil

In soil solution, pH values in both C2 and C1 fluctuated between 7.52 ± 0.10 and 8.31 ± 0.15 , indicating a slightly basic pH. SSAD amendment seemed to induce more homogeneous pH values between layers, when compared to unamended column (C1). Top layer (0-0.1 m) of C2 maintained higher ($p < 0.05$) pH levels than C1 at the end of the experimental period. While deeper soil layers (0.1-0.3 m) in C2 showed lower ($p < 0.05$) pH than C1. Results showed no significant ($p > 0.05$) differences between C2 and C1 in terms of Eh potential in soil solution over time, values remained relatively constant between 317 ± 2 mV and 417 ± 1 mV in all layers. Results clearly suggest the influence of SSAD amendment in increasing concentration of DOC in soil ($p < 0.05$). At the beginning of the experimental period immediately after SSAD amendment, DOC concentration in the top layer (0-0.1 m) of C2 was 213% higher when compared to same layer in C1. DOC in top layer of C2 rapidly rose over time until reaching a peak at day 10, when it was 573% higher than the same layer in C1. Subsequently, DOC concentration in top layer of C2 progressively decreased until day 32, after which it seemed to resume an upward trend. A steady increase of DOC over time can be observed in deeper layers of C2, especially in middle layer (0.1-0.2 m), suggesting the DOC migration through the soil profile. At the end of the experiment, the DOC concentration in top, middle and bottom layer of C2 was $0.29 \text{ g}\cdot\text{L}^{-1}$, $0.24 \text{ g}\cdot\text{L}^{-1}$ and $0.11 \text{ g}\cdot\text{L}^{-1}$, respectively. These values were 475%, 376% and 132% higher than those observed in the corresponding layers of C1. Fractionation of DOC composition is presented in Figure S3. In top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers of C1 and C2, DOC was mainly composed by proteins and humic substances. Qualitatively, there were not differences between C1 and C2 until the end of the

experiment, when top (0-0.1 m) and middle (0.1-0.2 m) layers of C2 showed lower percentage of proteins while higher percentage of sugars and uronic acids when compared to the same layer of C1.

Anions analysis demonstrated the significant impact of SSAD on elevating SO_4^{2-} concentrations in the soil solution. Initially, the top layer (0-0.1 m) of C2 exhibited a remarkable 2300% increase in SO_4^{2-} concentration compared to the same layer in C1. The results also indicated the migration of SO_4^{2-} from the top layer (0-0.1 m) to the middle layer (0.1-0.2 m) in C2. While the concentration in the top layer steadily decreased over time, leading to an increase in the middle layer, a subsequent decline in concentration in the middle layer occurred after day 12. Additionally, SSAD amendment slightly elevated Cl^- concentrations in the soil solution of both the top (0-0.1 m) and middle (0.1-0.2 m) layers. However, no significant changes ($p > 0.05$) in F^- , NO_2^- , NO_3^- , and PO_4^{3-} concentrations in the soil solution were observed between C2 and C1.

At day 0, C2 exhibited significantly higher concentrations of Na^{2+} , NH_4^+ , K^+ , Ca^{2+} , and Mg^{2+} in the top layer (0-0.1 m) compared to C1 (unamended column), with NH_4^+ and Ca^{2+} showing particularly elevated levels. Over the initial 5 days post-SSAD amendment, NH_4^+ and Ca^{2+} concentrations in the top layer (0-0.1 m) of C2 increased slightly, while Na^{2+} , K^+ , and Mg^{2+} remained relatively stable. Subsequently, NH_4^+ , K^+ , and Ca^{2+} concentrations gradually decreased, with NH_4^+ and Ca^{2+} exhibiting pronounced declines. Between days 12 and 24, Ca^{2+} concentrations in the top layer of C1 were higher than in C2, suggesting it did not originate from the SSAD. Results indicate migration of NH_4^+ and K^+ through the soil profile. Also, Ca^{2+} showed an initial increase in the bottom layer (0.2-0.3 m) of C2, followed by a progressive decline. At the end of the experiment, NH_4^+ , Na^{2+} and K^+ concentrations in all layers of C2 were higher ($p < 0.05$) than in C1. Specifically, NH_4^+ concentrations in C2's top, middle, and bottom layers were $179 \text{ mg}\cdot\text{L}^{-1}$, $184 \text{ mg}\cdot\text{L}^{-1}$, and $111 \text{ mg}\cdot\text{L}^{-1}$, respectively, contrasting with consistently low levels ($0 - 0.1 \text{ mg}\cdot\text{L}^{-1}$) in C1. Na^{2+} concentrations in C2's layers were $39 \text{ mg}\cdot\text{L}^{-1}$, $36 \text{ mg}\cdot\text{L}^{-1}$, and $37 \text{ mg}\cdot\text{L}^{-1}$, compared to $4.5\text{-}5 \text{ mg}\cdot\text{L}^{-1}$ in C1. K^+ concentrations in C2's layers were $66 \text{ mg}\cdot\text{L}^{-1}$, $58 \text{ mg}\cdot\text{L}^{-1}$, and $65 \text{ mg}\cdot\text{L}^{-1}$, compared to $8.9\text{-}10 \text{ mg}\cdot\text{L}^{-1}$ in C1.

4.3.A.1.2 Addition of *P. virgatum* to SSAD amended soil

The introduction of the plant (C3) induced a decrease ($p < 0.05$) in pH in top layer, which was maintained over time with some fluctuations. When plant was present (C3), a sharp drop ($p < 0.05$) in Eh values can be observed in all layers, especially in top and middle layers. By the end of the experiment (day 45), the Eh values had reached $202 \pm 1 \text{ mV}$,

224 $\pm$ 1 mV and 309 $\pm$ 2 mV in top, middle and bottom layers, respectively. The concentration of DOC in the top layer of C3 exhibited a similar pattern as in C2 until the introduction of *P. virgatum* into C3 on day 14. Subsequently, after day 14, the DOC concentration showed an increasing trend, diverging from the behavior observed in C2. Similar to C2, the concentration of DOC in the middle layer of C3 displayed a continuous and more noticeable increase over time. Bottom and leachate the same. At the end of the experiment, the DOC concentration in top, middle and bottom layer of C3 was 0.54 g·L⁻¹, 0.48 g·L⁻¹ and 0.16 g·L⁻¹, respectively. These values were 89%, 101%, 45% higher than those observed in the corresponding layers of C2. Regarding DOC fractionation, no significant differences were observed between C2 and C3 $p < 0.05$ over time and depth.

In the case of C3, the concentrations of SO₄²⁻, F⁻, NO₂⁻, NO₃⁻, and PO₄³⁻ in all layers displayed a pattern similar to that observed in C2. The introduction of *P. virgatum* seemed to induce a sharper decrease in the concentration of NH₄⁺, K⁺ and Ca²⁺ in the top layer (0-0.1 m), compared to C2. The concentrations of Na²⁺, NH₄⁺, K⁺, Ca²⁺ and Mg²⁺ in the middle layer (0.1-0.2 m) exhibited a similar behavior pattern to that observed in C2, with some fluctuations between day 18 to 26 in the case of NH₄⁺. Concerning the bottom layer (0.2-0.3 m), significantly higher concentrations of NH₄⁺ were observed towards the end of the experiment in C3 ($p < 0.05$). Additionally, the Ca²⁺ concentration in the soil solution of the bottom layer exhibited distinct behavior patterns between C2 and C3. In C2, Ca²⁺ gradually decreased over time following an initial increase. In contrast, in C3, initial Ca²⁺ concentrations were notably lower ($p < 0.05$) and increased progressively over time, reaching a peak before subsequently declining. At the end of the experiment the concentrations of NH₄⁺ and K⁺ were significantly lower in the top layer (0-0.1 m) of C3, measuring 43 mg·L⁻¹ and 27 mg·L⁻¹, respectively, when compared to the same layer in C2. In contrast, in the bottom layer (0.2-0.3 m) of C3, NH₄⁺ and K⁺ concentrations were significantly higher than the same layer in C2, measuring 273 mg·L⁻¹ and 95 mg·L⁻¹ respectively. Na²⁺ in top (0-0.1 m) and middle (0.1-0.2 m) and bottom (0.2-0.3 m) layer were similar between C2 and C3.

4.3.A.2 Trace metals

The results of Mn, Ni and Cu concentrations in soil solution over time across the top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers, as well as in the leachate of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + plant) are presented in Figure 23. Results of Al, Cr, Fe, Co, Zn, Pb and As are shown in Figure S4.

4.3.A.2.1 Addition of SSAD to soil

Overall, the results show the impact of SSAD amendment over both time and depth, demonstrating a notable initial increase in soil solution concentrations of Cr, Mn, Fe, Co, Ni, Cu, and As when compared to the unamended column (C1) ($p < 0.05$). SSAD amendment induced no significant ($p > 0.05$) changes in soil solution concentrations of Al, Zn and Pb. Results suggest Mn, Fe, Co, Ni, Cu, and As in soil solution were primarily confined in the first two layers of soil (0-0.2 m) throughout the 45-day experimental period, showing migration in solution from the top layer (0-0.1 m) to the layer underneath (middle layer, 0.1-0.2 m). A slight migration to deeper layers (0.2-0.3 m) was found for Mn, Co, Ni and Cu. In general, soluble Mn, Fe, Co, Ni and As concentration in top layer (0-0.1 m) decreased with time leading to an increase of their concentration in the layer underneath (middle, 0.1-0.2 m). This trend continued until it eventually reversed. For Cu, results suggest an initial migration to middle layer, which exhibited an upward trend until peaking at day 15. Subsequently, this trend was reversed, indicating an immobilizing effect. In the case of Cr, the application of SSAD resulted in a significant increase ($p < 0.05$) in its soluble concentration in the bottom layer of soil (0.2-0.3 m) immediately after the amendment. However, over the course of the experiment, soluble Cr concentration in bottom layer of C2 exhibited a progressive decrease over time, suggesting an immobilization process (0-0.2 m).

At the end of the experiment, Mn, Fe, Co, Ni, Cu, Zn, As and Pb concentrations in soil solution of C2 were considerably higher ($p < 0.05$), particularly in top (0-0.1 m) and middle (0.1-0.2 m) layers, when compared to the values found in the corresponding layers of C1. These results will be further described in the following section.

4.3.A.2.2 Addition of *P. virgatum* to SSAD amended soil

Overall, the presence of *P. virgatum* further increased the concentrations of Mn, Fe, Co, and Ni in the soil solution of top and middle layers, when compared to C2 (SSAD amended column - plant) ($p < 0.05$). Cr, Cu and As behaviour in C3 was similar to C2. Al, Zn and Pb exhibited consistent behaviour in all columns (C1, C2 and C3). Similar to C2, TMs were mainly concentrated within the first two layers of soil (0-0.2 m).

At the end of the experiment, Mn, Fe and Co concentrations in top (0-0.1 m) and middle (0.1-0.2 m) layers were greater in C3 when compared to C2. These results will be further described in the following section.

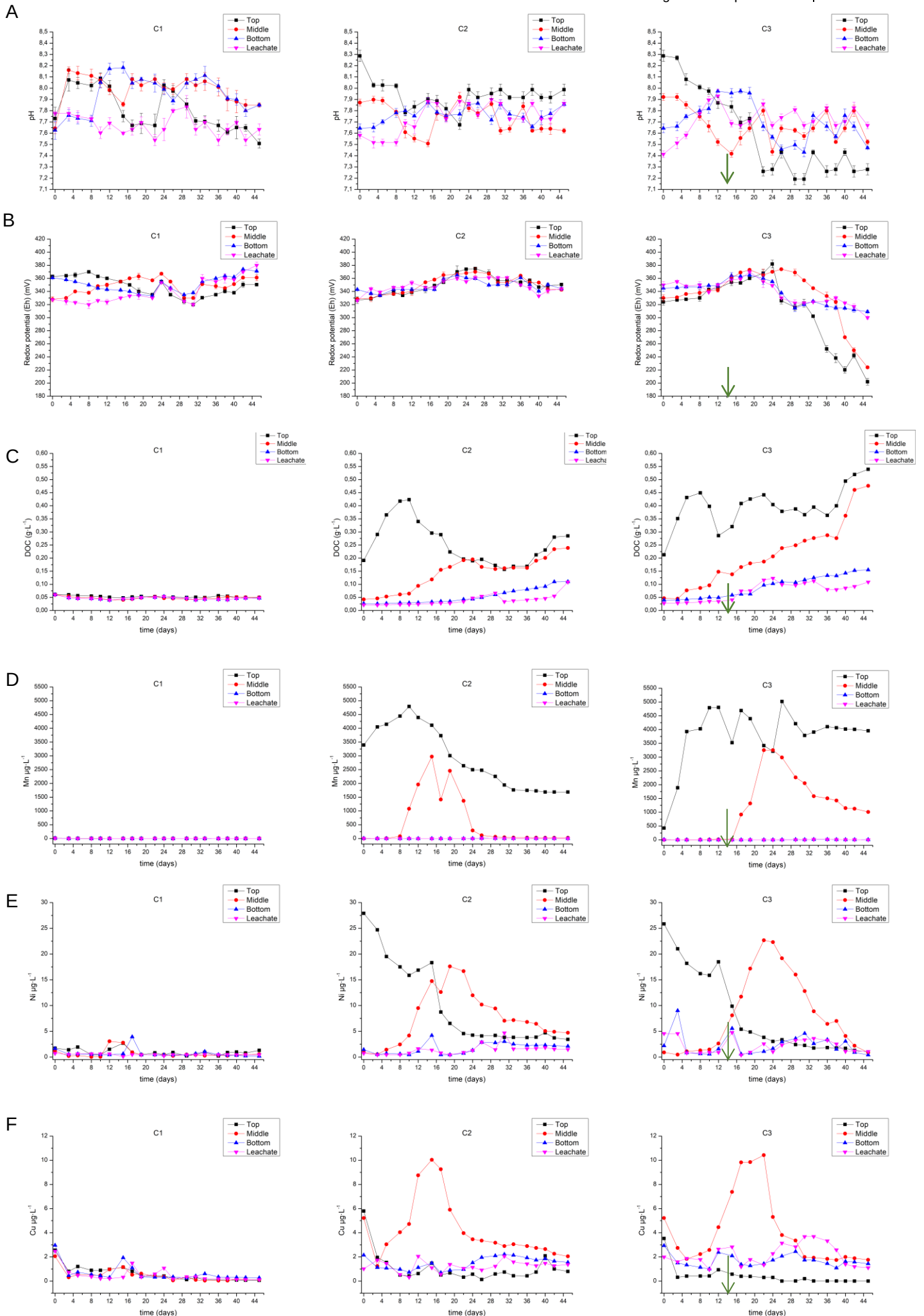


Figure 23. (A) pH, (B) Eh (mV), (C) dissolved organic carbon (DOC) ($\text{g} \cdot \text{L}^{-1}$), (D) Mn ($\mu\text{g} \cdot \text{L}^{-1}$), (E) Ni ($\mu\text{g} \cdot \text{L}^{-1}$) and (F) Cu ($\mu\text{g} \cdot \text{L}^{-1}$) in soil solution of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + *P. virgatum*) over time (from day 0 to day 45) and depth (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers). The green arrow indicates the date when plant was transplanted in C3 (day 14). The error bars in the pH and Eh graphs represent the standard error of the mean based on three measurements ($n = 3$). For Mn, Ni, and Cu, only one measurement was taken ($n = 1$).

4.3.B Soil-SSAD-plant system after 45 days

4.3.B.1 Physical chemical properties

pH, CEC, and SOC concentration measured in solid samples of top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + plant) at day 45 are presented in Figure 24 and in supplementary material Figure S6. SOM composition is shown in supplementary material Figure S5. DOC concentration, pH of soil solution and Eh at day 45 are shown above in Figure 23. By day 45, C2 (SSAD-amended column) exhibited significantly higher pH values ($p < 0.05$) in the top (0-0.1 m), middle (0.1-0.2 m), and bottom (0.2-0.3 m) layers compared to C1 (unamended column). In C3 (SSAD-amended column + plant), the pH of the top (0-0.1 m) and middle (0.1-0.2 m) layers was lower ($p < 0.05$) than both in C2 (SSAD-amended column) and C1 (unamended column). CEC and SOC values were higher ($p < 0.05$) in C2 (SSAD-amended column) compared to C1 (unamended column), while C3 (SSAD-amended column + plant) showed higher values than C2 (SSAD-amended column). Both CEC and SOC presented a downward trend from top to bottom in C2 (SSAD-amended column) and C3 (SSAD-amended column + plant).

Qualitatively, there were no significant differences ($p > 0.05$) observed in the composition of SOM between columns (C1, C2 and C3) and layers. Across all layers, a consistently higher presence of proteins ($\sim 35\text{-}40\% \pm 1\text{-}5$) and humic substances ($\sim 25\text{-}32\% \pm 0.5\text{-}6$) was noted.

4.3.B.2 Trace metals

Following 45 days of SSAD soil amendment, different methods to estimate TMs bioavailability were applied, namely (i) TMs concentration in exchangeable fraction (which shows the amount of weakly bound metals which are readily soluble in water. Therefore, considered to be most mobile and available fraction for the plant; (ii) TMs

concentration in soil solution and (iii) DGT-measured fluxes. Analyses were performed in top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers of C1 (unamended column), C2 (SSAD-amended column) and C3 (SSAD-amended column + plant). A comparative analysis of these methods was conducted to gain a more comprehensive understanding of the potential availability of TMs in soil subjected to SSAD amendment and under the conditions of phytomanagement of SSAD-amended soil. Results are presented in Figure 24 and in supplementary material Figure S6. Complete sequential extraction fractionation results of the three layers of C1, C2 and C3 at the end of the experiment are shown in Figure S7, Appendix III.

All the three measurements (TMs concentration in exchangeable fraction, TMs concentration in soil solution and DGT-measured fluxes) suggest higher availability of Mn, Fe and Co in top (0-0.1 m) and middle (0.1-0.2 m) layers of C2 and C3 when compared to C1, suggesting an effect of SSAD amendment. Concentration in soil solution and DGT-measured fluxes show statistically higher values ($p < 0.05$) of Mn, Fe and Co in presence of *P. virgatum* (C3) when compared to C2, suggesting an effect of the plant. Overall, DGT fluxes of Zn, Cu, Ni, Co, Fe, Mn and Pb exhibited a similar migration pattern through the soil profile when compared with their respective concentrations in the soil solution in all three columns. However, the DGT fluxes differed from their concentrations in the exchangeable fraction. Regarding Al and Cr, big differences were observed when comparing their DGT fluxes, concentrations in soil solution and concentrations in exchangeable fraction. These variations make it difficult to draw a definitive conclusion.

Mn, Fe, Co, Ni, Zn and Al in top (0-0.1 m) and middle (0.1-0.2 m) layers of C2 and C3 presented higher DGT fluxes when compared to Cr, Cu and Pb ($> \text{LOD}$), with Mn and Fe showing particularly higher fluxes. According to the DGT analysis, by day 45, in top layer (0-0.1 m) of C3 (where roots were present), Mn (flux: $4.16 \cdot 10^{-5} \pm 2.02 \cdot 10^{-6} \mu\text{g} \cdot \text{s}^{-1}$), Fe (flux: $3.36 \cdot 10^{-5} \pm 1.65 \cdot 10^{-6} \mu\text{g} \cdot \text{s}^{-1}$) showed the highest DGT fluxes, followed by Al (flux: $8.02 \cdot 10^{-7} \pm 2.90 \cdot 10^{-8} \mu\text{g} \cdot \text{s}^{-1}$), Zn (flux: $3.12 \cdot 10^{-7} \pm 1.59 \cdot 10^{-8} \mu\text{g} \cdot \text{s}^{-1}$), and Co (flux: $1.35 \cdot 10^{-7} \pm 2.82 \cdot 10^{-8} \mu\text{g} \cdot \text{s}^{-1}$). Moreover, Fe and Mn also displayed the highest concentrations in the soil solution ($17285 \pm 12 \mu\text{g} \cdot \text{L}^{-1}$ and $3956 \pm 10 \mu\text{g} \cdot \text{L}^{-1}$, respectively), followed by As ($59 \pm 3.1 \mu\text{g} \cdot \text{L}^{-1}$), Co ($10.22 \pm 1.05 \mu\text{g} \cdot \text{L}^{-1}$), Al ($7.24 \pm 0.90 \mu\text{g} \cdot \text{L}^{-1}$) and Zn ($4.00 \pm 0.32 \mu\text{g} \cdot \text{L}^{-1}$). In terms of the exchangeable fraction, Fe exhibited the highest concentrations ($201 \pm 4.00 \mu\text{g} \cdot \text{g dw}^{-1}$) followed by Al ($66.57 \pm 1.00 \mu\text{g} \cdot \text{g dw}^{-1}$), Mn ($60.10 \pm 0.89 \mu\text{g} \cdot \text{g dw}^{-1}$) and Zn ($6.89 \pm 0.64 \mu\text{g} \cdot \text{g dw}^{-1}$). However, Co and As concentrations in exchangeable fraction were very low ($0.80 \pm 0.19 \mu\text{g} \cdot \text{g dw}^{-1}$ and $0.82 \pm 0.08 \mu\text{g} \cdot \text{g dw}^{-1}$).

These results suggest that at day 45, Fe, Mn, Al, and Zn may be more readily available for uptake by plants in the specified soil layers, as evidenced by their higher DGT fluxes, soil solution and exchangeable fraction concentrations.

Complete sequential extraction results (Figure S7, Appendix III) showed that by day 45, the application of SSAD amendment led to a significant increase ($p < 0.05$) in the proportion of Mn, Co, and Zn within the exchangeable fraction in the top layer (0-0.1 m) of both C2 and C3, as compared to the unamended column (C1). Additionally, elevated percentages of Mn and Zn in the middle layer (0.1-0.2 m) were observed in C2 and C3. Notably, the percentage of Cu associated with the oxidizable fraction (bound to organic matter) was notably higher in both the top (0-0.1 m) and middle (0.1-0.2 m) layers of C2 and C3 in comparison to C1, with a slight yet significant difference ($p < 0.05$) between C3 and C2 in the top layer. These findings suggest a complexation of Cu with the organic matter. No significant differences ($p > 0.05$) were observed in the fractionation of other TMs between C2 and C3 across layers at the end of the experiment.

4.3.B.3 Plant trace metal content

After 45 days of SSAD amendment, *P. virgatum* was harvested from C3 (SSAD amended column + plant). Concentrations of TMs ($\mu\text{g}\cdot\text{g}^{-1}$ in dry weight basis) in roots and leaves, were measured and compared to initial concentrations. Results are illustrated in Figure 25 and in supplementary material Figure S8.

After 45 days of SSAD amendment, a significant rise ($p < 0.01$) was observed in the concentrations of all studied TMs in *P. virgatum* roots. Particularly noteworthy were the substantial increases in Fe, Al, Mn, As and Zn, with increments of $4001 \pm 3 \mu\text{g}\cdot\text{g}^{-1}$, $1560 \pm 5 \mu\text{g}\cdot\text{g}^{-1}$, $106 \pm 1 \mu\text{g}\cdot\text{g}^{-1}$, $15 \pm 0.5 \mu\text{g}\cdot\text{g}^{-1}$, and $14 \pm 0.5 \mu\text{g}\cdot\text{g}^{-1}$, respectively. In the case of *P. virgatum* leaves, an overall increase in TM concentrations was noted compared to the initial concentrations ($p < 0.05$), except for Ni, Cu, and As, which remained unchanged. The most remarkable raises were observed also for Al, Fe, Mn, and Zn with increases of $434 \pm 3 \mu\text{g}\cdot\text{g}^{-1}$, $196 \pm 2 \mu\text{g}\cdot\text{g}^{-1}$, $143 \pm 1 \mu\text{g}\cdot\text{g}^{-1}$, $7 \pm 0.4 \mu\text{g}\cdot\text{g}^{-1}$ respectively, at the conclusion of the experimental period. It is worth noting that in the above section Mn, Fe, Al, and Zn were estimated to be the most bioavailable TMs.

Most abundant TMs in *P. virgatum* tissues at the end of the experiment were Fe, Al, Mn, Zn and As, with concentrations of $4085 \pm 7 \mu\text{g}\cdot\text{g}^{-1}$, $1673 \pm 4 \mu\text{g}\cdot\text{g}^{-1}$, $180 \pm 3 \mu\text{g}\cdot\text{g}^{-1}$, $36 \pm 0.8 \mu\text{g}\cdot\text{g}^{-1}$ and $15 \pm 0.4 \mu\text{g}\cdot\text{g}^{-1}$ in roots, and $389 \pm 5 \mu\text{g}\cdot\text{g}^{-1}$, $662 \pm 2 \mu\text{g}\cdot\text{g}^{-1}$, $303 \pm 1 \mu\text{g}\cdot\text{g}^{-1}$, $29 \pm 1 \mu\text{g}\cdot\text{g}^{-1}$ and $0.72 \pm 0.06 \mu\text{g}\cdot\text{g}^{-1}$ in leaves. Plant uptake and accumulation were only observed in the cases of Mn and Zn ($\text{BCF} > 1$ and $\text{TF} > 1$).

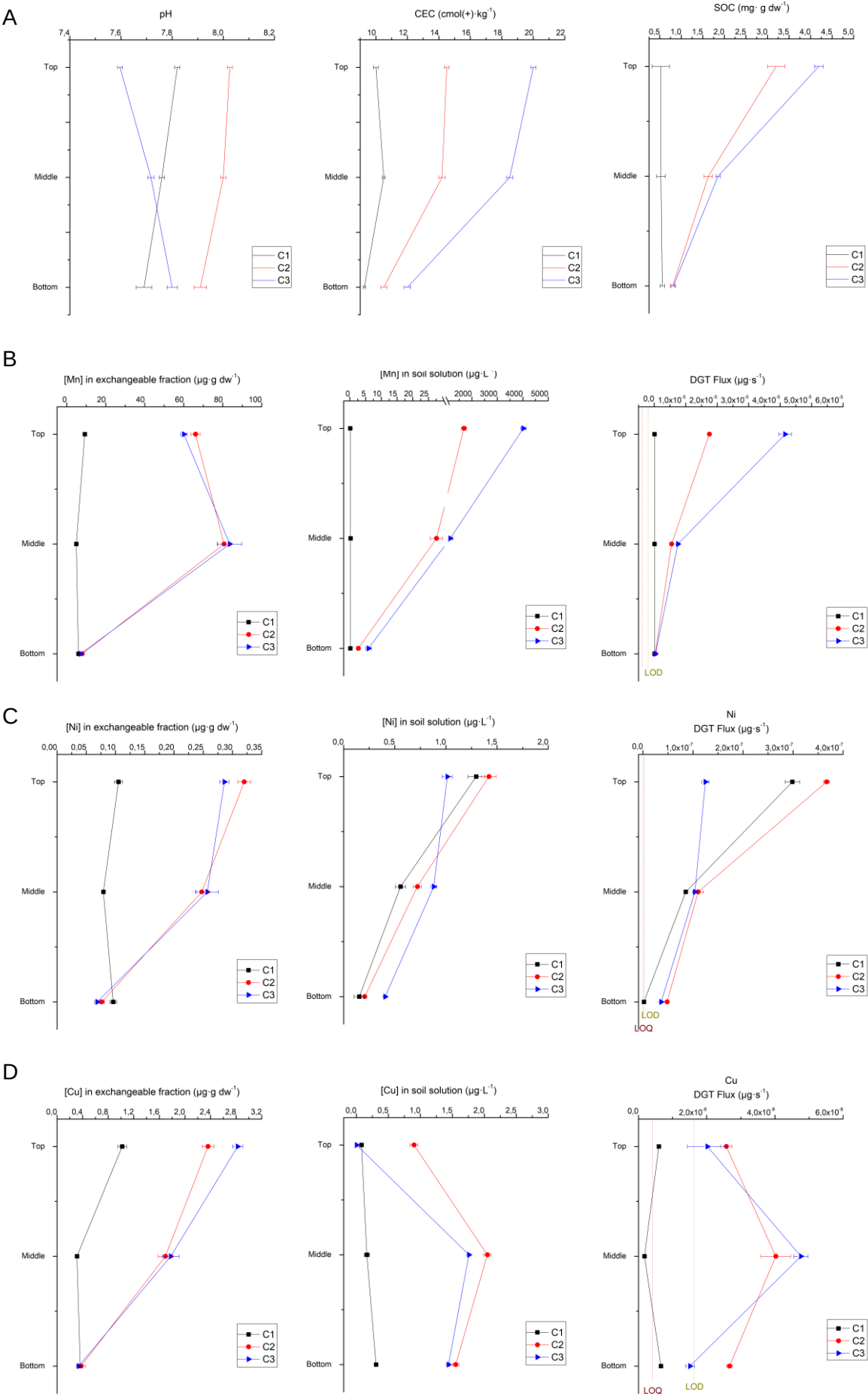
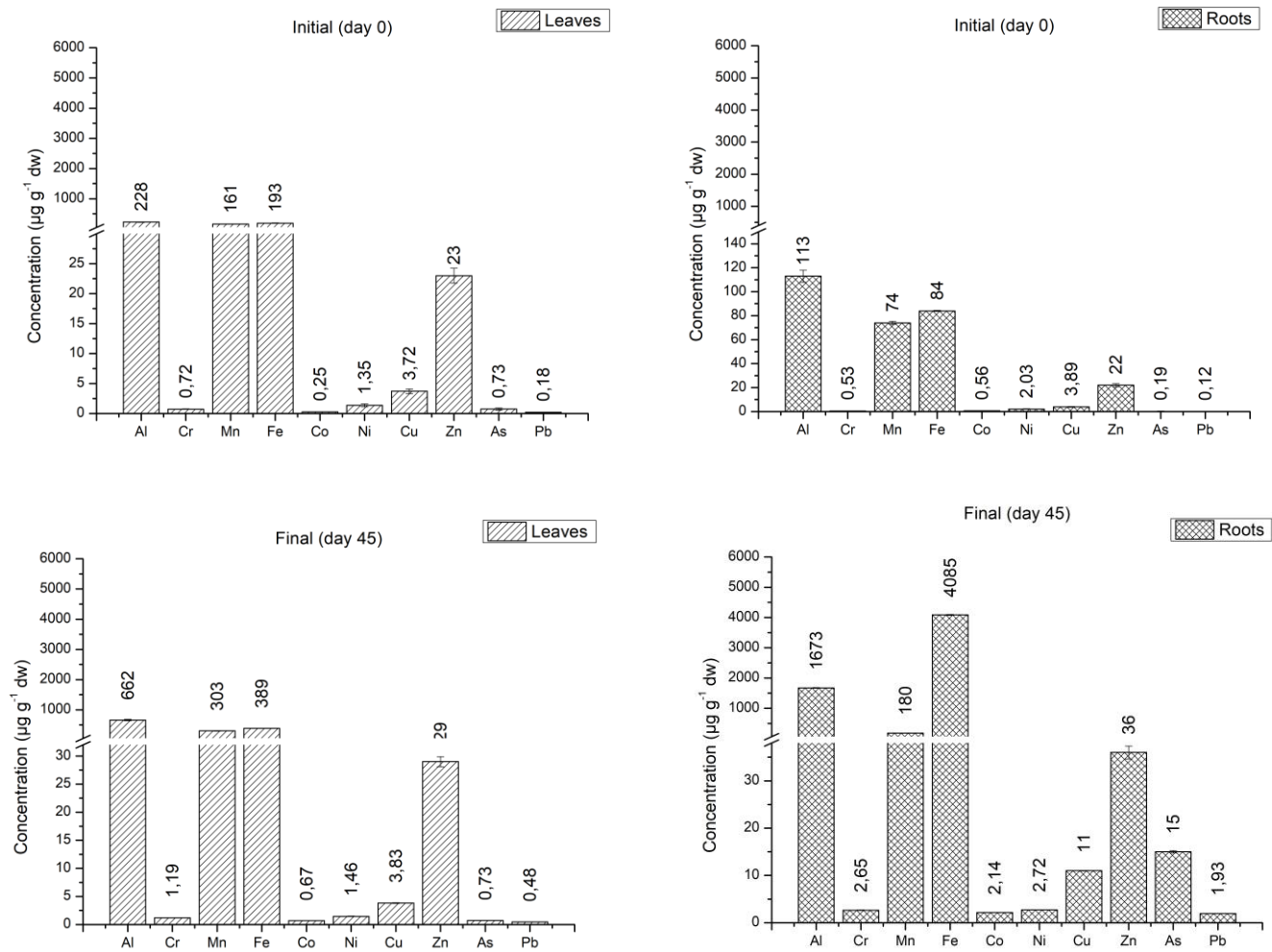


Figure 24. (A): pH, CEC and SOC over depth (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers) of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD-amended column + plant). (B), (C) and (D): Comparison of TMs' (Mn, Ni and Cu) (i) concentration in exchangeable fraction (ii) concentration in soil solution and (iii) DGT fluxes, over depth (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers) of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD-amended column + plant) at the end of the experimental period (day 45). Error bars indicate standard error of the mean of three measurements (n = 3).

A



B

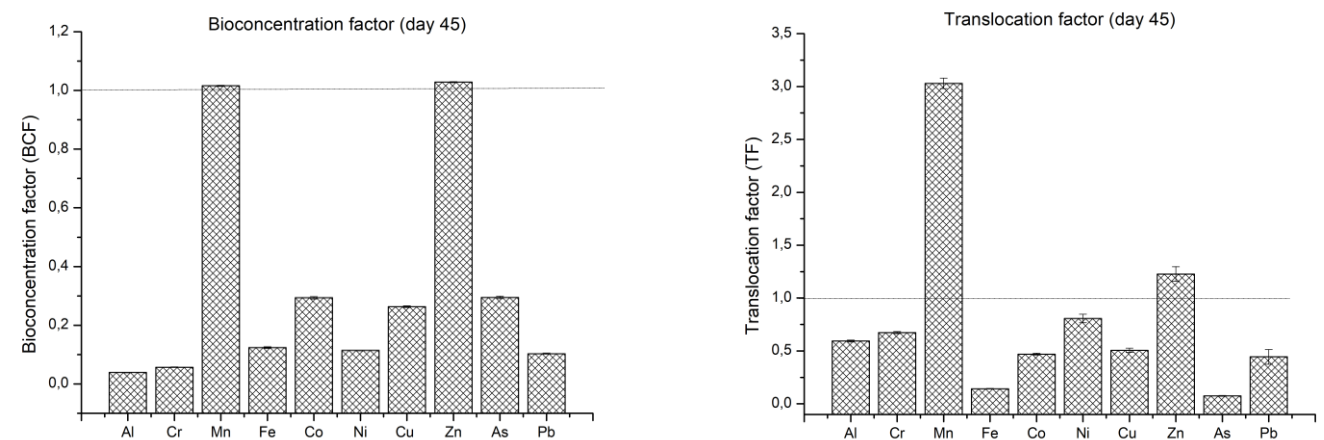


Figure 25. (A) TMs (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Pb) concentration ($\mu\text{g}\cdot\text{g}^{-1}$ in dry weight basis) in *P. virgatum* roots and leaves at the beginning (left) and end (right) of the experiment. (B) TMs bioconcentration factor (BCF) (left) and translocation factor (TF) (right) from *P. virgatum* at day 45. Error bars indicate standard error of the mean of three measurements ($n = 3$). Labels on the graphs indicate the mean of three measurements.

4.3.C Correlations

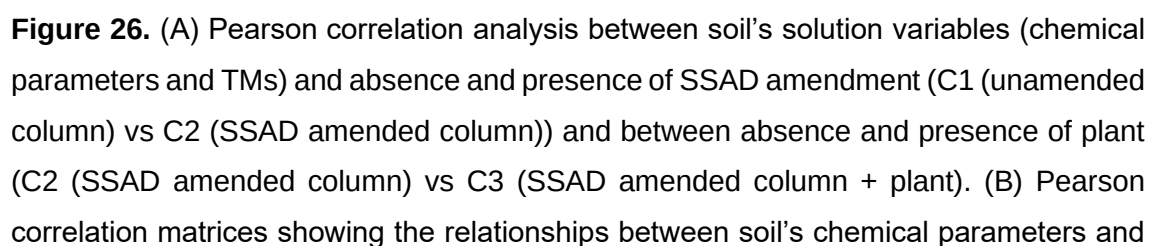
4.3.C.1 Dynamics over time in soil solution

Figure 26a presents a Pearson's correlation analysis between soil's solution variables (chemical parameters and TMs) and absence and presence of SSAD amendment (C1 (unamended column) vs C2 (SSAD amended column)) and between absence and presence of plant (C2 (SSAD amended column) vs C3 (SSAD amended column + plant)) over time and depth. Figure 26b depicts the relationships between soil's chemical parameters and TMs contents in soil solution over time and depth within each column over time and depth.

According to the results, the presence of SSAD amendment was significantly positive correlated with the concentrations in soil solution over time and depth of As, Mn, Fe, Co, Ni and Cu ($r = 0.47, p < 0.05$; $r = 0.49, p < 0.01$; $r = 0.37, p < 0.05$; $r = 0.47, p < 0.05$; $r = 0.53, p < 0.01$; $r = 0.47, p < 0.05$), while it was negatively correlated with Cr ($r = -0.42, p < 0.05$). The presence of SSAD amendment was also significantly positively correlated with DOC ($r = 0.50, p < 0.01$), proteins ($r = 0.60, p < 0.01$), humic substances ($r = 0.62, p < 0.01$), sugars ($r = 0.62, p < 0.01$), uronic acids ($r = 0.62, p < 0.01$), Na^+ ($r = 0.77, p < 0.01$), NH_4^+ ($r = 0.60, p < 0.05$), K^+ ($r = 0.70, p < 0.01$), Ca^{2+} ($r = 0.67, p < 0.05$), Mg^{2+} ($r = 0.75, p < 0.01$), F^- ($r = 0.75, p < 0.01$), Cl^- ($r = 0.80, p < 0.01$), NO_2^- ($r = 0.41, p < 0.05$), NO_3^- ($r = 0.59, p < 0.01$), PO_4^{3-} ($r = 0.45, p < 0.05$). Plant presence was positively correlated with concentrations in soil solution over time and depth of Mn ($r = 0.13, p < 0.01$), Fe ($r = 0.30, p < 0.01$), Co ($r = 0.14, p < 0.05$), and Zn ($r = 0.15, p < 0.05$), DOC ($r = 0.29, p < 0.05$), proteins ($r = 0.59, p < 0.05$), humic substances ($r = 0.30, p < 0.05$), sugars ($r = 0.30, p < 0.05$) and uronic acids ($r = 0.19, p < 0.05$). Negatively correlated with Pb ($r = -0.14, p < 0.05$), pH ($r = -0.33, p < 0.05$), and Eh (Redox) ($r = -0.33, p < 0.05$), Ca^{2+} ($r = -0.16, p < 0.05$), NO_2^- ($r = -0.33, p < 0.05$), NO_3^- ($r = -0.32, p < 0.05$).

Regarding correlations between soil's chemical parameters and TMs contents in soil solution within individual columns (Figure 26b), only strong (r value > 0.4) and significant correlations ($p \leq 0.05$) will be described in the text. In C1 As, Cr, Mn, Fe, Co, Ni, Cu, Zn, As and Pb concentrations in soil solution were mainly influenced by pH and DOC ($p \leq$

0.05). In C2, DOC was positively correlated with Mn ($r = 0.84$, $p < 0.01$), Fe ($r = 0.75$, $p < 0.01$), Co ($r = 0.89$, $p < 0.01$), Ni ($r = 0.73$, $p < 0.01$) and As ($r = 0.86$, $p < 0.01$), while negatively correlated with Cr ($r = -0.60$, $p < 0.01$). Proteins, humic substances, sugars and uronic acids followed the same trend. Na^+ , NH_4^+ , K^+ and Mg^{2+} were positively correlated with Mn, Fe, Co, Ni and As ($p \leq 0.05$) while negatively correlated with Cr ($p < 0.05$). pH was positively correlated with Al ($r = 0.41$, $p < 0.05$), Mn ($r = 0.42$, $p < 0.05$), and As ($r = 0.58$, $p < 0.05$). NO_2^- was positively correlated with Fe ($r = 0.42$, $p < 0.05$). NO_3^- was negatively correlated with Mn, Fe, Co, Ni, Zn and As ($r = \sim 0.4 - 0.5$, $p \leq 0.05$). Cl^- was positively correlated with Mn ($r = 0.57$, $p < 0.05$), Co ($r = 0.60$, $p < 0.05$), Ni ($r = 0.68$, $p < 0.05$), As ($r = 0.44$, $p < 0.05$), Pb ($r = 0.43$, $p < 0.05$). PO_4^{3-} was positively correlated with Fe ($r = 0.58$, $p < 0.05$) and negatively correlated with Cu ($r = -0.44$, $p < 0.05$). In C3 DOC was positively correlated with Mn ($r = 0.89$, $p < 0.01$), Fe ($r = 0.72$, $p < 0.01$), Co ($r = 0.75$, $p < 0.01$), and As ($r = 0.77$, $p < 0.01$), while negatively correlated with Cr ($r = -0.67$, $p < 0.01$) and Cu ($r = -0.43$, $p < 0.01$). Proteins, humic substances, sugars, and uronic acids followed the same rule. pH was negatively correlated with Fe ($r = -0.53$, $p < 0.05$). Eh potential (Redox) correlations with TMs in soil solution followed the same trend as pH. NH_4^+ was negatively correlated with Cr ($r = -0.52$, $p < 0.05$) while positively correlated with Co ($r = 0.73$, $p < 0.05$), Ni ($r = 0.90$, $p < 0.05$) and As ($r = 0.58$, $p < 0.05$). PO_4^{3-} was negatively correlated with Cu ($r = -0.48$, $p < 0.01$), and positively correlated with Mn ($r = 0.68$, $p < 0.01$) and Fe ($r = 0.76$, $p < 0.01$).

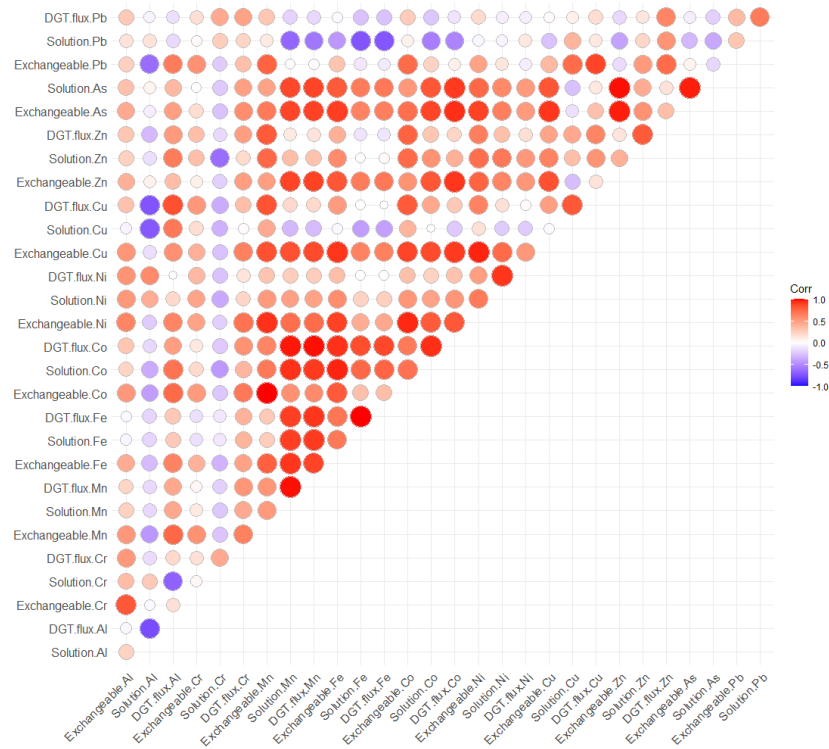


TMs contents in soil solution over time and depth within each column over time and depth.

4.3.C.2 Bioavailability measures at day 45.

Figure 27A shows a Pearson's correlation analysis between (i) concentration in soil solution, (ii) DGT flux and (iii) concentration in BCR exchangeable fraction of Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, Pb and As (except for DGT flux of As) at day 45 in C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD-amended column + plant). By day 45, DGT fluxes of Al, Cr, Mn, Fe, Co, Ni, Cu, Zn and Pb were significantly positive correlated ($p \leq 0.05$) to their concentrations in soil solution and exchangeable fraction. However, correlation coefficients were stronger for soil solution concentrations. In case of Mn, Fe, Co and Ni Pearson correlation coefficient between DGT fluxes and soil solution concentration were $r > 0.90$ ($p \leq 0.05$). Figure 27B shows a Pearson's correlation analysis between (i) the "bioavailability measures" (TMs DGT fluxes, TMs concentration in soil solution and TMs concentration in exchangeable fraction) in the top layer of C3 and C1 and (ii) the TMs concentration in plants tissues (leaves and roots) harvested from C3 and the control unamended pot on day 45. DGT fluxes showed robust positive correlations with the concentrations of all TMs in both the roots and leaves of *P. virgatum* ($r = 0.95-0.99$, $p \leq 0.05$). Additionally, concentrations of all TMs in the soil solution also demonstrated a positive correlation with their respective concentrations in plant tissues ($r > 0.70$, $p \leq 0.05$), especially Mn, Fe, Co, and Ni ($r > 0.90$, $p \leq 0.05$).

A



B

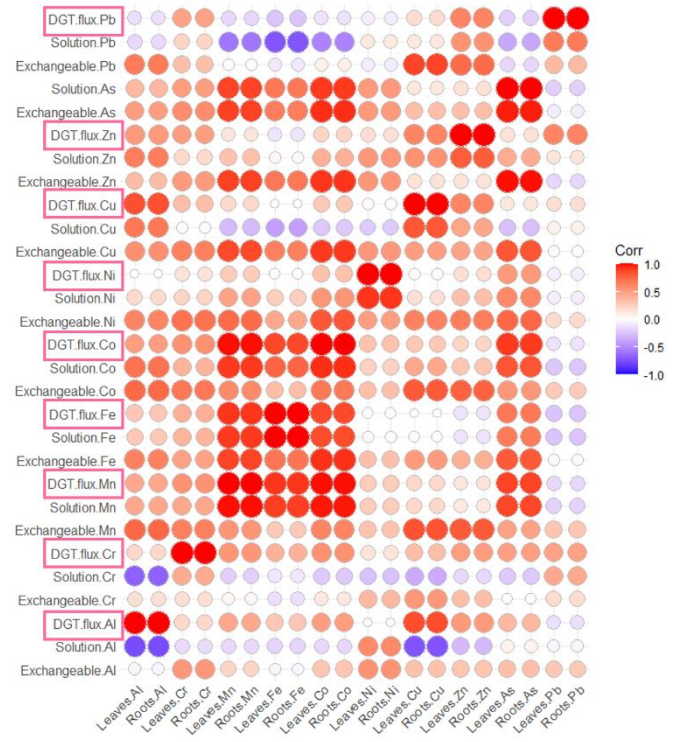


Figure 27. A) Pearson correlation analysis between DGT fluxes of TMs, TMs concentration in soil solution, and TMs concentration in exchangeable fraction at day 45. B) Pearson's correlation analysis between (i) the "bioavailability measures" (TMs DGT fluxes, TMs concentration in soil solution and TMs concentration in exchangeable fraction) in the top layer of C3 and C1 and (ii) the TMs concentration in plants tissues (leaves and roots) harvested from C3 and the control unamended pot at day 45. The colours represent the correlation coefficient, increasing from blue (negative) to red (positive).

4.4 Discussion

4.4.A Evolution of soil solution properties over time

4.4.A.1 Effect of SSAD amendment

Overall, SSAD amendment was positively correlated with As, Mn, Fe, Co, Ni and Cu concentrations in soil solution. SSAD amendment induced a high initial pulse of soil solution levels of Cr in bottom layer, Mn, Fe, Co, Ni and As in top and middle layer, and Cu in middle layer. Mn and Fe showed the highest concentrations in soil solution. No influence of SSAD amended was observed for Al, Zn and Pb concentrations in soil solution. The lack of effect of SSAD amendment on Al, Zn and Pb mobility could be attributed to their retention on soil particles. The results also suggest a downward migration of most TMs through the soil profile, which stabilized over time. However, no significant concentrations of TMs were observed in the leachate, except for Cu. The presence of Cu in the leachate could be explained by its well-known capacity to form mobile complexes with DOM (Alloway, 1995; Huang et al., 2018; Karlsson et al., 2006) or by the presence of other TMs and cations in the soil solution, competing with Cu for sorption sites in soil. In fact, SSAD amendment rose DOC concentrations in soil solution. The increase of DOC concentration due to the application of digestate-like products has been widely reported (Albuquerque et al., 2012; Cardelli et al., 2018; Cattin et al., 2021; García-Sánchez et al., 2016; Monard et al., 2020). The behaviour of DOC concentration in soil solution over time and depth showed a strong positive correlation with Mn, Fe, Co, Ni and As in soil solution, suggesting the formation of DOM-metal complexes (Weng et al., 2002). DOM showed relatively high percentages of proteins and humic substances. Several studies reported that humic-like and protein-like substances in DOM are associated with metal binding (Tipping, 2002; Ren et al., 2015; Weng et al., 2002). Conversely, a robust negative correlation was identified between DOC and Cr. The presence of DOM could enhance the sorption or complexation of Cr in soil's solid phase, resulting in lower concentrations in soil solution. However, there was not significant correlation between Cu and DOC, suggesting that the interaction between Cu and DOM could be complex.

SSAD amendment also induced an increase of anions and cations concentrations in soil solution. Möller & Müller (2012) reported high presence of anions and cations in digestate. Panuccio et al. (2021) reported an increase in Mg^{2+} and K^+ in digestate-treated soils. Correlations analysis suggest that the behavior of cations (Na^+ , NH_4^+ , K^+ , Mg^{2+}) and anions (NO_2^- , NO_3^- , Cl^- , PO_4^{3-}) concentrations in soil solution are correlated with most TMs' concentrations in soil solution. It is known that soil's solution ionic composition

changes can affect adsorption–desorption processes of TMs in soil and TMs concentration in soil solution. The presence of Na^+ , NH_4^+ , K^+ and Mg^{2+} could be blocking adsorption sites in soil, thus increasing TMs presence in soil solution (Strawn, 2021). Moreover, fractionation results showed that Mn, Fe and Co in SSAD amended soil were present in exchangeable fraction. These elements that can be released by ion-exchange processes (Filgueiras et al., 2002). Changes in the ionic composition induced by the SSAD amendment, could be affecting adsorption–desorption reactions and cause remobilization of Mn, Fe and Co from this fraction (Sutherland & Tack, 2000; Violante et al., 2010).

In the absence of *P. virgatum*, the initial increase of TMs concentration in soil solution induced by SSAD was not sustained over time, this could be explained by different processes (i) ion exchange processes (CEC levels were high due to SSAD amendment). (ii) precipitation and complex formation with inorganic or organic ligands into less soluble forms and (iii) sorption onto soil particles (the studied soil contained a high clay content) or organic matter (observed high content of SOC) (Vangronsveld et al., 2009). For example, the results from sequential extraction fractionation analysis revealed a higher percentage of Cu in the oxidizable fraction (bound to organic matter) within both the top (0-0.1 m) and middle (0.1-0.2 m) layers of C2 and C3, which aligns with a higher concentration of SOC. This could suggest the complexation of Cu from soil solution with the SOM (N. Bolan et al., 2003; Gondar & Bernal, 2009; Gustafsson et al., 2014). Other possible processes are: (iv) Microbial communities contributing to metal immobilization (H. Han et al., 2020; Xing et al., 2022). (v) Also, at > 300 mV Eh potential certain TMs can become less soluble as they undergo reduction to lower oxidation states that are less soluble or even precipitate as solid compounds (Alloway, 1995).

4.4.A.2 Effect of *P. virgatum* in SSAD- amended soil

The further increase of Mn and Fe concentrations in soil solution in presence of *P. virgatum* could be explained by (i) the observed increase of DOC in presence of *P. virgatum* (explained by rhizodeposition and/or destruction of aggregates by plant roots (Dijkstra et al., 2021; Huo et al., 2017). Thereby promoting the formation of soluble DOM-TMs complexes (Sauvé et al., 2000). (ii) The formation of complexes with root exudates (citric, oxalic, and tartaric acids) and/or (iii) the observed decrease in pH in presence of *P. virgatum* (which could be attributed to root exudation, oxygen release by roots, release of protons by roots, microbial activity in the rhizosphere, etc.). Moreover, Fe and Mn are known to be more bioavailable to the plant in reduced forms, a condition that aligns with the reduced redox conditions observed in the presence of *P. virgatum*.

Overall, Fe and manganese Mn are crucial micronutrients essential for sustaining various metabolic roles in plant growth and development (Alejandro et al., 2020; Murgia et al., 2022). Consequently, the plant may employ diverse strategies for their uptake.

4.4.B Bioavailability assessment on day 45: Determining the optimal measure of bioavailability.

While evaluating soil solution TM levels is generally deemed a suitable indicator of the plant-available metal pool in soil (Nolan et al., 2005), these assessments might not entirely reflect the soil's ability to sustain these solution concentrations. Furthermore, TMs in soil solution may exist in “non-bioavailable” forms, specifically when bound to either inorganic or organic ligands, thereby forming non-labile complexes (Lombi et al., 2003). As a result, relying solely on measuring total concentrations in soil solution may not accurately estimate the true bioavailability of TMs. Therefore, a multiapproach estimation of TMs bioavailability was applied at day 45 through the assessment and comparison of DGT fluxes, concentrations in soil solution and concentrations in exchangeable fraction. In this study, all the three measurements (TMs concentration in exchangeable fraction, TMs concentration in soil solution and DGT-measured fluxes) were positively correlated to each other, however higher correlation coefficients were observed between DGT fluxes and soil solution concentration.

According to DGT analysis, at day 45, in C3 (SSAD amended column + plant), Mn, Fe, Al, Zn, and Co exhibited the highest lability (As was not measured by DGT). Soil solution analysis indicated that Fe, Mn, As, Co, Al, and Zn were the most potentially bioavailable trace metals. Concentrations in the exchangeable fraction, determined through sequential extraction, highlighted Fe, Al, Mn, and Zn as the most potentially bioavailable trace metals, with As and Co showing low levels. All methods agreed in the higher potential bioavailability of Fe, Mn followed by Al and Zn, while soil solution also indicated As as potentially bioavailable. The disparity in the case of As, Co could be explained by speciation changes during sampling and extraction during the BCR procedure.

Concentrations of Fe, Al, Mn, As and Zn presented the highest increments in both roots and leaves of *P. virgatum* by day 45. Also, *P. virgatum* showed accumulation of Mn and Zn (BCF and TF > 1), which could be explained by the essential nature of Mn and Zn (Alejandro et al., 2020; Haydon & Cobbett, 2007; Viehweger, 2014). The obtained findings indicated that the assessed lability of Fe, Al, Mn, and Zn using the three methods aligned with elevated concentrations in *P. virgatum* tissues, including As, which was determined by sequential extraction and concentrations in soil solution but not by DGT.

However, this was not the case for Co, which was estimated to be bioavailable by soil solution analysis and DGT, but not by sequential extraction. Besides, DGT measurements strongly positively correlated with all TMs concentrations in *P. virgatum* roots and leaves. While concentrations of Fe, Mn, Co and Ni in *P. virgatum*'s roots and leaves were also positively correlated with their respective concentrations in soil solution. Several studies have reported significant positive correlations between concentrations in plant tissues with DGT measurements (Bai et al., 2023; L. Han et al., 2019; P. Ma et al., 2022; Tandy et al., 2011) and concentrations in exchangeable fraction (Fernández-Ondoño et al., 2017; Setia et al., 2021) while fewer studies perform comparisons with concentrations in soil solution (H. Zhang et al., 2001). The findings of this study suggest that DGT is an optimal method for the simultaneous determination of bioavailability of various TMs in soil amended with SSAD. Furthermore, soil solution concentration may also serve as a potential predictive indicator for the bioavailability of TMs, especially for Fe, Mn, Co, and Ni to plants under the studied conditions.

4.5 Conclusions

The study reveals a positive correlation between SSAD amendment and concentrations of As, Mn, Fe, Co, Ni, and Cu in soil solution. SSAD amendment induced an increase in DOC concentration and anions and cations concentrations, potentially influencing TMs behavior in soil solution. The presence of *P. virgatum* further increased Mn and Fe concentrations in soil solution, potentially associated with an increased DOC and a decrease in Eh and pH. A multi-approach estimation of bioavailability, particularly DGT fluxes and concentrations in soil solution, at the end of the experiment (day 45) indicated Mn, Fe, Co, Al, and Zn as the most available TMs, translating into higher concentrations in *P. virgatum* tissues. Moreover, a positive correlation was observed between all TMs DGT fluxes and concentrations in soil solution with their concentrations in *P. virgatum* tissues. The study underscores the relevance of DGT fluxes and soil solution concentrations in estimating the plant-available metal pool within the studied.

Authors contribution

NB-G: conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing—original draft. VR: conceptualization, investigation, methodology, data curation, supervision, validation, visualization, and writing—review and editing. IB: investigation, methodology, data curation, validation, visualization, and writing—review and editing. RB: methodology, data curation, validation, visualization, and writing—review and editing. FB: methodology, validation, visualization, and writing—

review. GG: conceptualization, funding acquisition. MA: conceptualization, funding acquisition, supervision, writing–review and editing. AM: conceptualization, funding acquisition, supervision.

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5. Chapter 5: Evolution of OM characteristics and Cu(II)-OM complexation strength in digestate amended soil-plant mesocosms

This chapter is based on the manuscript prepared for submission:

Neus Bonet-Garcia, Isabelle Bourven, Valentin Robin, Sylvain Bascle, Ana Paula Mucha, C. Marisa R. Almeida, Gilles Guibaud. Evolution of OM characteristics and Cu(II)-OM complexation strength in digestate amended soil-plant mesocosms.

Abstract

To comprehend the impact of organic matter (OM) on the behaviour of trace metals (TMs) in soil amended with digestate and managed by bioenergy crops, the nature and reactivity of "bound" OM of a marginal soil amended with sewage sludge anaerobic digestate (SSAD) and phytomanaged by *P. virgatum* was investigated. "Bound" OM was obtained by extraction with the Cation Exchange Resin (CER) method. The investigation used both colorimetric methods and fluorescence excitation emission matrix (EEM) methodology, focusing on two fluorescence indexes, HIX (humification index) and r(A,C) (aging index). The reactivity of the bound OM towards Cu (II), a common element of interest in soil management practices, was assessed using EEM coupled with fluorescence titration quenching. Results revealed that SSAD addition changed soil OM quantitatively but not qualitatively. SSAD increased the concentration of soil organic carbon (SOC), primarily in the form of proteins (tyrosine and tryptophan-like protein) and soluble microbial-like products. The fresh OM introduced by SSAD exhibits higher binding capacities (measured through the quenching binding constant) in comparison to the mature soil-borne OM. The incorporation of *P. virgatum* into the SSAD-amended soil yielded three primary effects: (i) a reduction in the migration of OM through the soil profile; (ii) an augmentation of tyrosine-like proteins content in the topsoil; and (iii) an enhancement in the binding capacity of these proteins in the topsoil, likely influenced by the lower humification and aging indexes of the OM introduced by the roots. These findings underscore the potential significance of tyrosine-like proteins in the complexation dynamics with Cu(II) within the studied conditions and underline the synergy between the molecular context (degree of fresh OM) and the complexation capacity of the studied fluorophores.

5.1 Introduction

Digestate, a by-product of the anaerobic digestion process, poses challenges in management and disposal (Cesaro, 2021; Lamolinara et al., 2022). Applying digestate as a soil amendment presents an environmentally beneficial solution, reintroducing essential organic matter (OM) and macronutrients into the soil, countering global soil degradation (FAO, 2019). This approach is crucial for maintaining adequate soil OM content, supporting soil ecosystem functions such as carbon sequestration, nitrogen mineralization, soil aggregation, plant health promotion, and compound retention (Alburquerque et al., 2012; Leno et al., 2021; Möller & Müller, 2012; Nkoa, 2014; Panuccio et al., 2021; Slepetiene et al., 2020; Tambone et al., 2009).

However, using digestate as a soil amendment may introduce contaminants, including trace metals (TMs), into the soil (Foster & Prasad, 2021; Malhotra et al., 2022; Saveyn & Eder, 2014), potentially leading to physical and chemical transformations affecting TM speciation and mobility (Ashworth & Alloway, 2008; Dragicevic et al., 2017; Fang et al., 2016; Pivato et al., 2016). The management of digestate-amended soils through green strategies, such as phytoremediation, is currently gaining interest. Phytomanagement strategies aim to address elevated concentrations of TMs in digestate-amended soil through two primary approaches: phytoextraction and phytostabilization. Phytoextraction targets the removal of excessive TM concentrations by utilizing plants to uptake and accumulate these TMs. On the other hand, phytostabilization involves the containment of contaminants within the vadose zone. This is achieved through mechanisms such as the accumulation of TMs by plant roots or precipitation within the rhizosphere. By doing so, the strategy aims to prevent off-site contamination resulting from the migration of TMs (Awa & Hadibarata, 2020; Bhat et al., 2022; Bolan et al., 2011; Tangahu et al., 2011; Wei et al., 2021).

Our previous research, using *P. virgatum* for the phytomanagement of a marginal soil amended with sewage sludge anaerobic digestate (SSAD) (Chapter 4), indicated that SSAD amendment and plant presence influenced the availability and mobility of TMs in soil, with OM increase being a potential key factor. During the process of phytomanagement of a marginal soil amended with digestate, OM is introduced into the soil environment in various forms. OM of digestate streams is typically composed by microbial biomass, extracellular polymeric substances (EPS, a complex mixture of macromolecules located outside cell surfaces) (Bourven et al., 2011; Ramesh et al., 2006), soluble microbial products (soluble compounds released during the growth/decay of microorganisms) (Barker & Stuckey, 1999), and recalcitrant exogenous compounds.

After digestate-borne OM enters soils, it can form metal organic complexes due to the abundant carboxyl, hydroxyl, phenolic di, amine and other active functional groups on OM surface which have high affinity for metal ions (M. Huang et al., 2018). Plant roots serve as an additional source of OM, as they release labile organic compounds into the soil through rhizodeposition, which includes root exudates (mostly low-molecular-weight organic compounds, including amino acids, organic acids, phenolics and sugars) and sloughed-off root cells (Jones et al., 2009; Pausch & Kuzyakov, 2018). Low-molecular-weight organic compounds released by plant's roots have been reported to be reactive with metals (Y. Chen et al., 2003; Koo et al., 2010; Luo et al., 2008; Montiel-Rozas et al., 2016). Moreover, this pool of organic compounds incorporated to the soil can function as an energy source for microorganisms, thereby boosting microbial biomass and activity. Microorganisms, in turn, can influence TM behaviour by, for example, secreting EPS, which play a significant role in chelating metal cations (Gupta & Diwan, 2017; Yin et al., 2019; Zeng et al., 2020).

Overall, these complexation reactions of metals-OM will have an influence on metal mobility, speciation and bioavailability (Ashworth & Alloway, 2008; Harter & Naidu, 1995; Sauvé et al., 1997). While numerous studies emphasize the role of the digestate-borne dissolved OM (DOM) in metal mobility (X. Guo et al., 2012, 2020; X.-J. Guo et al., 2019; He et al., 2014; C. Huang & Wang, 2014; M. Huang et al., 2018; Y. Huang et al., 2020; Zhu et al., 2021), the present research focuses on the concurrent effects of the OM introduced by both digestate and plants on TM complexation. Moreover, the study centers on the bound OM, providing insights into a more persistent fraction of OM that will ultimately influence soil processes and metal interactions over time. Specifically, this work aims to investigate the influence of SSAD amendment and the presence of *P. virgatum* on the nature of "bound" OM in soil and its reactivity with Cu(II). Indeed, Cu(II) was selected based on our prior findings. Results suggested that, in a soil amended with SSAD and phytomanaged by *P. virgatum*, the interactions of Cu(II) in the soil solution with soil OM were complex. Also, the percentage of Cu(II) in the oxidizable fraction (bound to OM) increased with SSAD amendment and *P. virgatum* presence (Chapter 4 and Figure S1 in Appendix III). This was more pronounced in the presence of *P. virgatum*. This suggests that bound OM could play a role in reducing the potential mobility of Cu(II) under the studied conditions. Results are presented in Appendix IV (Figure S1).

These findings prompt the following questions: (i) What is the reactivity of the bound OM towards Cu(II) under the studied conditions? (ii) Is the increased percentage of Cu(II) in the oxidizable fraction, observed in the presence of *P. virgatum* influenced by the

increased OM induced by the plant? We hypothesized (i) quantitative and qualitative changes in soil OM induced by SSAD amendment and accentuated by plant presence, and (ii) high reactivity of SSAD- and plant- borne OM reactivity towards Cu(II).

5.2 Material and methods

5.2.A Soil column mesocosm experiments

Soil and SSAD used in this study are described in Chapter 4. Vertical soil column mesocosm experiments were performed. Two treatment conditions were evaluated in separated columns: C2 (soil + SSAD) and C3 (soil + SSAD + *P. virgatum*), described in Chapter 4 and Figure 28. Solid samples were collected at the beginning of the experiment (initial samples: soil and mixture SSAD + soil) and at the end of the experiment (final samples: top, middle, and bottom layer of C2 and C3). Samples were stored in polyethylene tubes at 4°C, for subsequent analysis.

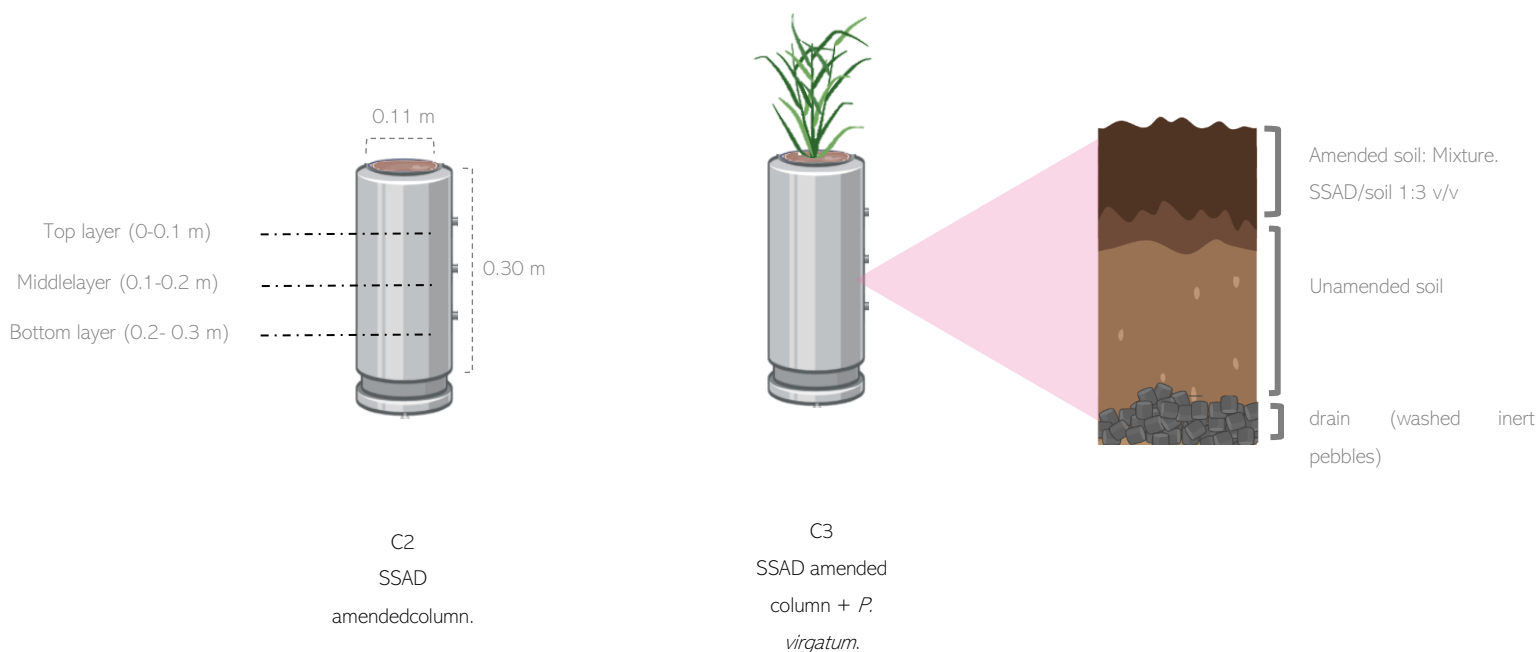


Figure 28. Schematic representation of vertical soil column mesocosm experiments. Where SSAD: sewage sludge anaerobic digestate. Figure created with Biorender.com. License agreement number EF26A4MNI.

5.2.A.1 Sample collection and organic matter extraction

Solid samples were collected at the beginning of the experiment (initial samples: soil and mixture SSAD + soil) and at the end of the experiment (final samples: top, middle, and bottom layer of C2 and C3). Samples were stored in polyethylene tubes at 4°C, for subsequent analysis.

The extraction procedure was based on cation exchange using a cation exchange resin (CER). This CER method is actually specific for OM extraction from biofilm in biological sludge (Frolund et al., 1996) or soil (Redmile-Gordon et al., 2014). The amount of CER required was calculated according to volatile solid (VS) content in soil samples (Frolund et al., 1996; Liu and Fang, 2002; Redmile-Gordon et al., 2014). VS content was measured following the loss on ignition method (LOI), previously dried samples (105 °C for 24 h) were weighted before and after ignition in an oven at 550 °C for 2 h (Nelson and Sommers, 1996). For the extraction, 70 g of CER (Sigma Aldrich, DOWEX Marathon, sodium form, 20-50 mesh; Sigma Aldrich, Midland, MI) per gram of VS were added to 40 mL of buffer solution (2 mM Na₃PO₄ + 4 mM NaH₂PO₄ + 9 mM NaCl + 1 mM KCl adjusted to pH 7.0 with HCl 2 M) in 50 mL polypropylene centrifuge tubes, capped, and cooled to 4 °C over night. Next, soil samples were transferred to the CER-containing 50 mL tubes. Agitated at 600 tr/min for 1 h at 4°C in an orbital shaker and centrifuged twice at 20,000 g for 20 min at 4°C. Supernatants were collected, transferred to new 50 mL polypropylene centrifuge tubes, and stored at -20 °C until analysis.

5.2.B Organic carbon determination and colorimetric assays for organic matter fractionation

Organic carbon content was quantified in triplicate by a Shimadzu® TOC-L TOC (Japan) with a limit of quantification (LOQ) of 0.5 mC·L⁻¹. Humic-like substances (HS), proteins (PN), polysaccharides (S), and uronic acids (UA) contents were measured by applying the colorimetric method described in D'Abzac et al. (2010) (Table 1). Specifically, the HA content and the PN content of the extracted OM were determined using the Frølund et al. (1995) protocols. The UA and S contents were corrected using the Dubois et al. (1956) and Blumenkrantz and Asboe Hansen (1973) protocols.

Table 1. Summary of parameters used in colorimetric assays.

Extract content	Calibration curve concentration (mg L ⁻¹)	Wavelength (nm)	Reagent used	Standard
Proteins	40–200	650	Folin's reagent Copper sulfate 0.5% (w/w)	Bovine albumin serum (96%, Sigma Aldrich)
Humic compounds	40–200	650	Folin's reagent	Humic acids (Sigma Aldrich)
Polysaccharides	20–100	492	Sulfuric acid 95% Phenol 5% (w/w)	Glucose (pure Recaptur Prolabo)
Uronic acids	50–500	520	Sodium tetraborate 12.5 mM Sulfuric acid 95%	Glucuronic acid (99%, Aldrich)

5.2.C Three-dimensional (3D) Excitation Emission Matrix (EEM) fluorescence spectroscopy analysis and HIX and R(A,C) indexes determination

5.2.C.1 Fluorescence EEM analysis

Fluorescence emission-excitation matrices (EEMs) were acquired on the CER-extracted OM using a spectrofluorometer (RF 5301, SHIMADZU, Japan) at 20 ± 1 °C, with a 5-nm excitation wavelength increment scan within an excitation range of 220–450 nm and an emission range of 230–550 nm with a 1 -nm increment. The excitation source used was a 150-W Xenon lamp. The scanning speed was set on fast with high sensitivity. The fluorescence data were processed using the software Panorama Fluorescence (LabCognition, Japan). Samples were diluted (1:20) in phosphate buffer 50 mM (25 mM NaH₂PO₄ + 25 mM Na₂HPO₄) at pH 6.5 (± 0.1). Origin software (OriginLab Corporation) was used to build the figures.

5.2.C.2 Humification index (HIX) and aging index (r(A,C))

The value for HIX was estimated from the EEM data at 254 nm excitation as the area under the emission spectra in the range from 435 to 480 nm, divided by the peak area at 300–345 nm (Ohno et al., 2002) using the R package *eemR* (v. 1.0.1).

The EEM data was also used to derive the aging index ($r(A,C)$), representing the ratio between the peak fluorescence intensities of fluorophores type A (fulvic-region III (§ 5.3.A. 2.2.)) and type C (humic-region V (§ 5.3.A. 2.2.)) (IA/IC), (Mounier et al., 2005).

5.2.D Complexation between Cu (II) and organic matter

5.2.D.1 Fluorescence quenching experiment

Before titration, samples were first diluted (1:20) in phosphate buffer 50 mM (25 mM NaH_2PO_4 + 25 mM Na_2HPO_4) at pH 6.5 (± 0.1). Titration experiments were conducted in triplicate, by adding 50 μL of a series of $CuCl_2$ solutions to each of the samples to generate a series of samples containing Cu(II) concentrations of 0, 20, 50, 80 and 100 $\mu mol/L$. 0 $\mu mol/L$ represented the initial fluorescence intensity of the samples between 550 and 800 (A.U.).

After each addition of Cu (II), the solution was mixed using a magnetic stirrer for 15 min at room temperature to ensure equilibration of the complexation reaction prior to fluorescence measurement. EEMs were obtained following the procedure described above.

5.2.D.2 Processing quenching data

To obtain the quenching rate constants, the fluorescence quenching data, were fitted by the Stern–Volmer equation (Eq. 1):

$$\frac{F_0}{F} = 1 + K_{sv} \cdot [Q]$$

where F_0 and F are fluorescence intensities in the absence and presence of the metal, respectively. K_{sv} represents the Stern–Volmer constant, and $[Q]$ the concentration of the quencher, which is Cu(II). The fluorescence intensity, without adding copper solution does not precisely signify the absence of Cu (II) since it was present in the samples (Figure S1, Appendix IV), F_0 fluorescence intensity, as well as K_{sv} were determined using the Statistica software (version 8.0) nonlinear model, least squares method $p < 0,05$ with the equivalent equation 1 (Eq. 2):

$$F = F_0 / (1 + K_{sv} \cdot [Q])$$

The higher affinity of OM for Cu (II) will be linked to the higher constant value of K_{sv} .

5.2.E Statistical analysis

Significance of the differences among and between C2 and C3 samples were evaluated through a parametric one-way analysis of variance (ANOVA). Significant differences ($p < 0.05$) were detected by a Tukey's Honestly Significant Difference (HSD) post hoc test.

Pearson correlation test was performed to determine the correlation between the evolution of binding capacity of the identified fluorophores and the degree of humification and maturity of the OM over soil depth, using R-package *corr* (version 0.4.4).

5.3 Results and discussion

5.3.A Study of the evolution of the CER-extracted OM along the columns at times 0 and 45 days

5.3.A.1 Concentration of organic carbon, proteins, humic substances, sugars, and uronic acids in CER-extracted OM at day 0 and day 45

Results are presented in Table 2, Table 3, and Appendix IV (Figure S2). Initial SSAD amended soil represents top layer (0-0.1 m) of C2 and C3 at day 0, while unamended soil represents middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers of C2 and C3 at day 0.

5.3.A.1.1 Day 0

The SSAD amended soil presents approximately a 5-fold increase ($p < 0.05$) in SOC (from $0.6 \pm 0.1 \text{ mg g}^{-1}$ dry weight to $2.6 \pm 0.1 \text{ mg g}^{-1}$ dry weight). The increase in SOC is mainly supported by the increase in protein (PN) intake, which showed a 6-fold increase ($p < 0.05$) (from $0.2 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight to $0.7 \pm 0.1 \text{ mg g}^{-1}$ dry weight) compared to a 3-fold increase for humic substances (HS), polysaccharides (S), and uronic acids (UA). Therefore, it can be concluded the primary contribution of CER-extracted OM through the SSAD amendment is predominantly in the form of proteins.

5.3.A.1.2 Day 45

By day 45, in the top layer (0-0.1 m) of C2, the concentration of SOC ($3.2 \pm 0.2 \text{ mg g}^{-1}$ dry weight) showed a 1.3 -fold increase ($p < 0.05$) when compared to the initial SSAD amended soil (above described). This increase in SOC concentration was mainly

provoked by PN ($1.3 \pm 0.3 \text{ mg g}^{-1}$ dry weight) that presented a 2-fold increase ($p < 0.05$) when compared to the initial SSAD amended soil (above described). At the same time, in the presence of plants (C3) the top layer's (0-0.1 m) concentrations of SOC ($4.2 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight) and PN ($1.7 \pm 0.1 \text{ mg g}^{-1}$ dry weight) were significantly higher ($p < 0.05$) than in C2. Plant presence (C3) had also induced higher concentrations of HS, S and UA ($1.3 \pm 0.4 \text{ mg} \cdot \text{g}^{-1}$ dry weight, $0.7 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight and $0.6 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight, respectively). The rise in SOC and PN concentration over time may be attributed to an increased microbial biomass, soluble microbial products and EPS induced by the SSAD amendment (Muscolo et al., 2017; van Midden et al., 2023), plus microbial necromass becoming part of the soil organic matter pool (Miltner, Bombach, Schmidt-Brucken, & Kastner, 2012). Moreover, this could also indicate a higher retention of OM in soil over time, which could become part of the persistent fraction of OM in soil. The further increase of PN, HS, S and UA in presence of plants could mean that (i) the plant induces a higher retention of OM in soil, for example by increasing soil aggregation (Rillig et al., 2015; Poirier et al., 2018; Tisdall et al., 2020; Xiao et al., 2020); (ii) a rise of OM due to rhizodeposition (Nguyen et al., 2003; Uren et al., 2001) and/or (iii) an increase of microbial communities' abundance induced by the plant and thus soluble microbial products and EPS (Pang et al., 2022). Overall, the observation that, by day 45, (i) the SOC content in the top layer of C3 (Appendix IV Figure S2) is nearly 1.5 as large as that in C2, and 2) the modification of the ration fraction where Cu(II) is present in the solid phase (Appendix IV Figure S1) in C3 compared to C2, suggested that the presence of the plant (C3) contributed to a higher presence of OM and to a modification of the OM compared to the amended soil in absence of the plant (C2). This could result in both (i) the OM being less readily solubilized in the soil solution and/or (ii) being retained by the top layer in the column with the plant (C3).

The middle layer (0.1- 0.2 m) of both C2 and C3, which on day 0 consisted in unamended soil, showed a 3-fold increase ($p < 0.05$) in SOC concentration ($1.7 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight and $1.9 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight, respectively) when compared to the initial unamended soil (above described). Concentrations of PN ($0.6 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight and $0.7 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight in C2 and C3, respectively) and HS ($0.5 \pm 0.2 \text{ mg} \cdot \text{g}^{-1}$ dry weight and $0.6 \pm 0.2 \text{ mg} \cdot \text{g}^{-1}$ dry weight in C2 and C3, respectively) showed a 5-fold and 4-fold increase ($p < 0.05$) when compared to the initial unamended soil (above described). Concentrations of both S ($0.3 \pm 0.2 \text{ mg} \cdot \text{g}^{-1}$ dry weight and $0.4 \pm 0.1 \text{ mg g}^{-1}$ dry weight in C2 and C3, respectively) and UA ($0.3 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight and $0.3 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$ dry weight in C2 and C3, respectively) showed a 2-fold increase when compared

to the initial unamended soil. These results suggest a migration of OM from the top layer (0 - 0.1 m) to the middle layer (0.1 – 0.2 m), mainly composed of PN. Moreover, these findings imply that the C3 column exhibits a proportionally lower transfer of OM, especially PN, from the top level to the middle level compared to C2. Specifically, the ratio of SOC between the top (0 - 0.1 m) and middle (0.1 – 0.2 m) layers is 2 for C2 and 2.2 for C3, while the ratio of PN contents is 2 for C2 and 2.4 for C3. In simpler terms, the introduction of the plant into the SSAD amended soil appears to mitigate the migration of OM, particularly PN, through the soil profile.

No significant ($p > 0.05$) differences were observed regarding SOC, PN, HS, S or UA concentrations between bottom layer of C2 and C3 on day 45 and the initial unamended soil.

Overall, results suggest an increase in SOC over time induced by SSAD amendment and plant presence, with PN identified as the primary contribution from both. Similarly, Slepetineet al. (2022) found that digestate increased SOC. However other studies reported that digestate did not significantly influence SOC (Barlóg et al., 2020; Möller et al., 2015), suggesting differences in digestate compositions. Moreover, previous research has already shown the presence of PN-like materials in digestate (Guo et al., 2012, Guo et al., 2020). Similarly, Yan et al. (2019) demonstrated that biogas slurry increased the PN content in soil OM. Findings also suggest that the incorporation of *P. virgatum* into the SSAD amended soil mitigates the OM transport, especially PN. Additionally, it appears to alter the fractionation of Cu(II) following sequential extraction (Figure S1). Hence, delving into the quality of OM and its capacity for Cu(II) complexation becomes an intriguing aspect that requires further investigation.

Table 2. Composition of the the CER-extracted organic matter (OM) of initial samples unamended soil and SSAD amended soil.

Sample	Dryweight (mg)	VS (mg g ⁻¹ dry weight)	SOC (mg g ⁻¹ dry weight)	Proteins (mg g ⁻¹ dry weight)	Humic-like Substances (mg g ⁻¹ dry weight)	Polysaccharides (mg g ⁻¹ dry weight)	Uronic acids (mg g ⁻¹ dry weight)
Initial unamended soil	4.5 ± 0.2	0.2 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Initial SSAD- amended soil	6.7 ± 0.3	0.5 ± 0.1	2.6 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.1

Where: VS (volatile solids); SOC (soil organic carbon)

Table 3. Composition of the CER-extracted organic matter (OM) in top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers of C2 and C3.

Layer	Column	Dry weight (mg)	VS (mg g ⁻¹ dry weight)	SOC (mg g ⁻¹ dry weight)	Proteins (PN) (mg g ⁻¹ dry weight)	Humic-like Substances (HS) (mg g ⁻¹ dry weight)	Polysaccharides (S) (mg g ⁻¹ dry weight)	Uronic acids (UA) (mg g ⁻¹ dry weight)
Top (0-0.1 m)	C2 (no plant)	7.5 ± 0.6	0.5 ± 0.5	3.2 ± 0.2	1.3 ± 0.2	0.9 ± 0.3	0.7 ± 0.1	0.5 ± 0.1
	C3 (plant)	7.8 ± 0.5	0.6 ± 0.5	4.2 ± 0.1	1.7 ± 0.1	1.2 ± 0.4	0.7 ± 0.1	0.6 ± 0.1
Middle (0.1-0.2 m)	C2 (no plant)	6.9 ± 0.4	0.5 ± 0.2	1.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
	C3 (plant)	7.2 ± 0.3	0.5 ± 0.3	1.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
Bottom (0.2- 0.3 m)	C2 (no plant)	5.2 ± 0.4	0.5 ± 0.2	0.8 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
	C3 (plant)	5.9 ± 0.2	0.5 ± 0.2	0.8 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

Where: VS (volatile solids); SOC (soil organic carbon)

5.3.A.2 Quality evolution of the CER-extracted OM from solid phase, assessed with fluorophore, HIX and r(A,C) analysis from the 3D EEM

5.3.A.2.1 HIX and r(A,C) evolution in CER-extracted OM on day 0 and day 45

In order to understand the degree of stability and maturity of the CER-extracted OM in SSAD-amended soil over time in absence and presence of *P. virgatum*, the HIX and r(A,C) ratios were calculated. Results of the HIX and r(A,C) of samples on day 0 and day 45 are shown in Table 4.

5.3.A.2.1.1 Day 0

A higher HIX was evident in the unamended soil (0.9 ± 0.1) compared to the soil amended with SSAD digestate (0.4 ± 0.1). This suggests that the OM in the recently SSAD-amended soil is in its initial decomposition state, with microbes actively breaking down the organic materials. It is noteworthy that digestate is commonly reported to contain a substantial amount of recalcitrant organic residues originating from anaerobic digestion processes (Fernández-Domínguez et al., 2021; Insam et al., 2015; Nkoa, 2014; Tambone et al., 2009). However, this does not appear to be the case in the current study. Contrary to the observations in this research that used SSAD from municipal wastewater,

Yan et al. (2019) demonstrated that swine biogas slurry increased the degree of OM humification in soil, highlighting variations in the effects of related organic amendments on humification processes.

The $r(A,C)$ ratio was also found to be higher ($p < 0.05$) in the unamended soil (2.7 ± 0.1) compared to the soil amended with SSAD digestate (1.9 ± 0.1). This ratio, suggested by different authors as a “maturity” or “aging index” (Hassouna et al., 2012; Mounier et al., 1999; Patel et al., 2002; Sierra et al., 2005), is associated with the extent of biological processing in dissolved organic matter (Saadi et al., 2006). According to these authors, an increase in this ratio reflects advanced alteration and a higher degree of oxidation. Therefore, the observed results here are meaningful, indicating that the SSAD digestate supplies fresh OM to the soil, leading to a decrease in the ratio value.

5.3.A.2.1.2 Day 45

By day 45, the upper layer (0-0.1 m) of C2 exhibited a significantly higher ($p < 0.05$) HIX value (0.6 ± 0.1) compared to the initial soil amended with SSAD. This elevation in HIX implies a potential stabilization of humification processes, leading to a more stabilized OM over time. Conversely, in presence of *P. virgatum* (C3), a lower ($p < 0.05$) HIX value was found (0.5 ± 0.1). This observation may be attributed to the stimulation of microbial activity in the rhizosphere, aimed at nutrient mining from soil OM, thereby accelerating OM decomposition (Kuzyakov & Xu, 2013). Similarly, Yan et al. (2019) observed that the cultivation of corn could weaken soil humification. In terms of the $r(A,C)$ ratio, a slight yet statistically significant ($p < 0.05$) increase was noted in the top layer (0-0.1 m) for both C2 and C3 compared to the initial SSAD-amended soil, reaching values of 2.1 ± 0.1 and 1.9 ± 0.1 , respectively. This suggested an increase of maturity of OM over time, especially for C2. The presence of *P. virgatum* may stimulate the synthesis of new OM by the microbial activity in the rhizosphere and/or through root exudates. This phenomenon may account for the slightly fresher quality of OM in the upper layers when *P. virgatum* is present.

Both the HIX and $r(A,C)$ ratios were higher ($p < 0.05$) over depth. These results imply a higher humification and maturity of OM in middle (0.1 – 0.2 m) and bottom (0.2 – 0.3 m) layers. It is noteworthy that these layers initially comprised unamended soil. When compared to the initial unamended soil, the observed values of both HIX and $r(A,C)$ in the middle layer (0.1 – 0.2 m) were significantly lower ($p < 0.05$). Also, $r(A,C)$ ratios in bottom layer (0.2 – 0.3 m) of C3 were significantly lower ($p < 0.05$) than initial unamended soil. This difference suggests a migration of fresh OM through the soil profile, impacting

the observed ratios as already observed when examining the evolution of OM quantity. Nonetheless, upon comparing C2 and C3, higher ($p < 0.05$) $r(A,C)$ and HIX ratios were evident in the middle (0.1 - 0.2 m) and bottom (0.2 – 0.3 m) layers of C3. This suggests a diminished migration of fresh OM in the presence of *P. virgatum*.

5.3.A.2.2 Identification of fluorophores in the CER-extracted OM on day 0 and day 45

Figure 29.1 (Figure 29) presents the EEM spectra of the CER-extracted OM of the initial samples: (i) SSAD-amended soil (which represents the initial top layer (0-0.1 m) of C2 and C3, and (ii) unamended soil (which represent the initial middle and bottom layers of C2 and C3). Figure 29.2 (Figure 29) shows the EEM spectra of CER-extracted OM of the top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers of C2 and C3 on day 45. EEM peaks were divided into five regions, including simple aromatic proteins such as tyrosine and tryptophan PN-like (Regions I and II), fulvic acid-like substances (Region III), related to soluble microbial by-product-like materials (Region IV), Humic acid-like organics (Region V) (Chen et al., 2003). Table 5 summarizes the excitation and emission wavelengths of the identified fluorophores. Table 4 shows the fluorescence intensity of the identified fluorophores and the ratios between them in each sample: *ratio a*, *b* and *c* represent $\text{Peak}_{\text{region I}}/\text{Peak}_{\text{region II}}$, $\text{Peak}_{\text{region I}}/\text{Peak}_{\text{region IV}}$, $\text{Peak}_{\text{region II}}/\text{Peak}_{\text{region IV}}$ respectively. As shown in Figure 26 and Table 4 three fluorophores were identified on day 0 and day 45 in all samples: $\text{Peak}_{\text{region I}}$, characterized by the excitation-emission wavelength pair of $230 \pm 5 \text{ nm} - 295 \pm 5 \text{ nm}$, $\text{Peak}_{\text{region II}}$ (ex-em: $240 \pm 5 \text{ nm} - 366 \pm 2 \text{ nm}$) and $\text{Peak}_{\text{region IV}}$ (ex-em: $275 \pm 5 \text{ nm} - 365 \pm 5 \text{ nm}$). The results of each EEM indicate fluorescent components composition influenced by tyrosine-like proteins (Region I), tryptophan-like proteins (Region II) and soluble-like microbial by-products (Region IV).

5.3.A.2.2.1 Day 0

In unamended soil, CER extractable OM was mainly composed of tyrosine-like proteins (*ratio a*: 1.5 and *ratio b*: 1.7). In SSAD-amended soil, extractable OM had comparable presence of tyrosine-like proteins, tryptophan-like proteins and soluble-like microbial by-products, with slightly higher ($p < 0.05$) presence of tyrosine-like proteins (*ratio a*, *ratio b* and *ratio c*: 1.2, 1.3 and 1.0, respectively). We can infer that the SSAD amendment primarily led to increased enrichment of tryptophan-like proteins (region II) and soluble microbial products (region IV) in the CER-extractable OM, as compared to the unamended soil. This is partially in agreement with the results of the colorimetry tests which indicated that the main contribution of SSAD amendment was proteins. Yan et al.

(2019) also found that the fluorescence intensity of region II increased with the increase in the biogas slurry dosage. The observed increase in soluble microbial products is expected as they constitute one of the main components of digestates (Fernández-Dominguez et al., 2023).

5.3.A.2.2.2 Day 45

On Day 45, the composition of CER-extractable OM in the top layer (0-0.1 m) of C2 seemed to show a more homogeneous distribution among tyrosine-like proteins, tryptophan-like proteins, and soluble-like microbial by-products, with *ratios a, b, and c* at 1.1, 1.1, and 1.0, respectively. In C3, a slightly higher ($p < 0.05$) presence of tyrosine-like proteins was observed, with *ratios a, b, and c* at 1.3, 1.3, and 1.0, respectively.

In the middle layer (0.1-0.2 m), C2 demonstrated an enrichment in tyrosine-like proteins (*ratios a, b, and c*: 1.3, 1.2, and 1.0, respectively) compared to the top layer, suggesting that tyrosine-like proteins migrate from the top to the middle layer in C2. Similarly, in C3, a parallel trend was observed, with an enrichment in tyrosine-like proteins (*ratios a, b, and c*: 1.3, 1.4, and 1.1, respectively) compared to the top layer. This trend persisted when comparing the middle layer with the bottom layer; C2 showed similar ratios to the middle layer (*ratios a, b, and c*: 1.2, 1.2, and 0.9, respectively), while C3 exhibited enrichment in tyrosine-like proteins (*ratios a, b, and c*: 1.5, 1.4, and 0.9, respectively) compared to the middle layer.

In summary, the identified fluorophores in the CER-extracted OM from each column were consistent. Moreover, the findings suggest that tyrosine-like proteins, tryptophan-like proteins, and soluble microbial by-products were initially present in the soil before amendment, but the addition of SSAD resulted in the enrichment of tryptophan-like proteins (region II) and soluble microbial products (region IV) in the CER-extractable OM. The presence of plants appeared to enhance the content of tyrosine-like proteins (region I) in SSAD-amended soil. The migration of OM from the top layer to the bottom in the amended soil consists of OM enriched in tyrosine protein-like fluorophore (ex-em: 230-295 nm), and this effect is more pronounced with the presence of plants.

The study of the capacity of the three fluorophores (tyrosine-like proteins, tryptophan-like proteins, and soluble microbial by-products) in complexing Cu(II) is described below.

Table 4. Fluorescence intensities of identified fluorophores, ratios of peak areas, humification index (HIX) and aging index ($r(A,C)$) of the CER-extracted organic matter (OM) of (i) initial samples (unamended soil and SSAD amended soil) and (ii) top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers of C2 and C3 on day 45.

		<i>Fint</i>			<i>ratio a: Fint</i> Peak _{region I} /Peak _{region II}	<i>ratio b: Fint</i> Peak _{region I} /Peak _{region IV}	<i>ratio c: Fint</i> Peak _{region II} /Peak _{region IV}	HIX	$r(A,C)=I_A/I_C$
Peak _{region I}		Peak _{region II}	Peak _{region IV}						
day 0									
Initial unamended soil		696 ± 9	462 ± 8	398 ± 8	1.5	1.7	1.2	0.87 ± 0.07	2.70 ± 0.03
Initial SSAD amended soil		814 ± 10	674 ± 9	646 ± 9	1.2	1.3	1.0	0.36 ± 0.05	1.93 ± 0.02
day 45 - Column samples									
Top layer (0-0.1 m)	C2 (no plant)	716 ± 9	647 ± 6	643 ± 6	1.1	1.1	1.0	0.57 ± 0.04	1.98 ± 0.01
	C3 (plant)	826 ± 5	648 ± 7	637 ± 4	1.3	1.3	1.0	0.50 ± 0.01	1.95 ± 0.01
Middle layer (0.1-0.2 m)	C2 (no plant)	768 ± 8	613 ± 9	639 ± 8	1.3	1.2	1.0	0.59 ± 0.03	2.05 ± 0.08
	C3 (plant)	576 ± 6	453 ± 10	405 ± 7	1.3	1.4	1.1	0.68 ± 0.02	2.17 ± 0.04
Bottom layer (0.2-0.3 m)	C2 (no plant)	673 ± 7	544 ± 6	579 ± 5	1.2	1.2	0.9	0.85 ± 0.02	2.10 ± 0.05
	C3 (plant)	521 ± 9	337 ± 10	361 ± 5	1.5	1.4	0.9	0.87 ± 0.01	2.22 ± 0.03

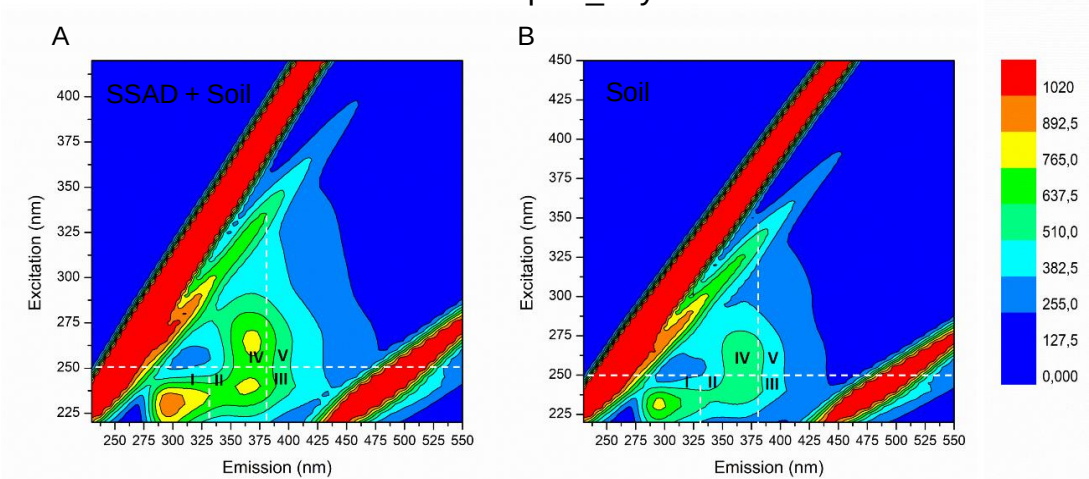
Where: *Fint* (fluorescence intensity); HIX (humification index).

Table 5. Excitation wavelength, emission wavelength and type of identified fluorophores.

Peak	λ Ex (nm)	λ Em (nm)	Type
Peak _{region I}	230 ± 5	295 ± 5	Tyrosine-like protein
Peak _{region II}	240 ± 5	366 ± 2	Tryptophan-like protein
Peak _{region IV}	275 ± 5	365 ± 4	Soluble microbial-like product

Where: λ Ex (excitation wavelength); λ Em (emission wavelength).

29.1 Initial samples_day 0



29.2 C2_day 45

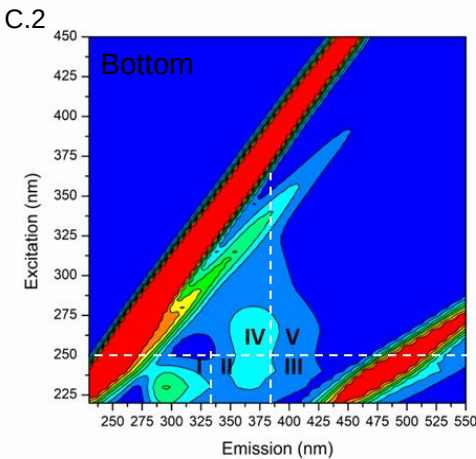
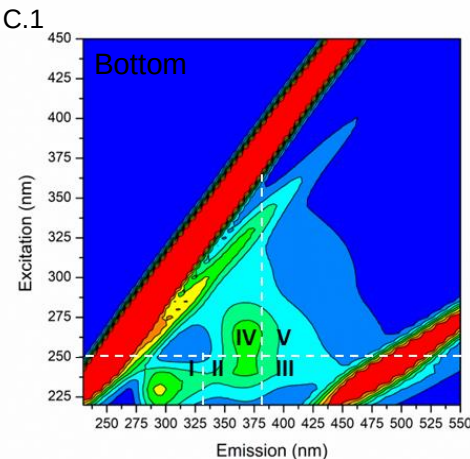
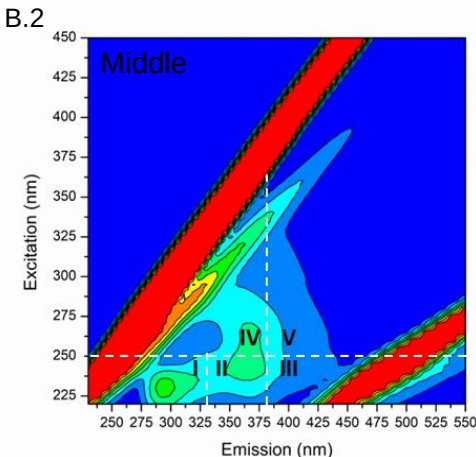
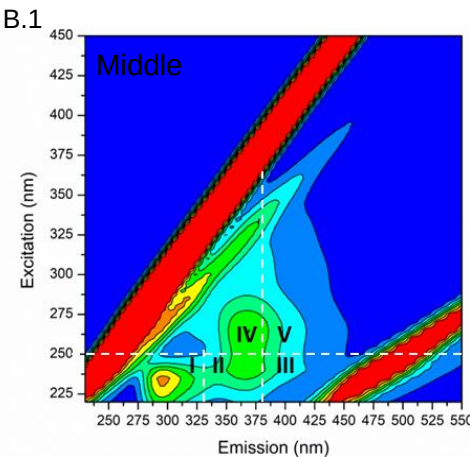
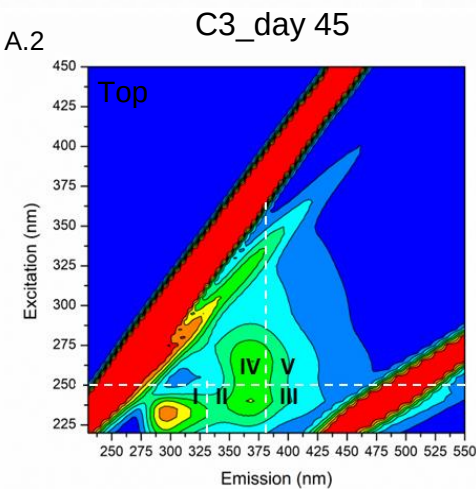
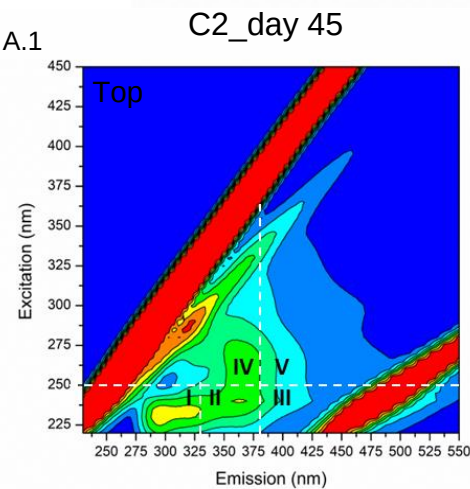


Figure 29. (29.1) Excitation emission matrix (EEM) spectra of the CER- extracted organic matter (OM) of (A) SSAD amended soil and (B) unamended soil on day 0. (29.2) EEM spectra of the CER-extracted OM of top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers of C2 (A.1, B.1 and C.1, respectively) and C3 (A.2, B.2, C.2, respectively) at the end of the experimental period (day 45). Dilution in phosphate buffer at pH 6.5 (dilution 1/20). Temperature 20 ± 2 °C. Numbers represent the five regions defined by Chen et al. (2003): I tyrosine-like proteins, II tryptophan-like proteins, III fulvic acid-like organics, IV soluble microbial by-product-like and V humic acid-like substances.

5.3.B Study of the evolution of the CER-extracted OM reactivity towards Cu(II) along the columns at times 0 and 45 days with the quenching method

The effects of increasing concentrations of Cu (II) (0 μ M, 20 μ M, 50 μ M, 80 μ M, and 100 μ M) in (i) the SSAD-amended soil on day 0, representing the initial top layer (0-0.1 m) of C2 and C3 and (ii) top (0 – 0.1 m), middle (0.1 – 0.2 m) and bottom (0.2 – 0.3 m) layers of C2 and C3 on day 45 are presented on Figure S3, Figure S4 and Figure S5, respectively. Overall, the findings reveal a significant reduction in the *Fint* of tyrosine-like and tryptophan-like proteins, as well as soluble-like microbial by-products derived from the CER-extracted OM with increasing concentrations of Cu (II). Similarly, Fine et al. (2018) indicated that the addition of Cu(II) decreased the fluorescence intensity of the dissolved OM but did not change the location of the peaks, indicating that formation of complexes between Cu and fluorophores in the dissolved OM quenches the fluorescence without forming new fluorescing moieties. Complexation of metal ions at the surfaces of bound OM might resemble complexation with dissolved OM compounds.

To determine quenching rate constants and assess the chemical stability of complexes, fluorescence quenching data were fitted using the Stern–Volmer equation (Eq. 1). The application of a nonlinear model through the least squares method (Eq. 2 derived from Eq. 1) was employed, with a significance level set at $p < 0.05$.

$$F = \frac{F_0}{(1 + K_{sv} \cdot [Q])}$$

Here, F_0 and F denote fluorescence intensities in the absence and presence of the metal (Cu(II)), respectively. K_{sv} represents the Stern–Volmer constant, and $[Q]$ is the concentration of the quencher (Cu(II)). In the model, variable 1 (v_1) is defined as $[Q]$, and variable 2 (v_2) is defined as F . For most cases experimental values fit with the model

(Appendix IV Figure S6 and Figure S7). The results are illustrated in Figure 30, and Ksv values are presented in log form to facilitate comparability with existing literature.

5.3.B.1 Day 0

On day 0, the mean log Ksv values for Peak region I (tyrosine-like proteins), Peak region II (tryptophan-like proteins), and Peak region IV (soluble microbial-like products) in the SSAD-amended soil (representing the top layer of both C2 and C3 on day 0) were 1.00 ± 0.1 , 1.20 ± 0.11 , and 1.40 ± 0.09 , respectively. These results suggest that, on day 0, soluble microbial-like products showed the highest ($p < 0.05$) binding capacity. Previous research has shown the capacity of soluble microbial products from sludge to bind metals (M. Huang et al., 2018).

5.3.B.2 Day 45

By day 45, the mean log Ksv value for the Peak region I (tyrosine-like proteins) in the top layer (0 – 0.1 m) of C3 was significantly higher ($p < 0.05$) compared to C2 (1.16 ± 0.05 and 0.95 ± 0.04 , respectively). Additionally, the value in C3 surpassed that of the initial SSAD-amended soil. Prior research has indicated the reactivity of proteins towards Cu (II) (X. Guo et al., 2012). Furthermore, NH_2 groups have been documented to exhibit high reactivity towards metals (Majumdar et al., 2008; Pagnanelli et al., 2009). In addition, Liu et al. (2015) demonstrated the adsorption of metals by the -OH and -COOH groups of sludge proteins. Similarly, Sheng et al. (2013) demonstrated that the presence of oxygen atoms within the -COOH groups of proteins played a significant role in the adsorption removal of Cu(II). These results emphasize the potential significant role of these proteins in the complexation dynamics with Cu under the studied conditions, especially in presence of *P. virgatum*. These results are in agreement with the higher presence of Cu(II) in the oxidizable fraction (bound to OM) in presence of the plant (Appendix IV Figure S1). On the other hand, the mean log Ksv values for Peak region II (tryptophan-like proteins) and Peak region IV (soluble microbial-like products) in the top layer (0 – 0.1 m) had significantly ($p < 0.05$) decreased to 0.91 ± 0.06 and 1.2 ± 0.3 , respectively, in C2, and 0.9 ± 0.3 and 1.1 ± 0.1 , respectively, in C3 when compared to the initial SSAD amended soil. There were no significant ($p > 0.05$) differences regarding log Ksv values of Peak region II (tryptophan-like proteins) and Peak region IV (soluble microbial-like products) between C2 and C3.

Within the middle layer (0.1 – 0.2 m) of C2, the mean log Ksv value for Peak region I (tyrosine-like proteins) stood at 1.15 ± 0.05 , significantly surpassing that of the top layer (0 – 0.1 m). Peak region II (tryptophan-like proteins) and Peak region IV (soluble

microbial-like products) within the middle layer (0.1 – 0.2 m) exhibited log K_{sv} values of 1.0 ± 0.2 and 1.0 ± 0.1 , respectively. These values were not significantly different ($p > 0.05$) from those in the top layer (0 – 0.1 m). In bottom layer (0.2 – 0.3 m) values of Peak region I (tyrosine-like proteins), Peak region II (tryptophan-like proteins) and Peak region IV (soluble microbial-like products) decreased ($p < 0.05$) to 0.8 ± 0.1 , 0.63 ± 0.08 and 0.7 ± 0.2 , respectively. By day 45, the mean log K_{sv} values of all components in middle layer (0.1 – 0.2 m) of C3 were lower than in C2 ($p < 0.05$). In bottom layer the mean log K_{sv} values of Peak region I (tyrosine-like proteins), Peak region II (tryptophan-like proteins) and Peak region IV (soluble microbial-like products) were significantly lower ($p < 0.05$) in C3 (0.7 ± 0.1 , 0.4 ± 0.2 and 0.5 ± 0.1 respectively) than in C2 (0.8 ± 0.1 , 0.6 ± 0.2 and 0.7 ± 0.2 , respectively).

In general, the binding capacities of the three fluorophores decreased from the top to the bottom of the column, reducing by around a factor of 2. This trend corresponds with the concurrent increase in the degree of humification and maturity of the OM over depth ($r = 0.9$, $p < 0.05$) (HIX and r(A,C) indexes Table 5). Different humification and maturity stages of the OM may potentially result in a loss or modification of functional groups capable of complexing Cu (e.g., a loss of NH_2 , -OH and -COOH groups). The findings suggest that fresh OM, provided by the SSAD, exhibits a greater binding capacity compared to the mature soil-borne OM. Also, this could potentially account for the observed distinctions between C2 and C3. Specifically, as above-mentioned, a higher binding capacity (log K_{sv}) of tyrosine-like proteins in top layer (0 – 0.1 m) was higher in presence of *P. virgatum*, this is linked to a lower HIX (Table 4), probably due to the supply of fresh OM released by plants roots. Also, lower log K_{sv} values for all fluorophores were noted in middle (0.1 – 0.2 m) and bottom (0.2 – 0.3 m) layers of C3 when compared to C2, likely stemming from a reduced migration of fresh organic matter in the presence of *P. virgatum*.

Ultimately, the chemical stability of the extracted OM-Cu(II) complexes determined in this study, appears notably lower when compared with previous investigations that studied dissolved OM-Cu(II) interactions in diverse environments. For instance, studies in sediments (Hur & Lee 2011) reported log K_{sv} values ranging from 4.8 to 6.1, working with a pH = 6. Guo et al. (2019) observed log K_{sv} values spanning from 4.42 to 5.64 in compost, working with a pH = 7 ± 0.05 . Similarly, studies in soil environments (Guo et al. 2015) yielded log K_{sv} values within the range of 5.11 to 5.75, working with a pH = 8 ± 0.05 . Anaerobic digestate derived from a straw substrate (Guo et al. 2020) showed log K_{sv} values ranging from 5.05 to 5.29, working with a pH = 7 ± 0.02 .

Fluorescence can detect mono- or polycyclic aromatic groups but may not capture the evolution of their molecular environment, as can happen during the maturation of OM, involving the loss of functional groups linked more or less distantly to the fluorescent molecule's structure. This study underscores the significance of OM "freshness" in determining the strength of fluorophore complexation with a ligand.

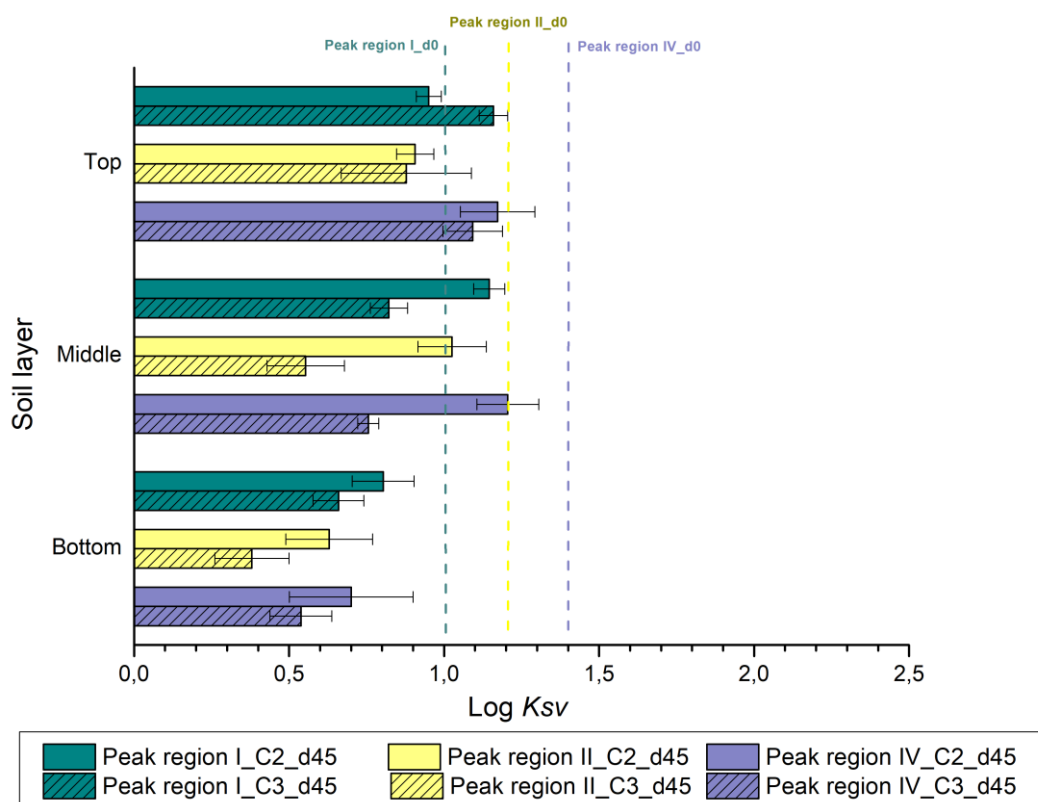


Figure 30. Variation in log K_{sv} constant of Peak region I (tyrosine-like proteins), Peak region II (typtophan-like proteins) and Peak region IV (soluble microbial-like products) in top (0 – 0.1 m), middle (0.1 – 0.2 m) and bottom layer (0.2 – 0.3) of C2 and C3 on day 45. Green, yellow and blue dashed lines represent the log K_{sv} of Peak region I (tyrosine-like proteins), Peak region II (typtophan-like proteins) and Peak region IV (soluble microbial-like products) in initial SSAD amended soil on day 0.

5.4 Conclusion

The findings highlight that SSAD induced substantial quantitative changes in the soil's "bound" OM. Specifically, SSAD leads to a noteworthy increase in SOC concentrations, predominantly in the form of proteins and soluble microbial-like products. Notably, the newly introduced OM from SSAD exhibits superior binding capacities compared to the mature soil-borne OM. The presence of *P. virgatum* in SSAD-amended soil induced three main effects: (i) a reduction in the migration of OM through the soil profile; (ii) an increase of tyrosine-like proteins concentrations in the topsoil; and (iii) an increase in the binding capacity of these proteins in the topsoil, possibly attributed to the lower humification degree of the OM introduced by the roots. These findings underscore the potential significance of tyrosine-like proteins in the complexation dynamics with Cu(II) under the examined conditions. Moreover, the molecular environment surrounding each fluorophore is notably influenced by the increase in the degree of OM maturity and humification. This, in turn, diminishes the OM's capacity to bind with the metal. Consequently, the ability of a fluorophore to form complexes with a metal should be evaluated in the context of its molecular maturity or freshness. These insights contribute to our understanding of how SSAD and *P. virgatum* collectively influence the dynamics of OM and Cu(II) complexation. This comprehensive understanding is crucial for addressing the environmental implications of sustainable digestate management practices.

Authors contribution

NB-G: conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing—original draft. IB: conceptualization, investigation, methodology, data curation, validation, visualization, supervision, and writing—review and editing. VR: investigation, methodology, data curation, validation, visualization, supervision, and writing—review and editing. SB: methodology, data curation and validation. MA: conceptualization, funding acquisition, supervision, writing—review and editing. AM: conceptualization, funding acquisition, supervision. GG: conceptualization, funding acquisition.

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6. Chapter 6: Biochemical methane production from *Panicum virgatum* and *Pennisetum alopecuroides* cultivated with metal-containing municipal solid waste digestate

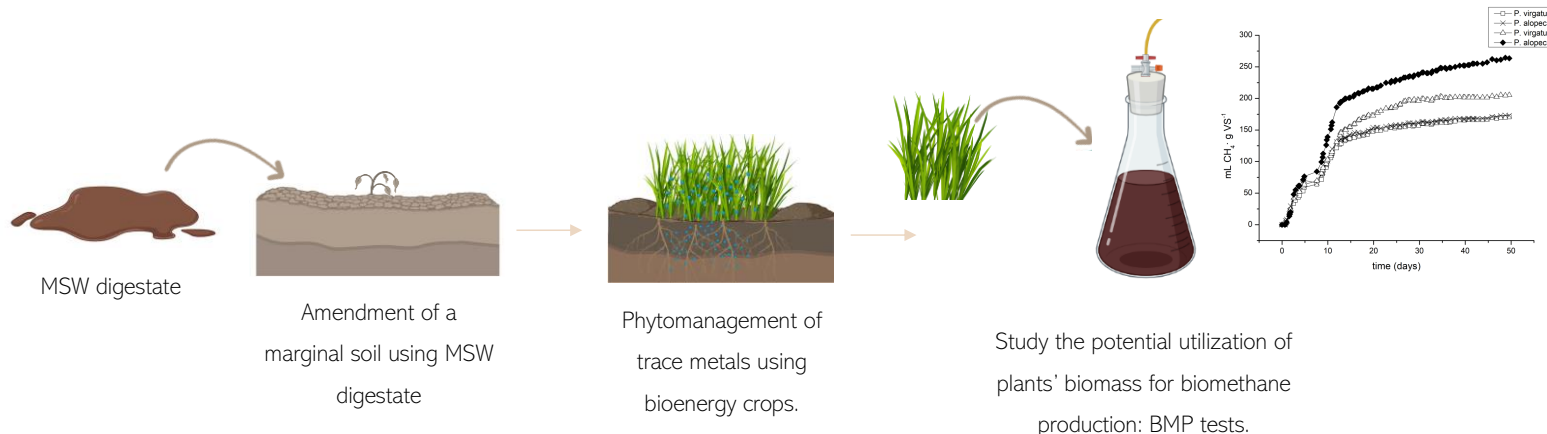
This chapter is based on the manuscript prepared for submission as:

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Abstract

Biochemical methane potential (BMP) tests were performed to assess the feasibility of utilizing post-phytomanagement biomass of *Panicum virgatum* and *Pennisetum alopecuroides* for biogas production. A significantly higher ($p < 0.05$) BMP performance was observed when utilizing *P. virgatum* or *P. alopecuroides* biomass cultivated in the presence of MSW as a substrate, in comparison to the biomass of *P. virgatum* or *P. alopecuroides* grown without digestate. A first-order exponential kinetic model was used to obtain the apparent kinetic constant of the processes assayed, which was similar between conditions. Values of pH, total solids (TS), volatile solids (VS), $\text{NH}_3\text{-N}$, soluble COD (chemical oxygen demand), alkalinity and volatile fatty acids (VFAs) indicated an effective process performance. Utilizing plant biomass could significantly aid in closing the material loop and fulfilling the demand for a circular economy.

6.1 Graphical abstract



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6.2 Introduction

The combination of anaerobic digestion and phytomanagement unfolds a symbiotic relationship with numerous benefits. The amendment of soil with digestate has been proven to improve soil's quality (reduce soil's bulk density, increase boost soil's organic carbon, increase saturated hydraulic conductivity, raise soil capacity of moisture retention, improve aggregate stability, stimulate crop yields, influence positively on the soil microbial diversity, etc.) (Möller & Müller, 2012; Nkoa, 2014). However, this practice can also lead to the introduction of digestate-borne contaminants into the soil, such as trace metals (TMs), or induce changes in soil physical chemical properties which in turn can result in changes on contaminants fate and distribution as well as, TMs bioavailability (Dragicevic et al., 2018; Kupper et al., 2014; Lamolinara et al., 2022).

The bioenergy crops cultivated in digestate-amended soil can act as a powerful phytoremediator, extracting, transforming, or stabilizing TMs present in the soil (Barbosa et al., 2015; Jha et al., 2017; Pandey et al., 2016; PP & Puthur, 2021; Prabha et al., 2021). Simultaneously, the crops can derive nutrients from the digestate (Antonkiewicz et al., 2017; Barłóg et al., 2020; Lee et al., 2021; Mucha et al., 2019). Furthermore, plants' biomass can afterward be used as a feedstock in the AD process (Gomes, 2012; Osman et al., 2020; Rheay et al., 2021; Tripathi et al., 2016), creating a closed-loop system that not only valorize the byproducts of anaerobic digestion but also provides feedstock for sustainable energy production (Haregu et al., 2023; Wang et al., 2021). Additionally, this plant biomass can act as a potential source of TMs for the AD process, therefore optimizing the biogas production yield and rate (Choong et al., 2016; Feroso et al., 2015; Moestedt et al., 2016; Thanh et al., 2016).

In our previous study (Chapter 2, Bonet-Garcia, 2023), we demonstrated that the bioenergy crops *P. virgatum* and *P. alopecuroides* effectively phytomanaged TMs from a marginal soil amended with municipal solid waste (MSW) digestate. The result was a significant reduction in the migration of TMs through the soil profile. These bioenergy crops not only mitigated the transport of TMs but also benefited from the improved soil quality resulting from the digestate amendment. Both plant species showed higher concentrations of Pb, Zn and Cu, especially Zn, in their tissues in presence of MSW digestate, when compared to plants grown in absence of MSW digestate, indicating that

they contribute for the removal of TM from the digestate, also protecting the soil from their toxicity.

The present study compares the methane production from the co-digestion of sewage sludge digestate with the biomass of two different bioenergy crops (*P. virgatum* and *P. alopecuroides*) that were grown in presence or absence of MSW digestate. The study aims to evaluate the potential to use *P. virgatum* and *P. alopecuroides*, previously used to phytomanage TMs from a soil amended with MSW digestate, in order to give use to the biomass and ultimately closing the AD material loop.

6.3 Material and methods

6.3.A Inoculum and substrate

The anaerobic biomass used as inoculum in the biochemical methane potential tests was sourced from an industrial-scale anaerobic reactor treating waste activated sludge from a municipal wastewater treatment plant operating at mesophilic conditions, located in Sevilla, Spain. The main characteristics of the anaerobic biomass were pH: 7.5 ± 0.2 ; total solids (TS): 4.09 ± 0.03 %, volatile solids (VS): 2.03 ± 0.07 % and total chemical oxygen demand (COD): 37164 ± 10 mg $O_2 \cdot L^{-1}$.

The harvestable parts of *P. virgatum* and *P. alopecuroides* grown for 83 days in presence or absence of digestate were used as substrate for the lab scale reactor (see section 6.3B), being obtained from a previous study (Bonet-Garcia et al 2023, Chapter 2). The main characteristics of the unamended soil and digestate amended soil where plants were grown are shown in Table S1. Plant biomass was harvested and subsequently air-dried at room temperature (22 ± 2 °C). Following the drying process, the plant biomass underwent chopping using an electric chopper, resulting in a mixed composition of particles spanning from 0 to 5 mm. To ensure uniformity, a 5 mm sieve was employed to ensure that all particles were <5 mm. The main characteristics of the harvestable biomass from *P. virgatum* and *P. alopecuroides* grown in presence (D) and absence (ND) of MSW digestate, are detailed in Table 6.

Table 6. Pb, Zn and Cu concentration ($\mu\text{g}\cdot\text{g}^{-1}$ dw) (adapted from Bonet-Garcia et al., (2023), Chapter 2), TS (%), VS (%) and COD concentration ($\text{mg O}_2\cdot\text{Kg}^{-1}$) $\pm$ SD in the leaves of *P. virgatum* and *P. alopecuroides* grown in presence (D) and absence (ND) of MSW digestate.

	[Pb] $\mu\text{g}\cdot\text{g}^{-1}$ dw	[Zn] $\mu\text{g}\cdot\text{g}^{-1}$ dw	[Cu] $\mu\text{g}\cdot\text{g}^{-1}$ dw	TS (%)	VS (%)	Total COD ($\text{mg O}_2\cdot\text{Kg}^{-1}$) ¹⁾
<i>P. virgatum</i> ND	0.86 ± 0.01	5.48 ± 0.08	<LOD	9.3 ± 0.1	8.50 ± 0.08	$6.3\cdot 10^5 \pm 2\cdot 10^3$
<i>P. alopecuroides</i> ND	0.86 ± 0.02	4.690 ± 0.004	<LOD	9.4 ± 0.1	8.60 ± 0.04	$6.2\cdot 10^5 \pm 1\cdot 10^3$
<i>P. virgatum</i> D	3.2 ± 0.1	58 ± 2	4 ± 1	9.42 ± 0.08	8.60 ± 0.06	$6.3\cdot 10^5 \pm 3\cdot 10^3$
<i>P. alopecuroides</i> D	2.9 ± 0.4	41 ± 2	3.5 ± 0.05	9.31 ± 0.07	8.45 ± 0.07	$6.2\cdot 10^5 \pm 9\cdot 10^2$

*LOD Cu $1.5 \mu\text{g}\cdot\text{g}^{-1}$

Where: VS: Volatile solids; TS : total solid; COD: chemical oxygen demand.

6.3.B Biochemical Methane Potential Assays

Trials of methane production by AD were carried out using the biochemical methane potential (BMP) tests. For that, 4 different substrate conditions in triplicate BMPs were tested (i: *P. virgatum* grown in soil in absence of MSW digestate (*P. virgatum* ND); ii: *P. alopecuroides* grown in soil in absence of MSW digestate (*P. alopecuroides* ND); iii: *P. virgatum* grown in MSW-digestate amended soil (*P. virgatum* D); iv: *P. alopecuroides* grown in MSW-digestate amended soil (*P. alopecuroides* D). A fifth condition served as a blank, containing inoculum without substrate.

BMP tests were performed in 250 mL Erlenmeyer flasks reactors with a working volume of 240 mL, using a multi-batch reactor system. Reactors were connected to a gas measuring cylinder. A plastic tube served as the connection between the reactor and an L-shaped glass tube with an 8 mm diameter, which, in turn, was linked to the gas measuring cylinder used for gas flow measurement (Figure 31). Rubber stoppers equipped with valves were placed into the reactor openings, ensuring that the valves were closed to maintain anaerobic conditions. BMP reactors were placed in a thermostatic water bath at mesophilic temperature (37 ± 1 °C) and continuously stirred by magnetic bars and an orbital shaker at 120 rpm.

The BMP reactors were loaded with 2.81 – 2.86 g of the substrate (2.40 – 2.41 g VS) (either *P. virgatum* or *P. alopecuroides* grown with or without digestate) and 237 g of

inoculum (4.80 g VS) (sewage sludge digestate). The inoculum-to-substrate ratio was at 2:1 based on the g of VS. The blank reactor was loaded with 240 g of inoculum (4.90 g VS). Before sealing the reactors with a rubber stopper, oxygen was displaced using N_2 to ensure anaerobic atmosphere conditions. The volume of produced biogas was measured employing a volumetric displacement method, as described by Esposito et al. (2012), and it was monitored daily until methane production ran out. The produced biogas was passed through a 2N NaOH solution to retain the CO_2 and to consider the remaining gas as methane. In particular, the produced methane passed as bubbles through a 2N NaOH solution. This action pushed the 2N NaOH solution level down in the cylinder, and the volume of displaced 2N NaOH was recorded as the volume of biomethane.

The BMP tests were carried out during the time interval required to exhaust the methane production (51-day period in the used conditions).

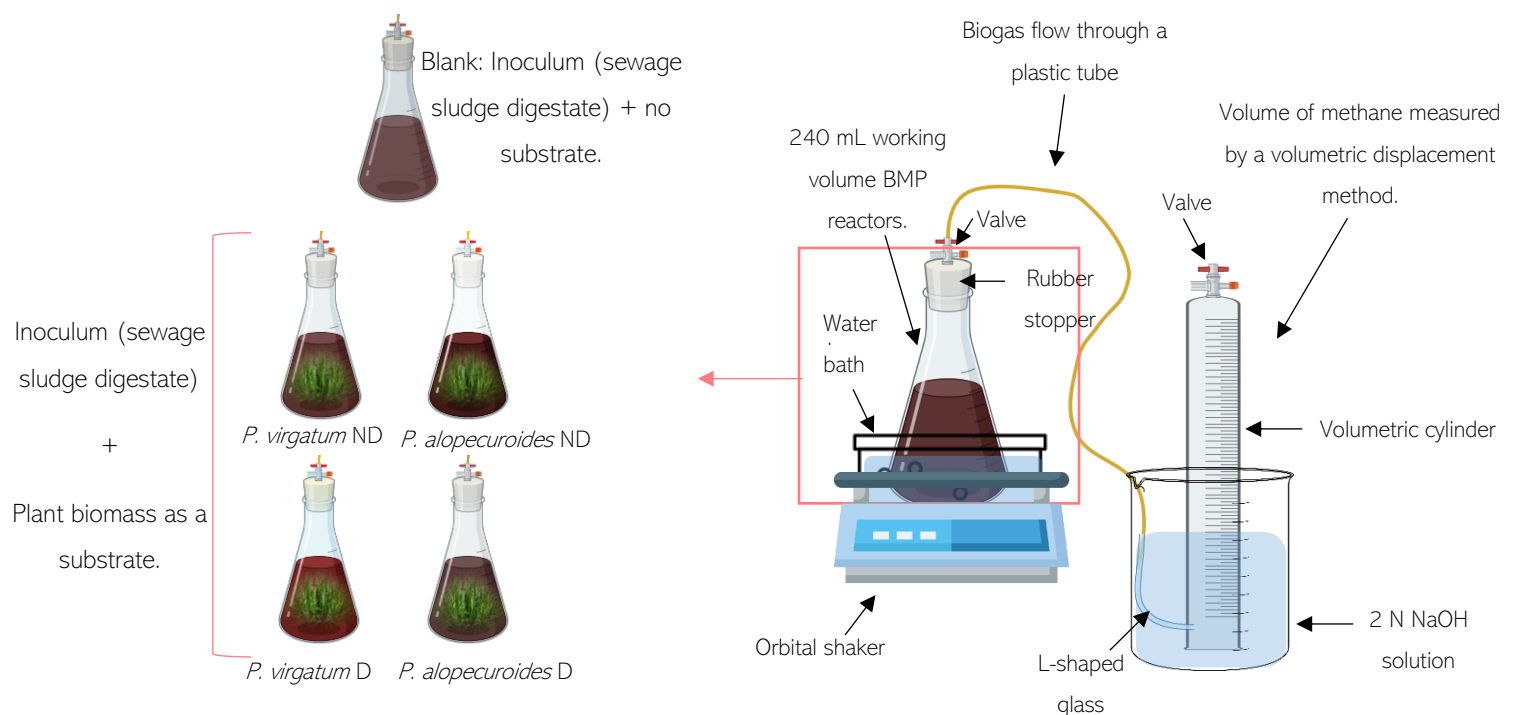


Figure 31. Schematic representation of the BMP tests. Four different substrate conditions: (i) *P. virgatum* grown in soil without MSW digestate (*P. virgatum* ND); (ii) *P.*

alopecuroides grown in soil without MSW digestate (*P. alopecuroides* ND); (iii) *P. virgatum* grown in MSW-digestate amended soil (*P. virgatum* D); and (iv) *P. alopecuroides* grown in MSW-digestate amended soil (*P. alopecuroides* D). A fifth condition served as a blank, consisting of inoculum without substrate.

6.3.C Analytical Methods

The concentration of TS (%), VS (%), alkalinity (mg CaCO₃ L⁻¹), ammonia nitrogen (NH₃-N) (mg L⁻¹), and pH were measured according to the recommendations of the Standard Methods of APHA (2005) (Eaton et al., 2005). Specifically, TS and VS were determined according to the Standard methods 2540B and 2540E, respectively. Alkalinity was determined by pH titration to 4.3. pH was analyzed using a pH-meter model Crison 20 Basic. Total COD (g kg⁻¹) was determined using the method described by Raposo et al. (2008), while soluble COD (sCOD; mg·L⁻¹) was determined by the closed digestion and the colorimetric standard method 5220D (APHA 2005) (Eaton et al., 2005). Individual volatile fatty acids (VFA) (acetic acid, butyric acid, propionic acid and valeric acid) were quantified using a gas chromatograph (GC) (Shimadzu GC-2014 gas) equipped with a 15 m × 4 mm Nukol-silica capillary column and a flame ionization detector. The oven temperature was gradually increased from 100 to 150 °C at a rate of 4 °C/min. Helium (28.6 kPa), Nitrogen (28.6 kPa), Hydrogen (14.3 kPa) and synthetic air (28.6 kPa) were used as carrier gas at a flow rate of 50 mL/min. The biogas composition at the end of the experiment was determined by GC (Shimadzu GC-2014 gas) using Argon as a carrier gas, and equipped with a thermal conductivity detector, a Valco GC valve and HAYESEP Q 80/100 packed column.

6.3.D Process kinetics

A first-order exponential model was used to correlate the evolution of the methane production with digestion time. This kinetic model is normally applied to assess the performance and kinetics of batch anaerobic digestion processes of easily biodegradable substrates (Li et al., 2012).

The first-order exponential model is given by the following expression (Eq 3):

$$B = B_{max} \cdot [1 - \exp(-k \cdot t)]$$

where: B ($\text{mL CH}_4 \cdot \text{g VS}^{-1}$) is the cumulative specific methane production, B_{max} ($\text{mL CH}_4 \cdot \text{g VS}^{-1}$) is the ultimate methane production, k is the specific rate constant or apparent kinetic constant (d^{-1}) and t (d) is the digestion time. The adjustment by non-linear regression of the pairs of experimental data (B , t) using RStudio (version 4.3) (package: *minpack.lm*) allowed the calculation of the parameters k and B_{max} for methane production.

6.3.E Statistical analysis

One-way ANOVA was used to establish significant differences between conditions. Significant differences ($p < 0.05$) were detected by a Tukey's Honestly Significant Difference (HSD) post hoc test.

6.4 Results and discussion

6.4.A Biochemical methane potential (BMP)

Figure 32 presents the evolution of methane production, expressed as mL of methane per g of VS ($\text{CH}_4 \text{ g VS}^{-1}$), for the BMP tests performed using as a substrate *P. virgatum* or *P. alopecuroides* grown in absence (ND) or presence (D) of MSW digestate, corrected with the blank (BMP without substrate). As can be observed in Figure 32, the methane yield for the conditions *P. virgatum* (D) and *P. alopecuroides* (D), reached $205 \pm 8.9 \text{ mL CH}_4 \cdot \text{g VS}^{-1}$ and $264 \pm 30 \text{ mL CH}_4 \cdot \text{g VS}^{-1}$, respectively. These values were $26 \pm 2\%$ and $53 \pm 6\%$ higher ($p < 0.05$) than the methane yields observed when using the plants that were grown in the absence of MSW digestate (*P. virgatum* ND and *P. alopecuroides* ND), which were $163 \pm 3 \text{ mL}$ and $173 \pm 6 \text{ mL}$, respectively. In all cases, around 80 % of the methane production occurred within the first 12 days. A higher fraction of CH_4 compared to CO_2 in the biogas mix was measured for all conditions (67 – 72 %) (Table 7).

The results here obtained agree with previous studies that reported a production of ~ 101 – 333 $\text{mL CH}_4/\text{g VS}$ in BMPs tests co-digesting different kinds of digestate and energy crops (Himanshu et al., 2018; Moset et al., 2017; Schroyen et al., 2015; Wahid et al., 2015). Also, Baute et al. (2018) reported a methane production of 186.5 ± 9.4 and $160.1 \pm 25.2 \text{ CH}_4 \cdot \text{g VS}^{-1}$ of *P. virgatum* co-digested with digestate in two different experiments. However, in the case of *P. alopecuroides*, Zhang et al. (2016) showed a lower methane production, specifically 37.7 and 42.4 L kg VS^{-1} , when using 5.8 kg of sludge and 0.3 kg and 0.5 kg of *P. alopecuroides*, respectively, in a 30-day batch experiment. These values are notably lower than the results observed in the present study.

Measurements of chemical parameters, including pH, TS, VS, $\text{NH}_3\text{-N}$, soluble COD, alkalinity and VFAs were performed in the effluent of the BMPs to assess the performance of the AD process, determine reaction stability, and identify potential inhibitory factors (Parkin & Owen, 1986) (Table 2). Similar results were observed in all conditions regarding pH (range: 7.41 ± 0.04 – 7.51 ± 0.09), which remained within the recommended range for an adequate methanogenic activity (Wheatley, 1990); TS (range: $3.31\% \pm 0.07$ – $3.55\% \pm 0.06$); VS (range: $1.61\% \pm 0.01$ – $1.89\% \pm 0.01$); $\text{NH}_3\text{-N}$ (range: $1401 \pm 20 \text{ mg N} \cdot \text{L}^{-1}$ – $1531 \pm 31 \text{ mg N} \cdot \text{L}^{-1}$); soluble COD (range: 34303 ± 201 – $39560 \pm 3215 \text{ mg O}_2 \cdot \text{L}^{-1}$) and alkalinity (range: $8752 \pm 943 \text{ mg} \cdot \text{L}^{-1}$ – $10071 \pm 352 \text{ mg} \cdot \text{L}^{-1}$). The pH values, along with elevated alkalinity and low VFAs concentration

observed at the end of the BMP tests, assured that acidification did not take place. Acidification could potentially inhibit the activity of methane-producing microorganisms (Serrano et al., 2017)(Xi et al., 2014; Serrano et al., 2017). Additionally, the concentrations of $\text{NH}_3\text{-N}$ were well below the inhibitory thresholds for AD microbial activity, as specified in the literature for the operational pH (Chen et al., 2008; Rajagopal et al., 2013).

Differences between D and ND conditions could be explained by biomass characteristics. Higher Zn concentration ($p < 0.05$) was present in *P. virgatum* and *P. alopecuroides* biomass grown in presence of MSW digestate (D) ($58 \pm 2 \mu\text{g}\cdot\text{g}^{-1}$ dw and $41 \pm 2 \mu\text{g}\cdot\text{g}^{-1}$ dw, respectively) when compared to plants grown in the absence of MSW digestate (ND) ($5.48 \pm 0.08 \mu\text{g}\cdot\text{g}^{-1}$ dw and $4.69 \pm 0.004 \mu\text{g}\cdot\text{g}^{-1}$ dw, respectively) (Table 1). Zn is essential for an optimal anaerobic digestion process (Fermoso et al., 2019; Thanh et al., 2016), as it has been identified as a cofactor of many microbial enzymes and coenzymes involved in the methane production (Jarrell & Kalmokoff, 1988; Mares et al., 2023; Sauer & Thauer, 2000).

Moreover, potentially higher concentrations of P and Mg were present in the biomass of the plants grown in presence of MSW digestate. In Table S1 it can be observed that soil amended with MSW digestate presented higher ($p < 0.05$) concentrations of P_2O_5 , MgO, TN and TOC, which ultimately influenced plants' growth (Bonet-Garcia et al., 2023), and potentially increased P and Mg contents in plant biomass (not measured in the current study). Jurgutis et al. (2021) reported that the use of digestate fertilization favored the P content in grass biomass. Głowacka et al. (2020) showed that digestate increased the content of P and Mg in *Panicum virgatum* biomass. P and Mg are recognized for their role in enhancing biogas production yields in AD as they are essential for microbial growth (Bożym et al., 2015; van Hullebusch et al., 2019). The potential increased presence of these elements in the biomass of plants grown in the presence of MSW digestate could explain the observed higher methane production.

Another potential explanation could be the differences in plant stages. As above-mentioned, in our previous study (Bonet-Garcia et al., 2023, Chapter 2) we reported that MSW digestate amendment stimulated *P. virgatum* and *P. alopecuroides* growth, most likely due to the higher presence of organic matter and nutrients, while in unamended soil plant growth was reduced. (Di Girolamo et al., 2013) reported that plant stage can influence methane production when using plant biomass as substrate on AD. On the other hand, it is known that lignin is involved in plant responses to various biotic and abiotic stresses, such as mineral deficiency (Barros et al., 2015; Cesarino, 2019; Moura

et al., 2010). When *P. virgatum* and *P. alopecuroides* were grown in absence of MSW digestate (ND) they may have experienced stress due to the low concentrations of organic matter and nutrients. Highly lignocellulosic biomass is recalcitrant to anaerobic degradation and ultimately results in lower biomethane yield (Sawatdeenarunat et al., 2015). Amaleviciute-Volunge et al., (2020) demonstrated strong negative correlation between cellulose content in perennial grasses and methane yield.

Table 7. pH, total solids (TS) (%), volatile solids (VS) (%), ammonia nitrogen (NH₃-N) (mg N L⁻¹), soluble chemical oxygen demand (COD) (mg O₂·Kg⁻¹), methane [CH₄] (μmol ·L⁻¹), carbon dioxide [CO₂] (μmol·L⁻¹), alkalinity (mg ·L⁻¹), acetic acid (mg·L⁻¹), propionic acid (mg·L⁻¹), butiric acid (mg·L⁻¹) and valeric acid (mg ·L⁻¹) in the BMP tests at the end of the experimental period.

	Blank	<i>P. virgatum</i> ND	<i>P. alopecuroides</i> ND.	<i>P. virgatum</i> D.	<i>P. alopecuroides</i> D.
pH	7.32 ± 0.03	7.51 ± 0.09	7.4 ± 0.1	7.50 ± 0.02	7.41 ± 0.04
TS (%)	3.1 ± 0.2	3.31 ± 0.07	3.54 ± 0.09	3.55 ± 0.06	3.48 ± 0.03
VS (%)	1.56 ± 0.00	1.61 ± 0.01	1.77 ± 0.02	1.81 ± 0.01	1.89 ± 0.01
NH ₃ -N (mg N · L ⁻¹)	747 ± 0.00	1401 ± 20	1433 ± 7	1531 ± 13	1512 ± 1
Soluble COD (mg O ₂ · L ⁻¹)	13558 ± 1608	34658 ± 1306	39560 ± 3215	34303 ± 201	38210 ± 1105
[CH ₄] (μmol ·L ⁻¹)	4385 ± 20	10891 ± 100	17068 ± 50	10560 ± 30	11731 ± 15
% CH ₄	52	72	67	72	70
[CO ₂] (μmol ·L ⁻¹)	4105 ± 10	4191 ± 20	8557 ± 12	4021 ± 11	4920 ± 18
% CO ₂	48	28	33	28	30
Alkalinity (mg ·L ⁻¹)	5721 ± 575	10071 ± 352	9507 ± 1261	8926 ± 203	8752 ± 943
Acetic acid (mg · L ⁻¹)	n.d.	n.d.	n.d.	n.d.	n.d.
Propionic acid (mg · L ⁻¹)	n.d.	n.d.	n.d.	n.d.	n.d.
Butiric acid (mg ·L ⁻¹)	n.d.	n.d.	n.d.	n.d.	n.d.
Valeric acid (mg ·L ⁻¹)	n.d.	n.d.	n.d.	n.d.	n.d.

Where: n.d., not detected; TS: total solids; VS: volatile solids; NH₃-N: ammonia nitrogen; COD: chemical oxygen demand; CH₄: methane; CO₂: carbon dioxide.

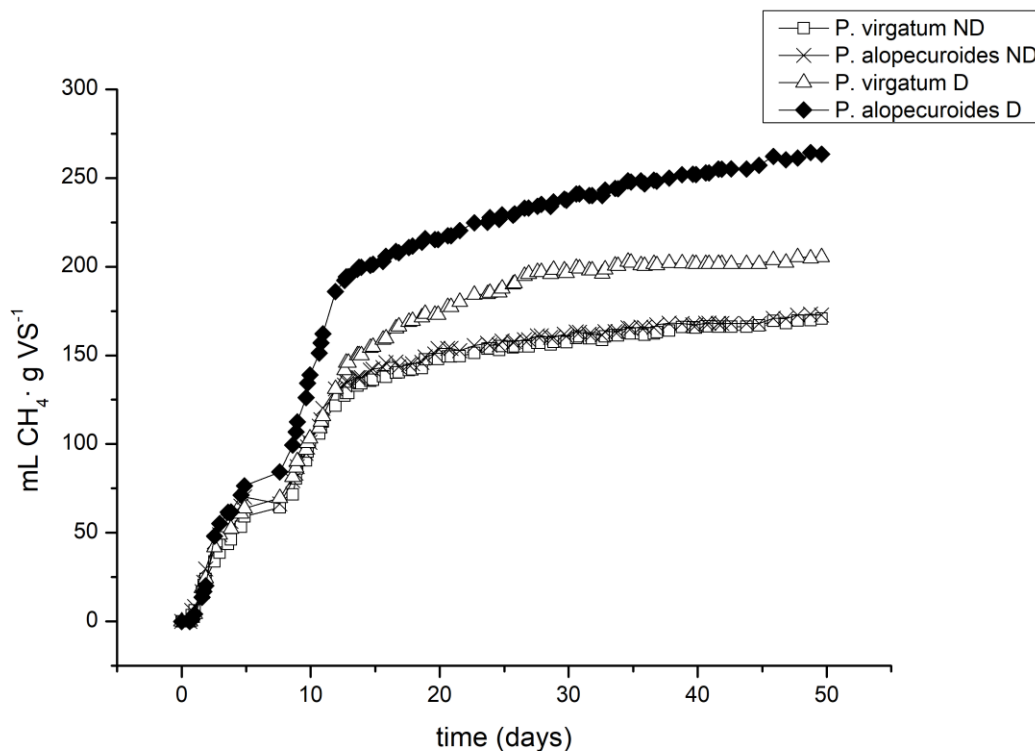


Figure 32. Cumulative methane production obtained from the anaerobic digestion using *P. virgatum* and *P. alopecuroides* cultivated in absence (ND) or presence (D) of digestate as a substrate. (n=2) Error bars are omitted in the graph for enhanced readability, full data is presented in Table S2 (Appendix V).

6.4.B Process kinetics

The calculated parameters k and B_{max} for methane production are summarized in Table 8. K values of the conditions *P. virgatum* D and *P. alopecuroides* D were very similar to *P. virgatum* ND and *P. alopecuroides* ND, respectively ($0.080 \pm 0.003 \text{ d}^{-1}$ and $0.090 \pm 0.004 \text{ d}^{-1}$ vs $0.120 \pm 0.003 \text{ d}^{-1}$ and $0.10 \pm 0.02 \text{ d}^{-1}$, respectively). This suggests that the biochemical processes responsible for methane production, such as hydrolysis, acidogenesis, acetogenesis, and methanogenesis, are occurring at comparable rates across the different conditions. These findings imply that the MSW digestate did not significantly alter the rate of methane production of *P. virgatum* and *P. alopecuroides*.

On the other hand, the values of calculated B_{max} were similar to those obtained from the BMP tests and suggest a higher CH_4 production using *P. virgatum* and *P.*

alopecuroides grown in presence of MSW digestate (D) ($224 \pm 3 \text{ CH}_4 \cdot \text{g VS}^{-1}$ and $288 \pm 4 \text{ CH}_4 \cdot \text{g VS}^{-1}$, respectively) than with *P. virgatum* and *P. alopecuroides* grown in absence of MSW digestate (ND) ($163 \pm 1 \text{ CH}_4 \cdot \text{g VS}^{-1}$ and $175 \pm 20 \text{ CH}_4 \cdot \text{g VS}^{-1}$, respectively). These results further suggest that the presence of MSW digestate enhances the methane production capacity of both *P. virgatum* and *P. alopecuroides*.

In practical terms, these findings suggest that while the rate of methane production may be similar across conditions, the total methane yield achievable is higher with *P. virgatum* and *P. alopecuroides* grown in presence of MSW digestate. This could be attributed to a higher concentration of nutrients present in plant biomass when grown in presence of MSW digestate, which could act as cofactors and support microbial growth and metabolism (Bożym et al., 2015; Fermoso et al., 2019; Jarrell & Kalmokoff, 1988; Mares et al., 2023; Sauer & Thauer, 2000; van Hullebusch et al., 2019;), as above discussed.

Table 8. Kinetic parameters obtained from Eq. (3) for the different conditions. Values following $\pm$ represent the standard error of each parameter.

	<i>Bmax</i> ($\text{mLCH}_4 \cdot \text{g VS}^{-1}$)	K (d^{-1})	R^2	S.E.E.
<i>P. virgatum</i> ND	163 ± 1	0.11 ± 0.03	0.981	10.49
<i>P. alopecuroides</i> ND	175 ± 20	0.10 ± 0.02	0.978	6.60
<i>P. virgatum</i> D	$224 \pm 3^*$	0.08 ± 0.003	0.975	5.67
<i>P. alopecuroides</i> D	$288 \pm 4^*$	0.09 ± 0.004	0.970	9.97

Where: *Bmax* is the ultimate methane production, *k* is the specific rate constant or apparent kinetic constant. Parameters from the nonlinear regression fit: R^2 : coefficient of determination. A good fit to the data is indicated by high R^2 values (above 0.95); S.E.E.: Standard Error of Estimate. The high values of the R^2 and the low values of the standard error of estimate demonstrate the goodness of the fit of experimental data to the proposed model. * $p < 0.05$ when comparing *P. virgatum* D with *P. virgatum* ND, and *P. alopecuroides* D with *P. alopecuroides* ND.

6.5 Conclusions

Enhanced methane production was achieved by utilizing *P. virgatum* or *P. alopecuroides* grown in MSW digestate as a substrate. Despite this, no significant difference was noted in terms of the reaction rate between conditions D and ND. While these findings represent an initial phase, they propose a promising approach for the sustainable and profitable utilization of MSW digestate in generating renewable biogas feedstock. Future research should delve deeper into the impact of MSW digestate on the chemical characteristics of *P. virgatum* and *P. alopecuroides* biomass, and their impact on methane production. Additionally, investigating the long-term and large-scale potential of MSW digestate fertilization to enhance grass yields and induce changes in soil properties is warranted. This comprehensive analysis would also facilitate the classification of *P. virgatum* and *P. alopecuroides* grown in MSW digestate, considering economic factors such as methane yield per hectare.

Authors contribution

NB-G: conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing—original draft. FF: conceptualization, funding acquisition, validation, visualization, supervision, and writing—review and editing. AM: conceptualization, funding acquisition, supervision. MA: conceptualization, funding acquisition, supervision, writing—review and editing.

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7. Chapter 7: Conclusions and future perspectives

Throughout this thesis, each chapter has provided valuable insights into the utilization of digestate for soil amendment and employing phytomanagement strategies with bioenergy crops, aiming at sustainable amended soil management and bioenergy production.

In Chapter 2, the research demonstrated that MSW digestate application enriched a marginal soil with organic matter and essential nutrients, promoting the growth of *Panicum virgatum* and *Pennisetum alopecuroides*. Despite the observed increase in trace metal (TM) concentrations in the upper soil layers following municipal solid waste (MSW) digestate application, their limited migration to deeper layers and low potential bioavailability suggested minimal risk of TM dispersion. Notably, the presence of *P. virgatum* or *P. alopecuroides* demonstrated a mitigating effect on TM migration, reducing it even more, particularly Zn, Cu, and Pb, indicating a protective role of these plants. No bioconcentration capacity was observed in either plant, suggesting their contribution to phytostabilization processes. These findings significantly contribute to our comprehension of digestate benefits and underscore its potential for enhancing soil health. Furthermore, these results highlight phytomanagement using bioenergy crops as a promising strategy for mitigating the TM migration in digestate-amended soils through soil profile. As we further explore the dynamics of digestate application and phytoremediation strategies, we gain valuable insights into sustainable soil management practices for future agricultural endeavors. In Chapter 3, the study revealed an increased prokaryotic diversity following 90 days of MSW digestate amendment, particularly enriched in the presence of *P. virgatum* and *P. alopecuroides*. By day 90, MSW digestate-amended was dominated by the prokaryotic phyla Proteobacteria, Bacteroidota, Firmicutes, Actinobacteriota, Planctomycetota, Verrucomicrobia, and the fungal phylum Ascomycota. The presence of *P. virgatum* and *P. alopecuroides* further intensified the abundances of beneficial soil prokaryotic phyla, including Planctomycetota, Chloroflexi, Verrucomicrobia, and Acidobacteria. Most prokaryotic genera observed in the amended topsoil were absent in plant root compartments, suggesting distinct microbial niches. The prokaryotic genus *Rhodanobacter*, and fungal genera *Fusarium*, *Penicillium*, and *Conlarium* present in root microbiomes both in presence and absence of MSW digestate, may constitute a core microbiome for these plants. These findings underscore the synergistic benefits of digestate amendment and

bioenergy crops in enhancing soil microbiome biodiversity. Furthermore, these results offer valuable insights into key prokaryotic and fungal genera that may contribute to promoting *P. virgatum* and *P. alopecuroides* growth and overall fitness. In Chapter 4, the study shows an increase of TM (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As and Pb) concentrations in soil solution following sewage sludge anaerobic digestate (SSAD) amendment, however low risk of TM leaching was observed. The presence of *P. virgatum* further increased the concentrations of Mn and Fe in soil solution. Results also underscored the potential influence of dissolved organic carbon (DOC) and anions and cations on the behavior of TMs in SSAD-amended soil phytomanaged by *P. virgatum*. Furthermore, a positive correlation was observed between TMs' DGT fluxes, soil solution concentrations, and their concentrations in *P. virgatum* tissues. These findings emphasize the importance of considering DGT fluxes and soil solution concentrations when assessing the bioavailability of TMs to plants in phytomanaged digestate-amended soils. Chapter 5 explores the influence of "bound" organic matter (OM) on TM behavior in a SSAD amended soil phytomanaged by *P. virgatum*. Results highlight significant increased soil organic carbon (SOC) concentrations induced by SSAD amendment, mainly in the form of proteins and microbial-like products. Newly introduced OM from SSAD demonstrated stronger binding capacities than mature soil-borne OM. *P. virgatum* in SSAD-amended soil reduced OM migration, increased tyrosine-like proteins in the topsoil, and enhanced their binding capacity, likely due to lower OM humification from root-derived OM. These insights underscore the significance of tyrosine-like proteins in Cu(II) complexation dynamics and highlight the importance of understanding the maturity degree of OM for evaluating its metal-binding capacity. Overall, the results enhance our understanding of SSAD and *P. virgatum*'s impact on the interactions between OM and Cu(II) (as an example of TM). This comprehension is crucial for addressing the environmental implications of sustainable digestate management practices. Finally, Chapter 6 highlights the potential of *P. virgatum* and *P. alopecuroides* grown in MSW digestate for enhanced biomethane production. These findings emphasize the promising applications of phytoremediation for sustainable MSW digestate management and sustainable energy generation, thus providing an example of route to close the loop of the circular bioeconomy, and potentially offering valuable insights for researchers and practitioners in the field.

To further advance the knowledge gathered in the present work, it is necessary to conduct studies to evaluate and optimize the selection of plant species in each particular case, based on their ability to uptake, translocate, and accumulate TMs from digestate-

amended soils while maximizing biomass production. Future research should also delve deeper into the impact of digestate on the chemical characteristics of plant biomass, and their potential impact on methane production. At the same time, investigating the underlying mechanisms involved in phytoremediation processes, including the role of root exudates, rhizosphere processes, and plant-microbe interactions, is essential for advancing our understanding of these remediation techniques. Furthermore, exploring the functional potential of microorganisms would provide valuable insights into their role in remediation processes. Screening the PGP traits and TM tolerance of identified microorganisms could facilitate the development of bioaugmentation possibilities, thereby enhancing the efficacy of bioenergy crops in managing digestate-amended soils. Moreover, utilizing powerful computing tools such as the PARAFAC technique to help interpreting the fluorescence excitation–emission matrix (EEM) data, could further enhance our understanding of the reactivity of digestate and plant-borne OM towards TMs. In addition, developing mechanistic models and predictive frameworks to simulate environmental factors-TMs interactions in phytomanaged digestate-amended soils is crucial, integrating factors such as soil properties, OM dynamics, and metal speciation. Also, investigating the potential introduction of additional contaminants associated with digestate amendment, such as emerging pollutants like pharmaceuticals, per- and polyfluorinated substances (PFAS), microplastics, among others, and their interactions with TMs, can provide valuable insights into the holistic impact of digestate application on environmental quality and human health, paving the way for more informed and sustainable waste management practices.

Last but not least, future research should prioritize long-term studies and field trials using different types of digestate aimed at validating the scalability, practicality, and assessing the sustained effects of digestate-amendment practices. Additionally, conducting comprehensive environmental risk assessments to evaluate the potential risks associated with these practices is essential. Also, conducting life cycle assessments (LCAs) is needed to evaluate the environmental and social impacts of biogas production from bioenergy crops cultivated in digestate-amended soils throughout the entire supply chain. This will help identify opportunities for enhancing sustainability, reducing greenhouse gas emissions, and improving overall system performance. Additionally, comprehensive economic analyses should be conducted to assess the cost-effectiveness and profitability of biogas production from various bioenergy crops grown in digestate-amended soil. Developing policy and regulatory frameworks that support the implementation of phytoremediation practices in digestate-amended soils is crucial. This

includes providing incentives for farmers and landowners, establishing guidelines for safe remediation practices, and implementing monitoring protocols for environmental protection. Such initiatives are crucial for contributing to global sustainability objectives, including the European Commission's proposal for healthy soils by 2030 and the United Nations Sustainable Development Goals.

Appendix I. This appendix contains supplementary material from Chapter 2: Metal mobility in an anaerobic-digestate amended soil: the role of two bioenergy crops and their metal phytoremediation potential.

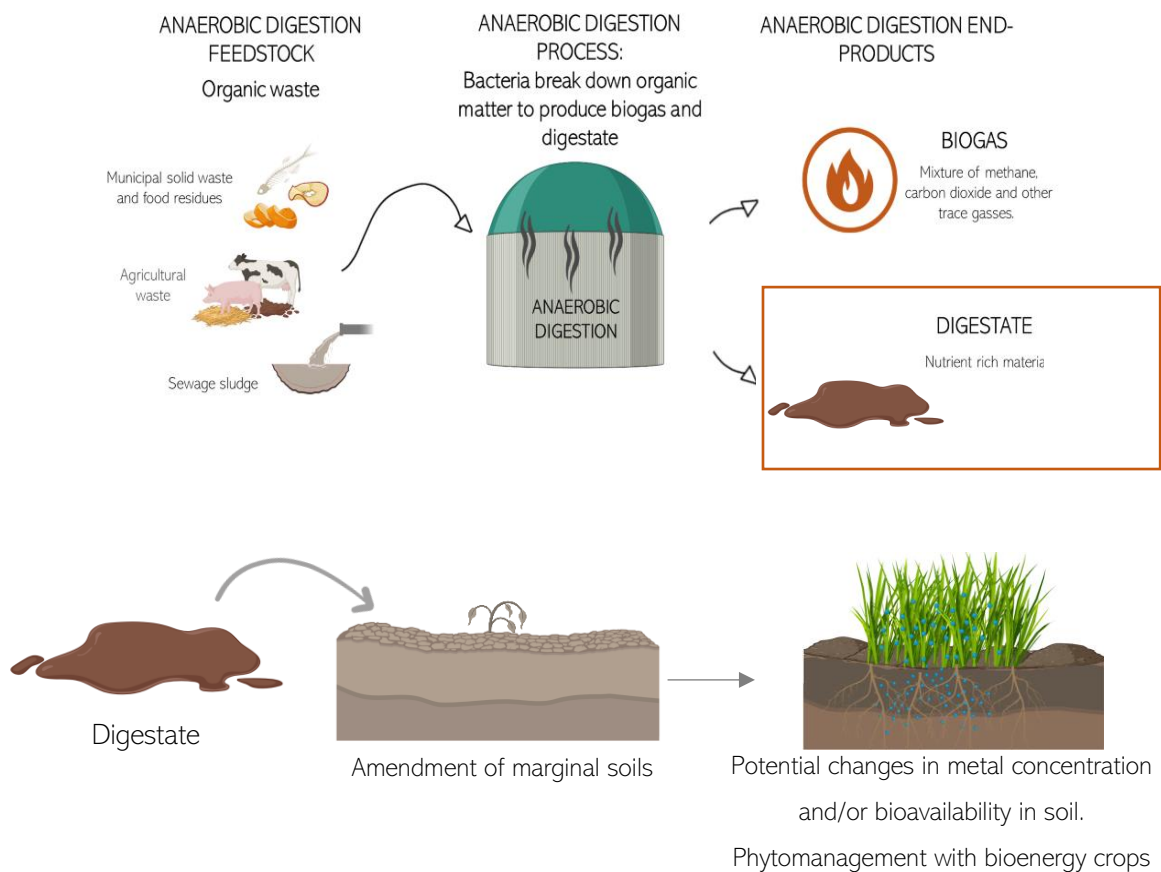


Figure S1. Scheme representing digestate utilization for marginal soil amendment and subsequent phytomanagement with bioenergy crops.

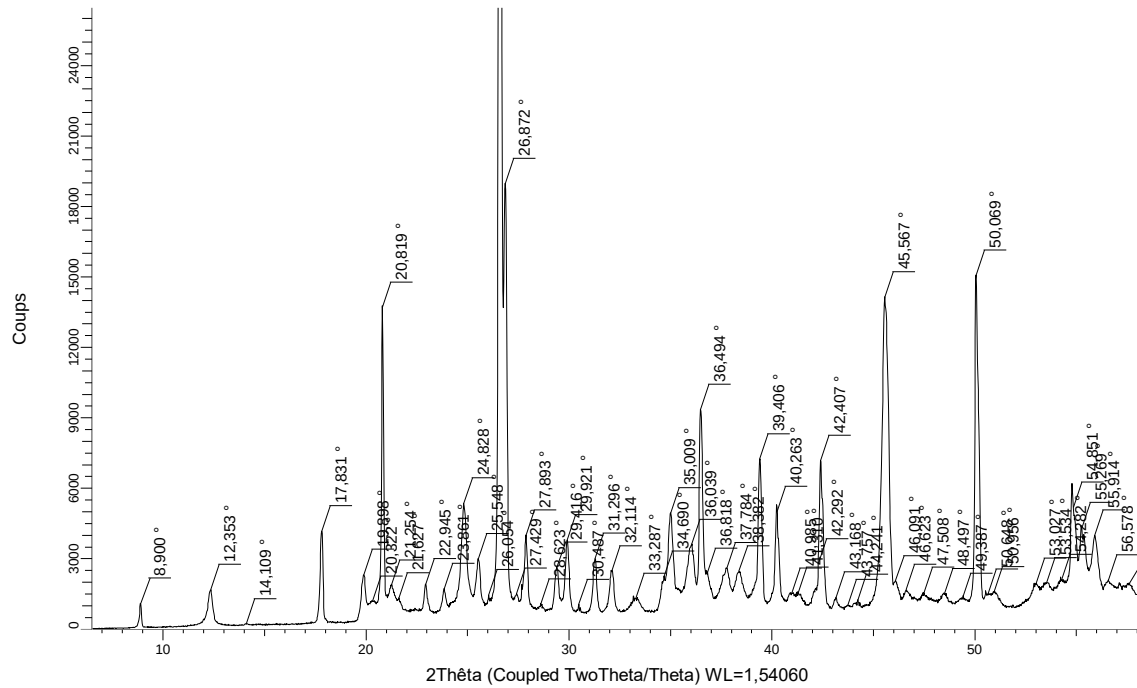


Figure S2. X-Ray Diffraction (XRD) pattern of soil used in this study. Major phases identified: quartz, kaolinite, muscovite.

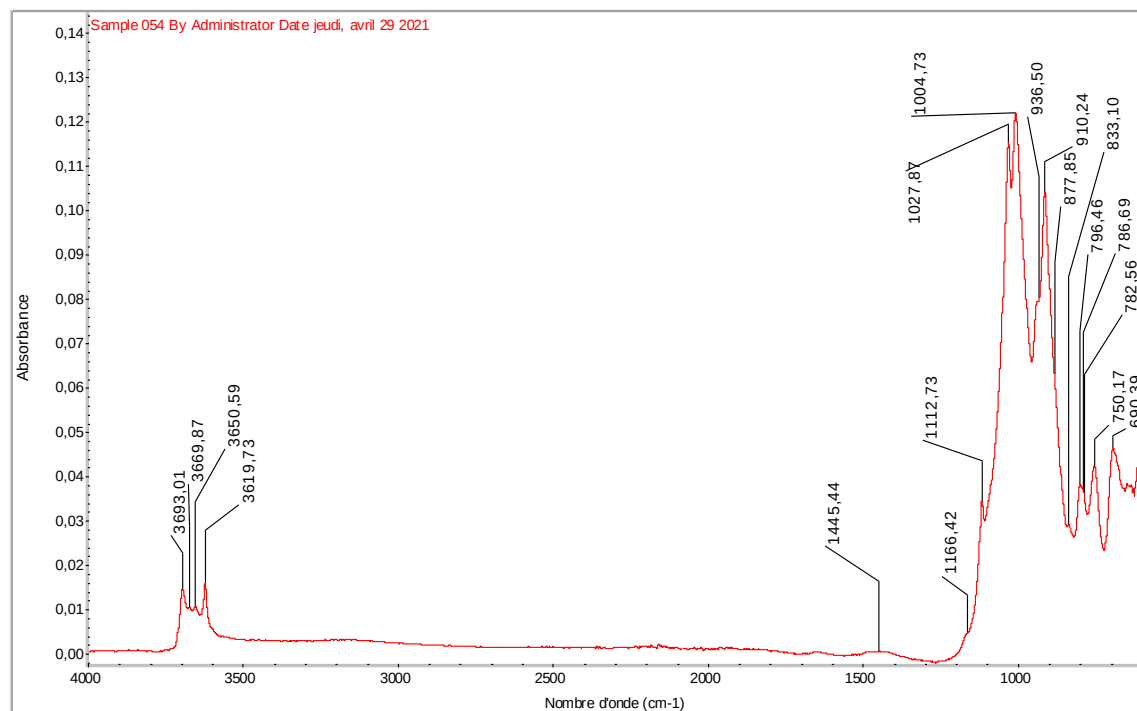


Figure S3. FTIR pattern of soil used in this study. 3620, 3650, 3670 et 3695 = OH stretching bands of kaolinite (vOH); Small bumps at 1650 and 3200 = water (bending

and stretching H_2O , respectively, δ and ν); 1440: possible traces of carbonates (CO_3); SiO kaolinite: 1005, 1028, 1113; Bending OH with AL-AL kaolinite: 937, 915; Other modes from kaolinite: 796, 750, 690; 878: bending OH with Al-Fe, probably contribution from muscovite; SiO quartz: shoulder at 780 and 800, 697, 1166.

Table S1. Overview of the influence of MSW digestate application on soil physical chemical properties. Values associated with * are significantly different among samples, at $p < 0.05$ level of significance by Tukey's multiple comparisons test.

Parameter	Initial Soil	Initial MSW Digestate	Initial Soil + MSW Digestate	Regulation EU n°2019/1009 (FPR)	Anaerobic digestates reported in the literature
Phosphorus (P_2O_5) (% dry matter)	0.10 ± 0.02	1.4 ± 0.1	$0.78 \pm 0.03^*$	1-2%	0.3 – 2.8 (Nkoa 2014)
Potassium (K_2O) (% dry matter)	2.7 ± 0.5	1.6 ± 0.2	2.09 ± 0.04	1-2%	1.3 – 5.3 (Nkoa 2014)
CaO (% dry matter)	1.3 ± 0.6	13 ± 1	$8.0 \pm 0.4^*$		
MgO (% dry matter)	0.3 ± 0.08	4.9 ± 0.4	$2.6 \pm 0.9^*$		
Total Nitrogen (TN) (% dry matter)	<0.1	1.8 ± 0.3	0.15 ± 0.02	1-2.5%	0.46–14.0 g L^{-1} (Chong et al., 2022; Dębowski et al., 2017; Fouda, 2011; Möller, 2015; Voča et al., 2005)
Total Sulphur (TS) (% dry matter)	<0.1	<0.1	<0.1		0.49– 1.95 g L^{-1} (Kluge et al., 2008; Okoro et al., 2018; Svensson et al., 2018)
Total Carbon (TC) (% dry matter)	0.40 ± 0.06	31 ± 2	7.3 ± 0.6		24.88–45.0 % dry matter (Möller, 2015; Okoro et al., 2018; Simeonov et al., 2012)
Total organic carbon (TOC) (% dry matter)	0.200 ± 0.005	19.0 ± 0.3	$4.4 \pm 0.7^*$	Min 15%	38.6–75.4 % dry matter (Möller, 2015; Möller & Stinner, 2009; Voča et al., 2005)
Loss on Ignition (% dry matter)	2.4 ± 0.1	22 ± 0.8	$6.5 \pm 0.1^*$		54.3- 98.5 (Svoboda et al., 2013; Teglia et al., 2011a, 2011b)
Water content (%)	12.5 ± 0.9	51 ± 3	$29 \pm 2^*$		20.3–53.4 (Teglia et al., 2011a, 2011b)
Water Holding Capacity (WHC)	24 ± 1	-	$53 \pm 4^*$		7.3–9.0 (Fouda, 2011; Kluge et al., 2008; Möller & Stinner, 2009; Tambone et al., 2009)
CEC (cmol+/kg)	4.4 ± 0.2	40 ± 3	$15.2 \pm 2^*$		
pH value	7.9 ± 0.2	8.8 ± 0.4	8.4 ± 0.1		
Particle density (g/cm^3)	2.54 ± 0.01	-	1.8 ± 0.9		
Bulk density (g/cm^3)	2.2 ± 0.3	-	$1.3 \pm 0.2^*$		
Porosity (%)	19.6 ± 0.3	-	23 ± 2		

Table S2. Maximum permissible values for the 'total' contents of metals in fertilizer material with organic components by class (milligrams per kilogram of dry matter), according to Portuguese legislation (Decreto -Lei n. 73/2011) and European Regulation (EU) 2019/1009

Portuguese legislation (Decreto -Lei n. 73/2011).					Regulation (EU) 2019/1009.
Metal	Fertilizer				Digestate amendment
	Class I	Class II	Class II A	Class III	
	mg kg ⁻¹ dw				
Cadmium (Cd)	0.7	1.5	3	5	1.5
Lead (Pb)	100	150	300	500	120
Copper (Cu)	100	200	400	600	300
Chromium (Cr)	100	150	300	400	2 (Cr (IV))
Mercury (Hg)	0.7	1.5	3	5	1
Nickel (Ni)	50	100	200	200	50
Zinc (Zn)	200	500	1000	1500	800

Specifications for Decreto -Lei n. 73/2011: Class I and II (Agriculture); Class II A (Tree and shrub agricultural crops, namely orchards, olive groves and vineyards; Forestry species); Class III (Soil where it is not intended to plant crops intended for human and animal food: Final coverage of landfills and dumps, quarries, and mines, with a view to restoring the landscape; Coverage of ditches and slopes, in the case of road construction (landscape integration); Fertilization of soils intended for forestry (species whose fruit is not used in human or animal food); Bioenergetic cultures; Gardening; floricultural production (excluding edible crops); Football and golf fields.)

Table S3. pH values determined in initial samples and soil column samples at layers 0-20 cm and 20-40 cm at the end of the experimental period (day 90). T0: control soil columns; T1: soil columns with *Panicum virgatum*; T2: soil columns with *Pennisetum alopecuroides*; T3: soil column with *Panicum virgatum* without MSW digestate; T4: soil column with *Pennisetum alopecuroides* without MSW digestate. For T0, T1 and T2 results are presented as the mean of three measurements per each duplicated column ($n = 2 \times 3$) $\pm$ SD. For T3 and T4 results are presented as the mean of three measurements per each column $\pm$ SD.

Sample		pH
Initial soil		7.9 $\pm$ 0.2
Initial MSW digestate		8.8 $\pm$ 0.3
Initial MSW digestate amended soil		8.42 $\pm$ 0.07
Soil Column – day 90	Layer	
T0	0-0.2 m	8.77 $\pm$ 0.01
	0.2-0.4 m	8.47 $\pm$ 0.01
T1	0-0.2 m	7.1 $\pm$ 0.03*
	0.2-0.4 m	8.29 $\pm$ 0.2*
T2	0-0.2 m	7.10 $\pm$ 0.02*
	0.2-0.4 m	8.29 $\pm$ 0.02*
T3	0-0.2 m	8.46 $\pm$ 0.01
	0.2-0.4 m	8.44 $\pm$ 0.05
T4	0-0.2 m	8.31 $\pm$ 0.04
	0.2-0.4 m	8.39 $\pm$ 0.06

Values associated with * are significantly different, at $p < 0.05$ Tukey's level of significance.

Table S4. Percentage of selected metals (Fe, Mn, Zn, Pb, Cu, Cr) in Exchangeable and Reducible fractions in initial samples (soil, MSW digestate and MSW digestate amended soil). Exchangeable (acid soluble and bound to carbonates); Reducible (bound to Fe and Mn oxides); Residual + Oxidizable (bound to the soil matrix and organic matter) $\pm$ relative standard deviation (RSD) of the mean of three measurements ($n = 3$). Values associated with * are significantly different among samples, at $p < 0.05$ level of significance by Tukey's multiple comparisons test.

Metal	Sample	Exchangeable fraction (%) $\pm$ RSD (%)	Reducible fraction (%) $\pm$ RSD (%)
Fe	Soil	0.04 ± 2.94	0.57 ± 15.36
	MSW digestate	5.1 ± 9.36	55.28 ± 9.14
	MSW digestate amended soil	46.2 ± 8.42 *	10.57 ± 12.76 *
Mn	Soil	5.52 ± 1.12	83.78 ± 87.68
	MSW digestate	30.41 ± 9.21	22.59 ± 10.35
	MSW digestate amended soil	56.53 ± 8.28 *	31.10 ± 30.44 *
Zn	Soil	26.08 ± 30.37	17.21 ± 10.29
	MSW digestate	7.46 ± 21.17	10 ± 0.93
	MSW digestate amended soil	18.38 ± 7.44	28.11 ± 4.52 *
Pb	Soil	0.193 ± 7.33	5.06 ± 2.04
	MSW digestate	0.042 ± 3.064	0.710 ± 0.20
	MSW digestate amended soil	0.025 ± 2.99	0.81 ± 0.11
Cu	Soil	2.58 ± 7.41	2.63 ± 18.84
	MSW digestate	0.36 ± 52.92	8.90 ± 1.50
	MSW digestate amended soil	0.147 ± 9.10	8.26 ± 16.46
Cr	Soil	0.60 ± 2.77	0.83 ± 5.38
	MSW digestate	0.29 ± 14.89	14.16 ± 11.53
	MSW digestate amended soil	0.45 ± 30.16	8.82 ± 36.43

Table S5. Pearson correlation coefficients among MSW digestate borne TMs (Zn, Cu, Cr, Pb, Ni, Fe and Mn) and between TMs and pH, in soil columns without plant (treatment T0)

	Pb	Mn	Zn	Ni	Cu	Fe	Cr	Time	pH	Depth	<i>P.alopecuroides</i>	<i>P.virgatum</i>
Pb	1.00	0.89 *	0.80 *	0.86 *	0.82 *	-0.88 *	0.41 *	0.01	-0.40 *	-0.84 *	0.01	-0.06
Mn		1.00	0.78 *	0.83 *	0.80 *	-0.84 *	0.48 *	0.06	-0.30 *	-0.80 *	-0.12	-0.04
Zn			1.00	0.74 *	0.80 *	-0.78 *	0.38 *	0.21	-0.41 *	-0.69 *	-0.07	-0.01
Ni				1.00	0.84 *	-0.73 *	0.49 *	-0.08	-0.16	-0.76 *	-0.08	-0.16
Cu					1.00	-0.72 *	0.59 *	-0.08	-0.21	-0.68 *	-0.19	-0.01
Fe						1.00	-0.37 *	-0.16	0.49 *	0.80 *	-0.09	0.01
Cr							1.00	-0.18	0.02	-0.42 *	-0.18	0.07
Time								1.00	-0.39 *	0.00	0.00	0.00
pH									1.00	0.40 *	-0.14	-0.16
Depth										1.00	0.00	0.00
<i>P. alopecuroides</i>											1.00	-0.50
<i>P. virgatum</i>												1.00

*Correlation is significant at 0.05 level

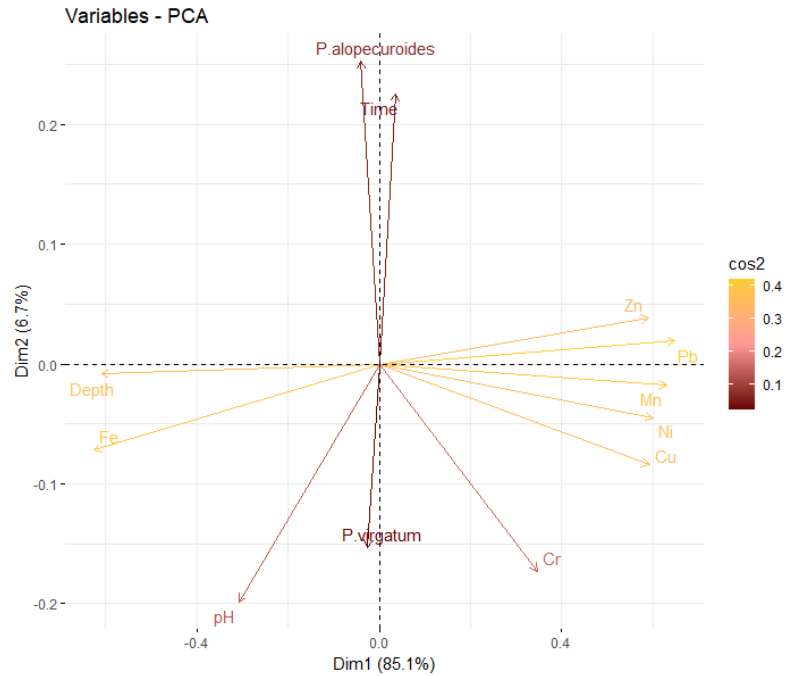
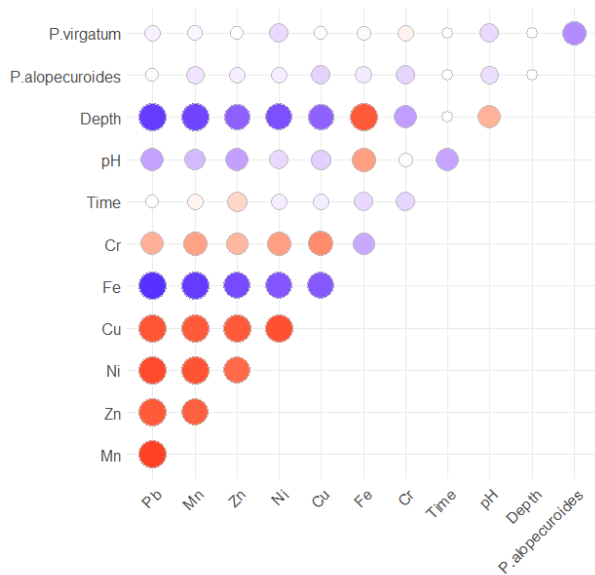


Figure S3. (A) Pearson correlation matrix, (B) principal component analysis (PCA) biplot (yellow: high $\cos^2$; pink: mid $\cos^2$, dark red: low $\cos^2$) among MSW digestate borne TMs (Zn, Cu, Cr, Pb, Ni, Fe and Mn) and between TMs and pH, in soil columns.

Appendix II. This appendix contains supplementary material from Chapter 3: Response of soil and plant microbiome to digestate amendment and phytomanagement of a marginal soil

Table S1: Results for the Adonis test for beta group significance with a Bray-Curtis distance matrix of topsoil samples from T0, T1 and T2 over time. The null hypothesis is that there is no interaction between our test variables and prokaryotic diversity and fungal diversity.

Variable	p-value	R ²
Prokaryotic community		
Time	< 0.01	0.81
Treatment	0.06	0.12
Fungal community		
Time	< 0.05	0.70
Treatment	0.05	0.24

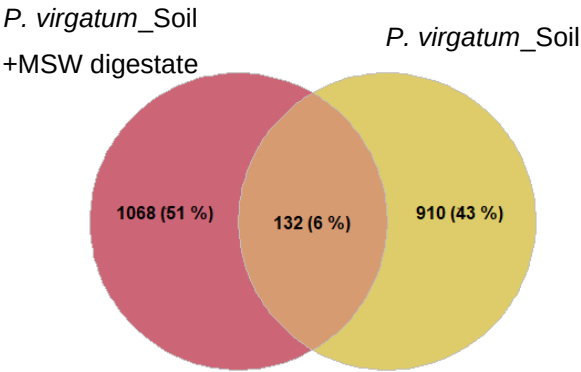
Table S2: Results for the Adonis test for beta group significance with a Bray-Curtis distance matrix of root microbiome from *P. virgatum* and *P. alopecuroides*. The null hypothesis is that there is no interaction between our test variables and prokaryotic diversity and fungal diversity.

Variable	<i>p</i> -value	R ²
Prokaryotic community		
Plant	0.07	0.34
MSW digestate presence	0.08	0.42
Root compartment	0.06	0.17
Fungal community		
Plant	0.05	0.62
MSW digestate presence	0.05	0.78
Root compartment	0.05	0.55

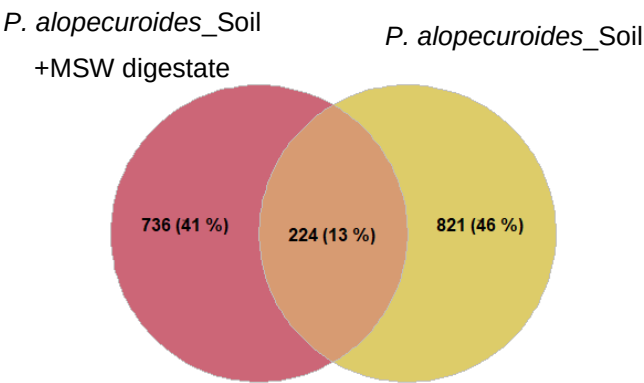
Endosphere

Prokaryotic community

A

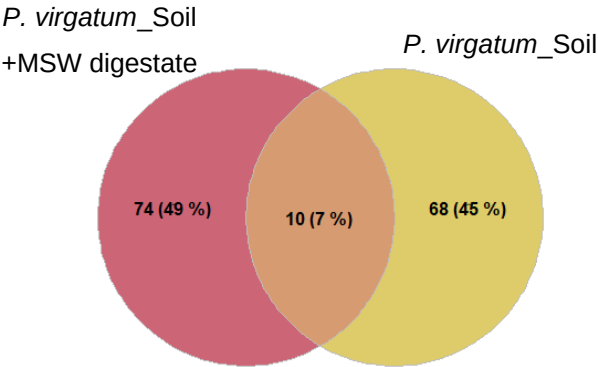


B



Fungal community

C



D

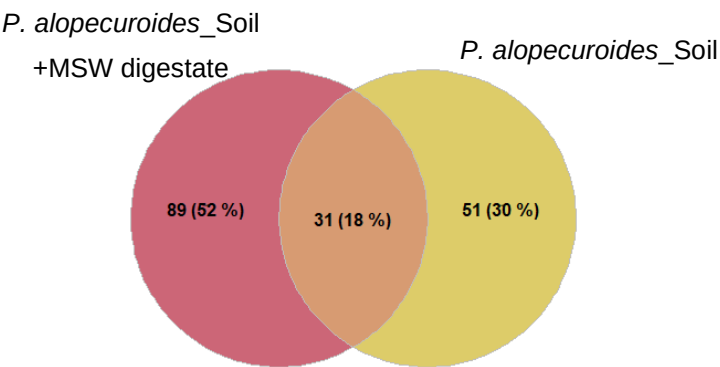
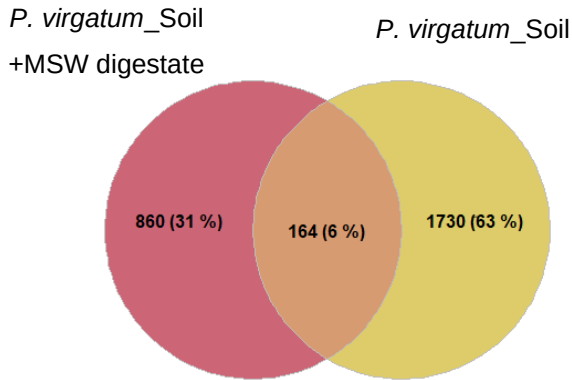


Figure S1. Shared core 16S v4 ASVs. Venn diagram showing the number and percentage of shared and unique prokaryotic ASVs (A and B) and fungal ASVs (C and D) in the endosphere of *P. virgatum* grown with MSW-digestate and without MSW-digestate and *P. alopecuroides* grown with MSW-digestate and without MSW-digestate.

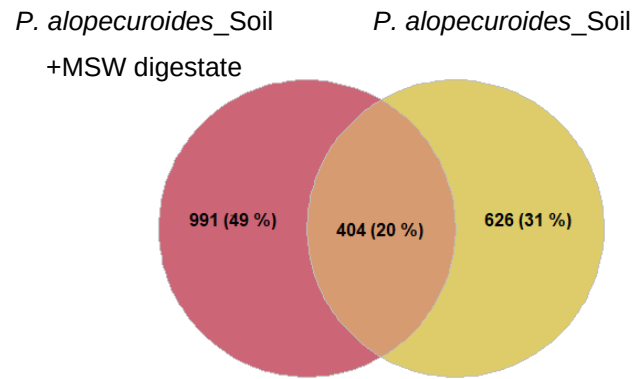
Episphere

Prokaryotic community

A

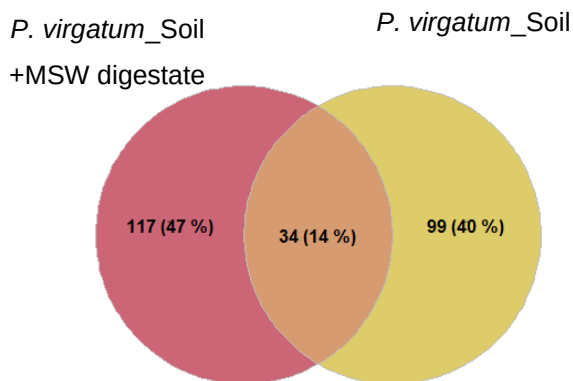


B



C

Fungal community



D

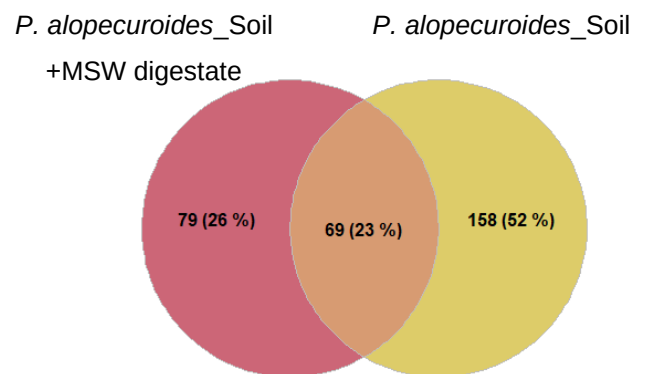
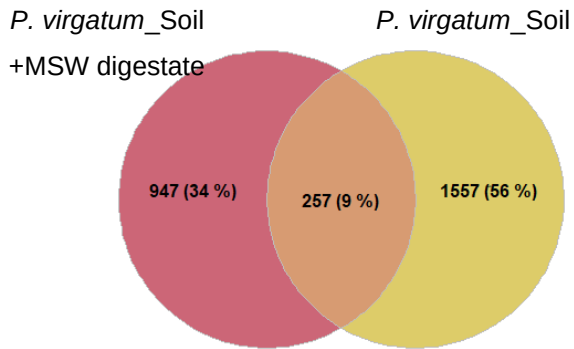


Figure S2. Shared core 16S v4 ASVs. Venn diagram showing the number and percentage of shared and unique prokaryotic ASVs (A and B) and fungal ASVs (C and D) in the episphere of *P. virgatum* grown with MSW-digestate and without MSW-digestate and *P. alopecuroides* grown with MSW-digestate and without MSW-digestate.

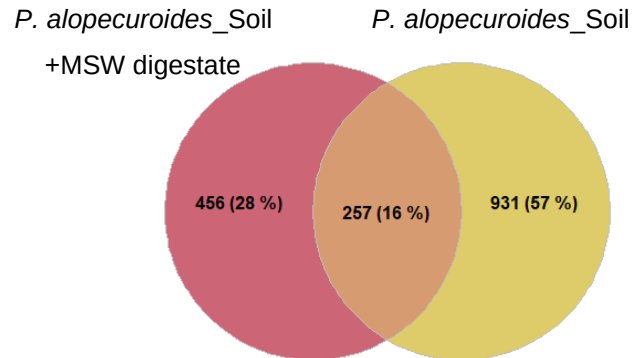
Rhizosphere

Prokaryotic community

A

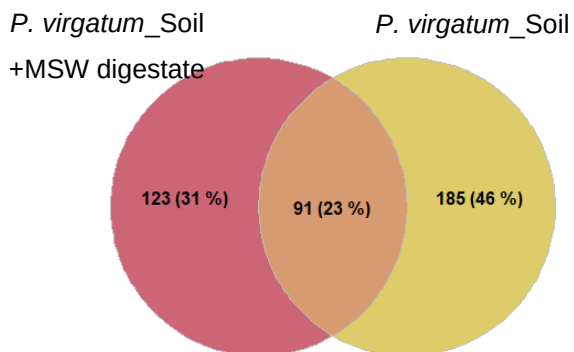


B



C

Fungal community



D

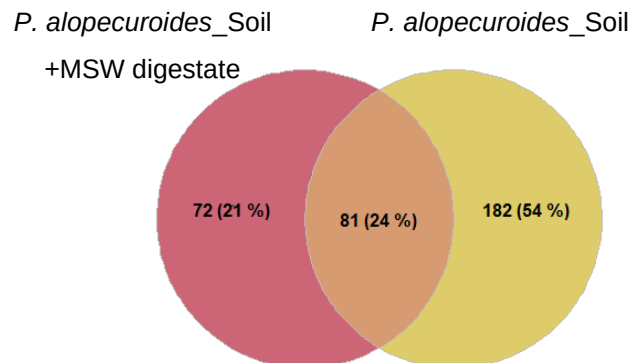


Figure S3. Shared core 16S v4 ASVs. Venn diagram showing the number and percentage of shared and unique prokaryotic ASVs (A and B) and fungal ASVs (C and D) in the rhizosphere of *P. virgatum* grown with MSW-digestate and without MSW-digestate and *P. alopecuroides* grown with MSW-digestate and without MSW-digestate.

Appendix III. This appendix contains supplementary material from Chapter 4: Trace metal mobility in soil-water-plant system with municipal sewage sludge digestate amendment. Experimental assessment of methodological approaches

Table S1. Overview of the chemical properties of initial samples (soil, SSAD and SSAD + soil 1:3 v/v).

Parameter	Initial Soil	Initial SSAD	Initial SSAD + soil (1:3 v/v)
Total Nitrogen (TN) (mg · g ⁻¹ dry matter)	<0.1	4.8 ± 0.3	0.15 ± 0.04
Total Hydrogen (TH) (mg · g ⁻¹ dry matter)	0.60 ± 0.06	4.5 ± 0.2	0.59 ± 0.07
Total Sulphur (TS) (mg · g ⁻¹ dry matter)	<0.1	0.73 ± 0.04	0.13 ± 0.03
Total organic carbon (TOC) (mg · g ⁻¹ dry matter)	0.520 ± 0.003	10.63 ± 0.2	2.9 ± 0.2
CEC (cmol+/kg)	9.3 ± 0.1	-	16.1 ± 0.2
pH value	7.80 ± 0.05	8.8 ± 0.4	8.10 ± 0.04

TMs concentration and sequential extraction fractionation in initial solid samples

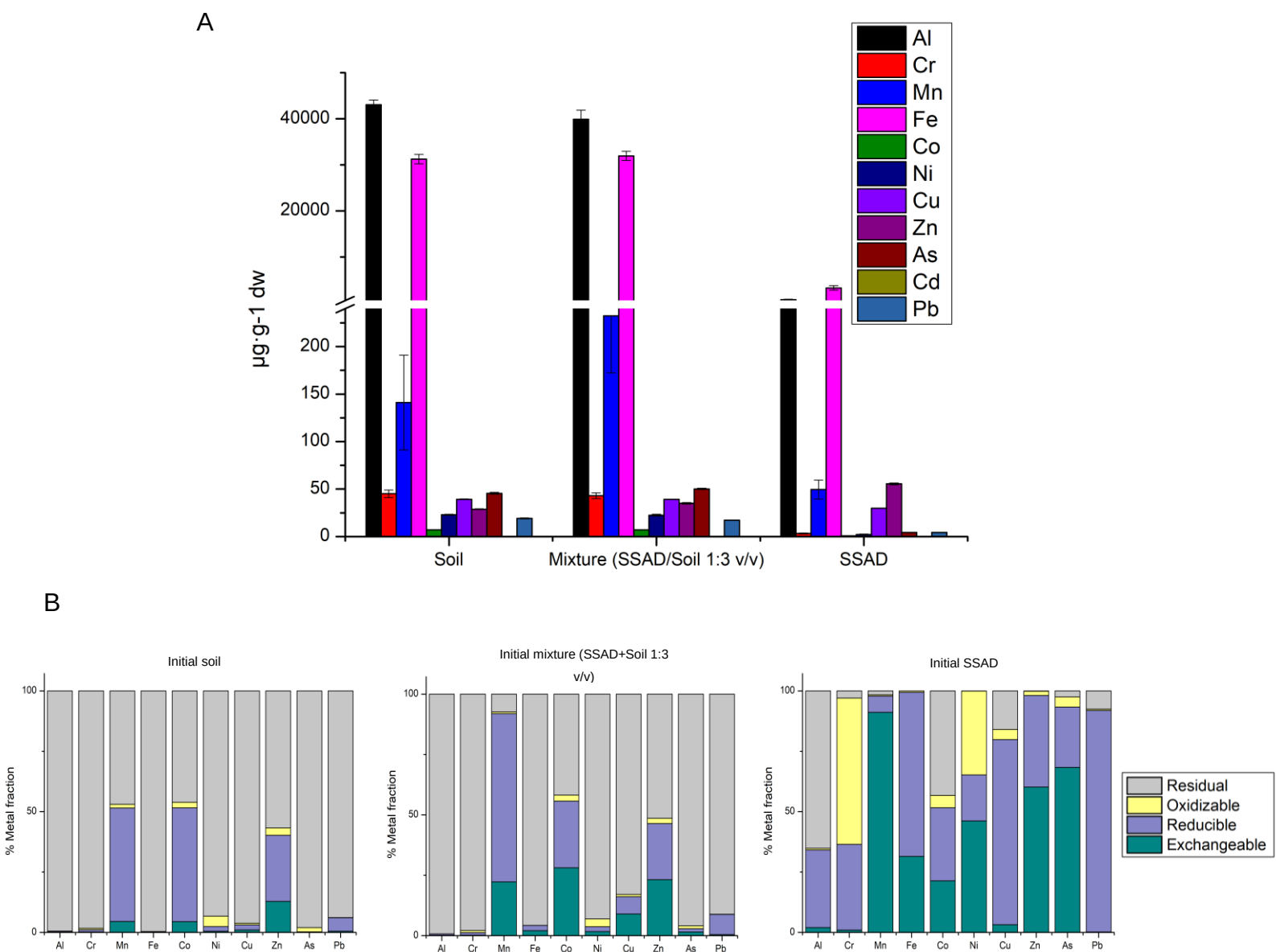


Figure S1. (A) TMs (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As and Pb) total concentration and (B) sequential extraction fractionation (Exchangeable, Reducible, Oxidizable and Residual) in initial samples (Soil, mixture (SSAD+ Soil) and SSAD). Error bars indicate standard error of the mean of three measurements (n = 3).

DOM composition and anions and cations concentrations in C1, C2 and C3 over time and depth (top, middle and bottom layers)

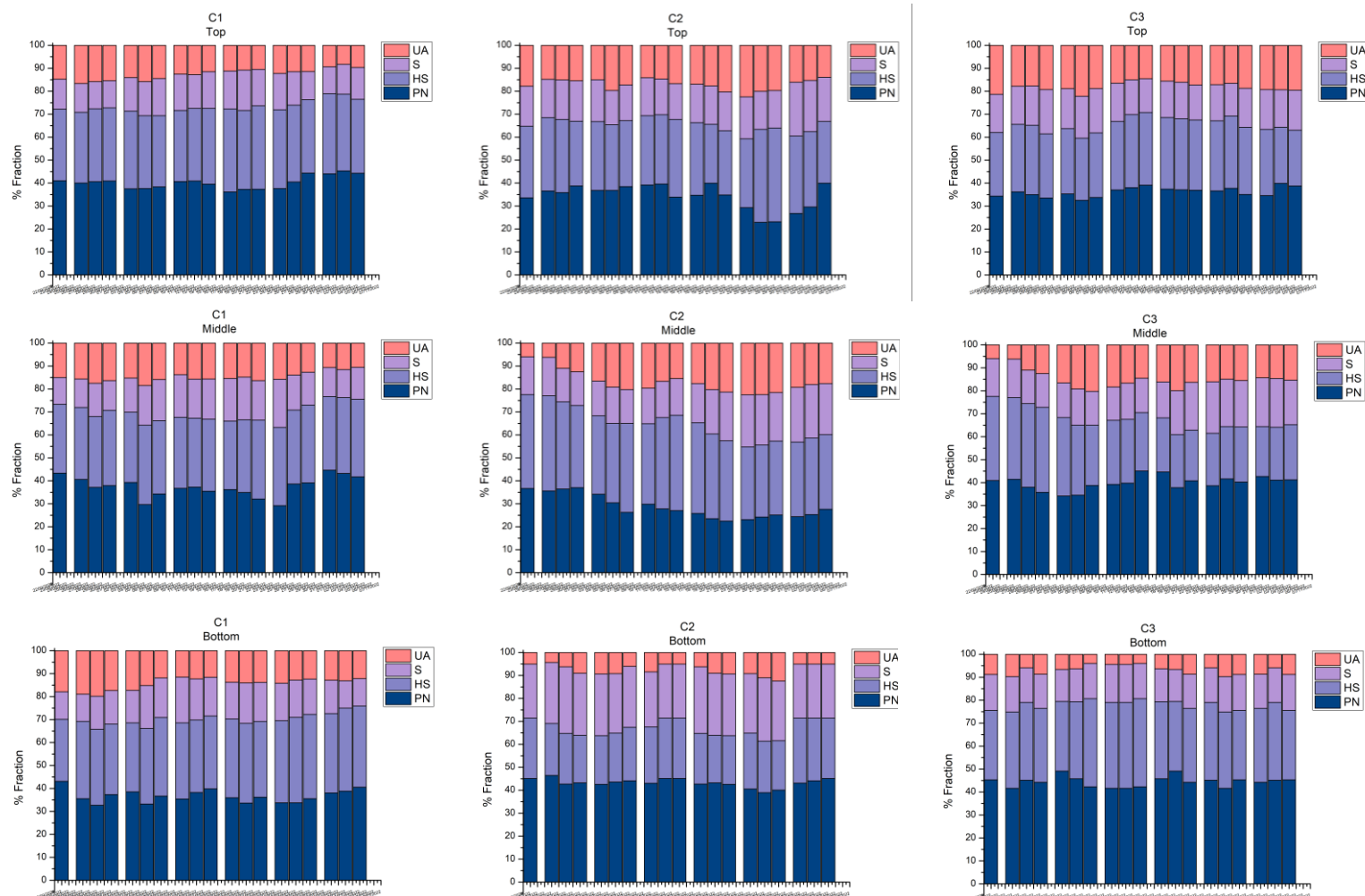
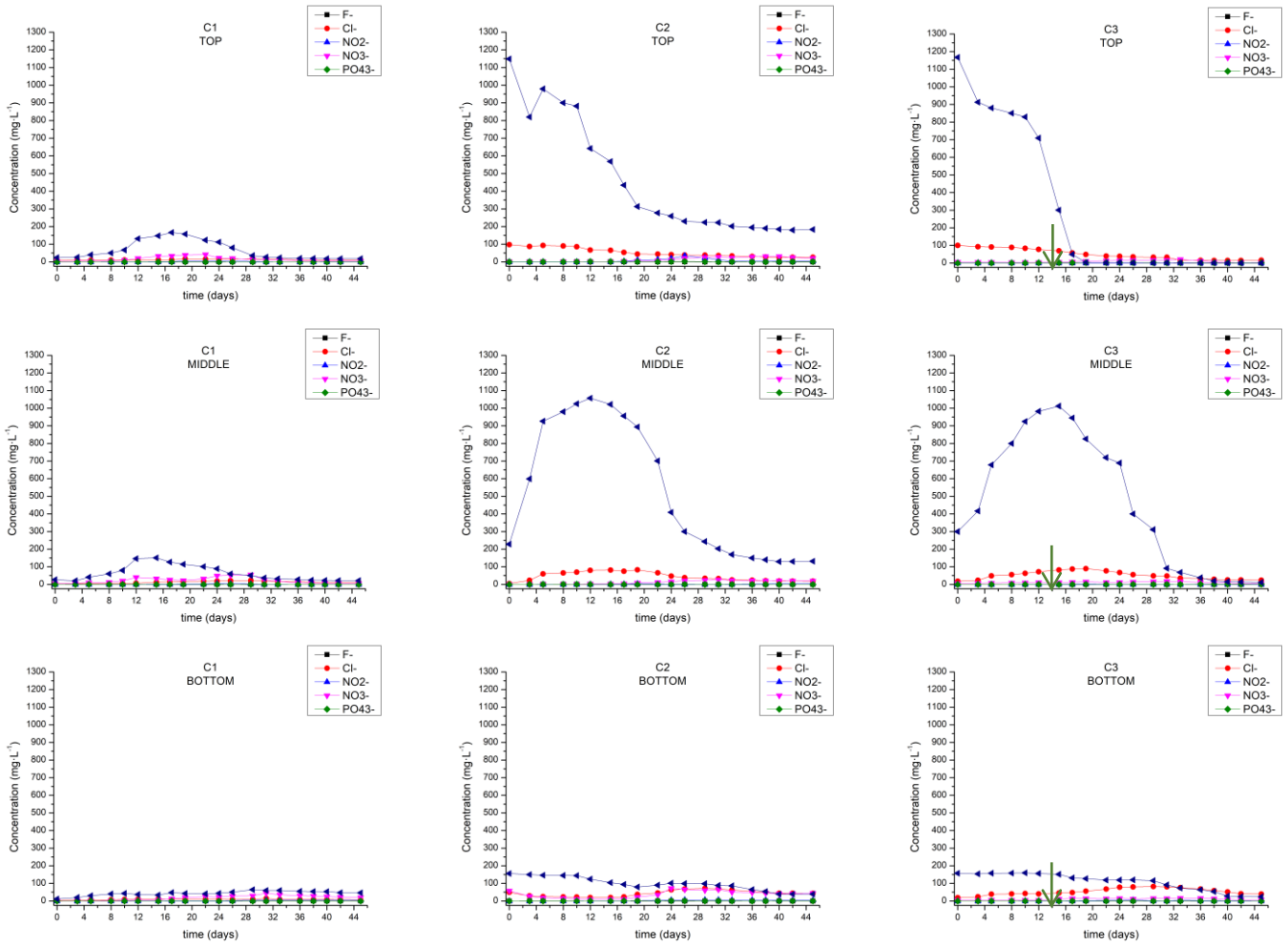


Figure S2. Proteins (PN), Humic Substances (HS), Sugars (S), Uronic Acids (UA) fractionation in soil solution of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + plant) over time (from day 0 to day 45) and depth (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers).

Anions

Phytoremediation of contaminated soils aiming metal recovery – including microorganisms' role and bioaugmentation possibilities



Cations

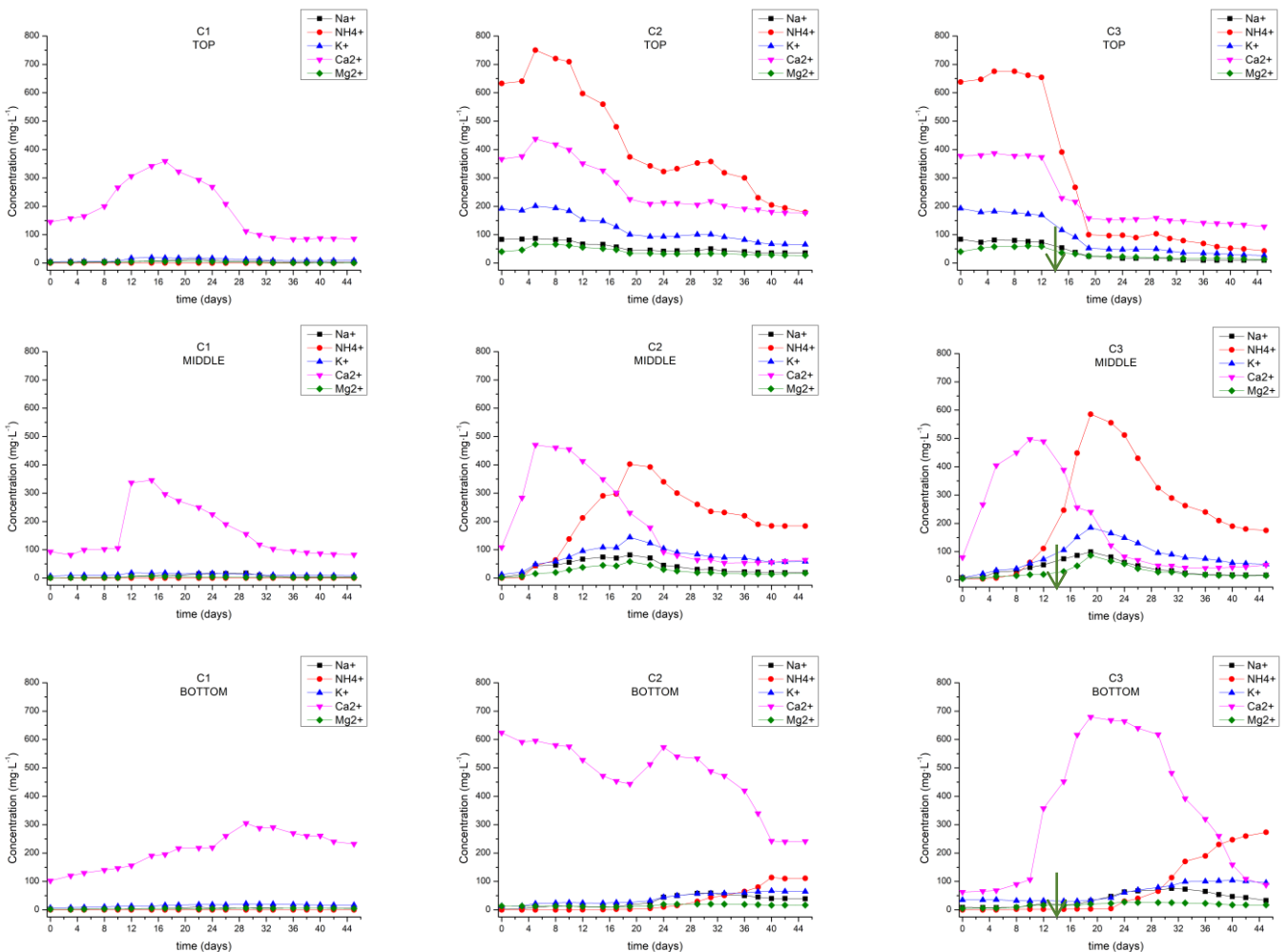
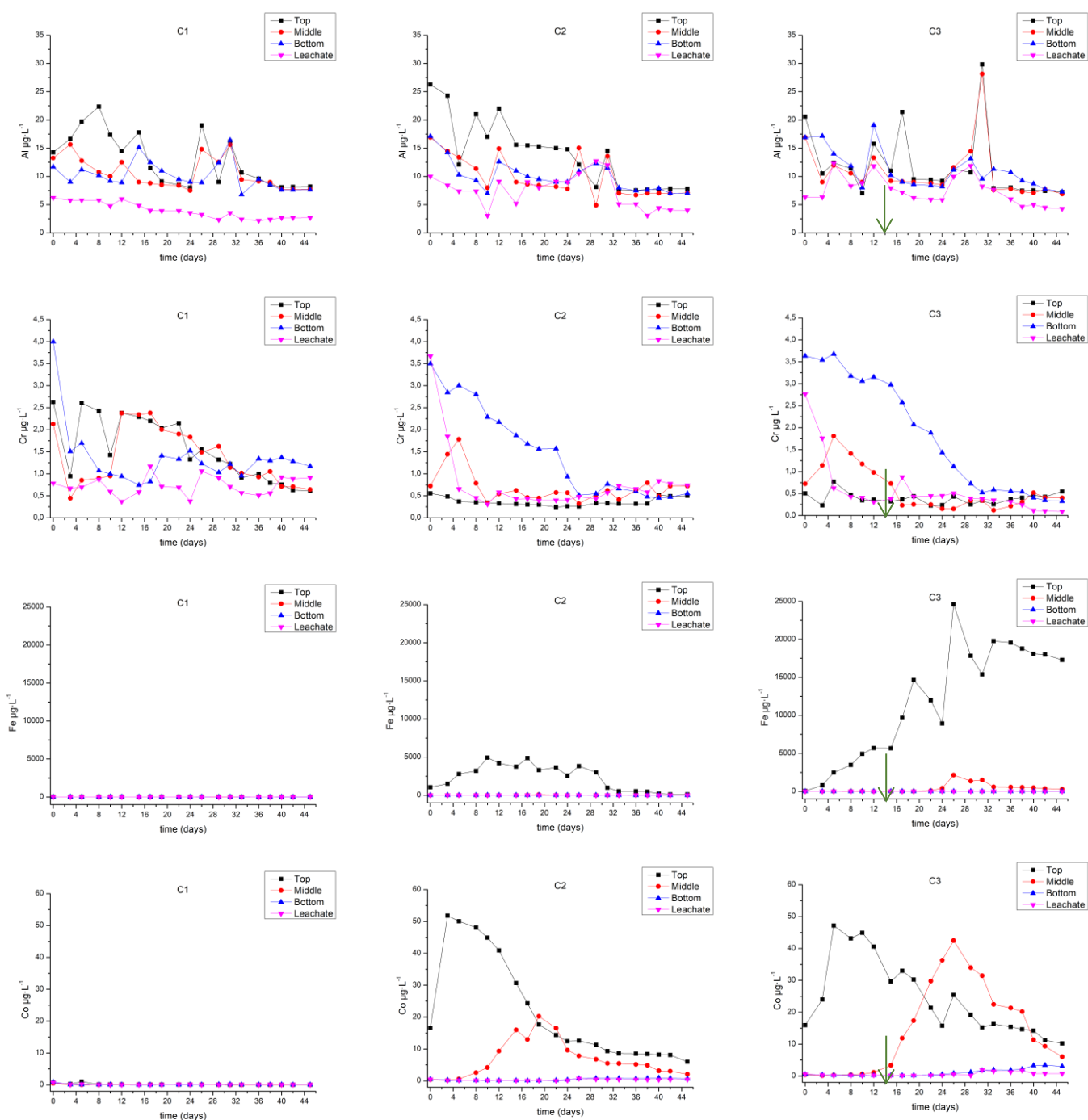


Figure S3. Concentrations of anions and cations in soil solution of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + plant) over time (from day 0 to day 45) and depth (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers). (n=1).



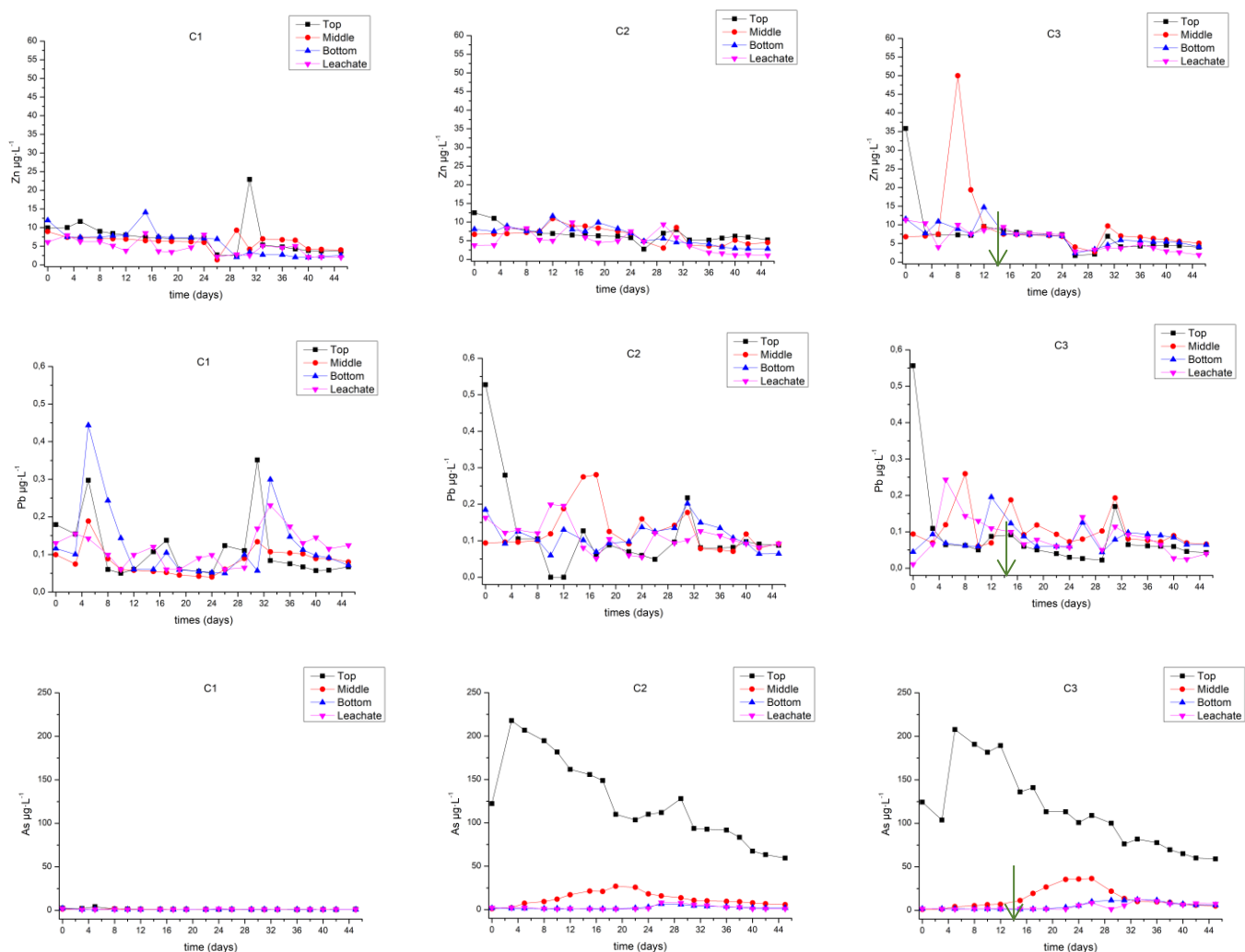


Figure S4. Concentrations of Al, Cr, Fe, Co, Zn, As and Pb in soil solution of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + plant) over time (from day 0 to day 45) and depth (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers). (n=1).

SOM fractionation at day 45

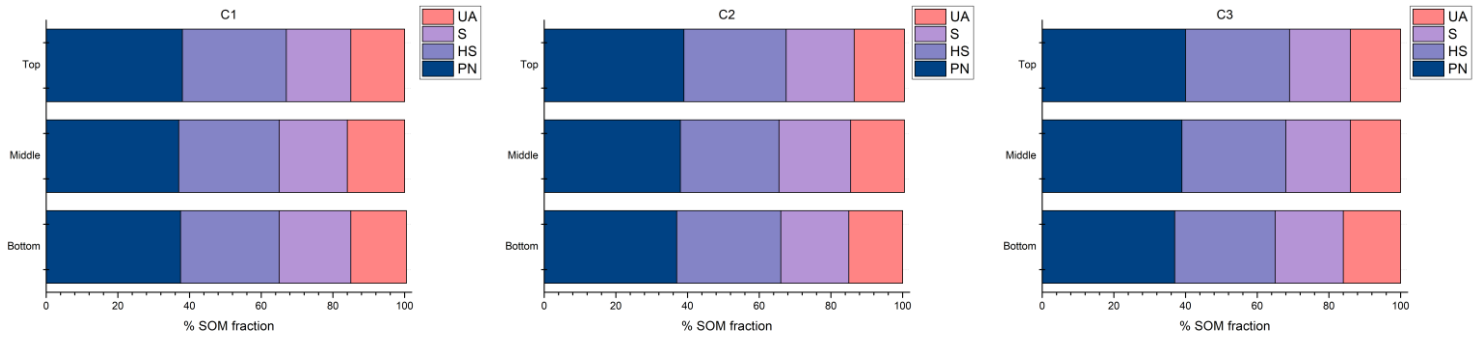
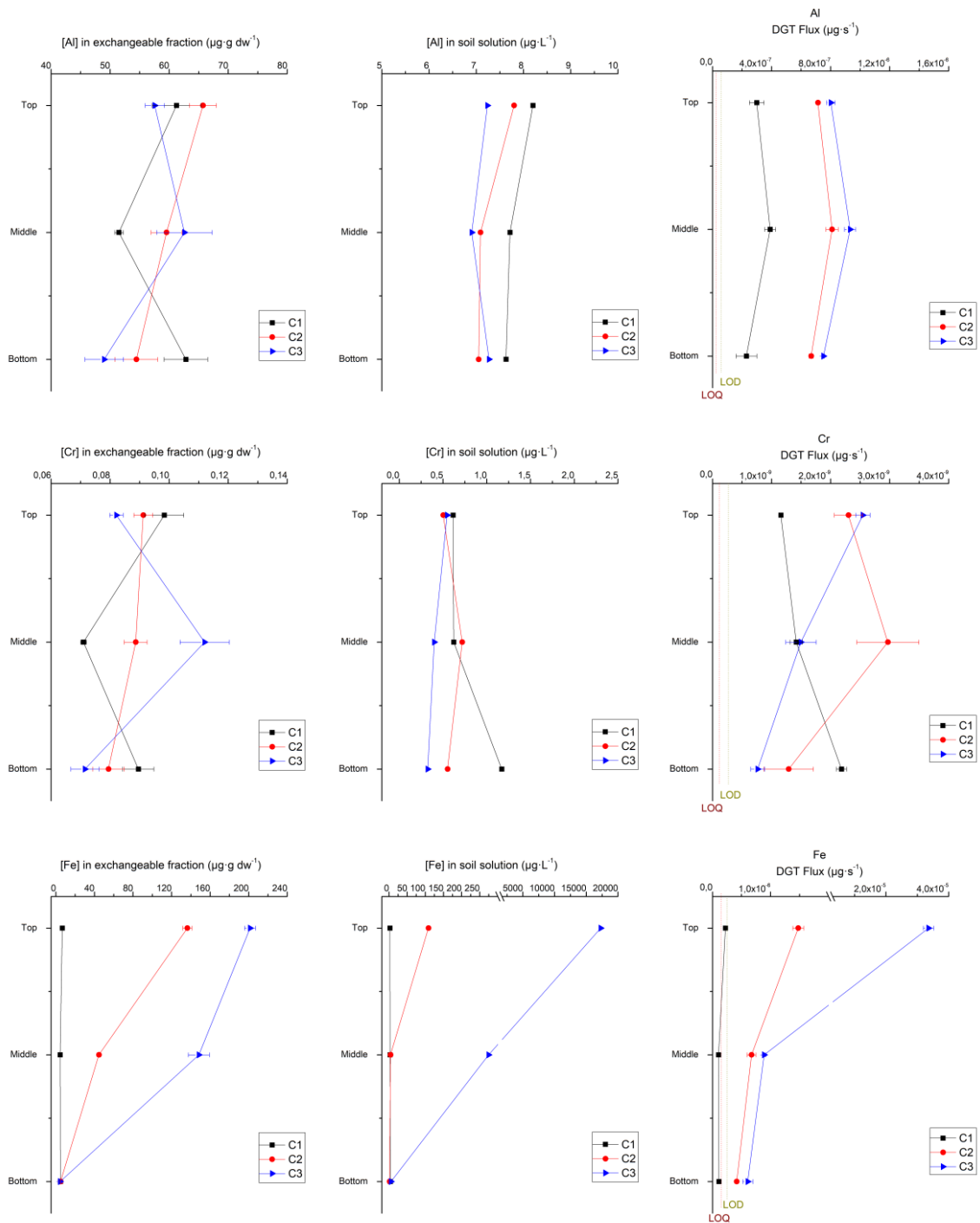


Figure S5. SOM fractionation (Proteins (PN), Humic Substances (HS), Sugars (S), Uronic Acids (UA)) in C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + plant) over depth (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers) at day 45. (n=3).

Comparison of TMs' (Al, Cr, Fe, Co, Cu, As and Pb) (i) concentration in exchangeable fraction (ii) concentration in soil solution and (iii) DGT fluxes at day 45



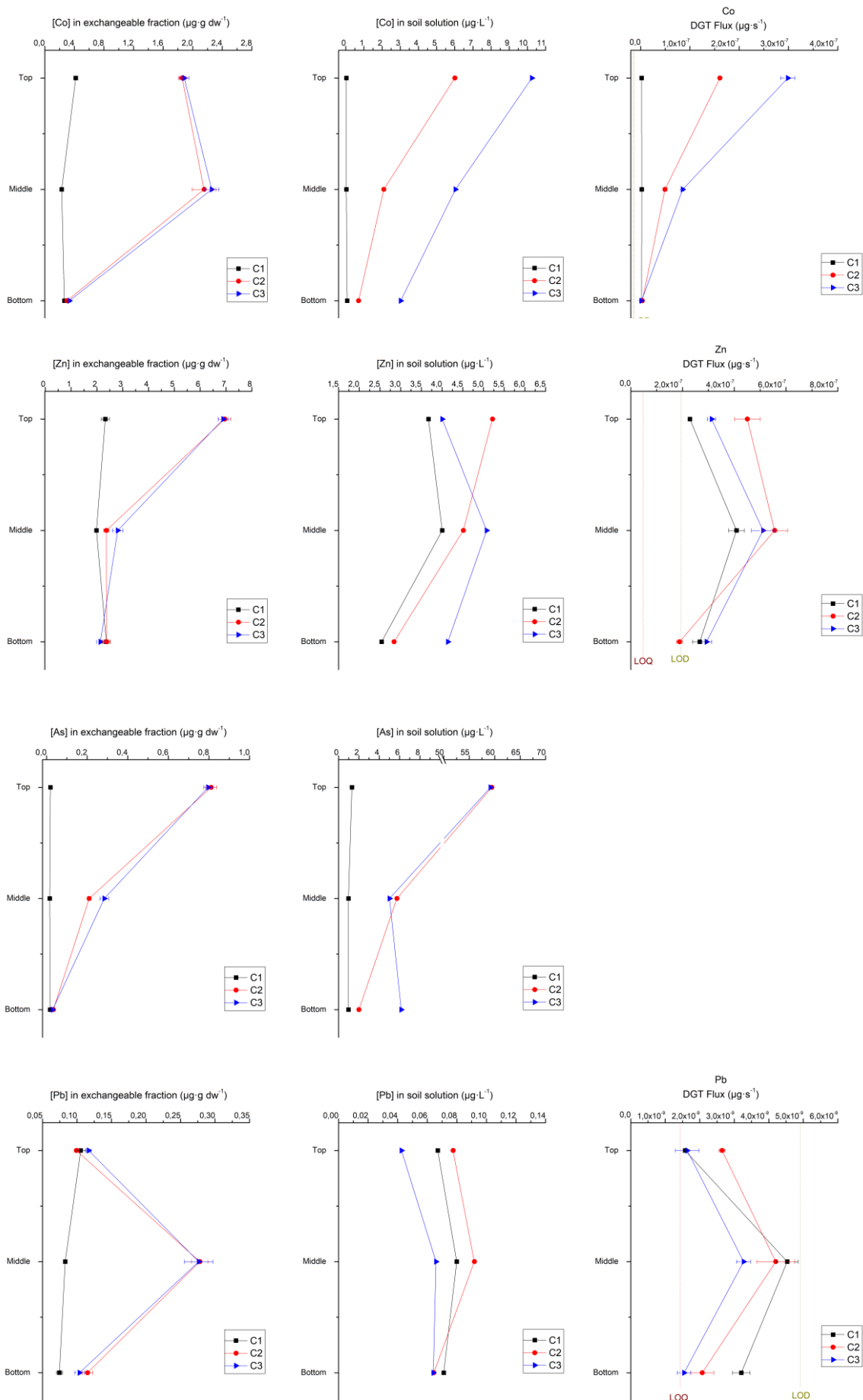


Figure S6. Comparison of TMs' (Al, Cr, Fe, Co, Zn, As and Pb) (i) concentration in exchangeable fraction (ii) concentration in soil solution and (iii) DGT fluxes, over depth (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers) of C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD-amended column + plant) at the end of the experimental period (day 45). Error bars indicate standard error of the mean of three measurements ($n = 3$). For DGT fluxes graphs- in yellow: limit of quantification (LOQ), in red: limit of detection (LOD). DGT fluxes for As are not presented since As does not bind to Chelex binding gel.

TMs (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As and Pb) sequential extraction fractionation in C1, C2 and C3 over depth (top, middle and bottom layers) at day 45

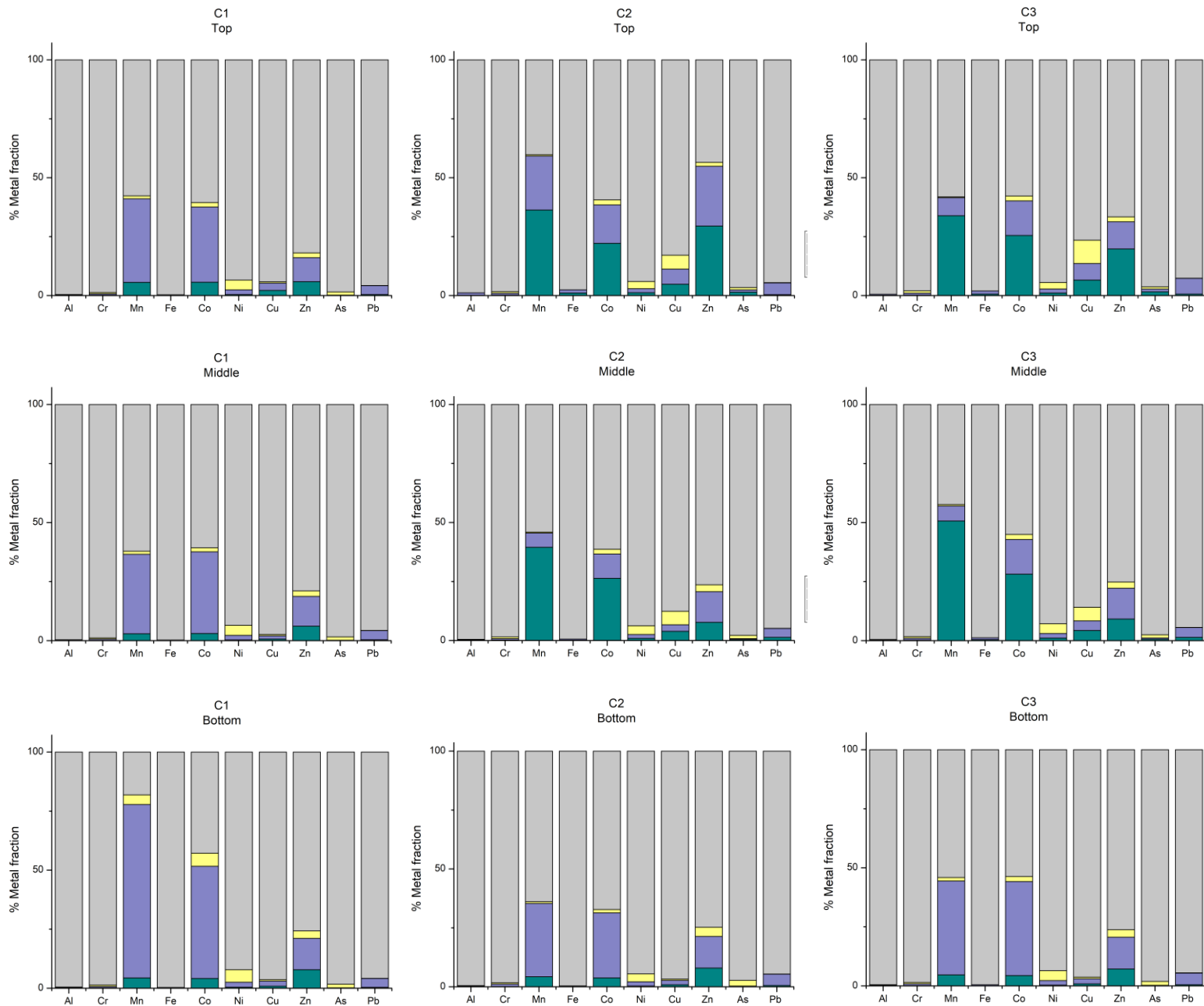


Figure S7. TMs (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As and Pb) sequential extraction fractionation in C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + plant) over depth (top (0-0.1m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers) at the end of the experiment (day 45).

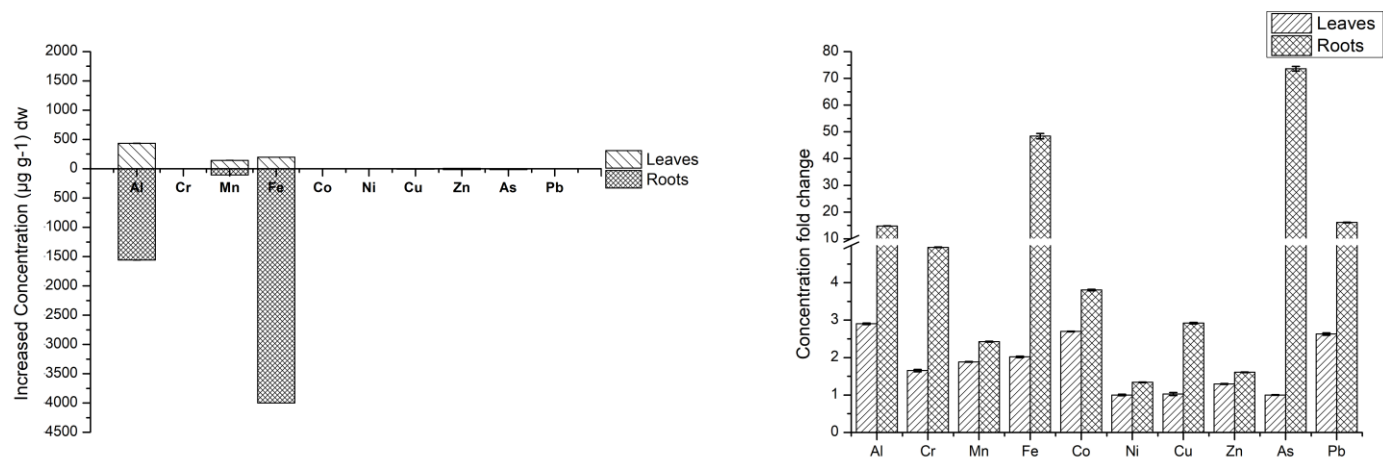


Figure S8. (A) TMs (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Pb) increased concentration ($\mu\text{g}\cdot\text{g}^{-1}$ in dry weight basis) (calculated as final concentration – initial concentration) in *P. virgatum* roots and leaves at day 45. (B) Fold change in the concentration of TMs (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Pb) (calculated as final concentration/Initial concentration) in roots and leaves of *P. virgatum* at day 45.

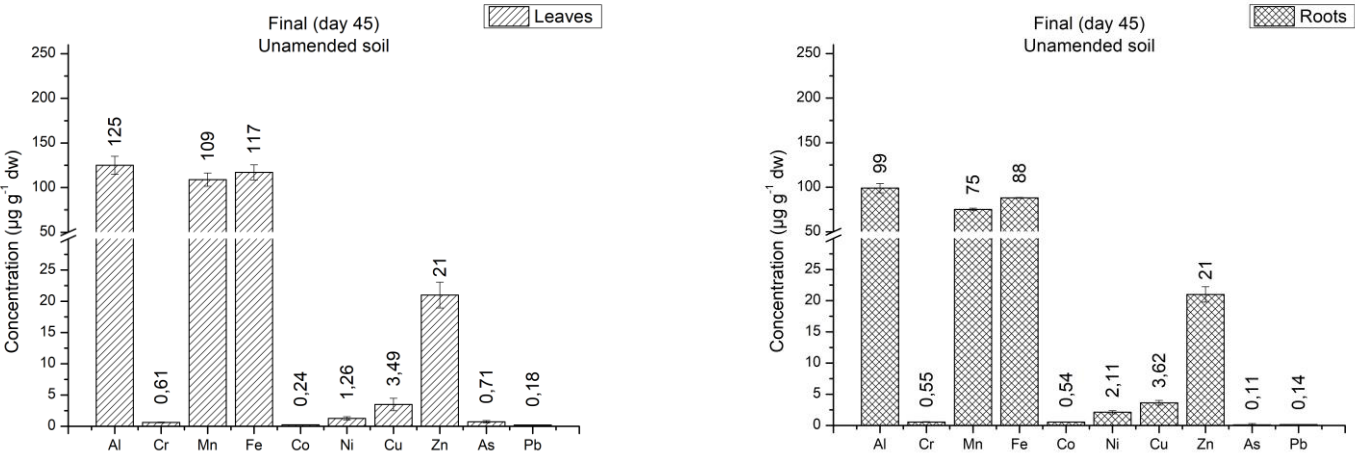


Figure S9. (A) TMs (Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Pb) iconcentration ($\mu\text{g}\cdot\text{g}^{-1}$ in dry weight basis) in roots and leaves of *P. virgatum* grown in unamended soil at day 45.

Appendix IV. This appendix contains supplementary material from Chapter 5: Evolution of OM characteristics and Cu(II)-OM complexation strength in anaerobic digestate amended soil-plant mesocosms

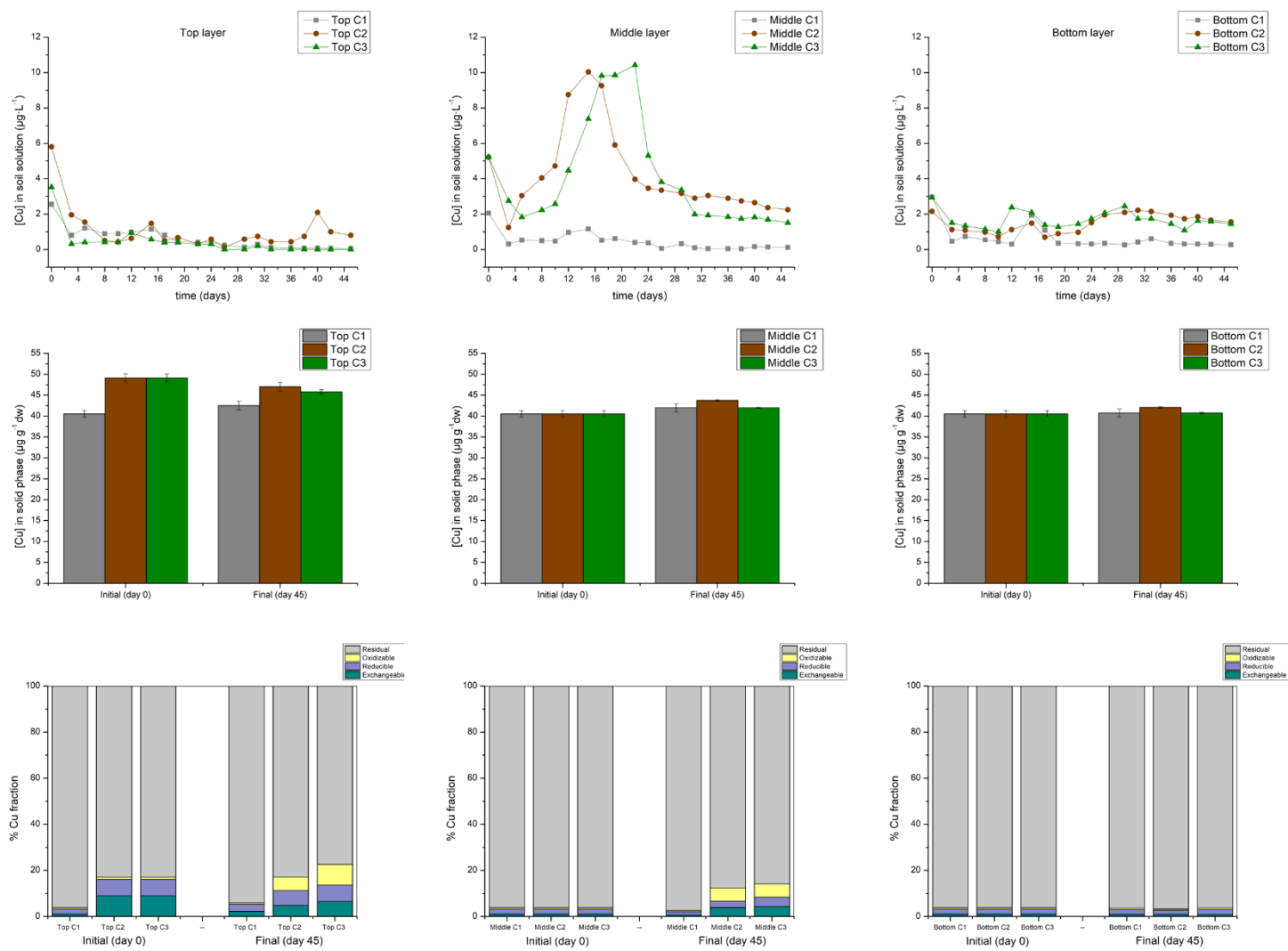


Figure S1. (A) Cu concentration in soil solution (B) Cu total concentration in soil's solid phase (C) Cu sequential extraction BCR fractionation in C1 (unamended column), C2 (SSAD amended column) and C3 (SSAD amended column + *P. virgatum*) over time (from day 0 to day 45) and depth (top (0-0.1 m), middle (0.1-0.2 m) and bottom (0.2-0.3 m) layers). Three measurements ($n = 3$) were performed for total Cu concentration and fractionation analysis. Error bars indicate the standard error of the mean of three measurements ($n = 3$). To enhance figure readability, error bars in fractionation graphs are omitted. One measurement ($n=1$) was performed for soil solution analysis.

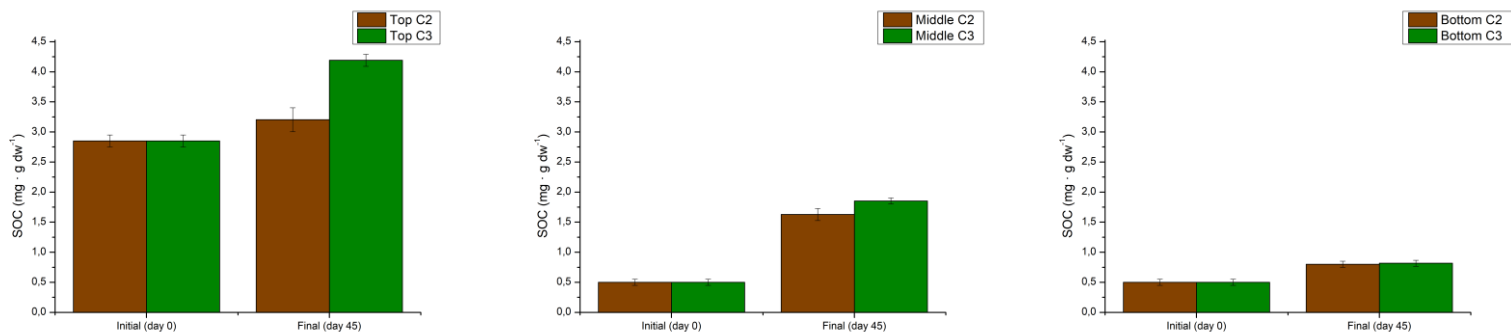


Figure S2. Soil organic carbon (SOC) concentration in top (0 – 0.1 m), middle (0.1 – 0.2 m) and bottom layer (0.2 – 0.3) of C2 and C3 on day 0 and day 45. Error bars represent the standard deviation ($n = 3$).

SSAD amended soil_day 0

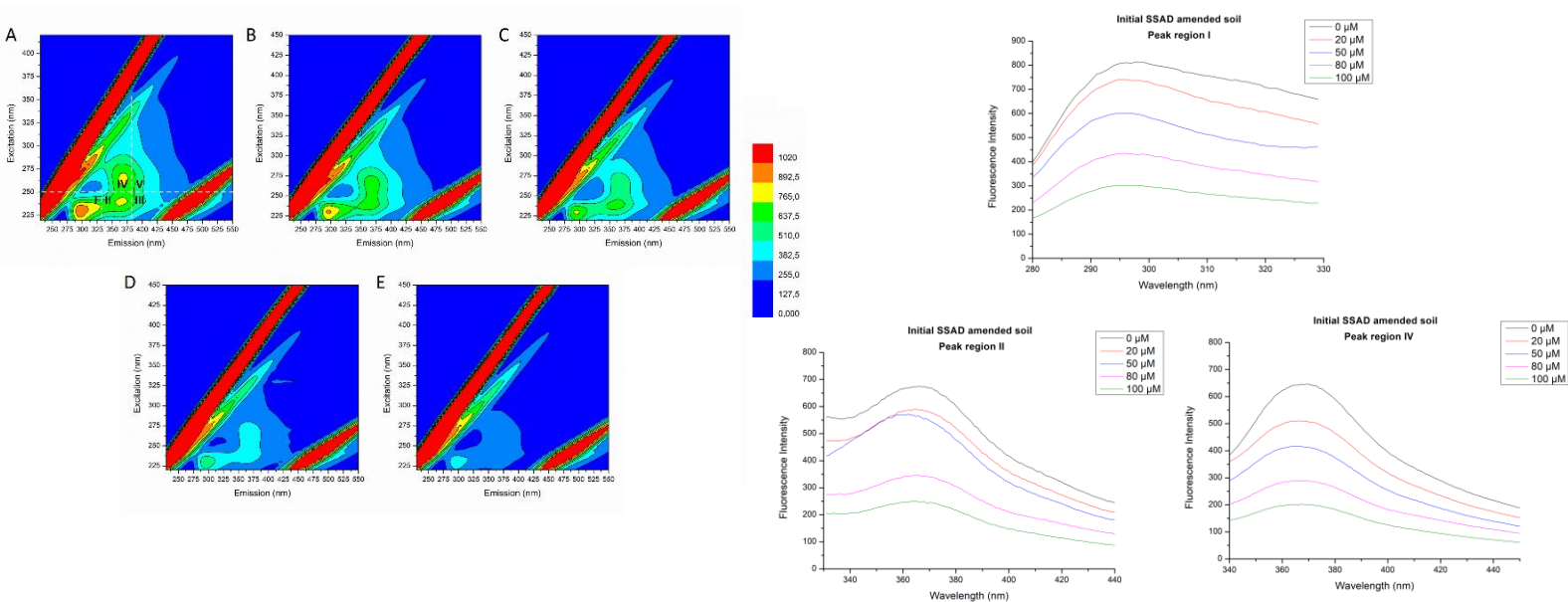
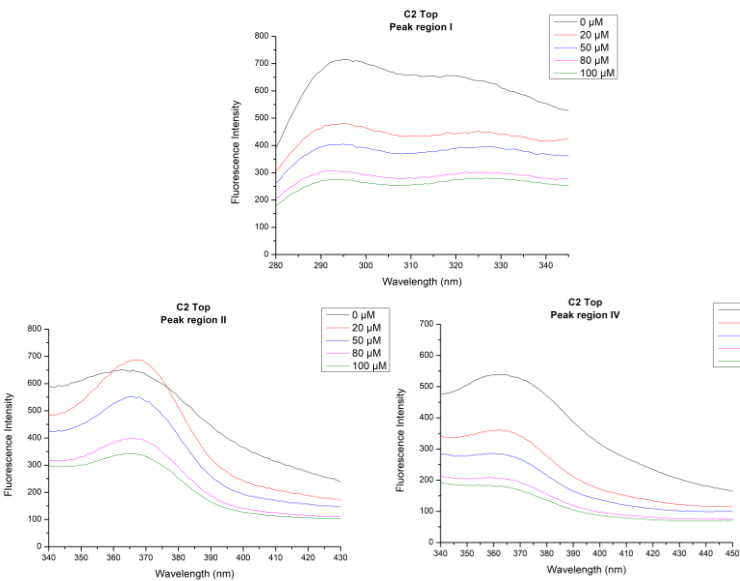
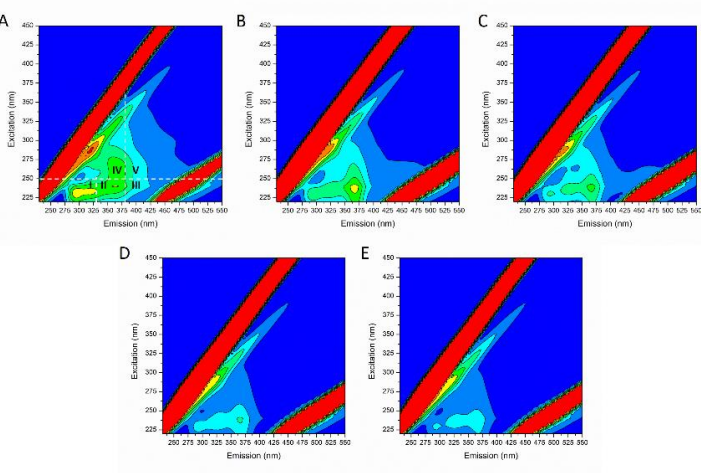


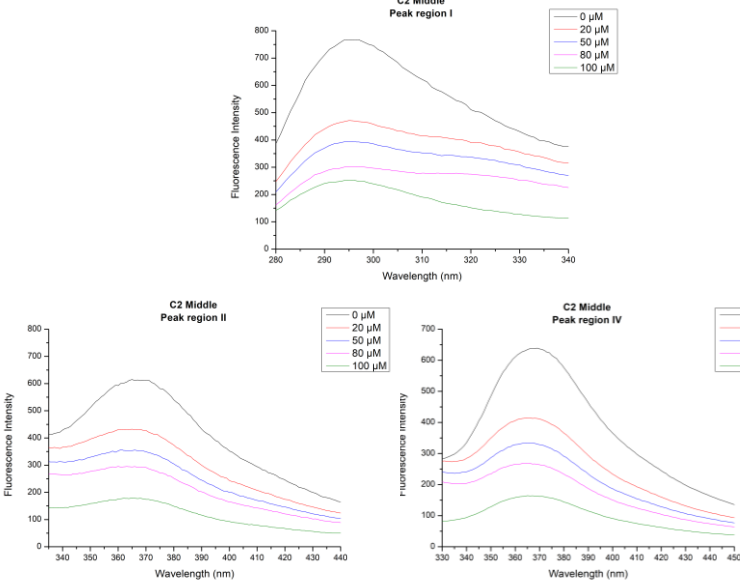
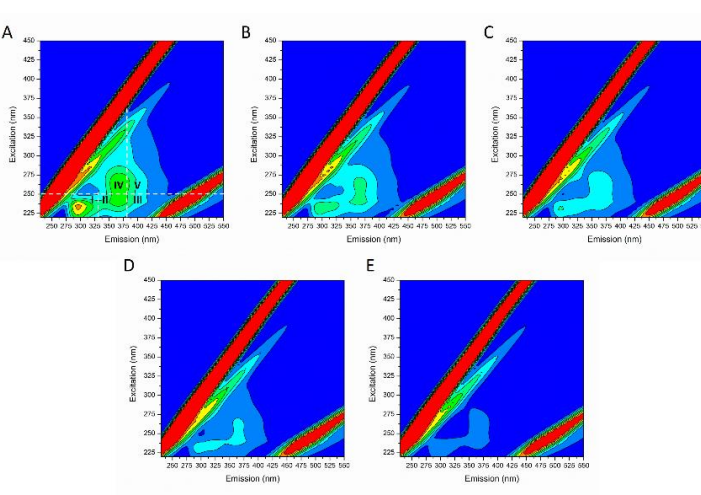
Figure S3. Left: EEM spectra of extracted OM, **right:** Fluorescence intensity of the identified peaks of the initial SSAD-amended soil at increasing added concentrations of Cu (A: 0 μM , B: 20 μM , C: 50 μM , D: 80 μM and E: 100 μM) at the beginning of the experimental period (day 0). Dilution in phosphate buffer at pH 6.5 (dilution 1/20). Incubation time: 15 min. Temperature $\sim 20 \pm 2$ $^{\circ}\text{C}$. Numbers represent the five regions defined by Chen et al. (2003): I tyrosine-like proteins, II tryptophan-like proteins, III fulvic acid-like organics, IV soluble microbial by-product-like and V humic acid-like substances.

C2_day 45

Top



Middle



Bottom

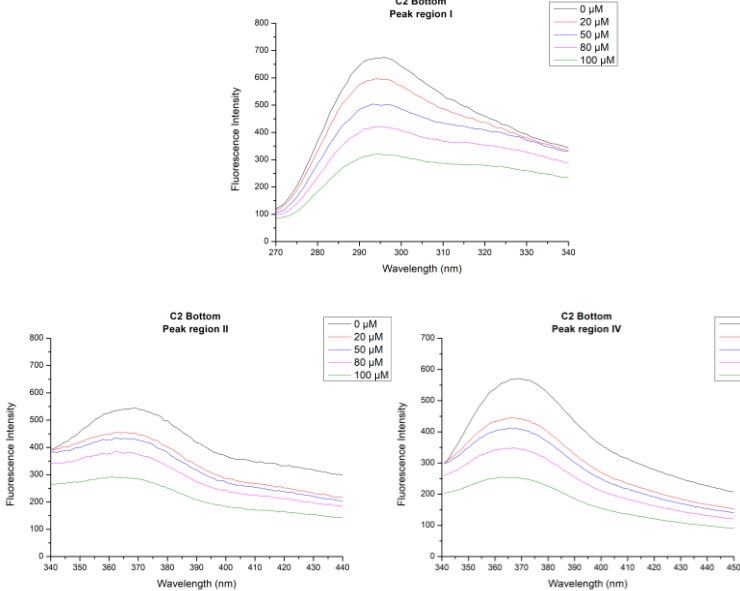
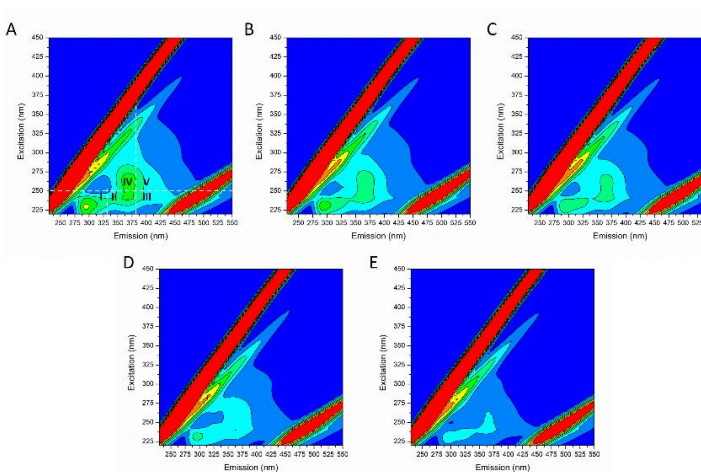
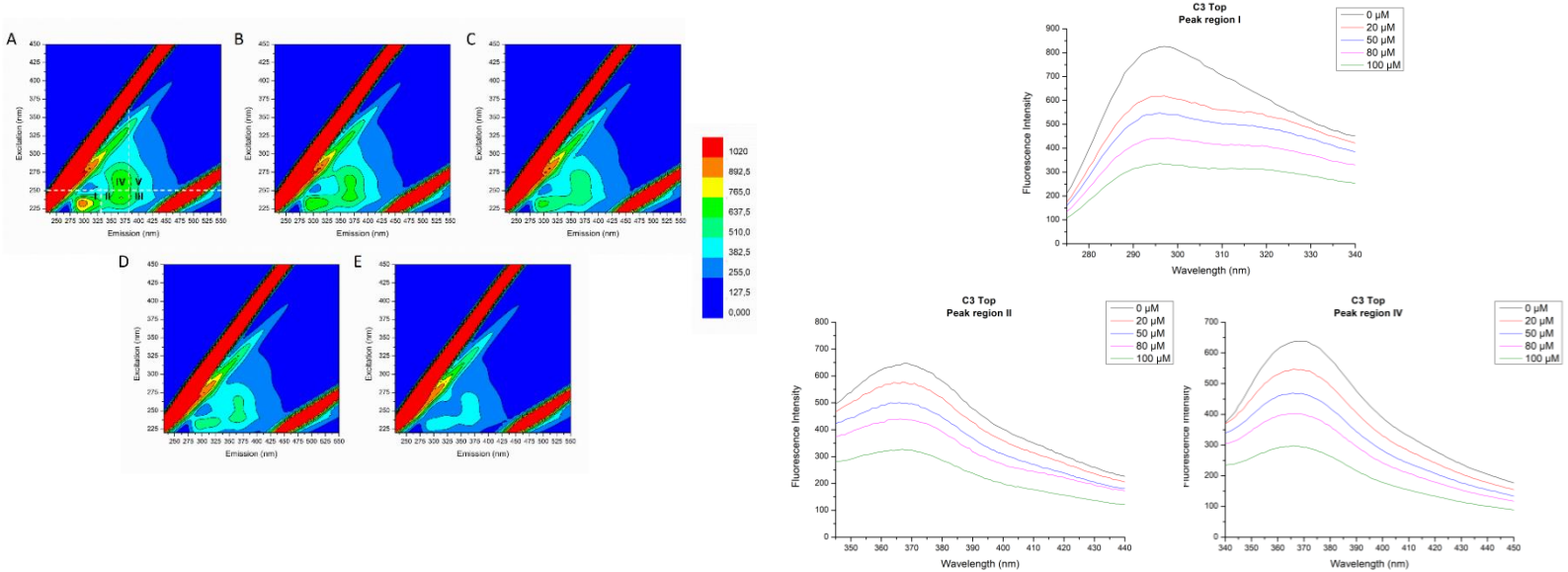


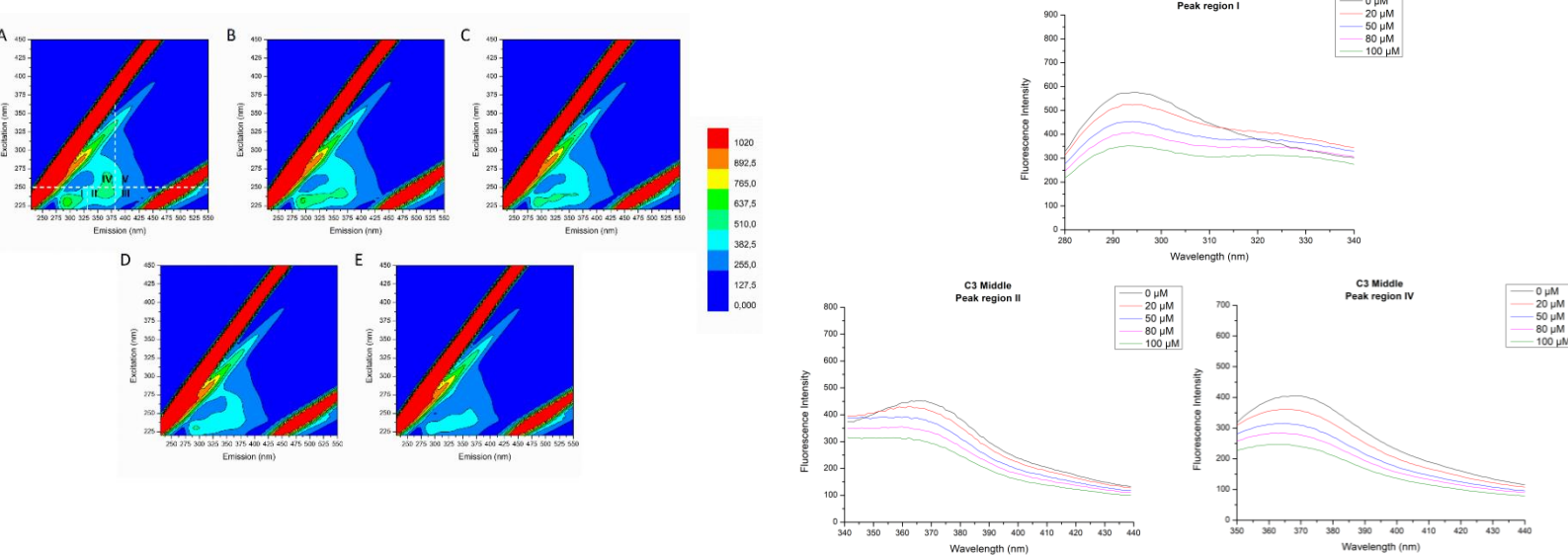
Figure S4. Left: EEM spectra of extracted OM, **right:** Fluorescence intensity of the identified peaks of the top (0 – 0.1 m), middle (0.1 – 0.2 m) and bottom (0.2 – 0.3 m) layers of C2 column (without *P. virgatum*) at increasing added concentrations of Cu (A: 0 uM, B: 20 uM, C: 50 uM, D: 80 uM and E: 100 uM) at the beginning of the experimental period (day 0). Dilution in phosphate buffer at pH 6.5 (dilution 1/20). Incubation time: 15 min. Temperature $\sim 20 \pm 2$ °C. Numbers represent the five regions defined by Chen et al. (2003): I tyrosine-like proteins, II tryptophan-like proteins, III fulvic acid-like organics, IV soluble microbial by-product-like and V humic acid-like substances.

C3_day 45

Top



Middle



Bottom

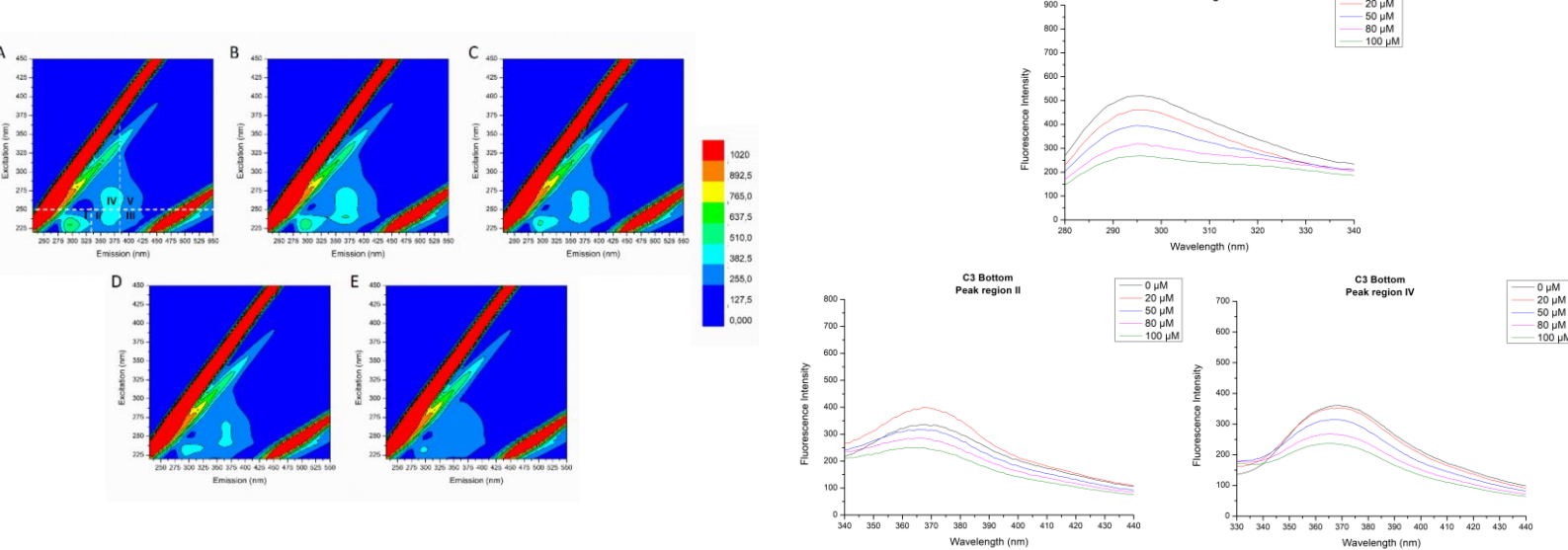
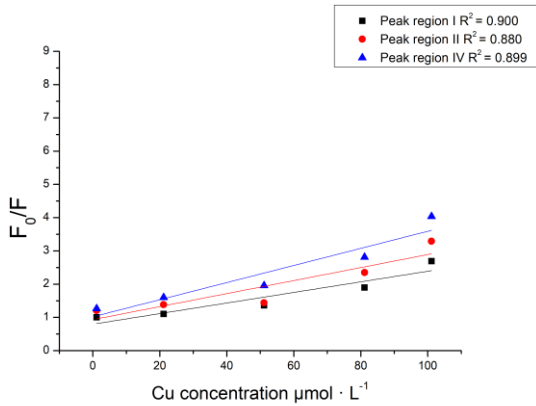
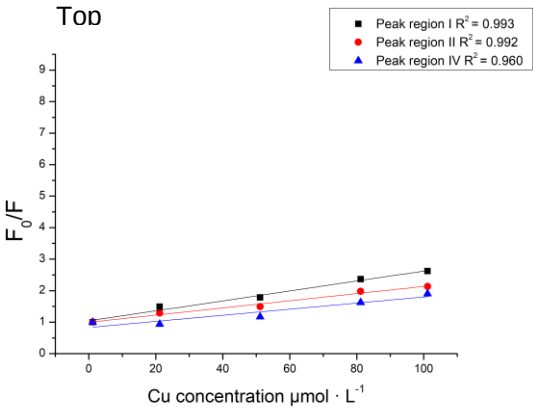


Figure S5. Left: EEM spectra of extracted OM, **right:** Fluorescence intensity of the identified peaks of the top (0 – 0.1 m), middle (0.1 – 0.2 m) and bottom (0.2 – 0.3 m) layers of C3 column (with *P. virgatum*) at increasing added concentrations of Cu (A: 0 uM, B: 20 uM, C: 50 uM, D: 80 uM and E: 100 uM) at the beginning of the experimental period (day 0). Dilution in phosphate buffer at pH 6.5 (dilution 1/20). Incubation time: 15 min. Temperature $\sim 20 \pm 2$ °C. Numbers represent the five regions defined by Chen et al. (2003): I tyrosine-like proteins, II tryptophan-like proteins, III fulvic acid-like organics, IV soluble microbial by-product-like and V humic acid-like substances.

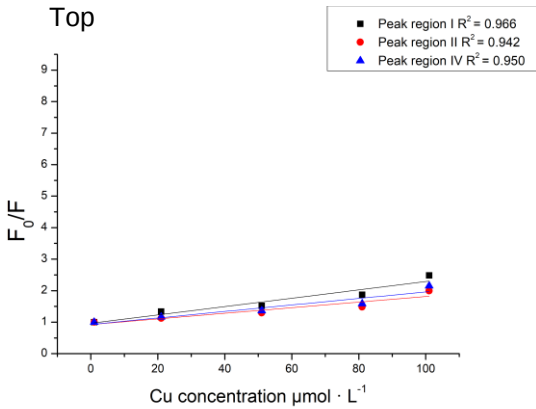
SSAD amended soil_day 0



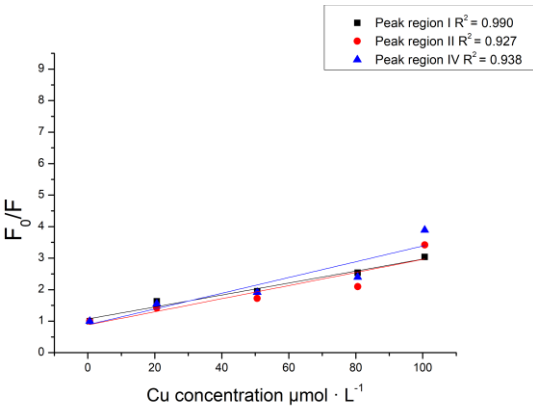
C2_day 45



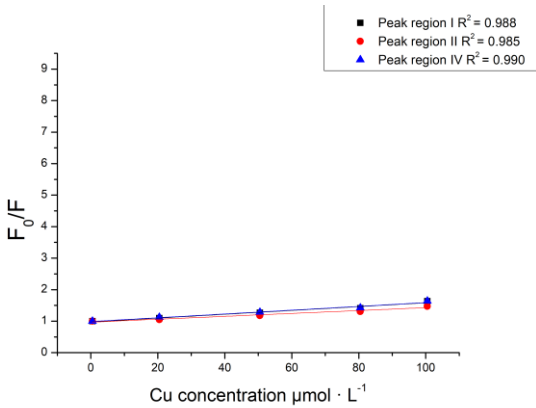
C3_day 45



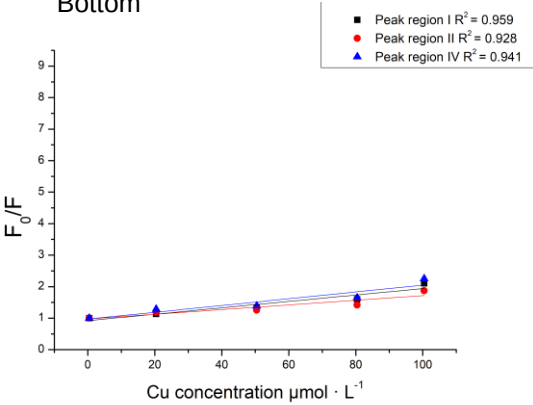
Middle



Middle



Bottom



Bottom

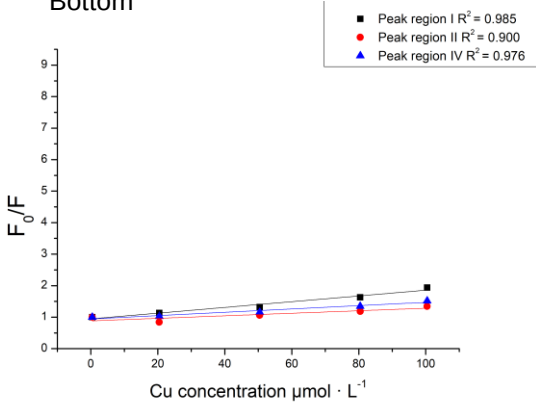


Figure S6. Stern-Volmer plots of Peak region I, Peak region II, Peak region IV of Initial SSAD amended soil (day 0) and top (0 – 0.1 m), middle (0.1 – 0.2 m) and bottom layer (0.2 – 0.3) of C2 and C3 on day 45.

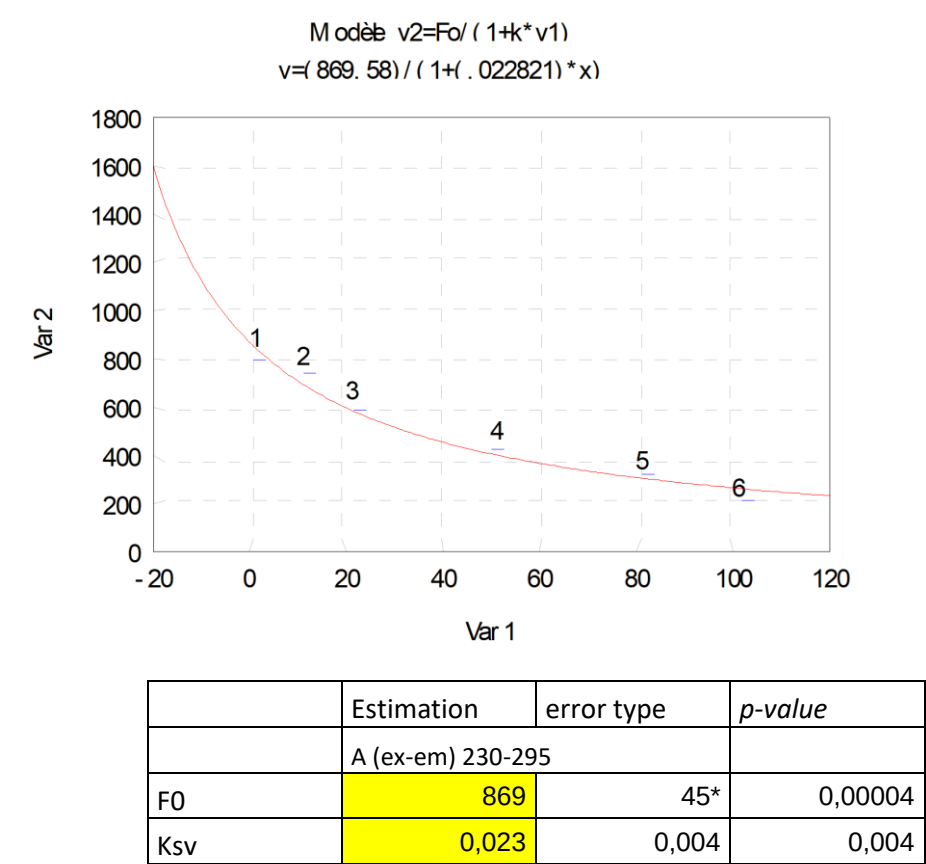


Figure S7: Example of the Ksv determination for tyrosine-like protein fluorophore in CER-extracted organic matter from the initial SSAD-amended soil. The determination of Ksv employs the Stern–Volmer equation (Eq 1) along with a nonlinear model and the least squares method with a significance level set at $p < 0.05$. In this determination, variable 1 ($v1$) is defined as $[Q]$ in $\mu\text{mol/L}$, and variable 2 ($v2$) is defined as F and F_0 (A.U.). The resulting Ksv is reported in units of $\text{L}/\mu\text{mol}$.

Appendix V. This appendix contains supplementary material from Chapter 6: Biochemical methane production from *Panicum virgatum* and *Pennisetum alopecuroides* cultivated with metal-containing municipal solid waste digestate

Table S1. Characteristics of the unamended soil and the MSW digestate amended soil where *P. virgatum* and *P. alopecuroides* were grown. Data from Bonet-Garcia et al. (2023).

Parameter	Unamended soil	Soil + MSW Digestate
Phosphorus (P ₂ O ₅) (% dry matter)	0.10 ± 0.02	0.78 ± 0.03*
Potassium (K ₂ O) (% dry matter)	2.7 ± 0.5	2.09 ± 0.04
CaO (% dry matter)	1.3 ± 0.6	8.0 ± 0.4*
MgO (% dry matter)	0.30 ± 0.08	2.6 ± 0.9*
Total Nitrogen (TN) (% dry matter)	<0.1	0.15 ± 0.02
Total Carbon (TC) (% dry matter)	0.40 ± 0.06	7.3 ± 0.6*
Total organic carbon (TOC) (% dry matter)	0.20 ± 0.01	4.4 ± 0.7*
pH value	7.9 ± 0.2	8.4 ± 0.1*

Table S2. Cumulative methane production obtained from the anaerobic digestion using *P. virgatum* and *P. alopecuroides* cultivated in absence (ND) or presence (D) of digestate as a substrate. SD represents the standard deviation (n=2).

Time	<i>P. virgatum</i> ND	SD	<i>P. alopecuroides</i> ND	SD	<i>P. virgatum</i> D	SD	<i>P. alopecuroides</i> D	SD
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.79	4.22	2.99	6.33	2.99	2.11	0.00	1.06	1.49
1.01	6.34	5.98	8.46	2.99	4.23	0.00	4.23	0.00
1.58	16.77	5.93	16.77	0.00	18.87	0.00	13.63	7.41
1.72	23.25	11.96	22.20	7.47	21.14	0.00	16.91	5.98
1.87	27.42	14.92	29.53	11.93	24.26	1.49	20.04	7.46
2.55	35.55	16.26	44.96	14.79	41.82	1.48	48.09	4.44
2.95	40.51	16.37	48.93	13.39	48.93	1.49	55.24	4.46
3.60	45.84	20.86	56.37	14.90	53.21	1.49	61.64	7.45
3.83	50.07	22.36	56.39	16.40	52.17	1.49	61.66	8.94
4.60	57.62	20.74	64.95	13.33	60.76	1.48	71.24	10.37
4.87	62.74	20.88	70.12	13.42	63.79	1.49	76.44	7.46
7.61	62.15	14.90	66.36	11.92	69.52	4.47	84.27	10.43
8.60	72.03	22.31	79.39	8.92	81.49	5.95	99.37	13.38
8.87	79.47	19.35	83.68	10.42	86.84	5.95	106.84	16.37
8.97	84.15	17.85	87.31	10.41	90.46	5.95	112.55	19.34
9.65	89.44	20.83	94.70	10.42	96.81	4.46	126.27	22.32
9.76	96.35	17.87	98.45	8.93	100.56	2.98	134.25	26.80
9.95	102.07	13.39	101.02	8.93	103.12	2.98	138.90	29.76
10.66	109.09	13.42	110.15	8.94	109.09	1.49	151.25	31.30
10.80	113.80	11.92	113.80	8.94	112.74	1.49	157.00	34.27
10.95	115.87	13.41	120.08	10.43	115.87	4.47	162.21	34.26
11.92	123.77	8.94	131.14	4.47	131.14	10.43	185.91	52.14
12.63	127.98	8.94	133.25	7.45	141.67	13.41	192.23	49.16
12.75	130.09	11.92	135.35	10.43	145.89	13.41	194.34	52.14
12.92	130.09	11.92	135.35	10.43	145.89	13.41	194.34	52.14
13.63	134.30	11.92	137.46	7.45	150.10	13.41	198.55	46.18
13.75	136.41	8.94	137.46	7.45	150.10	13.41	199.61	47.67
13.92	136.41	8.94	137.46	7.45	150.10	13.41	199.61	47.67
14.63	136.41	8.94	141.67	1.49	154.31	13.41	200.66	49.16
14.83	136.92	8.97	142.20	1.50	154.89	13.46	201.41	49.34
15.60	139.00	8.90	143.20	2.97	158.93	16.32	202.99	48.96
15.83	138.51	8.94	145.89	4.47	159.58	14.90	205.93	47.67
16.60	139.90	7.47	146.23	4.48	165.24	16.42	208.53	44.80
16.83	140.34	5.88	143.46	1.47	166.33	16.17	207.91	45.58
17.60	141.62	5.93	144.77	1.48	168.90	17.80	210.86	44.51
17.90	142.15	5.96	145.31	1.49	169.53	17.87	211.65	44.67
18.63	142.83	5.98	146.00	1.50	171.39	19.45	213.71	43.39
18.88	148.12	7.48	149.18	5.98	173.51	16.46	215.83	40.40
19.63	147.59	7.48	150.76	2.99	172.98	16.46	215.30	40.40

19.88	147.59	7.48	153.94	1.50	172.98	16.46	215.30	40.40
20.63	148.65	5.98	153.94	1.50	177.21	16.46	217.42	37.41
20.88	148.65	5.98	153.94	1.50	177.21	16.46	217.42	37.41
21.56	147.44	5.98	152.72	1.49	180.20	16.44	220.37	37.37
22.69	149.26	6.01	155.63	3.00	184.31	16.53	224.68	37.56
23.71	151.59	3.01	156.91	1.50	184.57	16.55	224.99	37.61
23.90	152.51	5.99	155.68	1.50	185.34	19.47	227.70	37.44
24.60	151.67	5.98	155.89	0.00	185.49	17.94	226.71	37.37
24.83	152.77	4.50	158.07	3.00	187.78	18.00	229.15	37.51
25.69	152.04	4.48	157.32	2.99	190.05	16.42	229.12	35.84
25.81	152.51	4.49	157.80	3.00	190.63	16.48	229.82	35.95
26.58	152.51	4.49	158.86	1.50	194.87	16.48	233.00	37.44
26.90	152.51	4.49	158.86	1.50	195.93	14.98	233.00	37.44
27.58	154.62	4.49	160.98	1.50	196.99	16.48	234.05	38.94
27.90	154.62	4.49	160.98	1.50	196.99	16.48	235.11	37.44
28.60	153.73	4.48	160.07	1.49	195.99	16.44	234.03	37.35
28.83	154.84	3.00	160.14	1.50	198.32	16.50	236.50	37.50
29.67	155.52	4.49	161.87	1.50	197.84	16.46	238.05	37.41
29.83	154.58	2.96	160.87	0.00	196.50	14.82	237.37	37.05
30.60	156.91	3.01	163.29	0.00	199.46	15.04	240.95	37.61
30.83	157.79	2.99	163.07	1.49	198.96	13.43	241.18	34.33
31.60	156.74	2.99	162.02	1.49	197.90	13.43	240.12	34.33
31.83	156.74	2.99	162.02	1.49	197.90	13.43	240.12	34.33
32.60	155.16	2.97	161.45	2.97	196.04	13.34	240.07	34.10
32.83	157.12	3.00	163.49	3.00	198.52	13.51	243.11	34.53
33.60	157.12	3.00	163.49	3.00	200.64	13.51	244.17	36.03
33.83	157.01	3.00	163.38	3.00	200.51	13.50	244.00	36.01
34.60	158.18	1.50	165.61	3.00	202.77	13.51	248.42	36.03
34.83	157.75	1.50	165.16	2.99	202.21	13.48	247.74	35.93
35.60	158.54	1.49	165.93	2.99	200.81	13.45	248.37	32.88
35.90	157.46	0.00	165.86	2.97	200.51	13.36	246.69	31.18
36.60	158.46	1.48	165.81	2.97	201.49	11.87	248.71	31.17
36.83	158.84	0.00	167.25	2.98	200.91	11.90	248.25	31.24
37.79	159.72	0.00	168.21	3.00	202.17	12.01	249.92	31.52
38.79	161.67	0.00	168.03	3.00	201.96	11.99	251.79	31.49
39.63	160.92	0.00	167.21	5.93	201.81	10.38	252.13	31.13
39.88	160.76	0.00	167.04	5.92	201.60	10.37	251.87	31.10
40.63	160.76	0.00	168.09	7.41	201.60	10.37	252.92	32.58
40.88	160.76	0.00	168.09	7.41	201.60	10.37	252.92	32.58
41.63	160.56	0.00	167.91	7.42	201.49	10.39	255.01	32.65
41.88	160.56	0.00	167.91	7.42	201.49	10.39	255.01	32.65
41.60	160.56	0.00	167.91	7.42	201.49	10.39	255.01	32.65
42.60	160.61	0.00	167.96	7.42	201.55	10.39	255.09	32.66
43.79	160.61	0.00	167.96	7.42	201.55	10.39	255.09	32.66
44.75	161.66	1.48	167.96	7.42	201.55	10.39	257.19	35.63
45.88	163.43	1.50	170.86	6.00	203.76	10.51	262.13	39.02

46.83	162.27	1.49	170.70	7.45	202.31	10.43	260.26	38.74
47.75	163.32	2.98	172.80	7.45	205.47	8.94	261.31	37.25
49.60	164.37	2.98	172.80	5.96	205.47	10.43	263.42	38.74
51.00	162.80	2.96	173.27	5.92	204.68	8.88	264.35	39.98