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Trace metal's fate and speciation in soils after contamination – interactions with organic micropollutants and effects on bioremediation processes

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The Supervisor,

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Metal-Micro exploitation

MARIE CURIE ACTIONS

Dedicated to

The ones who gave me

their unconditional love and support.

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Abstract (Portuguese)

Este estudo explorou a distribuição de contaminantes no solo após a utilização de digerido (um dos subprodutos da digestão anaeróbica de resíduos) como fertilizante, no contexto da bioeconomia circular e recuperação de solos. Este estudo investigou a distribuição de metais vestigiais (Zn, Cu, Pb, Ni, Cr, Co) em solos fertilizados com digerido considerando a influencia de microcontaminantes orgânicos, como compostos farmacêuticos (metformina e lamotrigina), e os seus possíveis efeitos nas comunidades microbianas do solo.

Este estudo centrou-se na aplicação de digerido contaminado com metais, proveniente da fração orgânica de resíduos sólidos municipais não separados na fonte, num solo argiloso e alcalino. Ao longo desta investigação, realizaram se experiências em colunas de solo (período experimental de 90 dias) investigando a distribuição de metais vestigiais, nomeadamente Zn, Cu, Pb e Cr, num solo fertilizado com digerido ao longo do tempo e a potencial influência do antidiabético metformina. Os resultados mostraram que os metais vestigiais apenas se transferiram da camada superior do solo fertilizado para a camada subjacente devido à maior quantidade de metais vestigiais presentes em formas mais biodisponíveis. Os resultados sugeriram que a metformina não influenciou significativamente a distribuição de metais vestigiais ao longo do perfil do solo, no entanto, interações com outros contaminantes potencialmente presentes devem ser consideradas.

Posteriormente, foi realizado uma experiência em coluna de solo (período experimental de 28 dias) para avaliar os potenciais impactos ambientais da aplicação de digerido. Esta parte do estudo centrou-se na distribuição de metais vestigiais e na potencial influência de dois compostos farmacêuticos, nomeadamente metformina e lamotrigina (um antiepilético), tanto no comportamento dos metais vestigiais quanto nas comunidades microbianas do solo. Os resultados mostraram que não ocorreu mobilidade significativa de metais vestigiais ao longo do perfil do solo durante o período experimental. Os resultados revelaram também uma rápida estabilização da comunidade microbiana, com uma mudança nos filos bacterianos dominantes ao longo do tempo.

As experiências em coluna de solo revelaram o potencial do digerido como fertilizante para a recuperação de solos e estratégias eficazes de economia circular, enfatizando a aplicação de estratégias de monitorização e remediação de contaminantes, como a fito/biorremediação. Além disso, este estudo explorou o comportamento de sorção de metformina e lamotrigina, classificados como contaminantes emergentes, num solo fertilizado com digerido. Experiências de adsorção revelaram uma capacidade aumentada de adsorção de contaminantes devido à adição do digerido ao solo e uma diferença no comportamento de adsorção devido à diferente natureza química dos compostos. A influência de metais vestigiais na adsorção de produtos farmacêuticos foi também investigada, destacando-se a importância de considerar as suas potenciais interações no modelo de economia circular de transformação de resíduos em recursos.

A adsorção e as interações dos contaminantes no solo fertilizado com digerido foram adicionalmente exploradas através da modelação matemática. O modelo competitivo de Langmuir foi utilizado para estudar a adsorção competitiva, observada experimentalmente, entre os metais vestigiais e a metformina no solo fertilizado com digerido. Em geral, o modelo competitivo de Langmuir demonstrou um bom ajuste aos dados experimentais, suportando a incorporação em um modelo de transporte de contaminantes para simular o destino desses contaminantes no solo fertilizado com digerido. O modelo aplicado salientou a importância de incorporar fenômenos de adsorção cinética (tempo-dependente) para obter simulações mais precisas sobre o comportamento e transporte dos contaminantes no solo. Além disso, os resultados sugeriram a necessidade de mais investigação em modelos cinéticos mais complexos para aprimorar as previsões de distribuição e transporte de contaminantes no solo.

No geral, este estudo forneceu dados cruciais sobre a potencial aplicação de digerido proveniente da fração orgânica de resíduos sólidos municipais não separados na fonte em solos marginais, abordando tópicos relacionados com a mobilidade de metais vestigiais, dinâmica da comunidade microbiana do solo e comportamento de sorção de compostos farmacêuticos. Os resultados obtidos destacam a necessidade de estratégias cuidadosas de gestão e remediação para garantir a utilização segura e eficaz de digerido em práticas sustentáveis de recuperação de solos dentro de uma abordagem de bioeconomia circular.

Palavras-chave: Digestato de resíduos sólidos municipais, emenda de solo, contaminantes emergentes, metais vestigiais, produtos farmacêuticos, biodisponibilidade de metais, comunidade microbiana do solo, interações solo-metal-fármaco, adsorção, transporte de contaminantes no solo.

Abstract (Italian)

Questo lavoro di ricerca ha esplorato la distribuzione dei contaminanti nel suolo a seguito dell'uso di digestato (uno dei sottoprodotti del processo di digestione anaerobica) come fertilizzante, nel contesto di bioeconomia circolare e di risanamento del suolo. Il lavoro si è focalizzato sulla distribuzione e trasporto di metalli in traccia (Zn, Cu, Pb, Ni, Cr, Co) nei suoli ammendati con digestato considerando l'influenza di contaminanti microorganici, come i composti farmaceutici (metformina e lamotrigina), e i loro effetti sulle comunità microbiche del suolo.

Il lavoro si è concentrato sull'applicazione di digestato, intrinsecamente contaminato, derivante dalla frazione organica dei rifiuti solidi urbani non differenziati su un suolo sabbioso limoso alcalino. Durante la ricerca, è stato eseguito un esperimento di 90 giorni mediante colonne di suolo per esplorare la variazione temporale della distribuzione dei metalli in traccia, in particolare Zn, Cu, Pb e Cr, nei suoli ammendati con digestato e la potenziale influenza del farmaco antidiabetico metformina. I risultati hanno dimostrato che i metalli in traccia sono stati trasferiti dallo strato di suolo ammendato con digestato allo strato di suolo sottostante dovuto alla maggiore quantità di metalli in traccia presenti in forme più biodisponibili. I risultati hanno indicato che la metformina non ha avuto nessun effetto significativo sulla distribuzione dei metalli in traccia lungo il profilo del suolo; tuttavia, interazioni con altri contaminanti potenzialmente presenti nel sistema dovrebbero essere prese in considerazione. Successivamente, è stato condotto un esperimento di 28 giorni mediante colonne di suolo per analizzare i potenziali impatti ambientali derivanti dall'applicazione del digestato sul suolo. Questa parte del lavoro si è focalizzata sulla distribuzione dei metalli in traccia nel suolo e sugli effetti dei composti farmaceutici, in particolare metformina e lamotrigina (antiepilettico), sui metalli in traccia e sulle comunità microbiche del suolo. I risultati hanno rilevato che i metalli in traccia non sono stati soggetti a nessun trasferimento significativo lungo il profilo del suolo durante il corso dell'esperimento. Inoltre, hanno rivelato una rapida stabilizzazione della comunità microbica del suolo con una variazione nel tempo delle phyla batteriche dominanti. Gli esperimenti eseguiti mediante colonne di suolo hanno evidenziato il potenziale del digestato come ammendante per il ripristino dei suoli, nonché come risorsa fondamentale per strategie circolari nel campo bioenergetico, enfatizzando la necessità di tecniche di monitoraggio e risanamento, come il fitorisanamento e il biorisanamento.

Questo lavoro di ricerca ha inoltre studiato l'adsorbimento sui suoli ammendati con digestato dei farmaci metformina e lamotrigina, classificati come contaminanti emergenti. Gli esperimenti di adsorbimento hanno rivelato un aumento della capacità di adsorbimento del suolo dovuto all'applicazione di digestato e una differenza nel comportamento di adsorbimento dei contaminanti dovuto alla loro diversa natura chimica. In aggiunta, è stato indagato l'effetto dei metalli in traccia sull'adsorbimento dei composti farmaceutici sul suolo, evidenziando l'importanza di tenere in considerazione le loro potenziali interazioni nel modello di economia circolare, da rifiuto a risorsa.

L'adsorbimento e le interazioni dei contaminanti nel suolo ammendato con digestato sono stati ulteriormente approfonditi con la modellazione matematica. Il modello competitivo di Langmuir è stato utilizzato per studiare l'adsorbimento competitivo, riscontrato sperimentalmente, tra i metalli in traccia e la metformina sia sul suolo naturale che sul suolo ammendato con digestato. In generale, il modello competitivo di Langmuir ha dimostrato un buon adattamento ai dati sperimentali, portando all'integrazione in un modello di trasporto di contaminanti per simulare il destino e trasporto dei contaminanti nel suolo ammendato con digestato. Le modellazioni hanno sottolineato l'importanza di incorporare fenomeni di adsorbimento cinetico (tempo-dipendenti) per ottenere simulazioni più accurate sul comportamento e trasporto dei contaminanti nel suolo. I risultati hanno indicato la necessità di ulteriore ricerca su modelli cinetici più complessi al fine di migliorare le previsioni di distribuzione e trasporto di contaminanti nel suolo.

Complessivamente, questo studio ha fornito informazioni fondamentali sul potenziale uso di digestato, proveniente dalla frazione organica non differenziata dei rifiuti solidi urbani, su terreni marginali affrontando problematiche relative alla mobilità dei metalli in traccia, alla dinamica delle comunità microbiche del suolo e all'adsorbimento e all'effetto di composti farmaceutici. I risultati hanno sottolineato l'importanza nell'adottare strategie di gestione, monitoraggio e risanamento, appropriate per garantire un utilizzo sicuro ed efficace del digestato nelle pratiche sostenibili di recupero del territorio marginale all'interno di un contesto di bioeconomia circolare.

Parole chiave: Digestato rifiuti solidi urbani, contaminanti emergenti, metalli in traccia, composti farmaceutici, biodisponibilità dei metalli, comunità microbica del suolo, interazioni suolo-metallo-farmaco, adsorbimento, trasporto di contaminanti nel suolo.

Abstract (English)

This study explored the fate of contaminants in soils amended with digestate, one of the end-products of anaerobic digestion, within the context of circular bioeconomy and land restoration. This research work delved into the distribution, fate and transport of trace metals (Zn, Cu, Pb, Ni, Cr, Co) in digestate-amended soils considering the influence of microorganic contaminants, such as pharmaceutical compounds (metformin and lamotrigine), and its effects on soil microbial communities.

This study focused on the application of inherently contaminated digestate, originating from the organic fraction of non-source-separated municipal solid waste, on an alkaline loamy sand soil. Over the course of this research, a 90-day soil mesocosm column experiment was conducted to investigate the distribution and fate of trace metals, namely Zn, Cu, Pb and Cr, in digestate-amended soil throughout time and the potential influence of the antidiabetic drug metformin. The results showed that trace metals only transferred from the top-amended soil layer to the underlying soil layer due to greater quantity of trace metals present in more bioavailable forms. The results suggested that metformin did not significantly influence trace metals' distribution along the soil profile, however interactions with other potentially present contaminants should be considered. Subsequently, a 28-day soil mesocosm column experiment was carried out to assess the potential environmental impacts of digestate application. This part of the study focused on the distribution and fate of trace metals and the influence of pharmaceutical compounds, namely metformin and lamotrigine (antiepileptic) on both trace metals and soil microbial communities. The results showed no significant mobility of trace metals along the soil profile throughout experiment time. They also revealed fast stabilization of the microbial community, with a shift of the dominant bacterial phyla over time. The soil column experiments revealed the valuable use of digestate for soil restoration and effective circular bioenergy strategies, emphasizing the application of contaminant monitoring and remediation strategies, as phytoremediation and bioremediation.

Additionally, this research work explored the sorption behaviour of metformin and lamotrigine, classified as emerging contaminants, in digestate-amended soil. Bench-scale adsorption experiments revealed increased contaminant adsorbing capacity due to digestate amendment and difference in the adsorption behaviour due to the compounds' different chemical nature. The influence of trace metals on pharmaceutical adsorption was also investigated, highlighting the importance of considering their potential interactions in the waste-to-resource circular economy model.

The sorption behaviours and interactions of contaminants in digestate-amended soil were further assessed using mathematical modelling. The competitive Langmuir model was applied to evaluate the competitive sorption behaviour, experimentally determined, between trace metals and metformin in both digestate-amended soil and natural soil. In general, the competitive Langmuir model showed high goodness-of-fit, leading to its integration into a multi-contaminant transport model for simulating contaminants' fate in the digestate-amended soil environment. The modelling underlined the importance of incorporating time-dependent sorption for more accurate predictions of contaminant behaviour and transport in soil. Further research is recommended to explore more complex kinetic models, aiming to improve simulations and predictions of contaminant distribution and fate in the soil environment.

Overall, this study provided crucial insights into the potential application of digestate originating from the organic fraction of non-source-separated municipal solid waste on marginal land, addressing concerns related to trace metal mobility, soil microbial community dynamics, and the sorption behaviour of pharmaceutical compounds. The findings stress the need for careful management and remediation strategies to ensure the safe and effective utilization of digestate in sustainable land restoration practices within a circular bioeconomy approach.

Keywords: Municipal solid waste digestate, soil amendment, emerging contaminants, trace metals, pharmaceuticals, metal bioavailability, soil microbial community, soil-metal-pharmaceutical interactions, adsorption, soil contaminant transport.

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List of abbreviations

AA	ATOMIC ABSORPTION
AAS	ATOMIC ABSORPTION SPECTROSCOPY
AD	ANAEROBIC DIGESTION
ADE	ADVECTION-DISPERTION EQUATION
ANOVA	ANALYSIS OF VARIANCE
ASV	AMPLICON SEQUENCE VARIANT
BCR	BUREAU COMMUNUATUAIRE DE RÉFÉRENCE
CEC	CATION EXCHANGE CAPACITY
CHNS	CARBON-HYDRGEN-NITROGEN-SULPHUR
CLM	COMPETITIVE LANGMUIR MODEL
DNA	DEOXYRIBONUCLEIC ACID
EC	EMERGING CONTAMINANTS
ET-AAS	ELECTROTHERMAL - AAS
EU	EUROPEAN UNION
F-AAS	FLAME - AAS
FDS	FINITE DIFFERENCE SCHEME
HPLC	HIGH PERFORMANCE LIQUID CROMATOGRAPHY
HSD	Tukey's HONESTLY SIGNIFICANT DIFFERENCE
ICP-MS	INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY
IPD	INTRAPARTICLE DIFFUSION

ISO	INTERNATIONAL ORGANIZATIONFOR STANDARDIZATION
ITN	INTERNATIONAL TRAINING NETWORK
LMT	LAMOTRIGINE
LOI	LOSS-ON-IGNITION
MANOVA	MULTIVARIATE ANALYSIS OF VARIANCE
MET	METFORMIN
MF	MOBILITY FACTOR
MSW	MUNICIPAL SOLID WASTE
MTE	METALIC TRACE ELEMENT
NGS	NEXT GENERATION SEQUENCING
OECD	ORGANIZATION for ECONOMIC COOPERATION and DEVELOPMENT
OFMSW	ORGANIC FRACTION MUNICIPAL SOLID WASTE
OM	ORGANIC MATTER
PERMANOVA	PERMUTAIONAL MULTIVARIATE ANALYSIS OF VARIANCE
PPCP	PHARMACEUTICAL AND PERSONAL CARE PRODUCTS
PT	PORTUGAL
RMSE	ROOT MEAN SQUARE ERROR
rRNA	RIBOSOMIAL RIBONUCLEIC ACID
R^2	COEFFICIENT OF DETERMINATION
TM	TRACE METALS

UV	ULTRAVIOLET SPECTRUM
UV-HPLC	UV HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY
UV-VIS	ULTRAVIOLET-VISIBLE SPECTRUM
WWTP	WASTEWATER TREATMENT PLANT

Chapter 1

Introduction

The present research work investigated the distribution and fate of trace metals (TM) in contaminated soils amended by digestate, assessing the influence of microorganic contaminants and their impacts on soil microbial communities. This research work falls within the context of the M2ex research programme, investigating metal-microbe applications and exploiting them to expand the circular economy centred on anaerobic digestion.

1.1 M2ex project

The M2ex research programme is a European Union (EU) Horizon 2020 Innovative Training Network (ITN) Marie Skłodowska-Curie Action Joint Doctorate Programme that aimed to provide an innovative and exciting environment for the education and training of a new generation of environmental scientists and engineers with deep, and multidisciplinary, knowledge and skills to address the complex challenges of EU's circular bioeconomy. Furthermore, with a commitment to fostering international collaboration, the M2ex programme targeted to have a pivotal role in shaping the future of research and innovation centred around the bioeconomy.

The significant challenges posed by global economic development, population growth and increasing waste generation, necessitate advanced waste management strategies based on innovative technologies centred on waste reduction, reutilization, resource recovery and bioenergy production, to meet environmental quality standards and maintain competitiveness and social well-being. This has prompted an imminent shift towards circular economies that consume recovered resources, as carbon, nutrients, bioenergy, and fresh water, and avoid the use of fossil-fuels and raw natural materials. Most of these new circular economies, as the one centred around anaerobic digestion (AD), rely on the activity of microorganisms which depends on specific key metal-microbe interactions, making it imperative to fully understand their role and how they affect the process. Compared to other possible treatment routes, AD is proven as one of the most economical and effective technologies for waste treatment and valorisation. It offers distinctive benefits, such as production of energy-rich biogas, high-quality fertiliser, electricity, heat, complete waste recycling, greenhouse gas reduction and environmental protection, in addition to reduced operating costs and smaller physical, and 'environmental', footprints. In EU, many countries have recognised the fundamental role of AD in organic solid waste treatment and management, and have enacted diverse Directives, including the Renewable Energy Directive[1], the Landfill Directives[2], and the

Water Framework Directive[3], which stimulated further research and application of AD practices for organic waste treatment. Still challenging and poorly-studied areas of AD research are (i) understanding of the role of TM in enabling biogas production and (ii) understanding their potential recovery.

The M2ex programme stemmed from this major knowledge gap and scientific challenge involving TM chemistry, TM interactions with solids, microbiology, and technology optimization. The project's main objectives were:

(i) To develop and apply new approaches to determine the bioavailable fractions of complex TM mixtures and matrices.
(ii) To link TM speciation and bioavailability with the physiological and metabolic activity of individual microbial cells.
(iii) To decipher and model the interactions between bioavailable trace elements and biofilm development, structure, and function in AD systems.
(iv) To investigate the role of trace elements in regulating biogeochemical mechanisms in soils and sediments exposed to AD digestates.
(v) To explore the potential of TM management for valorisation of wastes and residues in the bioeconomy.

In order to achieve the defined scientific objectives, the M2ex project established 8 work packages, five of which were designed to intertwine and tackle the different critical aspects of the metal bioeconomy. Amongst these work packages, the one that encompasses the present research work is the work package entitled 'Closing the loop to enable the bioeconomy'.

1.1.1 Work package 6: Closing the loop to enable the bioeconomy

Within the M2ex project framework, this work package aimed to develop new circular bioeconomy strategies focusing on the generated wastes, the removal and recovery of TM from the generated wastes, and their transformation into valuable resources.

The waste generated by AD, known as digestate, is a chemically-complex matrix containing carbon, nutrients, metals, and other contaminants deriving from a pool of different sources. The TMs' fate in the environment hinges on their speciation, their mobility, and their bioavailability, which are characteristics influenced greatly by the applied waste production and disposal processes. Thus, this work package aimed to understand the effects that digestate disposal could have on element-cycling and soil microbial communities responsible for relevant biogeochemical processes, as well as the interactions between TM and other (micro)pollutants potentially present in the environment or in the waste. In addition, it looked at waste characterization trying to identify the matrix fractions interacting with TM, and investigated the influence of the soil's physico-chemical properties on TM speciation once digestates were applied in the environment. Finally, it focused on the key knowledge gaps on waste reuse and TM recovery, presenting an opportunity to develop new strategies for waste valorisation.

In this context, the present research work aimed specifically to investigate the distribution and fate of TM in contaminated soils amended by digestate, taking into account the influence of (micro)organic contaminants on their behaviour and their impacts on soil microbial communities.

1.2 From current knowledge to research drivers

World economic evolution and population increase are two main drivers of the global issues our society faces today. A few of these issues are rising energy demand, primary resource scarcity, waste generation, and soil degradation. In the last decades, many efforts have been made worldwide to provide legislation that would encourage and promote sustainable, resilient, and climate-neutral societal and economic growth[4–7]. In this frame, the EU stands as one successful example of this ongoing transition, thanks to the implementation of the European Green Deal[6] and its new circular economy action plan[4]. These initiatives set the prerequisites for the transition to a zero-emission circular economy, allowing to decouple economic growth from resource use and fossil energy[8].

Despite these governmental efforts, one pressing problem of today's society is the increasing generation of municipal solid waste (MSW), expected to continue rising with population growth[9]. One of the waste fractions contributing critically to this problem is biowaste (organic waste), constituting up to 50 % of MSW across EU[10,11]. The Waste Framework Directive[3] and the European Green Deal[6] push to phase out landfilling, still accounting for > 24 % of MSW disposal, and incentivize other waste management strategies as incineration (accounting for 21 %), composting and AD. In addition, these directives mandate a separate collection of waste, including biowaste from 2024, to promote waste recycling and waste reduction. However, recent reports indicate that EU is still behind schedule in achieving its ambitious recycling and zero waste targets for 2030 and 2050[12]: recycling at least 65 % of all MSW and reducing to < 10 % landfill disposal[4]. On average, only 34 % of yearly produced household biowaste (food and garden waste) is effectively collected, and a mere 17 % is recycled into value-added products, as compost and digestate[10,11,13]. For these reasons, more focus should be placed on biowaste and circular waste management strategies to strengthen local circular bioeconomies and transform undesired biowastes into high-quality market materials[14,15].

From a circular bioeconomy standpoint, the biorefinery centered around AD is a well-known strategy for waste-to-resource conversion[5,16–24]. AD is a biological waste treatment process where specific microorganisms degrade organic waste through consecutive metabolic reactions producing biogas, used as renewable energy source, and digestate, used directly or indirectly in agronomic and industrial activities[24,25]. Biogas is generally composed of methane (45-60 %) and carbon dioxide (40-55 %), which is rarely valorised. In the realm of renewable energy, biogas has emerged as a significant player, with a production potential exceeding 100 bcm by 2050 [26–28]. Whereas digestate just recently gained renewed attention as a potential resource for soil restoration, trace element and nutrient recovery, and production of value-added biomolecules, as bioplastics, proteins, and enzymes [16,19,20,23,29–31]. There are also new processes being developed that use digestate for microalgae cultivation and production of biochar, used for soil remediation to increase soil carbon stocks and improve soil structure [32–38].

The organic waste used as feed for the AD may be agricultural waste, livestock waste (e.g. manure), wastewater biosolids and municipal biowaste (food and garden), which may be collected separately at the household or together with the non-recyclable waste. Digestates originating from source-separated municipal biowaste are considered of higher quality compared to digestate originating from non-source-separated municipal biowaste (referred to as organic fraction of MSW or OFMSW) because the latter can have greater biotic and abiotic contamination levels [39–41]. However, in EU and many world countries, there are numerous cases where non-source-separated OFMSW is utilized as feed for the AD process. Considering the EU, about 46 million tons out of 180 million tons of yearly produced digestate originate from non-source-separated OFMSW, while only 7 million originate from source-separated biowaste [11]. Hence, digestate originating from non-source-separated OFMSW holds a great potential for strengthening the bioeconomy [4,5,8,24,28]. Therefore, it is important to investigate its properties and potential applications to enhance circular bioeconomy strategies, crucial for addressing the abovementioned societal challenges and the pressing need for sustainable resource management [16,19,29,42].

One of the most widespread strategies for digestate use has been its application on land[16,18,42–45]. In recent years, this practice has further solidified, not only in response to the aforementioned directives[1,4–6,15,24,46,47], which advocate for circularity, zero-waste, and sustainability but also as a response to the call-to-action on soil health defined by the European Mission for Soil [48–50]. Soil, a fundamental natural resource for humanity, is under increasing stress due to human activities such as intensive agriculture and farming, deforestation, urbanization, and extreme weather events [7,50]. All of which have led to land degradation and soil impoverishment, which threaten the livelihood of entire populations. These issues have been addressed by the United Nations with the definition of the Sustainable Development Goals [7]. As a result, the concept of "soil health" gained importance, becoming a key element of the EU Green Deal[6] defined by the EU Mission for Soil: aiming for 70 % of healthy soils by 2030 [48–50]. It has been understood that soil provides essential ecosystem goods and services, habitats for biodiversity, and contributes to climate mitigation and resilience, making it imperative to reclaim degraded land, reduce pollution, restore soil quality and fertility, and put in place proper management strategies to protect and conserve this finite resource for future generations[51]. As so, there is a big push towards the use and application of organic waste-derived soil amendments, as digestates.

Even though land application of waste-derived organic amendments is considered a virtuous practice, it has led over the years to an increase in soil pollution. Amongst the contaminants found in the waste-derived organic amendments, there are priority substances[52], as metals, emerging contaminants (EC)[53] and inert impurities, as micro/nanoplastics [54–57]. The concentration of these contaminants in the amendments is dependent on several parameters, as their concentration in the treated waste, the waste's origin, the addition of essential micronutrients during AD, and the treatment technologies applied [58]. While these concentrations typically adhere to legislation limits (regulated compounds only)[3,53,59], they may significantly increase due to a change in waste content and seasonal variability, posing potential environmental risks when applied to soil. TM concentration in digestate varies specifically according to feed material and addition of essential micronutrients (Fe, Ni, Co, Zn, Mo, Se, W) to sustain AD process efficiency [16,60]. It is globally acknowledged that TM become priority contaminants when present in excessive amounts due to natural or anthropogenic activities because they become highly toxic for ecosystems and humans [52,60]. Moreover, they are persistent and can accumulate in the environment and bioaccumulate in living organisms. The leaching extent of applied digestate is influenced by seasonal changes of meteorological elements (as temperature and rainfall), and hydrologic properties (e.g. infiltration, water retention, drainage) [61]. As consequence, these factors can affect the soil's and digestate's physico-chemical properties, such as porosity, carbon content, mineral fractions (carbonates, iron and aluminium oxides, clays), microorganisms, pH, cation exchange capacity (CEC) and redox potential. These, in turn, can alter the redox state of the TM and influence their sorption behaviour, including all interactions between TM and soil constituents, affecting their speciation, distribution, and thus mobility and bioavailability in the soil environment[61-64]. Microplastics, defined as plastic debris with a diameter of less than 5 mm, can enter the soil system primarily through the application of waste-derived amendments containing inherent plastic particles [66,67]. Microplastics may interact with other contaminants, as metals and organic compounds, through adsorption/desorption mechanisms, leading to pollutant transport, pollutant leaching, and possible disrupting effects on soil biogeochemical cycles and soil microbial community activity [68,69]. Pharmaceutical and Personal Care Products (PPCP) are a class of EC that has risen great awareness because most compounds, within this class, are inefficiently removed by conventional waste treatment processes (including AD) and may pose unknown risks to human and ecosystem health[58,70–80]. Once introduced into the soil environment, EC may undergo sorption/desorption and transformation processes, driven by abiotic and biotic reactions, with biodegradation playing a dominant role [81]. Sorption is one of the most important mechanisms that controls the fate and transport of contaminants in the soil, which can be affected by the soil's physico-chemical properties (pH, CEC, surface area, mineral composition, organic matter content), the compound's nature and the presence of other contaminants [82]. EC can also exert toxicological effects: they can be uptook by microorganisms and plants (parent compounds and transformation products), leading to bioaccumulation and translocation in soil macrofauna and the trophic chain [83–85]. For example, two pharmaceutical compounds that have been detected in different environmental compartments and still poorly studied are the antidiabetic drug metformin (MET) and the antiepileptic drug lamotrigine (LMT). More specifically, MET and LMT have both been found in waste-derived amendments and in soils with concentrations up to the mg/L and µg/L, respectively [86–89], constituting a risk to human well-being and ecosystem health. When waste-derived amendments are applied to soil, it is critical to understand the behaviour and fate of these contaminants in the soil system, to assess their risk of transfer to water and biota and to apply proper remediation strategies to prevent and mitigate their impacts on the environment.

Nevertheless, there are strict regulations at the EU level governing the market availability and the intended applications of permitted organic waste-derived amendments [15,46,53,59,90]. These legislations impose quality standards to ensure safety and value of the organic waste-derived amendments, and protection of the environment in which they are applied [42]. Recently, digestate has been included in the list of regulated soil amenders and soil improvers [15], increasing its market value and promoting its use. However, OFMSW digestate is excluded from the list of approved digestates due to the potential presence of biological and chemical contaminants deriving from the feedstock [25,39–41,60,61]. Given that OFMSW digestate makes up 26 % of the yearly produced digestate in the EU[11], new strategies must be implemented to enhance the reuse of this material, transforming it into a resource.

One strategy that has been proposed by the scientific community is the use of OFMSW digestate as soil amendment for the restoration of marginal land coupled with nature-based solutions, as bio/phytoremediation, to remediate the soil, recover specific elements of interest, and monitor the soil environment [16,91–95]. The studies conducted on the application of OFMSW digestate on marginal land, looking at its effects on soil quality and soil microbial communities, yielded contrasting results [16,18,22,44,45,96–105]. Overall, although it was shown that OFMSW digestate improved the soil structure and the soil properties, some studies found no significant effect on soil microbial community, limiting its potential to enhance biodiversity [97,101,105], while others observed an enhanced bacterial growth and microbial activity [102,106]. Few studies were conducted on the application of OFMSW digestate centering on the fate of TM in the soil system, showing that OFMSW digestate, compared to other waste-derived amendments, reduced the risk of TM leaching [104,107–109]. However, there remains a possibility of TM transport and redistribution within the soil due to interactions with other contaminants and changes in their speciation, potentially increasing their bioavailability and mobility [44,62-65,96,104,107–111]. In this regard, no study in literature has focused on the effects that OFMSW digestate has on the behaviour and fate of TM in soil in presence of micro-organic contaminants and inert impurities, as MET and LMT pharmaceutical compounds and microplastics, respectively. Thus, there is an impellent need for further research to deepen the knowledge on this topic and give insights to support the development of new strategies that can be integrated in the circular bioeconomy.

All the emphasized knowledge gaps mentioned above, acted as the primary driving force behind the current research work.

1.3 Research Aims and Objectives

The present research work's overarching aim was (i) to investigate the distribution and fate of TM in contaminated soil and digestate-amended soil, considering the presence of microorganic contaminants, and (ii) to evaluate the effects of digestate amendment and potentially present contaminants on the soil microbial community.

Going into more detail, the specific objectives of the research project were defined as follows:

i. Understand the influence of soil physico-chemical properties on the fate and distribution of TM present in soils and contaminated amended soils through soil mesocosm column experiments, including in-depth soil analysis, assessment of metal concentrations, metal fractionation, and presence of other organic contaminants, as pharmaceuticals and micro-plastics.
ii. Assess specific soil metal contamination sources, as AD digestate amendments, and evaluate TM effects on contaminant behaviour in soil and soil microbial community composition, also considering the presence of micro-organic contaminants (as pharmaceutical compounds).
iii. Study the sorption behaviour on soil and digestate-amended soil of TM and pharmaceutical compounds through batch adsorption and desorption tests, aiming to elucidate the mechanisms governing metal-soil and metal-pharmaceutical-soil interactions.
iv. Develop a mathematical model to simulate the fate of TM in soils, originating from a top digestate-amended soil layer, by applying different models, also considering the presence of micro-organic contaminants (as pharmaceutical compounds)

Moreover, these research goals and objectives help establish a solid framework for the development of sustainable soil management strategies by safely utilizing OFMSW. This includes bioremediation and phytoremediation strategies coupled with proper monitoring practices, contributing to a more sustainable and resilient society.

1.4 Thesis outline

The whole thesis comprises 6 chapters, with the current one, Chapter 1, serving as introduction to the topic.

The following 4 chapters comprise of the research work contributing to a comprehensive exploration of the research's aim. Chapter 2, " Trace metal fate in soil after application of digestate originating from anaerobic digestion of non-source-separated organic fraction of municipal solid waste", focuses on the fate of TM in soil after digestate application, presenting empirical findings and insights obtained through soil mesocosms column experiments. Chapter 3, "Effects of digestate soil amendment on the fate of trace metals and on the soil microbial community", extends the discussion to the effects that TM and pharmaceutical compounds, present in the digestate, may have on the soil's microbial community evaluated through more in depth soil mesocosm column experiments. Chapter 4, "Metformin and lamotrigine adsorption on a biogas digestate amended soil in presence of trace metal contamination", deepens the understanding on the fate of TMs in amended soil through sorption batch tests and adsorption kinetic studies, giving more insights on the behaviour and mechanisms controlling TM fate in contaminated soil. Chapter 5, "Modelling metformin and trace metals transport in a digestate amended alkaline soil", centres on modelling contaminant transport in digestate amended soil providing a tool to predict their behaviour in the soil system, allowing to support and optimize future soil mesocosm and field experimental designs.

The cumulative knowledge generated across these chapters culminates in the final chapter, "Conclusions & Future Research", where the key findings are summarised, their implications are discussed, and future research paths are proposed. Overall, this research work aspires to make a contribution to the field of TM fate and distribution in soil after the application of contaminated digestate, enriching the basic scientific knowledge and providing valuable insights for researchers, practitioners, and policymakers alike.

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Chapter 2

Trace metal fate in soil after application of digestate originating from non-source-separated organic fraction of municipal solid waste

This chapter is based on the publication:

Baldasso V, Bonet-Garcia N, Sayen S, Guillon E, Frunzo L, Gomes CAR, Alves M, Castro R, Mucha AP, Almeida C. Trace metal fate in soil after application of digestate originating from the anaerobic digestion of non-source-separated organic fraction of municipal solid waste. *Frontiers in Environmental Science.* 2023; 10. https://doi.org/10.3389/fenvs.2022.1007390

Abstract

Attention towards anaerobic digestion (AD) is growing as global demand for renewable energy increases. Apart from biogas, the AD process produces also digestate that frequently is treated as waste discarded in landfills because it does not meet the quality standards imposed by legislations. In the frame of a circular bioeconomy, new applications for digestate are needed. One possible application is its use as soil amendment for marginal land restoration coupled with sustainable energy crop cultivation. For this reason, the fate of contaminants potentially contained in digestate must be studied. The present work focused on the fate of trace metals namely Zn, Cu, Pb, Cr, along the soil profile after digestate application, and their potential interactions with soil constituents and micro-organic pollutants (as pharmaceuticals). A 90-day soil column experiment was conducted in acrylic plastic columns filled with fine loamy sand soil and a top layer of amended soil, with a soil to digestate proportion of 14 to 1 (dry weight). The digestate was collected in an AD plant treating non-source-separated organic fraction of municipal solid waste. Two experimental conditions were tested: soil amended with digestate, and soil amended with digestate doped with the antidiabetic drug metformin. Soil samples taken at 5 depths on days 1, 7, 21, 35 and 90, were analysed for total trace metals concentrations and trace metals fractionation via atomic absorption spectroscopy. Results showed that Zn, Cu, Pb ad Cr were transferred from the amended soil layer to the underlying soil layer over time, whereas no transfer was detected to the deeper soil layers. Trace metals in soil were predominantly in immobile and less bioavailable forms, most likely associated with mineral groups (e.g. silicates). In the amended soil, metals were in more bioavailable forms. The results suggested that metformin did not have a significant influence on trace metal behaviour during the experiment duration; however, interaction with other potentially present contaminants (e.g. microplastics) should be considered. To use digestate in new bioenergy circular economy strategies, appropriate management of metal contamination needs to be implemented, taking in account their physico-chemical interactions in soil.

2.1 Introduction

Anaerobic digestion (AD), an anaerobic biological process in which specific microorganisms degrade organic material of different origins through consecutive metabolic reactions [1], not only produces biogas but also tackles the growing problem of waste generation and treatment. In fact, the organic waste that would be discarded in landfill is treated to produce different value-added products strengthening local circular bioeconomies [2].

The value-added products produced by AD are biogas and digestate [3]. For many years, digestate has been discredited and not considered during AD design, which focused merely on biogas production. However, with the increasing degradation of land and soil due to intensive agriculture and poor farming practices, digestate has been re-evaluated as a potential resource for sustainable farming, soil quality improvement and land restoration [4], as this semi-stabilized organic matter (OM) contains macronutrients as nitrogen, phosphorous and potassium, and micronutrients as trace metals (TM) [5].

There are strict regulations at the European Union (EU) level that define the permitted types of soil amendments originating from AD and their applications [6]. These legislations impose quality standards to ensure safety and value of the digestate, and protection of the environment in which they are applied [7]. Organic fraction of municipal solid waste (OFMSW) digestate is still classified as waste, which can be transformed into product after appropriate treatment providing the necessary quality standards to safeguard society, ecosystems, and environment [6,8–13]. Given that the majority of municipal solid waste (MSW) digestates originates from non-source-separated OFMSW [14], it is important to investigate the properties and potential applications of OFMSW digestate, exploring strategies that would transform it into a resource, enhancing circular bioeconomy strategies, promoting its use, similarly to digested sludge, compost or OW digestate [7]. For instance, a simple circular bioeconomy strategy involving OFMSW digestate (whole or solid fraction) could be its application on marginal lands for soil restoration [5] coupled with bio-energy crop cultivation. This would allow to cultivate energy crops needed as feed material for AD reactors while remediating the soil and decreasing the competition between land for food and land for energy [15,16].

A concern regarding OFMSW digestate is the presence of contaminants [1,17,18], such as metals [5,12,17], inorganic and organic impurities (as plastics) [19–22], micro-organic contaminants (as PPCP [23,24]) not degraded during the AD process [25–31] and greenhouse gases, as discussed in Chapter 1, paragraph 1.2 From knowledge to research drivers. For these reasons, once digestate is applied to soil, it is critical to understand the behaviour of the contaminants along the soil profile to mitigate and prevent their risks towards ecosystems and human health [18,28–37].

Soil physico-chemical properties play a role in contaminant distribution in the soil as well as in the reactions and processes between contaminants and soil constituents [1]. One of the main processes was found to be sorption, including adsorption, absorption, and desorption [38,39]. For instance, TM as Cd, Ni, Zn, Cu, Pb at low pH and redox-potential have shown higher solubility and thus higher mobility and availability [40–42]. Biosolid amendment can also induce a downward movement of TM in soil and their redistribution amongst soil fractions, with an increase in TM bioavailability and concentration in soil leachate due to complexation with dissolved organic carbon [43,44]. These phenomena were found to be lower in amendments originating from OFMSW due to the decrease of dissolved organic carbon with OM mineralization [45], and an increased adsorption onto OM in the soil [46–48].

Only a few studies have been conducted on evaluating the possibility to use OFMSW digestate as a marginal soil amendment [49], which is a subject that deserves research. Combined with lignocellulosic waste, OFMSW digestate was shown to meet quality requirements as soil fertilizer while mitigating the risk of metal leaching, immobilizing metals in soil and valorising nutrient content [50]. It was also found to have presumably good agronomical characteristics and higher biomass yield, when compared to mineral fertilizers [51]. To our knowledge there is no study that focuses on the potential contamination of soils with TM, persistent micro-organic contaminants, and anthropogenic impurities, as microplastics, originating from OFMSW digestate, and their possible interactions and transformations within the soil system. For this reason, we conducted a soil mesocosm column experiment aiming to understand the fate and mobility of TM (mainly Zn, Cu, Pb, Cr) in marginal land soils after application of OFMWS digestate contaminated with TM, metformin (MET) as a proxy for micro-organic pollutants, and microplastics. The results will increase the knowledge on the possible safe and sustainable use of digestate in bioenergy circular economy strategies.

2.2 Materials and Methods

2.2.1 Materials and reagents

All chemical compounds used in the study were of the highest available purity. The chemical compounds used were ethanol puriss. p.a. > 98% (603-002-00-5, Honeywell), analytical grade hydrogen peroxide > 30% w/v (H/1800/15, Fischer Scientific), nitric acid puriss. p.a. 65% (84380-M, Sigma-Aldrich), acetic acid glacial 100% p.a. (100063, Merck), hydroxylamine hydrochloride ACS reagent 98.0% (255580, Sigma-Aldrich), ammonium acetate ACS reagent grade > 97% (238074, Sigma-Aldrich) and metformin hydrochloride (PHR1084, Sigma-Aldrich). Metal stock standard solutions were also acquired from Sigma-Aldrich. Deionized water (conductivity < 0.1 mS cm^{-1}) was used throughout the experiments, for solution preparation and dilutions. All standard laboratory glassware and plastic equipment used was thoroughly washed, placed overnight in a nitric acid bath (20 % (v/v)), and thoroughly rinsed with deionised water prior to utilization.

2.2.2 Soil and OFMSW Digestate

The soil was collected in a residential area in Ermesinde, located in the Porto city district (NW Portugal; 41°12'25.7''N, 8°32'26.7''W), from an excavation site for building construction. The soil texture was identified through INFOSOLO, the Portuguese online database for soil profile data [52]. Three soil profile data points were used to identify the soil texture around the sampling area (INFOSOLO soil profiles identification codes: 355V, 351V and 27762200). The soil was classified as a Cambisol, deriving from metamorphic Schist parent rock.

The OFMSW solid digestate (referred to as digestate from now on) was collected at a Portuguese full-scale municipal solid waste treatment facility, equipped with an AD unit for the treatment of OFMSW. The solid fraction of the digestate was collected at the end of the solid-liquid separation process line, at the outlet of the centrifugation unit.

The soil and the digestate were characterized for pH, water content, OM content, TM total concentration and TM fractionation. The soil was also characterized for cation exchange capacity (CEC), elemental composition, total carbon, total organic carbon, nitrogen and sulphur content. This can be found in Appendix - A. Microplastics in soil and digestate were also quantified and characterised with the methodology reported in Appendix - A.

Before being used in the experiments, the soil was homogenized and sieved to <2 mm.

2.2.3 Soil Mesocosm Column Experiment

The experiment was a short-term experiment of 90 days duration. The experimental conditions defined were: (E1-Dig) soil amended with digestate and (E1-Dig-Met) soil amended with digestate spiked with MET. Each experimental condition was conducted in duplicate. The soil column experiments were carried out in transparent acrylic plastic soil columns designed and constructed in the facilities of the Physics Department of the Faculty of Science of University of Porto, Portugal. The columns, 60 cm in height and 19.4 cm in diameter, had 6 evenly spaced lateral sampling ports, and a bottom sampling port positioned at the centre of the column base (Figure 1) for leachate collection when needed. Each column was assembled with three layers: (i) a 2 cm bottom layer of inert gravel (diameter < 1 cm) to allow drainage and prevent clogging, (ii) a 37 cm middle layer of soil, and (iii) a 15 cm top layer of digestate amended soil. The digestate amended soil was prepared by thoroughly mixing soil and digestate for 30 min with a soil to digestate proportion of 14 to 1 (dry weight). This was done to simulate a potential real-case scenario of digestate application on marginal land followed by plowing and tillage operations for energy crop planting. The different materials were packed in the columns with 5 cm layers at a time, manually compacting them and scarifying their surface to ensure hydraulic conductivity [53]. For E1-Dig-Met, the digestate amended soil was spiked with a 2 mg/L MET aqueous solution (by thoroughly mixing it with the amended soil) before placing it on top of the soil layer of the soil column. The concentration chosen simulated MET concentration found in digestates and AD sludges [54,55]. The columns were completely covered with aluminium foil to prevent sunlight exposure (with exception of the top base), and they were kept in an indoor open-space with natural sunlight exposure at ambient temperature (22 ± 1 °C). The water content of the soil columns was kept constant at 80 % water holding capacity, by watering each column once a week with 200 mL of deionized water.

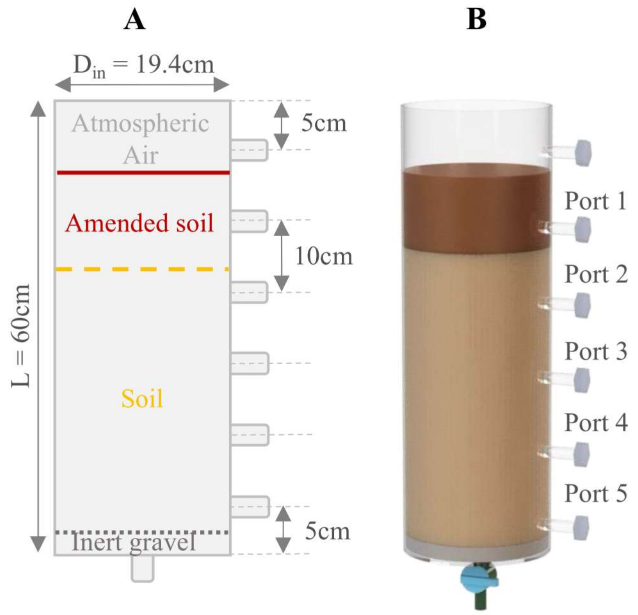

Figure 1: (A) Soil mesocosm schematic with dimensions and identification of the different internal layers; (B) 3D representation of the soil column. Dark brown layer represents digestate amended soil.

Soil samples were collected along the soil profile, through the column lateral sampling ports from 1 to 4 (Figure 1) on days 1, 7, 21, 35, and 90. The samples collected from each port were immediately stored at -20 °C for sample preservation until further analysis. All solid samples were analysed for TM total and fractionation concentrations. During experiment duration, leachate from the base port of each column was also collected, acidified with HNO_3 and analysed for total metal concentrations.

2.2.4 Analytical determinations

Water content was determined by heating solid samples at 105 °C until constant weight was reached, and subsequently calculating the difference of mass between the initial sample and the dried one. OM content was determined through Loss on Ignition (LOI) method, by heating a 2 g dry solid sample in a muffle furnace at 500 °C for 4 h and then calculating the difference of mass between the initial sample and the dried one. The soil pH was measured following the ISO method for Soil quality - pH determination (NF ISO 10390). Analyses were carried out in triplicate for each sample.

Total metal concentrations (Fe, Mn, Ni, Zn, Cu, Pb, Cr, Cd and Co) in solid samples were analysed by atomic absorption spectroscopy (AAS) after a high-pressure digestion in an advanced microwave system (ETHOS 1, Milestone Inc), following a laboratory validated procedure described by Almeida et al (2004) [56]. For soil, 0.25 g of sample was weighted into a microwave Teflon vessel and 5 mL HNO_3 were added; while for digestate and digestate amended soil 0.50 g of sample was weighed and 1 mL of HNO_3 along with 5 mL of H_2O_2 were added. After the microwave digestion, the vessels were allowed to cool down to room temperature; the solution was transferred into 50 mL falcon tubes and volume was made up to 15 mL with deionized water. The solutions were analysed either with AAS with flame atomisation (F-AAS), using an AAnalysit 200 AA spectrometer system (PerkinElmer), or with AAS with electrothermal atomisation (ET-AAS), using a PinAAcle 900Z AA spectrometer (PerkinElemer), depending on TM concentration values and instrument range of operation. The concentrations were obtained by external calibrations with aqueous metal standard solutions conducted prior to each analysis. Acidified leachate samples were analysed directly either with F-AAS or ET-AAS.

For TM fractionation, the sequential extraction BCR procedure described by Rauret et al. (1999) [57,58] was used with few minor modifications. For initial soil, digestate and digestate amended soil samples, fractionation was carried out for Fe, Mn, Zn, Cu, Pb and Cr, whereas for the samples collected during the soil column experiment it was carried out for Zn, Cu, Pb and Cr. Initially, 0.50 g of each sample was weighed and placed into 50 mL falcon tubes. The first extraction was carried out with 20 mL of a 0.11 mol/L acetic acid solution for the determination of the soluble and exchangeable metal fraction. The second sequential extraction was carried out with 20 mL of a 0.5 mol/L hydroxylamine hydrochloride solution for the determination of the reducible iron/manganese oxides fraction. The third sequential extraction, for the determination of the oxidizable OM and sulphide fraction, was carried out with a 1 mol/L ammonium acetate solution after sample oxidation with hydrogen peroxide aqueous solution 30 % (v/v). The extractions were carried out in an end-over-end shaker (Unitronic Reciprocating Shaking Bat, JP Selecta) at room temperature. TM concentrations in the extract solutions were analysed by F-AAS or ET-AAS.

2.2.5 Data Analysis

For each solid sample, the TM total and fractionation concentrations were represented as the mean and standard deviation of replicate samples. Statistical analyses were performed using RStudio, version 1.4.1103 [58]. Data collected from the soil column experiment was analysed via two-way ANOVA statistical analysis to evaluate significant differences in TM total and fractionation concentrations through time and between experimental conditions. TM concentrations were defined as the dependent variable, while time and experimental conditions as the independent variables. The significant results (p value < 0.05) were further assessed by pairwise comparison with post-hoc Tukey's Honestly Significant-Difference (HSD) test to understand which groups were significantly different from one another.

2.3 Results

2.3.1 Soil, Digestate and Digestate Amended Soil Characterization

The soil texture was determined to be a fine loamy sand with a proportion of sand, silt, and clay of 78.7%, 14.8% and 6.5%, respectively, using the soil texture triangle (Appendix - A, Figure A1). Soil pH was (8.1 ± 0.5) in average. The soil initial water content was (11.9 ± 0.6) wt% and the OM content was (3.02 ± 0.06) dw%. The digestate had a dry matter content of (50 ± 6) wt% and an OM content of (24 ± 4) dw%.

The soil, digestate and digestate amended soil TM concentrations, Fe, Mn, Ni, Zn, Pb, Cu, Cr, Co, and Cd, are reported in Figure 2. The results showed that in general the TM concentrations were higher in the digestate than in the soil. The concentrations of Mn, Zn, Cu and Pb in the digestate were significantly higher than those in the soil, potentially posing a risk of metal transport along the soil profile. These high concentrations are probably due to the origin of the digestate, which derives from non-source-separated OFMSW. For Cr and Ni, concentrations were also slightly higher in digestate than in soil but lower than those of the other mentioned TM. Only Fe, considered as a major element, had a higher concentration in the soil due to its mineral origin. Concentrations for Co were low in both soil and digestate, while Cd was below the limit of detection (5.8 µg/g) in all samples analysed. Thus, these two TM were not considered in the soil column experiments. The TM concentrations in the digestate amended soil showed the dilution effect that the soil had on the digestate, although some TM concentrations namely those of Zn, Cu and Pb continued to be more than double of those in the soil. The difference in TM concentrations between soil and digestate amended soil led to use the digestate without adding TM, allowing to simulate a potential real case scenario of digestate application on soil.

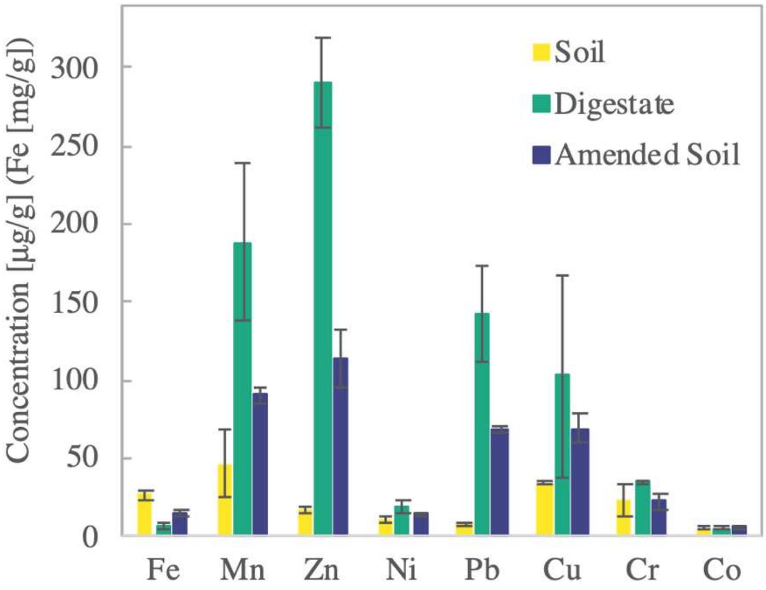

Figure 2: Total trace metals concentrations in the initial soil, digestate, and digestate amended soil used in the soil column experiment (mean ± standard deviation, n=2).

The soil, digestate and digestate amended soil TM fractionation analysis allowed to evaluate TM concentrations within different operationally defined chemical forms present in the solid samples: the exchangeable, the reducible (bound to Fe and Mn oxides), the oxidizable (bound to organic) and the residual fraction. It was decided to combine the oxidizable and residual fractions because the quantity of TM in the oxidizable fraction (bound to OM) was, in general, very low compared to the quantity present in the other fractions (Appendix - A, Figure A2). The fractionation results are reported in Figure 3 (A – C) as percentage of the total TM concentration measured. The results for soil showed that Fe, Pb, Cu and Cr were found mainly in the residual + oxidizable fraction at 99, 93, 95, and 98 % respectively. Zn and Mn were found in all the fractions: Zn mainly in the residual + oxidizable (50 %) and the exchangeable (30 %) fractions, and Mn mostly in the reducible fraction (81 %). The digestate showed similar results with Pb, Cu, Cr and Zn found mainly in the residual + oxidizable fraction at 99, 90, 99, and 58 %, respectively. Fe was mostly bound to Fe/Mn oxides (55 %) and in the residual fraction (39 %), whereas Mn was found in all fractions with 30 % in exchangeable fraction, 22 % in the reducible fraction, and 47% in the residual fraction. In the digestate amended soil Pb, Cu and Cr were found mostly in the residual + oxidizable fraction at 98, 81 and 91 %. Cu and Cr were also found in small percentage in the reducible fraction. Fe, Mn and Zn were found in all fractions: in residual + oxidizable (52, 27 and 49 %), bound to Fe/Mn oxides (12, 29 and 30 %), and exchangeable (35, 43 and 21 %) fractions. The distribution in the digestate amended soil was influenced by the mixing between the soil and the digestate. It can be estimated that the initial substrates contained Pb, Cu and Cr mainly in immobile forms, and Zn in both readily bioavailable and immobile forms. As previously mentioned, TM fractionation was not carried out for Co, Cd and Ni as their total concentrations were too low to detect or to follow any possible variation.

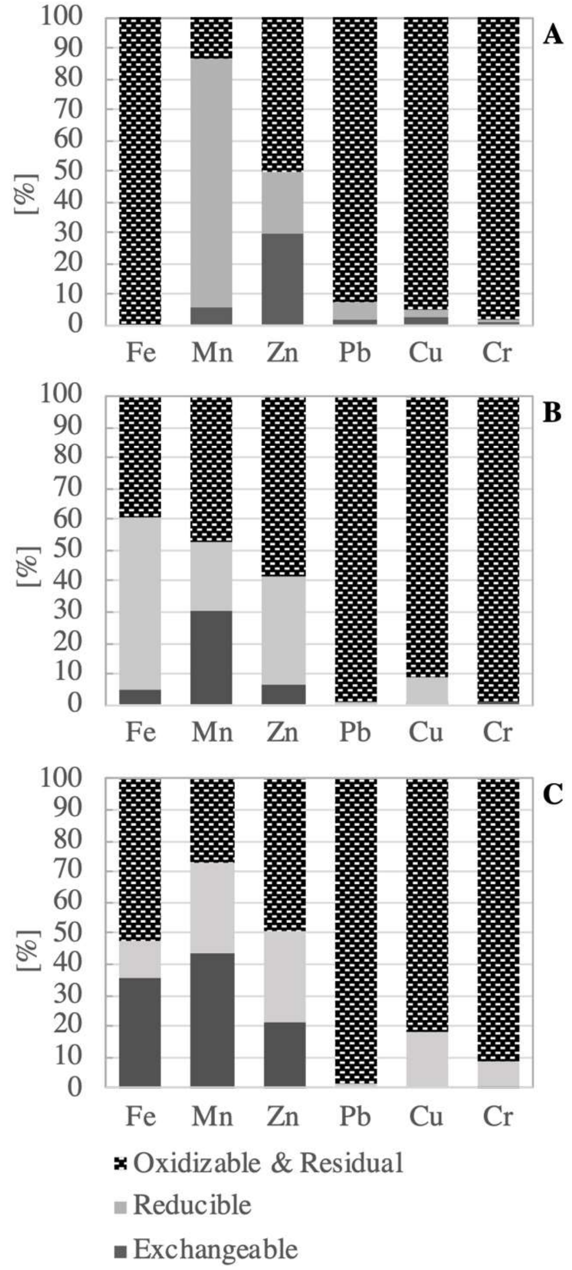

Figure 3: Trace metals fractionation in the initial soil (A), digestate (B) and digestate amended soil (C) (mean ± standard deviation, n=2).

2.3.2 Soil column experiments: pH and leachate TM concentrations

pH was measured in the digestate amended soil layer and in the different soil layers at the beginning (day 1) and at the end of the experiment (day 90). The average pH of the soil layers over time varied between (8.1 ± 0.2) on day 1 and (8.4 ± 0.1) on day 90, indicating a slight non-significant increase. The average pH of the digestate amended soil layer over time varied between (9.1 ± 0.5) on day 1 and (8.2 ± 0.7) on day 90, indicating a slight non-significant decrease.

Regarding leachate samples, overall, no TM were detected or quantified in the collected leachate solutions.

2.3.3 Soil column experiments: Total TM concentrations

TM concentrations along the column profile throughout experiment duration (90 days) for both experimental conditions E1-Dig and E1-Dig-Met are reported in Figure 4 for Zn, Pb, Cu and Cr and in Appendix - A (Figure A3) for Fe, Mn and Ni. For both experimental conditions, TM concentrations measured in the deeper soil layers (port 3 and 4, Figure 1B) were in the same range of those of the initial soil, indicating that TM from the digestate amended soil layer (port 1, Figure 1B) were not able to reach the deeper soil layers throughout the experiment duration. Additionally, TM concentrations overall had a decreasing trend by the end of the experimental period in the first layer (port 1, Figure 1B) and an increasing trend in the second layer (port 2, Figure 1B) through time, with the exception of Fe, which was present in higher amount in soil than in digestate, and Cr (Figure 4, G and H), whose concentrations in the soil (24 ± 10 µg/g) and in the digestate amended soil substrate (23 ± 5 µg/g) were statistically identical. Moreover, no statistically significant difference between the trend of total TM' concentrations in the soil columns of the two experimental conditions (E1-Dig and E1-Dig-Met) were observed.

The statistical analysis results (independence of variables, normality, and homoscedasticity tested and verified) on the effect of time and experimental condition (presence/absence of metformin) on TM total concentrations are reported in Appendix - A (Table A4). The statistical analysis was carried out only for Zn, Cu, Pb and Cr as these were the TM of concern due to possible toxic effects when present in high concentrations, whereas Fe, Mn and Ni were not considered in the analysis. In fact, Fe and Mn are major elements commonly present in soils, while Ni had low concentrations not significantly different between soil and digestate amended soil, which prevented following a possible migration through the soil profile.

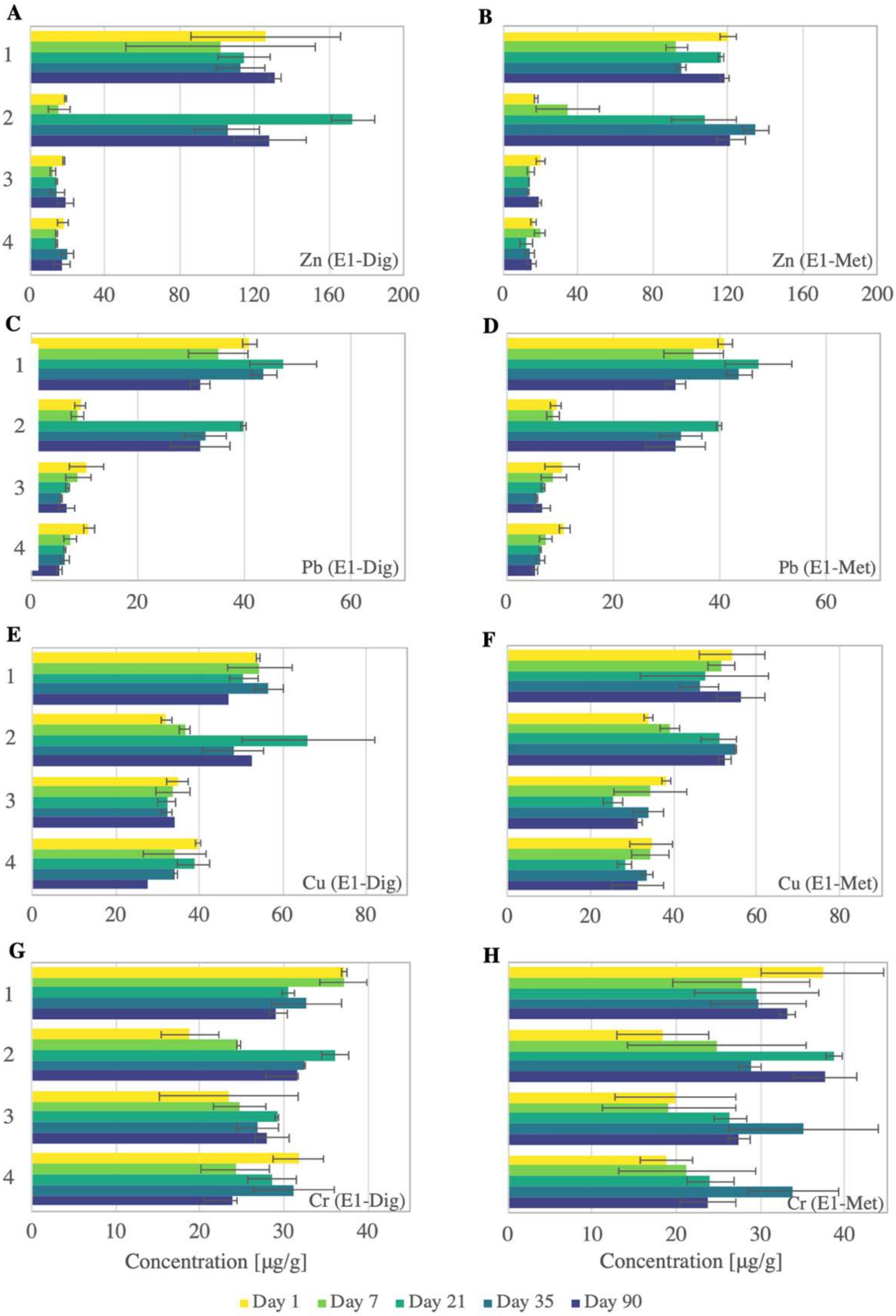

Figure 4: Zn, Pb, Cu and Cr total concentrations in the different column layers (ports 1, 2, 3 and 4) over time, for the two experimental conditions E1-Dig (soil amended with digestate (A, C, E, G)) and E1-Dig-Met (soil amended with digestate doped with metformin (B, D, F, H)) (mean ±standard deviation, n=2).

Overall, the statistical analysis revealed that there was not a statistically significant interaction between the effects of time and experimental condition, with exception for Zn in the second layer (port 2 Figure 1B). The simple main effects analysis showed that time had a statistically significant effect on the TM total concentration, especially in the second layer of the soil column. For experimental condition E1-Dig, the concentration of Zn in the second layer on days 1 and 7 was significantly lower than the concentration of Zn on days 21, 35 and 90, with a similar (non-significant) trend being observed for E1-Dig-Met. The variation of Cu concentration in the second layer was significant only for experimental condition E1-Dig-Met, with the concentrations on days 1 and 7 also significantly lower than those on days 21, 35 and 90; this (non-significant) trend was observed also for condition E1-Dig. Similarly, for experimental condition E1-Dig, Pb in the second layer on day 1 and day 7 was significantly lower than on days 21, 35 and 90; while for condition E1-Dig-Met Pb on day 1 was significantly lower than day 21. Moreover, for condition E1-Dig, Pb in the fourth layer (port 4) on day 1 was also significantly higher than on days 21, 35 and 90. Lastly, Cr in the second layer for condition E1-Dig on day 1 was significantly lower compared to Cr on days 21, 35 and 90; while Cr on day 7 was significantly lower compared to Cr on day 21. All these results indicated a significant increase in total TM concentrations in the second soil column layer around the third week of the experiment (day 21), with a non-significant variation of the concentration in the other layers.

In addition, the simple main effects analysis showed that experimental condition (presence/absence of metformin) did not have a statistically significant effect on the variation of TM total concentrations. In fact, the presence of metformin had a statistically insignificant effect on TM behaviour in soil during experiment time, when compared to the non-spiked digestate amended soil.

2.3.4 Soil column experiments: TM fractionation

TM fractionation along the column profile over time (90 days) for both experimental conditions, E1-Dig and E1-Dig-Met, are reported in Figure 5 and Figure 6, respectively. TM fractionation was carried out only for the four TM of concern as indicated above (Zn, Cu, Pb and Cr). As previously mentioned, the oxidizable and residual fractions are represented together because the quantity of metal bound to the organic fraction was very low compared to the quantity in the other fractions. TM fractionation showed that Zn was mostly found in an immobile form (residual + oxidizable fraction) in the lower soil layers (ports 3 and 4), while it was distributed among all fractions in the first and second layers (ports 1 and 2). Cr, Cu and Pb were mostly found in an immobile form in all soil column layers, with a small percentage also found in the reducible fraction (between 5 - 20 %) and exchangeable fraction (< 5 %). Over time, for Zn, the exchangeable and reducible fractions were subject to some variation with a slight increase of the quantity in the more bioavailable fractions and a consequent decrease in the residual fraction. Similarly, Pb and Cu had a variation of the quantity of TM over time within the different fractions, but no clear trend was observed. Whereas Cr showed no variation within the different fractions, remaining prevalently in the residual fraction over time. Additionally, no evident difference between E1-Dig and E1-Dig-Met was observed.

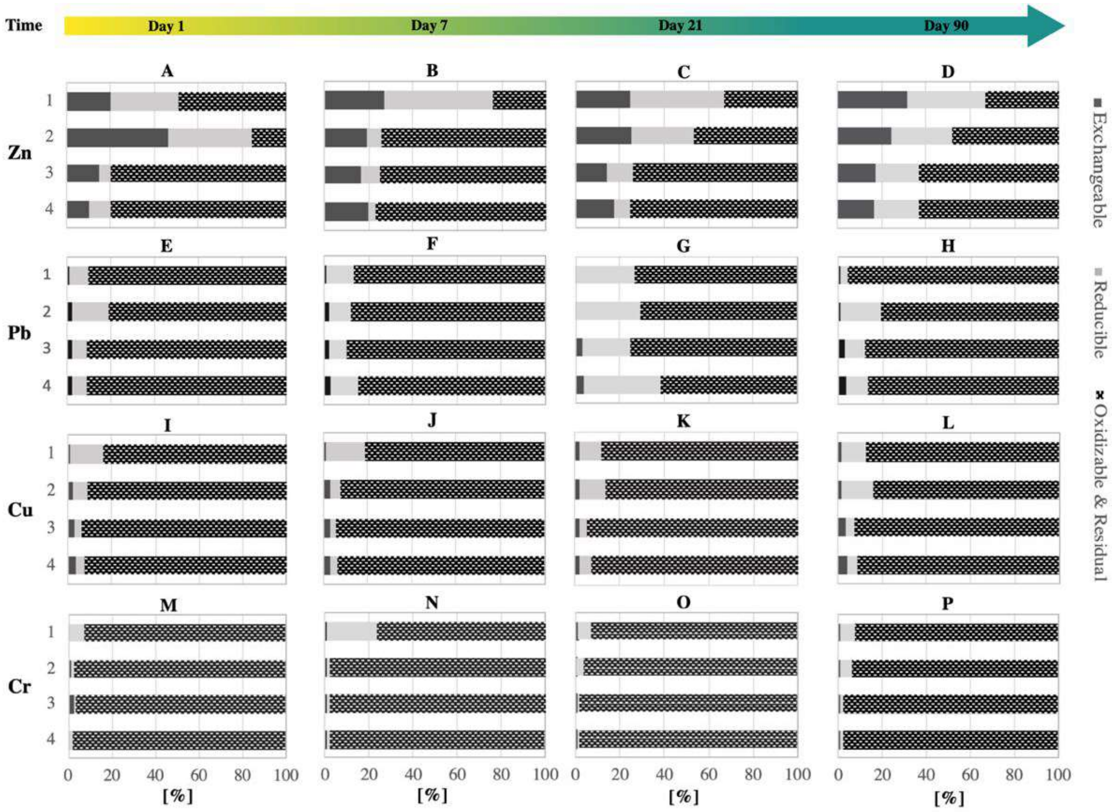

Figure 5: Metal fractionation in the different column layers over time for soil amended with digestate (E1-Dig): Zn (A – D), Pb (E – H), Cu (I – L), and Cr (M – P) (mean ±standard deviation, n=2).

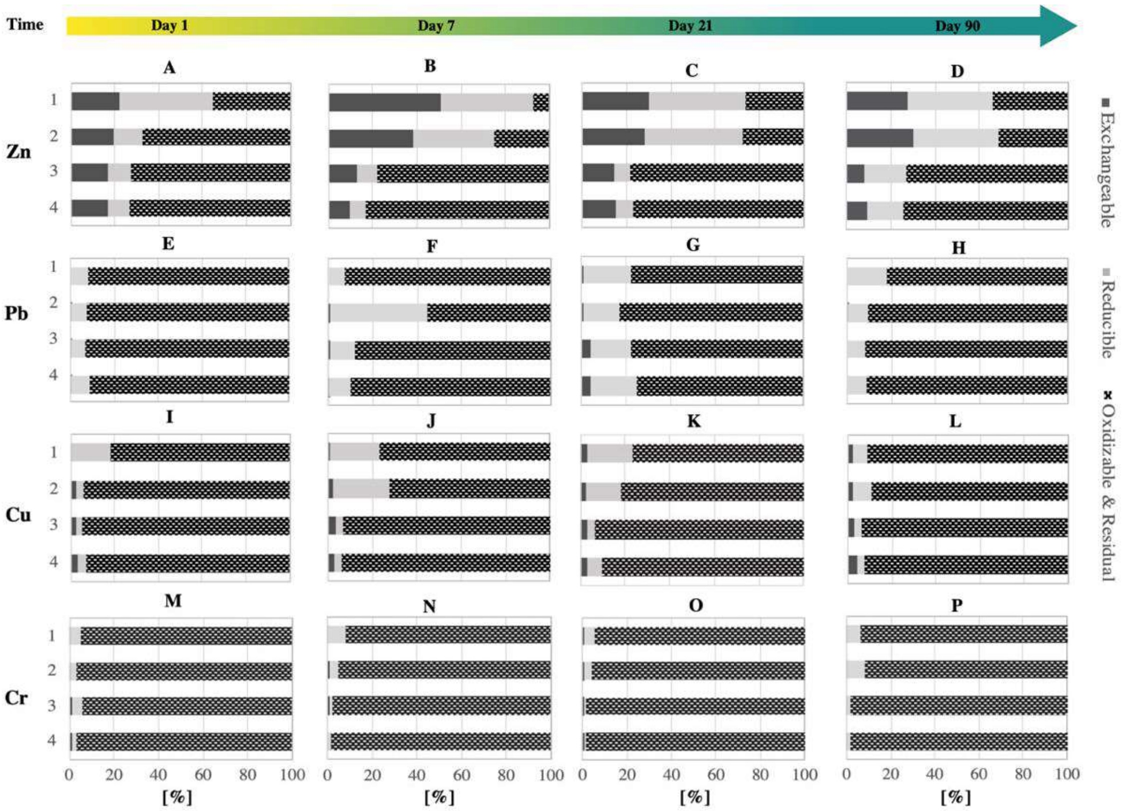

Figure 6: Metal fractionation in the different column layers over time for soil amended with digestate doped with Metformin (E1-Dig-Met): Zn (A – D), Pb (E – H), Cu (I – L), and Cr (M – P) (mean ±standard deviation, n=2).

The statistical analysis on the effect of time and experimental condition (presence/absence of metformin) on TM fractionation are reported in Appendix - A (Table A5). Overall, the analysis revealed that there was not a statistically significant interaction between the effects of time and experimental condition, with the exception for Zn in the fourth layer in the exchangeable and oxidizable-residual fractions. The simple main effects statistical analyses showed that, for some TM and soil layers, both time and experimental conditions had a statistically significant effect on TM fractionation. Specifically, Zn in the exchangeable fraction for experimental condition E1-Dig-Met in the second layer on day 1 was significantly lower than that on days 21 and 90; while Zn in the oxidizable-residual fraction for condition E1-Dig in the fourth layer on day 90 was statistically lower than that on the other days. Cu fractionation varied significantly in the first and second layers only for condition E1-Dig-Met. In the first layer, the Cu exchangeable fraction on days 1 and 7 was significantly lower than that on day 21, the reducible fraction on days 7 and 21 was significantly greater than that on day 90, and the oxidizable-residual fraction increased significantly between day 21 and day 90. In the fourth layer, Cu reducible and oxidizable-residual fractions on day 1 were significantly different compared to those on day 21, which showed an increase and decrease, respectively. Pb fractionation had a statistically significant variation in all column layers. In the first layer, the simple effect analysis showed that experimental condition had a significant effect on Pb reducible fraction on day 90, E1-Dig-Met had a greater quantity of Pb bound to Fe/Mn oxides compared to E1-Dig. In the second layer, Pb exchangeable fraction for condition E1-Dig on days 21 and 90 significantly increased compared to that on days 1 and 7. In third layer, the simple main effect analysis showed that neither time nor experimental condition had a significant effect on Pb fractionation. In the fourth layer, Pb reducible and oxidizable-residual fractions for condition E1-Dig-Met on day 1 and day 7 were significantly greater than those on day 21; while for condition E1-Dig-Met on day 90, Pb reducible fraction increased, and oxidizable-residual fraction decreased significantly compared to those on day 21. Cr fractionation varied significantly only for condition E1-Dig in the second layer: Cr exchangeable fraction on day 90 significantly decreased compared to that on day 1, Cr reducible fraction on day 90 significantly increased compared to that on days 1 and 7, and Cr oxidizable-residual fraction on day 90 significantly decreased compared to that on days 1, 7 and 21.

2.4 Discussion

Comparing with the EU maximum metal concentrations criteria for the use of compost and digestates [6], all TM concentrations in the digestate were below legislation limits with exception of Pb, which had a concentration of 142 mg/kg$_{dw}$ (120 mg/kg$_{dw}$ limit). Even though the OFMSW digestate total TM concentrations were within the legislation limits, they were more than double than those of the chosen soil which allowed to study a theoretical real-case scenario in which solid OFMSW digestate would be applied to a marginal land soil, with no addition of targeted metals.

The addition of OFMSW digestate on the selected naturally poor loamy sand soil led to an alkaline pH throughout the whole experiment duration with values ranging from 8.3 to 8.5, which does not favour metal mobility. In fact, it has been shown that metal sorption, metal complexation, precipitation and other reaction mechanisms in the soil system that govern metal mobility are highly dependent on pH: as pH increases, the retention of metal cations increases due to increase of global negative soil surface charge [48].

Generally, it has been observed that Pb had the strongest adsorption properties, Zn, Cu, and Cd intermediate, and Ni the weakest [18].

The soil mesocosm column experiments showed that total TM concentrations increased in the soil layer directly beneath the digestate amended-soil layer (top layer) throughout the 90-day experiment period, showing a downward movement through the first layers of the column. This behaviour could be due to a seepage of the digestate from the amended-soil layer into the underlying one, or a movement of TM by soil solution and colloidal particles due to convective and diffusive transport, as was observed by Gu and Bai after the application of sewage-sludge to mudflat soils [44]. As illustrated in Figure 4, no migration of TM to the deeper soil layers was observed, which was most likely determined by the TM forms.

TM fractionation analysis in the deeper soil column layers showed that the TM analysed were mainly found in the residual fraction (results for this fraction also include the oxidizable fraction which was negligible). This suggests that the TM were not subject to redistribution amongst the different fractions, and the transfer within the soil matrix through time was probably due to the strong bounds between TM and mineral groups of the soil, as kaolinite and muscovite (phyllosilicates) and quartz (silicate mineral). In fact, clay and silicate minerals not only favour the formation of high microporosities in the soil matrix but enhance the adsorption of metallic cations due to their negative surface charge [38,59].

In this study, the proportion of Zn in the deeper soil layers followed the order: residual > exchangeable > reducible fractions which changed through time with the reducible fraction having greater proportion than the exchangeable fraction on day 90. The proportion of Cu followed the order: residual > reducible > exchangeable fractions throughout the entire experiment time. Cu is known to have a high affinity towards stable soil OM [60]. Even though the soil used in the experiments had an OM content of 3.02 % dw, the percentage found in the oxidizable fraction for all TM was very low (< 4%) and considered negligible compared to the residual fraction. The proportion of Pb followed the same order as Cu: residual > reducible > exchangeable fractions with a slight increase of the proportion of the reducible fraction through time. This is in accordance with other studies that observed a high affinity of Pb to Fe, Mn and Al oxides and clay mineral groups (that can contribute to both mentioned fractions [61]), which are greatly present in the studied soil (Appendix A, Table A1), leading to its lower mobility and lower bioavailability [44]. In contrast, Cr was found primarily in the residual fractions with the exchangeable and reducible fractions being less than 1 % and 5 %, respectively, throughout the entire experiment duration. This could be due to the fast sorption kinetics in alkaline soils predominantly governed by precipitation of Cr hydroxide onto the soil [60].

TM fractionation in the first and second column layers, in which a downward transfer of TM was observed, showed that the TM were distributed differently amongst the assessed fractions compared to the deeper soil layers. In fact, the proportion of Zn was in average reducible = residual > exchangeable throughout time. This is in agreement with previous studies looking at soil amendment with different organic materials originating from source-separated OFMSW digestate, which observed that Zn was in greater quantities bound to Fe/Mn oxides [43,62]. This is probably due to the high affinity between Zn and Fe/Mn oxides at increasing pH [63]. This is also in contrast with the results found by Gu and Bai [44] which observed that Zn was the least mobile and bioavailable metal in mudflats amended with sludge. Pb, Cu and Cr in the first and second column layers followed the same order described for the deeper soil layers, with slightly greater quantities found in the reducible fraction. As mentioned above, this is probably attributed to the high affinity of these TM to silicate mineral groups, metal oxides and stable OM that leads to their low bioavailability [41]. This is in agreement with a previous study which found that Cu, Ni, and Zn originating from digestate showed lower metal mobility, thus a decrease of environmental impact, compared to those originating from animal manure or mineral fertilizer, resulting in metal leachate concentrations lower than the limits of regulation on drinking water [64]. As to leachate solutions, this study showed that no TM was detected in leachate samples over experiment time.

Additionally, attention must be given to anthropogenic impurities (inert materials as small DIY debris, glass, aluminium, and plastics) in the non-source-separated OFMSW digestate because they may increase the sorption surface for metals and contribute to the proportion of metals in the oxidizable-residual fraction. In the present study a considerable high number of microplastics were found in the digestate used (data in Appendix - A, Table A3). Recent studies have shown that the presence of microplastics in soil can impact the soil metal adsorption capacity, while increasing metal desorption and mobility due to the weak attraction forces and bonds between microplastics and metals that can easily be broken with changes in environmental conditions [27,29]. We could hypothesise that part of the TM found in the exchangeable fraction in the digestate amended soil layer originates from microplastics, which can increase the risk of (i) metal mobility due to their potential metal desorption in longer time frames (higher than the 90 day experiment) or under different environmental conditions, and (ii) metal leaching due to their transfer within the soil-groundwater system via plastic-metal complexes [29].

Organic contaminants, as EC, are also compounds that may affect the behaviour and the mobility of TM in the digestate amended soil and in the soil profile. Although, the soil column experiments conducted showed that the presence of metformin, pharmaceutical compound selected as proxy for polar organic contaminants, did not have a statistically significant effect on the mobility of the TM during experiment time, it cannot be concluded that the presence of metformin did not affect TM speciation and TM behaviour with soil constituents. In fact, in the presence of organic compounds TM may undergo complexation, forming coordination complexes that modify their speciation and their behaviour in the soil system [65], which may lead to an increase or decrease of the transfer of these contaminants within the soil system, posing additional environmental risks.

In general, the TM mobility and transfer through the loamy sand soil column profile determined in this study was most likely due to (i) the mixture of digestate and soil in the top layer that promoted sorption mechanisms in the amended soil and (ii) the alkaline nature of the loamy sand soil.

This study was made during a short time period (90-days); however it would be important to conduct a similar experiment with a long time frame to study the effect of metal aging in OFMSW digestate soil amendment. Furthermore, a more in-depth analysis should be conducted on the influence of emerging micro-organic contaminants on metal sorption mechanisms in the soil system. While this study looked at the macroscopic effect of a specific type of pharmaceutical compound (metformin) on the TM transfer along the soil profile through time, it is important to conduct a study at the microscopic level to evaluate the potential formation of coordination complexes and their influence on metal adsorption and transfer. More research should also be conducted on microplastics in the soil system to better understand their role in soil contaminant transfer and their effect on soil ecosystems, due to their increasing concentration in environmental matrices and the lack of knowledge concerning their behaviour and toxicity.

2.5 Conclusion

In this study, the fate of TM in marginal land soil after application of solid biogas digestate, originating from non-source separated OFMSW, was assessed. This work showed that total TM concentrations increased in the soil layer directly beneath the digestate amended-soil layer (top layer) throughout the experiment period, indicating a downward movement of the TM through the first column layers. Nevertheless, the TM were not able to reach the deeper soil layers most likely due to their speciation. In fact, TM fractionation showed that over time, in the deeper soil layers, TM were mainly found in the residual fraction, suggesting that they were not subject to redistribution amongst the other operationally defined fractions due to the strong bounds between metals and soil mineral groups (especially silicates). On the other hand, in the first few layers TM were found significantly also in the exchangeable and the reducible (bound to Fe/Mn oxides) fractions, suggesting a greater bioavailability and possible metal mobility through the soil profile, which may also increase due to the presence and interaction with micro-organic pollutants and anthropogenic impurities present in the digestate amended soil. Overall, the results showed a potential for the application of non-source-separated OFMSW digestate as marginal-land amendment due to the high TM sorption and low mobility of TM throughout time. Thus, this application would allow to increase marginal land soil quality through the supply of stable OM and nutrients, which coupled with the cultivation of energy crops would allow to further immobilize and accumulate the mobile and bioavailable fractions of TM present in the soil, as shown in previous studies using different types of digestates [50,51,66].

Associated content

The supporting information for this chapter is reported in Appendix A. This content includes further details on soil, digestate and amended soil characterization, the total metal concentration and fractionation results of initial solid matrices, microplastic isolation and characterization, and complementary soil column experiment results.

Author Contributions

Veronica Baldasso: Conceptualization, Methodology, Investigation, Data collection, Formal analysis (data interpretation and analysis), Writing – original draft. *Neus Bonet Garcia*: Investigation, Data collection. *Stephanie Sayen*: Conceptualization, Writing - review and editing. *Emmanuel Guillon*: Conceptualization, Writing - review and editing. *Luigi Frunzo*: Conceptualization, Writing - review and editing, Funding acquisition. *Carlos A. R. Gomes*: Conceptualization, Methodology, Writing - review and editing, Supervision. *Ana Paula Mucha*: Conceptualization, Methodology, Writing - review and editing, Supervision, Funding acquisition. *C. Marisa R. Almeida*: Conceptualization, Methodology, Writing - review and editing, Supervision, Funding acquisition. All authors have given approval to the final version of the manuscript.

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Chapter 3

Effects of digestate soil amendment on the fate of trace metals and on the soil microbial community

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

Baldasso V, Tomasino MP, Sayen S, Guillon E, Frunzo L, Gomes CAR, Mucha AP, Almeida CMR. Effects of digestate soil amendment on the fate of trace metals and on the soil microbial community.

Abstract

Digestate can be applied as a soil amendment for land restoration. However, concerns arise regarding potential soil and groundwater contamination and possible changes within the soil microbial community. A 28-day soil column experiment was conducted to assess: (i) the fate of trace metals (Zn, Cu, Pb) in a non-source-separated biogas digestate amended soil; (ii) the impact of this amendment on the soil microbial community; and (iii) the effect of two pharmaceuticals (metformin – antidiabetic and lamotrigine – antiepileptic) on both trace metals and microbial community behaviour. Three experimental conditions were defined: digestate, digestate spiked with metformin, and digestate spiked with lamotrigine. Soil samples were collected throughout time (day 1, 14 and 28) along the soil profile. Soil samples were analysed for trace metals' total concentrations and fractionation via atomic absorption spectroscopy, and the soil prokaryotic community was characterised through Illumina NGS technology. Results revealed no statistically significant mobility of trace metals through the soil profile over time due to their immobile, less bioavailable, forms. Initially, the soil microbial community in the digestate-amended soil was dominated by phylum Firmicutes, while at day 14 by Proteobacteria and Bacteroidota. Subsequently, the microbial community stabilized, showing minimal differences between days 14 and 28. The presence of metformin and lamotrigine, at the tested levels, did not significantly influence trace metals' behaviour or the prokaryotic community structure in the soil system. No significant difference in taxonomic composition was observed between treatment groups. This study suggests that contaminants may be immobilized in the digestate, rendering it a valuable resource for marginal land restoration. Nonetheless, proper monitoring and remediation strategies are crucial to ensure its safe application within circular bioeconomy strategies.

3.1 Introduction

Anaerobic digestion (AD) is a robust waste treatment process that treats organic waste producing biogas, used as a renewable energy source, and digestate [1,2]. In the last decade, thanks to the change in soil amendment and fertilizer regulations [3], the use of digestate as soil amendment gained a lot of attention. However, only few studies have shown non-source-separated municipal solid waste (MSW) digestate, comprising about 26 % of the yearly produced digestate in the Europe Union (EU) [4],to be a promising resource for marginal land restoration [5–9]. Digestate was shown to have promising agronomic properties, improving soil structure and increasing water retention capacity, potentially favouring greater biomass yield and soil biodiversity [9–11]. Digestates, originating from animal waste, manure and sewage sludge, were found to have high microbial abundance, with methanogenic species and microbial species of agronomical and environmental importance being detected in high and low abundance, respectively [11,12]. Moreover, some studies found digestate to stimulate the decomposer microbial species and favour microbial activity in soils, especially in the presence of plants [13,14]. While other studies found digestate to have had only a temporary impact on the soil microbial community's diversity, suggesting a low agronomical potential in terms of community diversity enhancement and indicating its potential to maintain and safeguard the native soil microbial species [11,15,16]. Like so, this value-added nutrient-rich organic material can be considered a valuable resource for solving the problem of unhealthy soils and degraded land in EU [17]. Furthermore, it was also found that compared to other biosolid amendments, non-source-separated MSW digestate reduced the risk of trace metal (TM) leaching due to a higher amount of mineralized organic matter (OM) and a lower quantity of dissolved organic carbon, favouring TM adsorption and immobilization onto the OM and mineral constituents of the soil [18–21]. However, high content of TM [22] and contaminants of emerging concern [23], like pharmaceutical and personal care products, can be a risk for the ecosystem and human health, as they can be toxic and/or give origin to toxic secondary compounds, and they can transfer through the soil system and accumulate in the flora and fauna [24–28]. Despite the moderately low risk of MSW digestates, there is still a possibility of transport and redistribution of contaminants inherently present in the digestate within the soil [24], making it essential to better understand how the fate of contaminants and the soil ecosystem are affected by MSW digestate application.

Some of the studies carried out on the potential use of digestate, originating from non-source-separated municipal biowaste (referred to as digestate from now on), as soil amendment have led to inconsistent results [7,9,29,30], making it a subject worthy of more investigation. Moreover, to the authors knowledge very few studies have focused on the effects that this type of digestate, specifically its solid fraction, can have on the soil microbial community. The present study centred on soil contamination by TM and pharmaceutical compounds deriving from the digestate, focusing on contaminants fate and effects on soil microbial community. Specifically, this study aimed to evaluate the bioavailability and distribution of Zn, Cu and Pb in a poor-quality soil after amendment with a digestate contaminated with these TM and with two pharmaceutical compounds classified as contaminants of emerging concern, namely metformin and lamotrigine. It is hypothesised that the TM found in the digestate can migrate through the soil profile, that their fate can be influenced by the presence of the pharmaceutical compounds, and that these contaminants affect the soil microbial community. Zn, Cu and Pb were selected because they were found in high concentrations in the digestate selected for this study, being TM that are easily uptook and stored by plants with a varying degree of toxicity [31–33]. Overall, this work contributes to deepen the understanding of (i) TM fate in soil after digestate application and (ii) digestate's effects on soil microbial community, both in the absence or presence of one of two pharmaceutical compounds. The two pharmaceutical compounds, metformin and lamotrigine, were selected as they are two of the most commonly used drugs for treating diabetes and epilepsy, respectively, and have been recently detected in biosolids, digestates and soils [34–38].

3.2 Materials and Methods

3.2.1 Materials

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

3.2.2 Solid matrices characterization

The soil employed for the experiment was sampled from a construction site in Ermesinde, Porto (PT), homogenized and sieved to <2 mm. The soil was classified as a Cambisol deriving from metamorphic Schist, with a fine loamy sand texture. The digestate was sampled at the end of the solid-liquid separation line of an MSW treatment plant, equipped with anaerobic digesters for the treatment the non-source-separated biowaste. The solid matrices were characterized for their main physical and chemical properties (Table 1) following the methodology reported in [39]. The soil and digestate were combined in a proportion of 14 to 1 (dry weight) and thoroughly mixed to form a homogeneous mixture of amended soil. The soil, digestate and amended soil were also characterized for total TM concentration and TM fractionation following the methodology described in section 3.2.4.

Table 1: Soil (S) and digestate (D) main physical-chemical properties (adapted from Baldasso *et al.*[39]).

Solid	pH (-)	CEC (cmol+/kg)	OM (%)	TC (%)	TOC (%)	CaO (wt%)	MgO (wt%)	Al_2O_3 (wt%)	Fe_2O_3 (wt%)	SiO_2 (wt%)
S	8.1	4.5	3.4	0.4	0.2	1.3	0.3	23.5	5.7	58.1
D	8.4	39.9	22.0	-	-	9.9	3.8	7.6	1.5	34.9

3.2.3 Soil Mesocosm Column Experiment

The soil mesocosm column experiment was conducted utilising transparent acrylic plastic columns (height of 60 cm and diameter of 19.4 cm) with 6 lateral regularly spaced-out sampling ports and a base port connected to a glass bottle for the collection of possible soil leaching (Figure 7). Soil columns were assembled by layering a first bottom layer of inert gravel (2 cm) for drainage, a mid-layer of soil (37 cm), manually packing the solid matrices at 5 cm strata at a time [40], and a final layer of soil amended with digestate (15 cm), to simulate digestate application on marginal land soil for energy crop cultivation. The soil columns were wrapped in tinfoil, to block sunlight, and placed in an outdoor greenhouse with an average temperature of 25 °C. The soil columns were irrigated with 200 mL of deionized water once a week to ensure a constant water content equal to 80 % of soil water holding capacity. The experiment had a duration of 28 days. Three experimental conditions were defined, each conducted in triplicates: soil amended with digestate (D); soil amended with digestate spiked with metformin (D-Met) and soil amended with digestate spiked with lamotrigine (D-Lmt). D-Met and D-Lmt experimental conditions were prepared by spiking with 2 mg/L metformin or 2 mg/L lamotrigine aqueous solution, respectively, the digestate and thoroughly mixing it. Then, this spiked digestate was used to prepare the digestate-amended soil amended soil, which was put on the top of the soil column immediately after preparation. The concentration of pharmaceutical compounds was selected based on the concentrations detected in biosolids, digestates and soils, reported in the literature [34–38].

Sampling of the soil columns was carried out on days 1, 14 and 28 by collecting soil samples from the column's 5 side ports (the 6th port was above the soil). Samples were stored at -20 °C until processing. During the 28 days of experiment no soil leaching was obtained from the bottom port. The solid samples were analysed for Zn, Cu and Pb total concentration and fractionation, and for soil prokaryotic community diversity and composition. The sampling days were chosen according to the findings of previous soil mesocosm experiment [39] showing no significant changes after day 21.

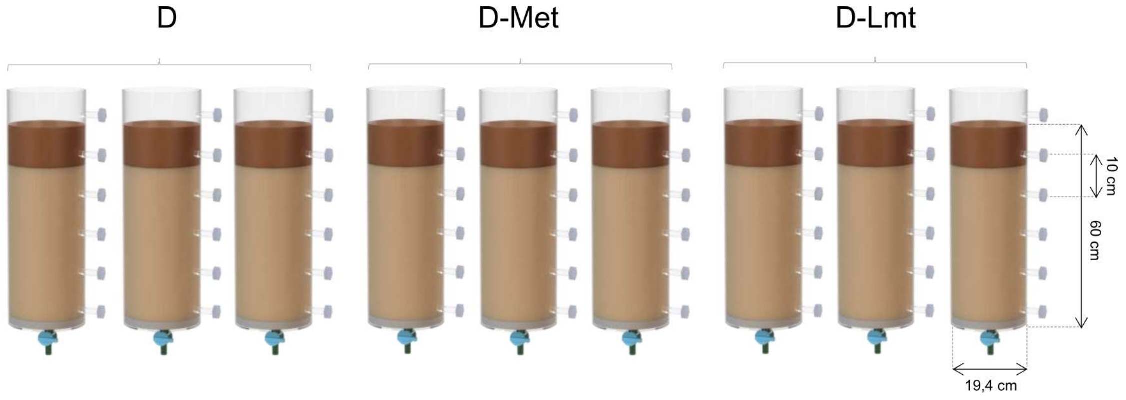

Figure 7: Soil column experiment set-up with the column's main dimension and the different packing layers, as inert gravel (grey), soil (light brown), and digestate amended soil (dark brown).

3.2.4 Analytical determinations

The solid samples' total TM concentrations were determined by atomic absorption spectroscopy (AAS) after a high-pressure microwave digestion (ETHOS 1, Milestone Inc) following the method found in [41]. The microwave digestion method used for soil samples, 5 mL of nitric acid added to 0.25 g of dry soil placed in a Teflon microwave vessel, differed slightly from that used for amended soil samples, 1 mL of nitric acid and 5 mL of hydrogen peroxide added to 0.50 g of dry amended soil. Before AAS analysis, the digestated samples, cooled to ambient temperature, were placed in 50 mL centrifuge tubes and diluted with deionized water to reach a final volume of 15 mL. The digestated samples were analysed by AAS with flame atomization (F-AAS) or AAS with electrothermal atomization (ET-AAS) using an AAnalysit 200 AA spectrometer system (PerkinElmer) and a PinAAcle 900Z AA spectrometer (PerkinElemer), respectively, depending on the analyte's concentration and the equipment's detection limits. The metal element concentrations were attained through calibration curves of aqueous standard metal solutions. The analysis was carried out in triplicate for samples of initial soil, digestate and amended soil, and for all single replicate samples of each soil column. Blank samples were also prepared and analysed following the same procedure to ensure no contamination occurred during the execution. Details on AAS method precision and analytical parameters can be found in Appendix B (Table B1).

TM fractionation followed the BCR sequential extraction method [41,42] to evaluate (i) the soluble and exchangeable fraction, (ii) the reducible iron/manganese oxides fraction, (iii) the oxidizable OM and sulphide fraction and (iv) the residual metal fractions. Preliminarily, 0.50 g of dry solid sample were weighed in a 50 mL centrifuge tube. Then, the extractions were carried out sequentially using 20 mL of acetic acid solution (0.11 mol/L) for the first extraction, 20 mL of hydroxylamine hydrochloride solution (0.5 mol/L) for the second extraction, and 25 mL of ammonium acetate solution (1 mol/L) after hydrogen peroxide (30 % v/v) oxidation for the third extraction. All extractions were carried out at ambient temperature using an end-over-end shaker (Unitronic Reciprocating Shaking Bath, JP Selecta). Metal concentrations in extraction solutions were analysed via F-AAS or ET-AAS, following the above-mentioned methodology. Fractionation results were reported as percentage of total TM concentration. The residual fraction was obtained by subtracting the other defined fractions from the total TM concentration. Blank samples were also prepared and analysed following the same procedure, confirming no contamination. TM fractionation was carried out in triplicate for samples of initial soil, digestate and amended soil, and for all single replicate samples of each soil column.

3.2.5 Data Analysis

Total TM concentrations and TM fractionations were assessed as average of three replicates with corresponding standard deviation. A statistical analysis was performed with SPSS Statistics software (version 28.0.1.0) to evaluate (i) significant differences along soil depth and (ii) significant effects of time and experimental conditions on total TM concentration and TM fractionation results. Firstly, an ANOVA followed by pairwise comparison with post-hoc Tukey HSD test was performed to evaluate if and which TM concentrations differed significantly by sampling depth. Secondly, a two-way MANOVA followed by a one-way MANOVA and pairwise comparison with post-hoc Tukey HSD test was performed to evaluate if time and experimental conditions had significant interaction and main effects on TM concentrations and which samples differed significantly. When data did not meet the assumption of normality and homoscedasticity, the non-parametric Kruskal-Wallis test followed by a non-parametric pairwise comparison test was performed. The different TM concentrations (total and fractionations) were set as dependent variables and sampling depth, time and experimental conditions were set as independent factor variables. Statistical significance level was set to 0.05.

3.2.6 Soil prokaryotic community

Prokaryotic community analysis was conducted in triplicate for initial soil, digestate and amended soil samples (taken at the start of the soil column experiment – day 0), and on one replicate for each soil column samples originating from the amended soil layer (port 1) and the mid soil layer (port 4) collected on days 1, 14 and 28. The analysis was carried out to evaluate changes in the soil prokaryotic community through time and through soil depth induced by digestate amendment and the presence of TM and pharmaceutical compounds (metformin and lamotrigine). The prokaryotic community analysis followed three steps: (i) DNA extraction, (ii) next generation sequencing (NGS) with Illumina MiSeq technology for 16S rRNA amplicons, and (iii) bioinformatics data analysis.

3.2.6.1 Environmental DNA extraction and 16S rRNA amplicon sequencing

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

For those samples, amplification was carried out using the specific primer pair, forward 515F-Y, 5'- GTGYCAGCMGCCGCGGTAA -3'; and reverse 806rB, 5'- GGACTACNVGGGTWTCTAAT -3', targeting the V4 hypervariable region of 16S rRNA gene.[43] At Genoinseq laboratories, amplification products were purified and normalized using Sequalprep™ 96-well plate kit (ThermoFisher Scientific, Waltham, USA).[44] Then, pair-end sequencing was conducted in the Illumina MiSeq® sequencer with the V3 chemistry (Illumina Inc., San Diego, California, USA).

3.2.6.2 Bioinformatic analysis

Raw fastq reads obtained from Illumina MiSeq® sequencing were quality-filtered by Genoinseq[45,46] using default parameters. The bioinformatic analysis consisted of an upstream and downstream workflow, all performed using RStudio (version 2023.03.1+446). The upstream analysis used raw fastq reads and followed the DADA2 pipeline (package version 1.26) with default settings: it included sample filtering, trimming (including primer removal), denoising, dereplication, providing an Amplicon Sequence Variance (ASV) table as output. ASVs representative sequences were then classified against the SILVA database (v.138.1)[47] for taxonomic classification. The downstream analysis investigated the diversity and the distribution of the prokaryotic community in and among the samples. The main packages used were phyloseq (version 1.42.0), vegan (version 2.6-4) and ggplot2 (version 3.4.2). Samples' α-diversity was evaluated through Shannon, inverse Simpson and Chao indexes coupled with a non-parametric ANOVA and pairwise Wilcoxon rank sum test. Samples' β-diversity was assessed through non-metric multidimensional scaling (NMDS) coupled with a PERMANOVA, and the samples' taxonomic profiles were visually assessed using barplots.

3.3 Results and Discussion

3.3.1 TM analysis

3.3.1.1 Total TM concentration

Zn, Cu and Pb were the TM selected for the experiment because they were the elements that had concentrations in the digestate more than double of those in the soil (Table 2), enabling to perform the experiment without spiking the amended soil with metals and reproduce a real-world scenario of digestate application on land. The total concentrations of TM found in the digestate were all within the metal limits imposed by EU regulation on soil improvers and growing media[48], suggesting that digestate from non-source-separated household biowaste can be a potential resource for land application and should not be officially classified solely based on its feed material [49].

Table 2: Soil, digestate and digestate amended soil TM total concentration (C) (mean ± standard deviation, n=3) and the percentage of exchangeable (Ex), reducible (Red), oxidizable (Ox) and residual fractions (Res) for metal fractionation.

Sample	Metal	C (µg/g)	Ex %	Red %	Ox %	Res %
Soil	Zn	26 ± 7	30	20	11	39
	Pb	28 ± 2	2	7	2	89
	Cu	33 ± 2	2	2	1	96
	Cr	30 ± 6	1	1	1	97
	Co	1.7 ± 0.3	-	-	-	-
	Cd	0.16 ± 0.01	-	-	-	-
	Ni	10.0 ± 0.6	-	-	-	-
	Fe	$(28 \pm 2) \cdot 10^3$	0.04	0.6	-	99
	Mn	48 ± 8	6	81	-	14
Digestate	Zn	235 ± 35	6	35	-	59
	Pb	53 ± 5	0.1	0.7	-	99.2
	Cu	64 ± 11	0.4	8.9	-	90.7
	Cr	15 ± 3	0.5	0.5	-	99
	Co	0.75 ± 0.01	-	-	-	-
	Cd	0.49 ± 0.01	-	-	-	-
	Ni	14 ± 1	-	-	-	-
	Fe	$(6.5 \pm 0.1) \cdot 10^3$	5	55	-	40
	Mn	129 ± 13	30	23	-	47
Amended soil	Zn	96 ± 29	27	36	8	29
	Pb	25 ± 8	0	1	4	95

Cu	44 ± 10	1	9	1	89
Cr	24 ± 6	1	8	1	91
Co	0.75 ± 0.01	-	-	-	-
Cd	0.12 ± 0.04	-	-	-	-
Ni	13 ± 3	-	-	-	-
Fe	$(18 \pm 1)\cdot 10^3$	35	12	-	53
Mn	83 ± 16	43	29	-	27

Zn, Cu and Pb total concentration along the soil column profile for day 1, day 14 and day 28 are reported in Figure 8.

The concentrations of Zn, Cu and Pb at day 1 were found to be similar to those of the initial soil and amended soil, evaluated prior to soil column assemblage (Table 2). Furthermore, Zn, Cu and Pb concentrations in the digestate amended soil layer (port 1) were found to be all significantly higher than their respective concentrations in the other soil column layers, (port 2, 3, 4 and 5) for all experimental conditions (presence/absence pharmaceutical compound) tested and sampling days (p-values < 0.05). In fact, Zn, Cu and Pb concentrations in the first layer ranged between 72 – 108, 28 – 41, and 24 – 37 µg/g, while in the other layers the concentrations ranged between 16 – 22, 28 – 36, and 18 – 29 µg/g, respectively. These results are also similar to those of the initial soil and amended soil used to assemble the soil columns. All these concentrations were found to be lower than the maximum permissible metal concentration in soil defined by EU regulation on the protection of the environment [50].

Considering the first soil column layers, it was found that neither experimental condition nor time had a significant effect on TM total concentrations (p-values > 0.05). Similarly, considering the other soil column layers it was found that Zn, Cu and Pb concentrations did not vary significantly throughout the soil column depth (p-value > 0.05) for each time and for each experimental condition. Looking at the effect of time and experimental condition on Zn total concentration on soil layers, it was found that sampling time did not have a significant effect (p-value > 0.05), while experimental condition had a significant effect resulting in Zn concentration of D being significantly higher than that of D-Lmt (p-value < 0.05). In contrast, Cu and Pb total concentrations were significantly affected only by sampling time (p-value < 0.05). Cu concentrations from samples collected on day 1 and day 14 (range 30 – 36 µg/g) were significantly higher than those collected on day 28 (range 28 – 34 µg/g) and Pb concentrations of day 1 (23 – 43 µg/g) were significantly higher (p-value > 0.05) than those of day 14 and day 28 (18 – 25 µg/g). The results of the statistical analyses are shown in Appendix B.

Overall, these results indicated no significant downward movement or migration of TM from the top amended soil layer to the deeper soil layers during the 28 day experiment, which agreed with previous studies where digestate had the lowest risk of metal leaching, compared to sewage sludge and mineral fertilizers, even during intense precipitation events [7,25]. This result can be explained by (i) the alkaline pH of the soil column system (average 8.6) which increases the soil's total negative surface charge favouring the retention of metal cations through different reaction mechanisms (precipitation, complexation, sorption) [19] and (ii) the chosen experimental set-up which kept the soil water content constant to 80 % water holding capacity, not leading to soil saturation and soil leaching, a key factor in colloidal particle transport [24,51]. In addition, the slight decrease in time of Cu and Pb concentrations detected in the deep soil layers, can be attributed to the intrinsic heterogeneity of the collected samples and/or to a subtle transport through preferential pathways occurring after column watering. Furthermore, the presence of metformin and lamotrigine did not have any statistically significant effect on TM total concentration along the soil profile through time, which agrees with the findings of Baldasso et al. (2023) [39] who studied the condition of digestate contaminated with metformin. Their study observed a slight TM migration from the top amended soil layer to the underlaying soil layer at the third experimental week (day 21) which was not observed in the current study. This difference can be attributed to the different column watering method and to an excess soil sampling, which in Baldasso et al. (2023) [39] might have induced soil leaching and mixing of the first two column layers.

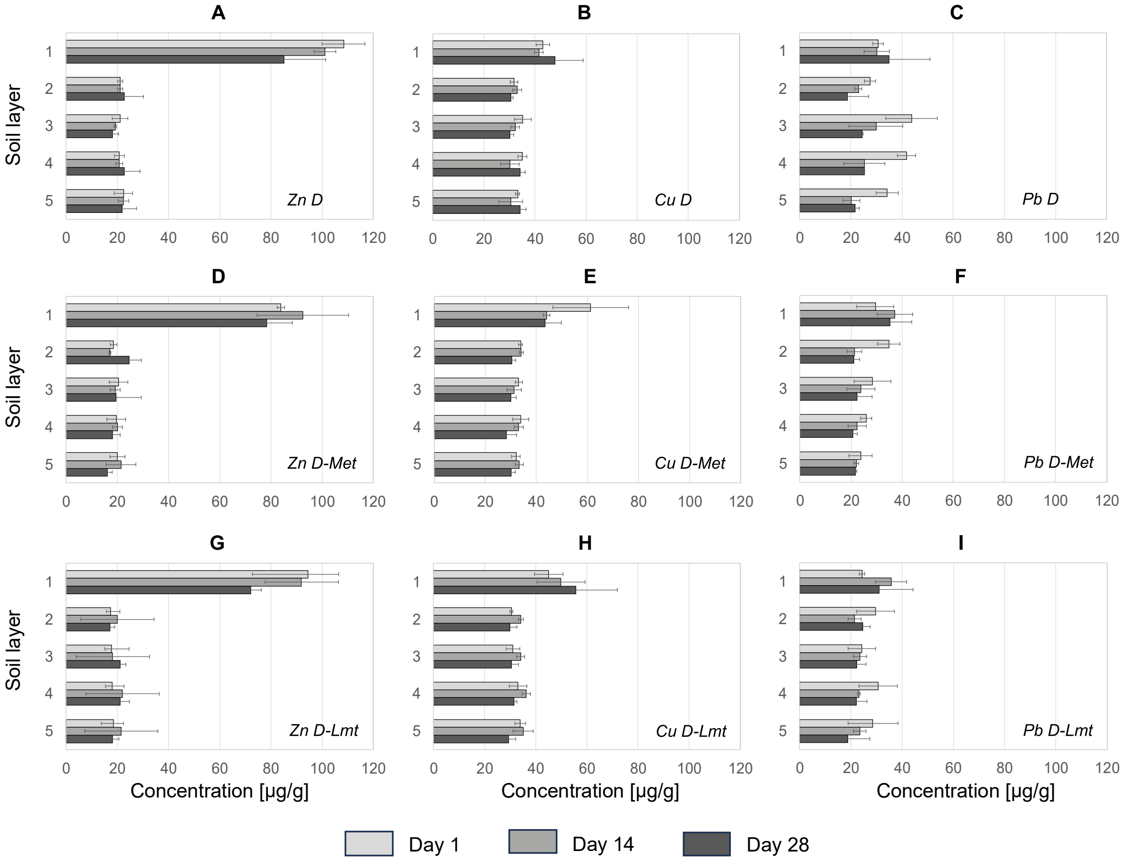

Figure 8: Zn, Cu and Pb total concentration along the soil profile throughout time for digestate-amended soil (A, B, C), digestate-amended soil with digestate previously spiked with metformin (D, E, F) and digestate-amended soil with digestate previously spiked with lamotrigine (G, H, I).

3.3.1.2 TM fractionation

Zn fractionation results for the different sampling depths (from port 1 to 5) for day 1, day 14 and day 28 are shown in Figure 9. Overall, Zn distribution in the second, third, fourth and fifth soil column layer for experimental condition D (Figure 9: A, B, C) followed the order Oxidizable-Residual > Exchangeable > Reducible, while in the first layer the distribution changed with time resulting in Oxidizable-Residual > Reducible > Exchangeable for day 1 and day 14 and Reducible > Exchangeable > Oxidizable-Residual for day 28. Zn distribution in the second, third, fourth and fifth soil column layer for the D-Met (Figure 9: D, E, F) followed the order Oxidizable-Residual > Exchangeable > Reducible, while in the first layer the distribution followed the sequence Reducible > Exchangeable > Oxidizable-Residual. Zn distribution in the second, third, fourth and fifth soil column layer for D-Lmt (Figure 9: G, H, I) followed the order Oxidizable-Residual >

Exchangeable ≈ Reducible, while in the first layer the distribution changed with time following the sequence Reducible > Oxidizable-Residual > Exchangeable for day 1 and day 14, and Exchangeable > Reducible > Oxidizable-Residual for day 28.

Going in more depth, for the first soil column layer, it was found that the exchangeable and reducible Zn fractions were significantly higher than the respective fractions measured in the other soil layers (p-values <0.05), while the oxidizable-residual fraction overall was not significantly different amongst the different layers (p-values >0.05). In fact, the exchangeable and reducible fractions in the first layer (digestate amended soil) varied from 20 % and 35 % to <10 % and 5 % in the soil layers, respectively. It was also observed that the exchangeable and oxidizable-residual fractions in the first layer were affected by sampling time but not by experimental condition, resulting in the exchangeable fraction of day 1 (20 - 32 %) and 14 (26 – 32 %) being significantly lower than that of day 28 (41 – 54 %), and the oxidizable-residual fraction of day 1 (20 - 45 %) and 14 (23 – 43 %) being significantly higher than that of day 28 (13 – 2 %). The reducible fraction was not affected by the analysed factor variables. Furthermore, considering the other soil column layers, it was seen that experimental condition and sampling time did not have any significant effect on Zn fractionation results (p-value < 0.05). These results reveal that Zn in the amended soil layer changed its form with time increasing its bioavailability and potential mobility, as was shown in other works on non-source separated MSW [39], source-separated MSW and sewage sludge amendment [7,30,52]. This can be a result of the high affinity of Zn toward Fe and Mn oxides, favoured by alkaline pH, and its propensity to form complexes with labile organic molecules [53]. This could also explain the greater increase over time of exchangeable Zn for D-Met and D-Lmt, as Zn might be in competition with the pharmaceutical compounds for soil adsorption sites [54].

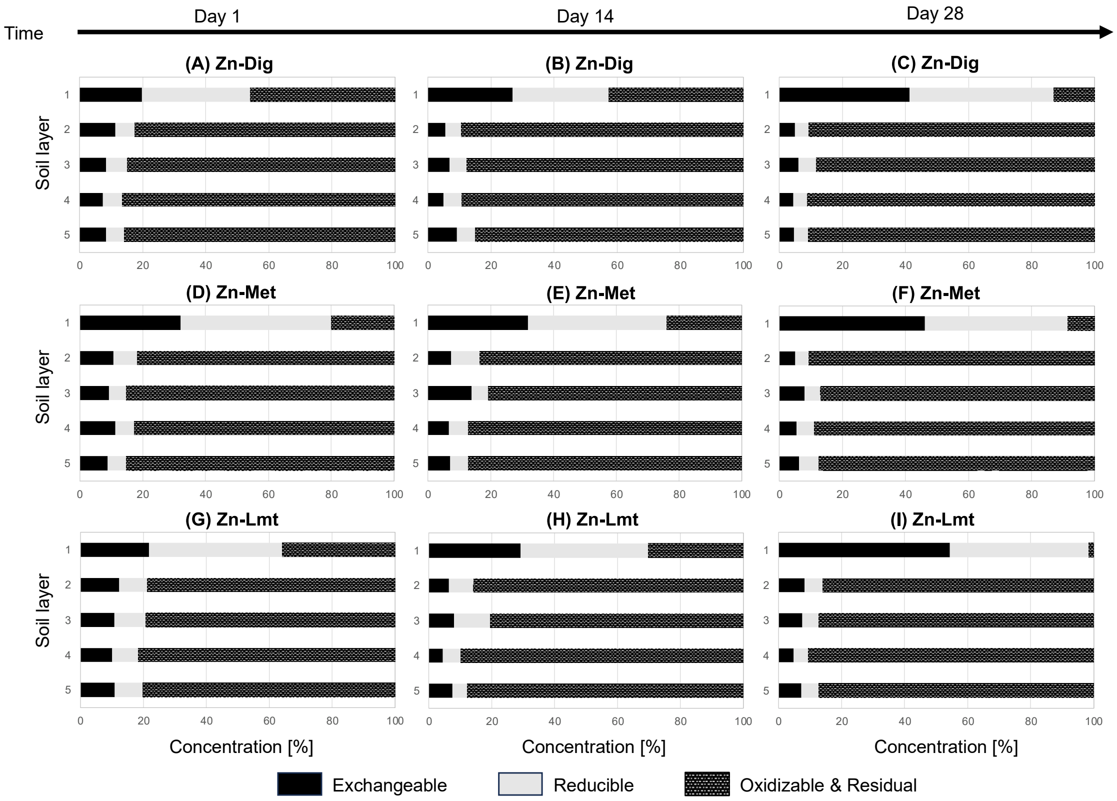

Figure 9: Zn fractionation along the soil profile throughout time for digestate-amended soil (A, B, C), digestate-amended soil with digestate previously spiked with metformin (D, E, F) and digestate-amended soil with digestate previously spiked with lamotrigine (G, H, I).

Cu fractionation in the different soil depths (from port 1 to 5) for day 1, day 14 and day 28 are shown in Figure 10. Overall, Cu distribution in all soil column layers for experimental condition D (Figure 10: A, B, C), D-Met (Figure 10: D, E, F) and D-Lmt (Figure 10: G, H, I) followed the order Oxidizable-Residual > Reducible > Exchangeable similarly to was observed in the study of Baldasso *et al.* (2023) [39]. More specifically, in the first soil column layer, it was found that the exchangeable fraction was significantly higher than the one in all the other soil column layers (p-value <0.05); the reducible fraction in the first layer was significantly higher compared to the one in the third, fourth and fifth soil column layer (p-value <0.05); while the oxidizable-residual fraction was significantly lower than those in all the other soil column layers (p-value <0.05). In fact, the exchangeable, reducible and oxidizable-residual fractions in the first layer varied from 1.2 – 2.3 %, 4.3 - 15 % and 82 – 94 % to 1.2 – 1.9 %, 1.6 – 3.7 % and 93 – 97 % in the other soil layers, respectively. This result agrees with past studies finding Cu availability and mobility to be directly linked to soil's OM content [7,31]. In fact, it is well accepted that Cu tends to form very stable complexes with OM, especially in alkaline environments [30], and has great affinity for silicate mineral groups. The loamy sand soil used in the study had an OM content of 3.4 % dw and a high content of silicate and clay minerals, explaining why Cu can be predominantly found bounded to OM and mineral groups (oxidizable-residual fraction).

More specifically, looking at the first layer, Cu fractions were not affected by experimental condition or sampling time with exception for the reducible fraction, which was significantly affected by time factor resulting in the quantity of day 1 (4.3 – 7.3 %) being significantly lower than that of day 14 (14 – 16 %) and day 28 (10 – 15 %). Considering the other soil column layers, neither the oxidizable-residual fraction (average 96 %) nor the reducible fraction (average 2.5 %) was affected by time resulting in similar values for all sampling days. These results highlight an increase in reducible Cu through time at the expense of oxidizable-residual Cu, which can be caused by OM mineralization leading to the release of Cu metallic cations that can readily react with available metal oxides [51]. Moreover, the presence of metformin and lamotrigine did not affect Cu fractionation throughout the soil profile most likely due to the synergistic adsorption behaviour on soil OM and mineral phases observed between Cu and the two pharmaceutical compounds[54].

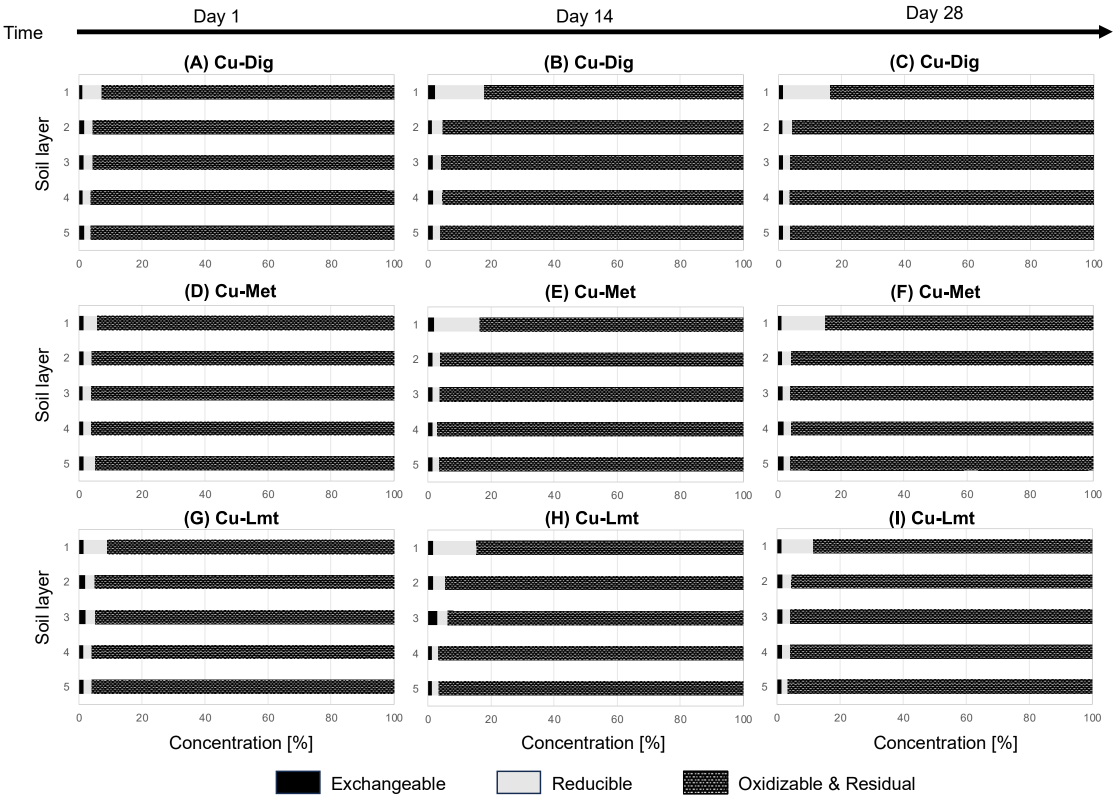

Figure 10: Cu fractionation along the soil profile throughout time for digestate-amended soil (A, B, C), digestate-amended soil with digestate previously spiked with metformin (D, E, F) and digestate-amended soil with digestate previously spiked with lamotrigine (G, H, I).

Pb fractionation results for the different soil depths (from port 1 to 5) for day 1, day 14 and day 28 are shown in Figure 11. Overall, Pb distribution in the second, third, fourth and fifth soil column layer for experimental condition D (Figure 11: A, B, C), D-Met (Figure 11: D, E, F) and D-Lmt (Figure 11: G, H, I) followed the order Oxidizable-Residual > Reducible > Exchangeable, in accordance with its low mobility and bioavailability [30,51,55]. Pb distribution in the first layer for all experimental conditions tested changed with time. Overall, Pb distribution on day 1 and day 14 was Oxidizable-Residual > Reducible > Exchangeable, and on day 28 Reducible > Oxidizable-Residual > Exchangeable for all experimental conditions D, D-Met and D-Lmt. This contrasts with the findings reported in Baldasso *et al.* [39], who found that Pb distribution in the amended soil layer was constant through time with Pb found dominantly in the oxidizable-residual fraction. Furthermore, the current study found that the exchangeable and the oxidizable-residual fractions did not significantly vary along soil column depth ($p > 0.05$, with exception of oxidizable and residual fraction of layer 1 and layer 3); whereas the reducible fraction of the first column layer was significantly higher than that of the other layers (p-value < 0.05). This fraction was between 31 – 92 % in the first layer and between 3.0 – 20 % in the other layers. It is important also to highlight that Pb distribution was not significantly affected by the presence of pharmaceutical compounds most likely due to Pb's greater affinity toward Fe/Al/Mn oxides, OM, silicate and clay minerals and its possible precipitation at alkaline pH [54].

Going in more detail and considering the first soil column layer, it was found that only the reducible fraction was significantly affected by time (p-value < 0.05), resulting in the amount in day 28 (average 73 %) being significantly higher than that of day 1 (49 %) and day 14 (38 %). Looking at the reducible fraction in all the other layers, time factor was also the only one that had a significant effect, with the reducible fraction of day 28 (average 12 %) being significantly higher (p-value < 0.05) than that of day 1 (average 5.0 %) and day 14 (average 6.0 %). Concerning the exchangeable and the oxidizable-residual fractions, only time had a significant effect (p-value < 0.05), with the exchangeable fraction of day 28 (average 2.6 %) being significantly higher than that of day 1 (average 1.0 %) and day 14 (average 1.2 %) and the oxidizable-residual fraction of day 28 (average 85 %) being significantly lower compared to that of day 1 (average 94 %). It is known that Pb behaviour in soil is affected by different mechanisms, predominantly adsorption onto specific soil and clay minerals characterized by Mn, Fe and Al oxides for which Pb exhibits the strongest affinity [55]. The soil used in the experiment presented high contents of quartz and kaolinite, with minor contents of calcite and muscovite, providing different reactive solid surfaces especially Al-oxides. Pb adsorption onto Al-oxides is known to be characterised by two phases: a first quick reaction on free surface site and a second reaction governed by diffusion in the mineral particles and lower site affinity [55]. This mechanism coupled with OM mineralization can explain the increase in Pb reducible fraction through time, with the simultaneous reduction in oxidizable-residual fraction [51].

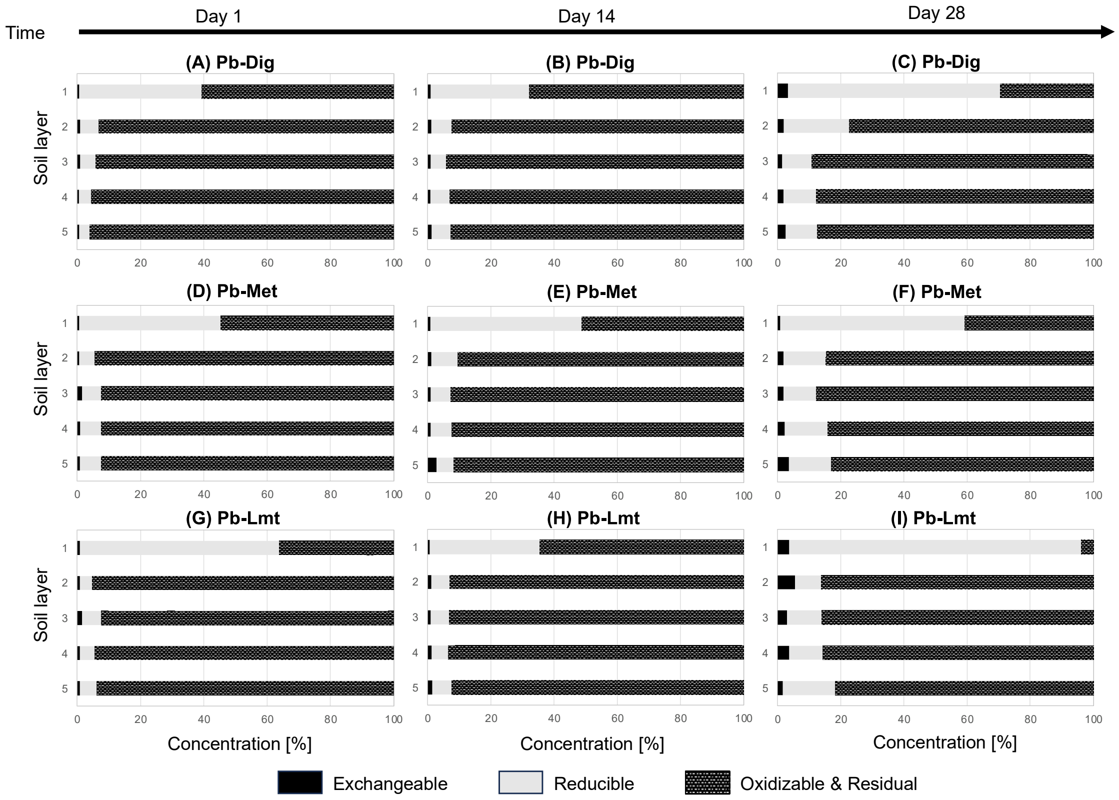

Figure 11: Pb fractionation along the soil profile throughout time for digestate-amended soil (A, B, C), digestate-amended soil with digestate previously spiked with metformin (D, E, F) and digestate-amended with digestate previously soil spiked with lamotrigine (G, H, I).

3.3.1.3 TM mobility factor

The TM mobility factor (MF) was calculated for the different sampling depths and sampling days to have a better understanding of the potential mobility of TM along the soil profile through time (Table 3). The MF was calculated using relative contents of the bioavailable TM fractions following equation 1 [56].

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

The MFs of Zn decreased along the soil profile for all experimental conditions tested. Zn MFs was the highest among the TM considered, with the ones observed for D-Lmt on day 28 being the greatest of all. In contrast, MFs for Cu and Pb did not follow any specific trend along the soil profile, with very little variation. For all experimental conditions, Zn MFs in the first layer increased with time, while those for the other layers decreased (even if slightly) over time. Cu MFs in the first layer, for all experimental conditions, increased from day 1 to day 14 and decreased from day 14 to day 28, while no specific trend was found for the other layers. Pb MFs for all experimental conditions and soil depths increased with time, with higher MFs on day 28. These results give a summary of what was observed with the in depth fractionation analysis. Overall, the digestate amendment seems to have increased the TM potential mobility in the soil through time, and thus probably their bioavailability. The TM had different behaviours, with Zn being potentially the most mobile element posing greater risk for the soil environment, followed by Cu and then Pb, generally agreeing with past findings [57,58]. However, considering the amount of TM in the exchangeable fraction, this mobility might not represent a significant risk as no significant increase in TM levels was observed on the soil layers beneath the digestate-amended soil layer.

Overall, despite the increase in total TM levels in the amended soil, due to amendment application characterized by greater TM levels than the soil, in this study case TM seemed to have been retained in the top digestate-amended soil layer with zero to very little TM redistribution through the soil profile over time. Moreover, the presence of pharmaceutical compounds, namely metformin and lamotrigine, did not significantly influence TM fate and distribution within the soil system.

Table 3: Trace metal mobility factor (%) assessed along the soil column profile through time for the different experimental conditions: digestate-amended soil (D), digestate-amended soil with digestate previously spiked with metformin (D-Met) and digestate-amended soil with digestate previously spiked with lamotrigine (D-Lmt).

	Trace metal	Zn			Cu			Pb		
	Day	1	14	28	1	14	28	1	14	28
Experimental Condition	Sampling depth	MF	MF	MF	MF	MF	MF	MF	MF	MF
E-Dig	1	20	27	41	1.2	2.3	1.4	0.7	1.0	3.4
	2	11	5.6	4.9	1.8	1.4	1.3	1.0	1.2	2.0
	3	8.4	6.8	6.1	1.5	1.6	1.5	1.0	1.1	1.4
	4	7.4	4.9	4.5	1.3	1.8	1.5	0.6	0.8	1.8
	5	8.4	9.2	4.6	1.7	1.7	1.4	0.8	1.3	2.6
E-Met	1	32	32	46	1.6	2.0	1.2	0.7	0.8	0.9
	2	11	7.4	5.2	1.6	1.5	1.5	0.8	1.2	1.9

	3	9.4	14	8.0	1.3	1.5	1.6	1.7	1.0	2.0
	4	11	6.6	5.5	1.4	1.5	1.9	1.0	1.0	2.4
	5	8.8	7.1	6.2	1.7	1.5	1.9	0.9	2.9	3.7
E-Lmt	1	22	29	54	1.6	1.8	1.4	0.8	0.7	3.8
	2	12	6.5	8.3	2.0	1.8	1.8	0.9	1.1	5.6
	3	11	8.0	7.5	2.2	3.1	1.8	1.6	1.0	3.1
	4	10	4.5	4.8	1.4	1.3	1.6	0.9	1.3	3.8
	5	11	7.6	7.3	1.7	1.3	1.4	0.9	1.4	1.7

3.3.2 Soil microbial community

Soil amendment with digestate might have an impact on soil microbial community, not only due to the input of OM and nutrients but also due to the potential presence of contaminants, such as TM or pharmaceutical compounds, either alone or together. Moreover, changes in the soil microbial community might occur over time.

In order to characterize the microbial community, DNA was extracted from the initial soil, digestate and digestate-amended soil, and in the soil column samples, collected in the first, second and fourth layer of each soil column (third layer was considered identical to the fourth layer), for each experimental condition (absence or spiking of the digestate with one of the two selected pharmaceuticals), over time.

Initial soil and soil column samples taken below the layer of soil that was amended with the digestate (first layer) presented very low amounts of DNA (Table B2, Appendix B), confirming that the soil selected for the experiment, a marginal soil from a construction site, was poor in terms of living organisms. This high difference in terms of DNA obtained when comparing amended with non-amended soil, demonstrated an important role of the amendment with digestate in increasing the microbial abundance in the selected soil.

However, the low amount of DNA obtained in the non-amended soil, prevented an effective amplification of the extracted DNA, compromising the prokaryotic community analysis for these samples. Therefore, the results reported below focus only on the prokaryotic community profile of the initial digestate and the digestate amended soil over time.

A total of 2,947,832 raw sequences were obtained from Illumina MiSeq sequencing, ranging from 35,181 to 211,092 sequences per sample, with an average of 89,328 sequences. A total of 1,926,007 cleaned sequences were obtained at the end of the upstream workflow, ranging from 23,182 to 141,030 sequences per sample with an average of 58,363 sequences, corresponding to 65 % of the average input (Appendix B, Table B3).

3.3.2.1 Prokaryotic community richness and diversity

Rarefaction analysis, based on ASVs, determined that sequencing reached saturation for all samples meaning that the number of sequences obtained was enough to have a good coverage of the community diversity (Appendix B, Figure B1).

The prokaryotic communities' alpha-diversity was evaluated through ASV abundance and alpha-diversity indices, as Chao, Shannon and Inverse Simpson (Figure 12), coupled with non-parametric Kruskal-Wallis test to evaluate the effect of sampling time and experimental conditions and Wilcoxon rank sum test for pairwise comparison (Appendix B, Table B4 and Table B5). The abundance of ASVs per sample was very similar to the results of Chao index showing that specie richness was similar amongst all the different samples. The close correspondence between the observed ASVs and the Chao index suggests that the rare biosphere was well represented in all samples, indicating that additional sampling would have unlikely revealed other unique species. It's important to consider that the Chao index provides a statistical estimate, and further investigations may provide additional insights into the dynamics of rare species within these communities, giving additional information on overall community structure. Moreover, neither experimental conditions nor time had a significant effect on the samples' species richness (p-value > 0.05). Shannon and Inverse Simpson indices showed variation among time (p-value < 0.05). Samples of day 0 (start of the experiment) had lower diversity compared to those of day 1, day 14 and day 28 according to Inverse Simpson index, whereas according to Shannon index samples of day 0 had lower diversity only compared to those of day 14 and day 28. Moreover, according to both indices, day 1 samples had lower diversity compared to samples of day 14 and day 28, while there was no significant difference between the diversity of samples of day 14 and day 28 (p-values > 0.05). These indices also showed that initial digestate (day 0) had slightly higher diversity than the initial amended soil (day 0). In addition, Inverse Simpson index showed variation amongst the experimental conditions tested (p-value < 0.05) indicating that the diversity of the initial amended soil (day 0) was significantly lower than that of the experimental conditions tested (D, D-Met and D-Lmt). However, considering that the digestate-amended experimental condition (D) used natural digestate-amended soil as the one constituting the initial amended soil samples, the observed significant difference among these samples was attributable solely to time variation.

The prokaryotic communities' beta-diversity is shown in the multidimensional scaling plot reported in Figure 13, indicating a clear prokaryotic community separation. The beta-diversity was combined with PERMANOVA and pairwise comparison tests to evaluate the possible effects of time and experimental conditions on the prokaryotic communities' beta-diversity (Appendix B, Table B6). Overall, beta-diversity was found to be significantly affected by sampling time (p-value < 0.05), with no significant difference among experimental conditions and with no significant interaction effect determined (p-value > 0.05). Considering sampling time, the initial samples (day 0) and samples of day 1 formed a cluster and samples of day 14 and day 28 formed a second cluster. Considering experimental conditions, the initial digestate and amended soil samples (day 0) formed two close but distinct clusters (not statistically significant). Results show that digestate amendment clearly increased the microbial abundance of the marginal soil selected for the experiment. This agrees with past studies which found that digestate application can clearly stimulate soil prokaryotic growth [13], leading to an increase in soil prokaryotic community abundance [14].

Characterization of the microbial community structure was only possible for the soil amended with digestate, and demonstrated a differentiation of the community with time, and a tendency for diversity increase throughout the experiment. At the beginning of the experiment, the amended soil showed a prokaryotic community that was very similar to the community present in the digestate. After 14 days, the community was clearly different from the beginning of the experiment, and quite similar to the community found at the end of experimental time, pointing to a stabilization of the prokaryotic community structure. These results may indicate that part of the microorganisms introduced by the digestate (e.g. anaerobic microorganisms) were, after some time, replaced by other microorganisms more adapt to the new environmental conditions (e.g. aerobic microorganisms). Other authors have already observed that part of the dominant prokaryotes from the digestate are not able to permanently colonize the soil [11,59,60]. On the other hand, it was not possible to observe a clear effect of the added pharmaceuticals, specifically metformin and lamotrigine, at the levels tested, in the prokaryotic community structure or diversity.

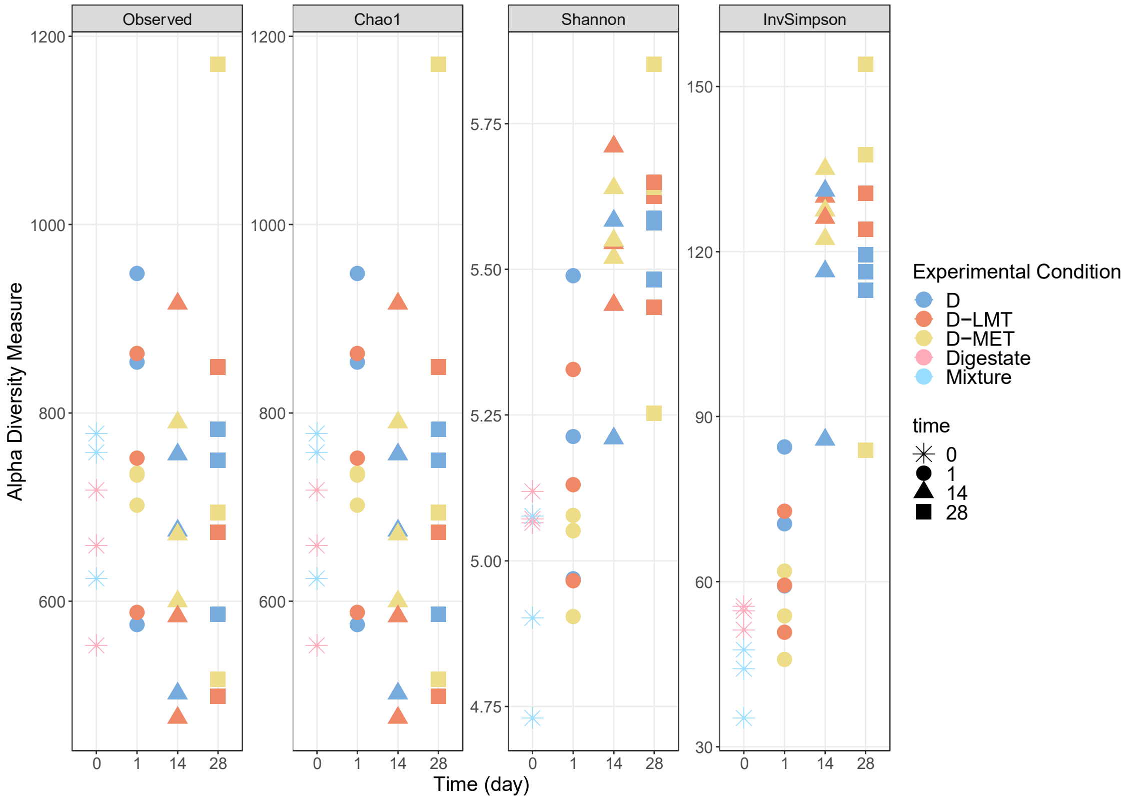

Figure 12: Richness and alpha diversity indices of the prokaryotic community of the initial substrates (digestate – pink, amended soil (mixture) – light blue) on day 0 and of first soil column layers through time (day 1, day 14 and day 28). Experimental conditions were digestate-amended soil (D in blue), digestate-amended soil with digestate previously spiked with metformin (D-MET in red), and digestate-amended soil with digestate previously spiked with lamotrigine (D-LMT in yellow).

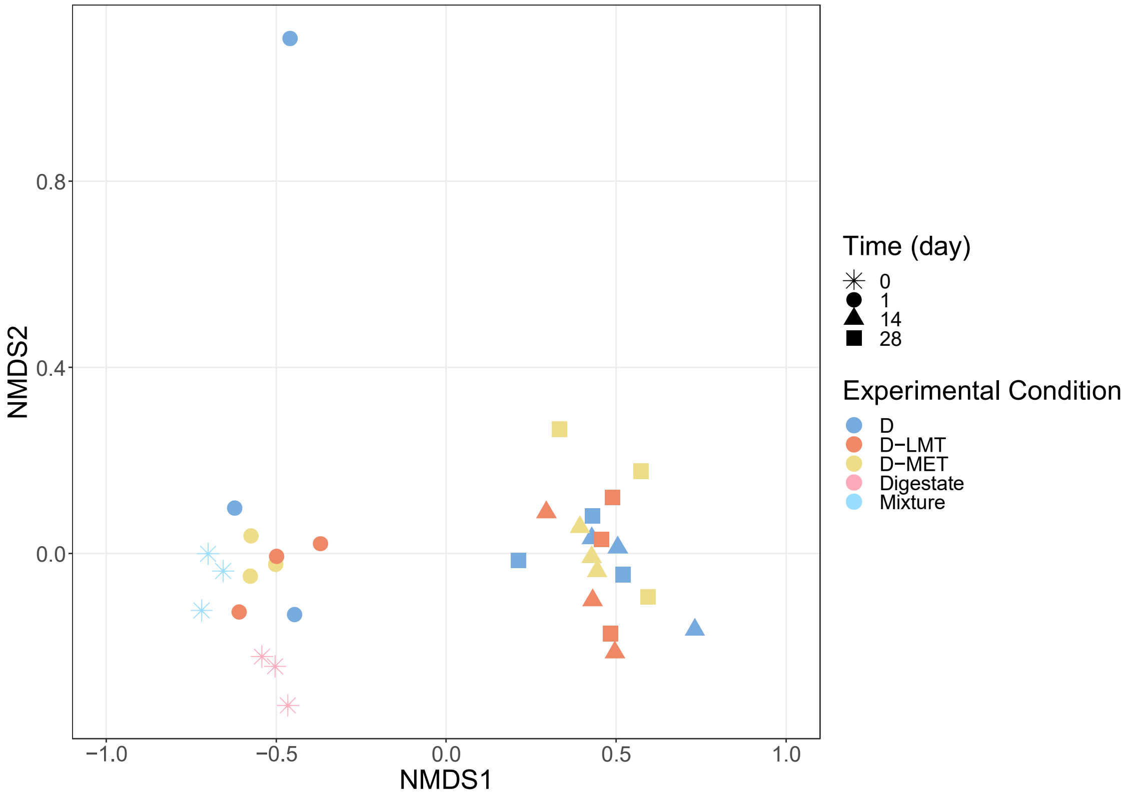

Figure 13: Beta diversity of the prokaryote community of the initial substrates (digestate – pink, amended soil – light blue) and of the first soil column layers through time (day 0 – circle, day 14 – triangle, day 28 – square). Experimental conditions were digestate amended soil (D in blue), digestate amended soil with digestate previously spiked with metformin (D-MET in red) and digestate amended soil with digestate previously spiked with lamotrigine (D-LMT in yellow).

3.3.2.2 Prokaryotic community taxonomic composition

It is important to highlight that on average 99.7 % of ASVs belonged to bacteria domain while < 0.15 % to archaea domain and < 0.13 % to unclassified species (Table B7, Appendix B).

For the archaea domain, the most abundant archaeal groups were the Methanobacteriaceae and Methanomicrobiaceae family followed by Nitrososphaeraceae, which have been detected in different digestates and liquid digestate amended grasslands and peatlands [11,61,62]. The abundance of these archaea groups (Table B8, Appendix B), calculated based on the total archaea abundance, varies between the digestate and the amended soil samples, with Methanobacteriaceae (52 %) > Methanomicrobiaceae (31 %) > Nitrososphaeraceae (17 %) in the digestate and Methanomicrobiaceae (40 %) > Methanobacteriaceae (38 %) > Nitrososphaeraceae (22

%) in the initial amended soil. The most abundant genera of the Methanobacteriaceae and Methanomicrobiaceae families were *Methanobacterium* (48 % in digestate, 36 % in amended soil) and *Methanoculleus* (31 % in digestate, 40 % in amended soil), respectively. These bacteria live in anaerobic conditions and can potentially increase the soil's methane emission through hydrogenotrophic methanogenesis pathways, specifically in soils favouring anaerobic condition (e.g. peatlands), however this was not the case for the current study which utilized a loamy sand soil [61,63].

Considering the bacteria domain, the samples' taxonomic composition was evaluated by comparing the relative abundance of reads on phylum and genus basis (Figure 14 and Figure 15). Results showed that the initial digestate, initial amended soil and samples collected on day 1 had similar bacterial community composition at phylum level with the main phyla being Firmicutes (54-62 %), Proteobacteria (7.6-15 %), Bacteroidota (7.6-17 %), Caldatribacteriota (6.8-11 %) and Actinobacteriota (3.5-9.2 %). For the samples collected on day 14 and day 28 for all experimental conditions, a similar bacterial composition was observed with the main phyla being Proteobacteria (30-46 %), Bacteroidota (21-34 %), Firmicutes (10-28 %), Actinobacteriota (4.9-8.4 %), Planctomycetota (1.7-6.6 %), Deinococcota (1.3-5.8 %), Patescibacteria (0.9-6.2 %) and Desulfobacterota (1.2-3.0 %). A few minor differences could be noticed among these samples such as (i) the appearance of the Desulfobacterota phylum on day 28 in most of the samples (2.1 – 2.9 %), (ii) a slight increase in the less abundant phyla as Actinobacteriota (on average from 5.8 to 7.2 %), Planctomycetota (on average from 2.8 to 3.6 %) and Deinococcota (on average from 2.3 to 2.9 %) and a decrease in Proteobacteria (from 36 to 33 %). When comparing the composition of initial samples and samples of day 1 with that of samples of day 14 and 28, a clear decrease in the phyla Firmicutes (on average from 56 to < 18 %) and Caldatribacteriota (on average from 8.5 to < 2 %) was observed. On the contrary, there was a clear increase in Proteobacteria (on average from 11 to < 34 %), while other phyla like Planctomycetota, Deinococcota, Patescibacteria and Desulfobacterota increased their relative abundance to values on average above 2 %.

The bacterial communities' composition in digestate are known to be intrinsically dependent on the operational conditions of the AD process, the feed material and the inoculum used [12,64]. Other studies also observed that Firmicutes, Bacteroidota and Proteobacteria were the dominating phyla in fresh digestate and compost. Moreover, a growth of Actinobacteria, Planctomycetota and Deinococcota with organic amendment aging has been reported [11,15,62,65]. Firmicutes and Bacteroidota (fermenting bacteria) are known to have a fundamental role in OM mineralization [66]. Firmicutes are more stress-resistant and capable of using recalcitrant C sources, while Bacteroidota are more environment-sensitive and mainly use labile OM sources [65–67]. These characteristics, together with a change from anaerobic to aerobic conditions, can explain the decrease in Firmicutes and the increase in Bacteroidota in amended soil samples [60]. Moreover, Proteobacteria and Actinobacteria are known to have key roles in soil carbon, nitrogen and sulphur cycling, the same as Planctomycetota, Patescibacteria and Desulfobacterota that have been positively correlated with soil C/N ratio [65]. All these phyla that presented a tendency to increase their relative abundance after a period of soil stabilization (around 14 days), have already been described as characteristic phyla of soil bacteria communities [67].

This variation in bacterial community composition was also found at the genus level (Appendix B, Figure B2). Overall, most of the genera found in the samples have been found also in other organic amendments and amended soils, even though many of the most common bacterial genera (*Clostridium*, *Bacillus*, *Rhizobium*, etc) detected in anaerobic digestates were found with < 1 % relative abundance [11,68]. Considering the taxonomic pattern of the top 10 genera (Figure 15), it was noticeable that the initial digestate, the initial amended soil and day 1 samples were similar to each other with the top 10 genera being *Fastidiosipila* (on average 8.5 – 9.7 %), *Candidatus_Caldatribacterium* (6.8 – 11 %), *Tepidimicrobium* (4.5 – 8.3 %), *Flavobacterium* (0.9 – 5.0 %), *Thiopseudomonas* (1.3 – 3.1 %), *Pusillimonas* (0.6 – 1.9 %), *Aquamicrobium* (0.4 – 1.1 %), *Gelidibacter* (0.7 – 0.8 %) and *Pseudomonas* (0.3 – 1.8 %). The taxonomic pattern of these samples resulted being different, in terms of relative abundance, to the taxonomic pattern of the samples collected on day 14 and day 28, which resulted being similar to one another, with the top 10 genera being *Flavobacterium* (3.2 – 12 %), *Gelidibacter* (0.3 – 8.8 %), *Aquamicrobium* (2.7 – 6.1 %), *Pseudomonas* (2.0 – 5.8 %), *Thiopseudomonas* (0.7 – 5.4 %), *Pusillimonas* (1.4 – 3.5 %), *Fastidiosipila* (1.0 – 3.4 %), *Tepidimicrobium* (0.3 – 3.6 %), and *Candidatus_Caldatribacterium* (0.2 – 3.4 %). Furthermore, the phyla of the top 10 genera confirmed the prokaryotic community composition found at the phylum level, with *Fastidiosipila* and *Tepidimicrobium* of the Firmicutes phylum being the dominant genera of initial and day 1 samples, and *Pusillimonas*, *Thiopseudomonas*, *Aquamicrobium* and *Pseudomonas* of the Proteobacteria phylum being the dominant genera of day 14 and day 28 samples. Going in detail, *Fastidiosipila* and *Tepidimicrobium* are known to be characteristic genera of the meso/thermophilic AD process, with the first being important for the conversion of complex organic molecules to volatile fatty acids and carbon hydroxide and the second being important in the hydrogenotrophic pathways of methanogenesis [2,62]. Their functions highlight their AD origins and explain why they were found in great abundance in the digestate samples. Moreover, these genera could lead to potential soil greenhouse gas emissions (carbon dioxide and methane) when found in favourable anoxic conditions, especially after heavy precipitation events with low soil drainage [67]. *Pusillimonas*, *Thiopseudomonas*, *Aquamicrobium* and *Pseudomonas* are all gram-negative bacteria that have been detected in diverse soil and man-made environments (wastewater treatment plants, anaerobic digestors, biofilters, etc). Amongst these genera, *Pseudomonas* is known to be a plant-growth-promoting genus [11,60], while *Aquamicrobium* and *Pusillimonas* are known to have pollutant-degrading capacities specifically towards hydrocarbons and petroleum derived compounds [69,70]. Additionally, other genera with lower abundances were also found to have plant-growth promoting effects, nitrogen fixing functions, denitrifying functions, contaminant degrading and biosorption capacities. Among these there are *Rhodopirellula* [71] and *Actinomyces* [72] proposed to contribute to the global carbon and nitrogen cycles, and several other processes, *Sporosarcina* [72] *and Leucobacter* [74] proposed as plant growth promoting species, *Marinobacter* [75] and *Taibaiella* [76] proposed to have a role in biogeochemical cycling of organics and metals, *Paenacalcigenes* [77] proposed as a potential plastic-polymer degrader. In addition, *Paenibacillus*, *Iamia* and *Streptococcus* are considered important metal biosorbents that have been applied successfully in contaminated soils; they have been found in the amended soil samples suggesting they can contribute to the immobilization of metals originating from the digestate, reducing their threat towards the soil environment [78].

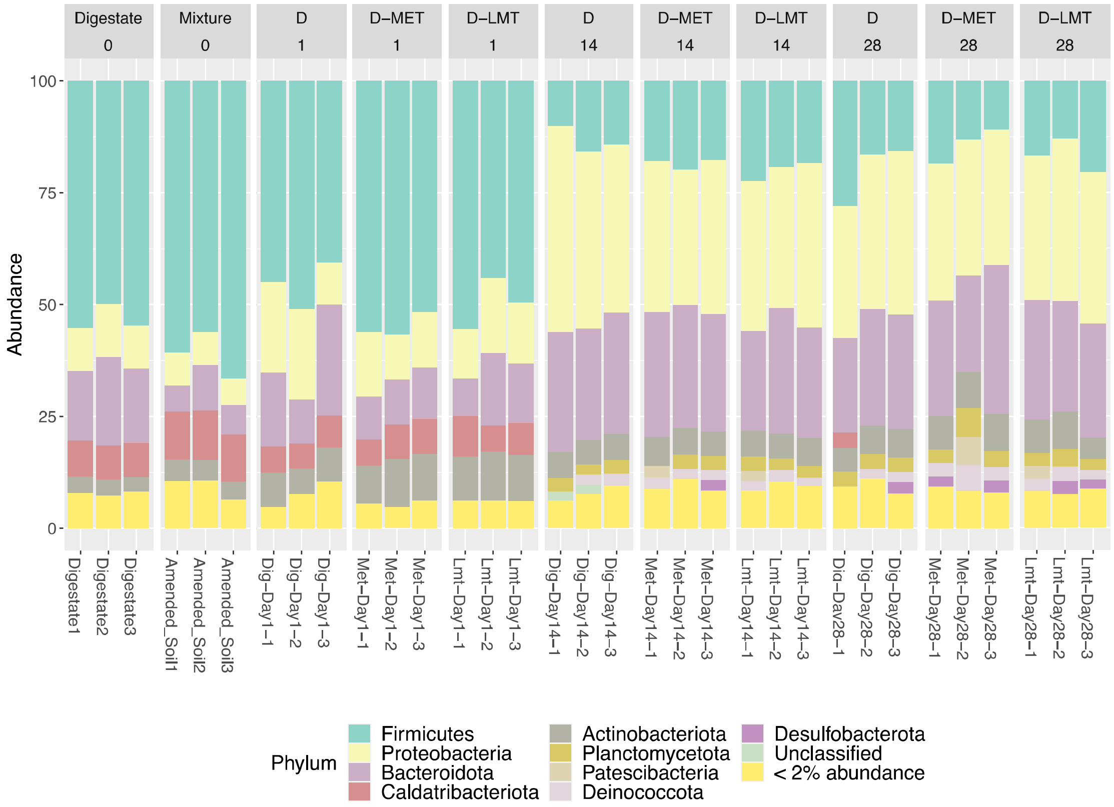

Figure 14: Taxonomic profiles at phylum level of the bacterial communities of initial digestate, initial amended soil and samples taken from the first soil column layers through time (day 1, day 14 and day 28) for the different experimental conditions (without (D) or with spiking of lamotrigine (D-LMT) or metformin (D-MET). Only phyla with ≥ 2% relative abundance are shown.

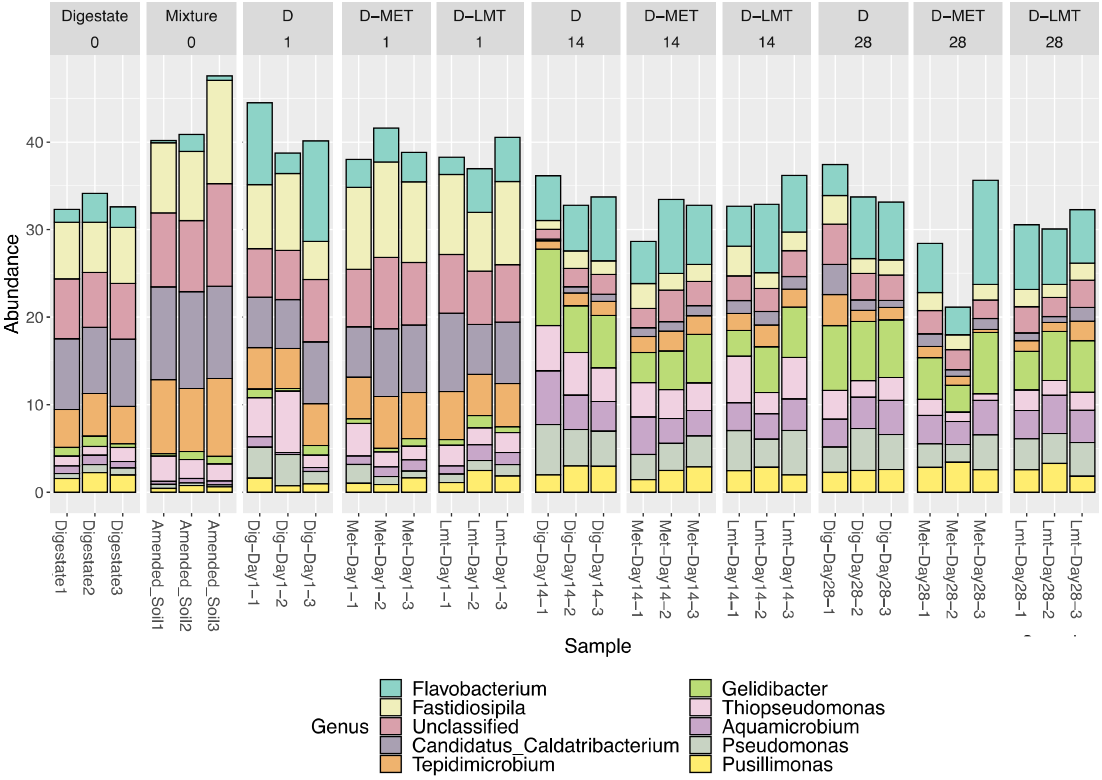

Figure 15: Taxonomic profiles of the top 10 genera of the bacterial communities of initial digestate, initial amended soil and samples taken from the first soil column layer over time (day 1, day 14 and day 28) for the different experimental conditions (without (D) or with spiking of lamotrigine (D-LMT) or metformin (D-MET), expressed in terms of relative abundance (%).

3.4 Conclusions

This study evaluated how the fate and mobility of TM in soil and the soil prokaryotic community was affected after the use of non-source-separated biowaste digestate as soil amendment. The present work showed that the TM originating from the digestate remained in the top amended soil layer without being transported to the deeper soil layers during the experimental time. TM in the soil layers were primarily found in the oxidizable-residual fraction through time, suggesting that TM were strongly bound to soil OM and soil mineral groups decreasing their mobility and preventing their redistribution among the other experimentally defined soil fractions. Contrarily, the TM in the top amended soil layer were found abundantly also in the exchangeable and the reducible fractions making them more bioavailable and potentially more mobile. Furthermore, the mobility factor determined that Zn was the TM element that had the greatest mobility potential followed by Cu and Pb, which had higher affinity to soil mineral and OM phases. The TM mobility potential increased during time in the amended soil layer, indicating a greater risk for TM transport and TM leaching with the aging of amended soil. The mobility with aging could also be affected by the presence and fate of other types of contaminants and anthropogenic impurities that can act as vectors for transport. This is an important point that deserves further investigation, especially to understand the effect of digestate aging on metal fate and mobility in soil. However, current results showed that the amount of TM in this fraction was low which can diminish the potential impact of digestate amendment. From a microbiological perspective, digestate amendment led to an increase in microbial abundance in soil, and a stabilization of the prokaryotic community after 14 days, that could potentially favour plant growth, organic contaminant degradation and TM immobilization. The presence of metformin and lamotrigine, at the tested levels, did not significantly influence TM behaviour and did not evidently affect the prokaryotic community structure in the soil system. Overall, non-source-separated household biowaste can be considered a valuable resource for soil restoration given its metal sorption and immobilization capacity, its high content of macro and micronutrients and its positive effect on soil microbial abundance, which coupled with other strategies, as plant cover for instance, can lead to effective restoration and remediation of marginal land, boosting local bioeconomies and empowering local communities.

Associated content

The supporting information for this chapter is reported in Appendix B. This content includes further details on soil, digestate and amended soil characterization, the total metal concentration and fractionation results of initial solid matrices, details of statistical analysis performed and further details on the soil microbial community characterisation.

Author Contributions

Veronica Baldasso: Conceptualization, Methodology, Investigation, Data collection, Formal analysis, Writing – original draft. *Stephanie Sayen*: Conceptualization, Writing - review and editing. *Emmanuel Guillon*: Conceptualization, Writing - review and editing. *Luigi Frunzo*: Conceptualization, Writing - review and editing, Funding acquisition. *Carlos A. R. Gomes*: Conceptualization, Writing - review and editing, Supervision. *Ana Paula Mucha*: Conceptualization, Methodology, Writing - review and editing, Supervision, Funding acquisition. *C. Marisa R. Almeida*: Conceptualization, Methodology, Writing - review and editing, Supervision, Funding acquisition.

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Chapter 4

Metformin and lamotrigine sorption on a biogas digestate amended soil in presence of trace metal contamination

This chapter is based on the publication:

Baldasso V, Sayen S, Frunzo L, Gomes A R C, Almeida M, Guillon E. Metformin and lamotrigine sorption on a biogas digestate amended soil in presence of trace metal contamination. *Journal of Hazardous Materials*. 2024, 466, 133635.
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Abstract

The antidiabetic drug metformin and antiepileptic drug lamotrigine are contaminants of emerging concern that have been detected in biowaste-derived amendments and in the environment, and their fate must be carefully studied. This work aimed to evaluate their sorption behaviour on soil upon digestate application. Experiments were conducted on soil and digestate-amended soil as a function of time to study kinetic processes, and at equilibrium also regarding the influence of trace metals (Pb, Ni, Cr, Co, Cu, Zn) at ratio pharmaceutical/metal 1/1, 1/10, and 1/100. Pharmaceutical desorption experiments were also conducted to assess their potential mobility to groundwater. Results revealed that digestate amendment increased metformin and lamotrigine adsorbed amounts by 210% and 240%, respectively, increasing organic matter content. Metformin adsorption kinetics were best described by Langmuir model and those of lamotrigine by Elovich and intraparticle diffusion models. Trace metals did not significantly affect the adsorption of metformin in amended soil while significantly decreased that of lamotrigine by 12 - 39%, with exception for Cu^{2+} that increased both pharmaceuticals adsorbed amounts by 5 – 8%. This study highlighted the influence of digestate amendment on pharmaceutical adsorption and fate in soil, which must be considered in the circular economy scenario of waste-to-resource.

4.1. Introduction

Soil is one of the fundamental natural resources for humanity, yet it is one of the most deteriorated and improperly managed [1,2]. The perception of the gravity of the situation led to the concept of “soil health” which in recent years has become one of the key elements of the European Union (EU) Green Deal [3], defined by the EU Mission: Soil Health and Food [1,4].

It is of fundamental importance to reclaim degraded land, restoring soil quality and fertility, and put in place proper management strategies to protect and conserve this resource for future generations [5]. In the last decades, with the establishment of circular economy and zero waste policies, there has been a great push towards the reuse of materials originating from waste streams such as sludges and digestates as a way to tackle the impelling issue of waste generation and management [3,6–9]. These biowaste derived materials are nutrient-rich organic matrices that have been found to improve fundamental soil properties such as water holding capacity, organic matter (OM) and nutrient content and soil structure [8,9]. In fact, the most widespread practice has been their application on land for agriculture practices and soil recovery, however this has led over the years to an increase in soil pollution and presence of persistent contaminants, e.g. metals and emerging contaminants (EC) [10,11].

Two ECs that have been detected in the soil system are the pharmaceutical compounds metformin (MET) and lamotrigine (LMT). MET is one of the most prescribed type-2 antidiabetic drugs worldwide that has been detected in wastewaters and WWTP effluents with concentrations between 1 – 47 μg/L, in sludges/digestate with concentrations between 0.55 – 3 μg/g, and different environmental compartments (surface waters, sediments, and soil) with concentrations ranging between 2 ng/g and 1 μg/g [12–16]. MET and its main metabolite guanylurea were found to have negative effects on plant growth and development [17], soil microbial communities [18,19], and endocrine disrupting effects on aquatic species [20]. MET was also found to have no significant risk at benchmark quotients, based on drinking water concentrations, however it was found that MET oxidation products could be harmful to human health [14,21,22]. LMT is extensively used as an antiepileptic and psychotherapy drug that has been detected in wastewaters and in the environment only in the last decade. Lamotrigine has been detected in WWTP effluents with concentrations of 0.5 – 1.5 μg/L, in natural waters with concentrations of 0.1 – 0.4 μg/L, in natural soils at 4 ng/kg and in agricultural soils in the range of 1.5 – 9 μg/kg [13,23–28]. LMT is excreted by the body mainly as its metabolite lamotrigine-N2-

glucuronide that, in favourable conditions, transforms back into LMT through deconjugation [28]. There is scarce information on toxicological effects of LMT on ecosystems and human health, however it is suggested that LMT may lead to drug-drug interactions reducing the effects of other prescribed drugs [24]. Moreover, recently it was shown that LMT could translocate in plants, reaching leaves, and affect the expression of stress genes [29]. There were only few studies present in literature on MET and LMT behaviour and fate in the environment, especially in soil [12–15,21,25–27], despite their reported occurrence. In the last years, due to an increasing use of sludge and reclaimed wastewater on land, which can be contaminated with ECs and thus be a source of contamination for soils, more research was conducted on these compounds showing their (bio)degradation pathways [14,27,28,30–33], and sorption mechanisms in agricultural soils[34–37]. However, there is still very little information on the effect of digestate soil amendment on MET and LMT sorption behaviour in soils and on the factors that might affect it, as the presence of other contaminants such as metals. The recent approval of digestate as a soil amendment by the EU [38] underlines the importance of understanding its effects on soil contaminant sorption and its potential impact in transferring ECs, particularly poorly studied pharmaceutical compounds as MET and LMT, to the soil and groundwater environmental compartments.

Sorption is one of the most important mechanisms that controls the transport and fate of contaminants in soils, which can be affected by the soil's physico-chemical properties (pH, cationic exchange capacity (CEC), surface area, mineral composition, OM content) and the contaminant's nature [39]. Electrostatic interaction was proposed as the main mechanism regulating MET soil adsorption via the negative charged surface sites of soil clay minerals [34]. It was also shown that MET adsorption decreased with the application of biosolids, probably due to the increase of soil pH [34]. In contrast, LMT soil adsorption was found to be mainly affected by soil OM, which can increase upon soil amendment with biosolids, and its high π-energy, allowing for π-π interactions forming weak bonds [25]. Thus, soil sorption behaviour can be affected also by soil amendments, as biogas digestate. The sorption behaviour of pharmaceutical compounds can be affected also by the presence of other contaminants, either in the soil or in the amendment itself, especially metallic trace elements (MTE) [10,40]. MTE are classified as priority substances [41] that can be found in the environment and in the applied amendments [42–45]. MTE can interact with pharmaceuticals through complexation via strong coordination groups as carboxyl, phenolic and amino groups [46,47] present on these organic molecules. The formation of these coordination complexes may modify the pharmaceutical behaviour in the soil system. MTE can also compete with emerging organic contaminants for sorption sites [10,25,46–49].

To our knowledge, there is currently no study in the literature on the effect of biogas digestate amendment on MET and LMT sorption on soils and on their sorption behaviour in the presence of MTE. Given that these EC can be present in the digestate together with MTE and that digestate application on land is likely to increase in the years to come, it is of fundamental importance to evaluate the sorption behaviour of these contaminants in digestate-amended soils inherently containing MTE. Thus, this became the aim of the current study. It is assumed that digestate amendment as well as the presence of MTE will change the soil sorption behaviour of the two selected ECs, ultimately affecting their transfer within the soil system. For these reasons, we conducted (i) a kinetic study using a natural soil and a digestate-amended soil to assess the effect of biogas digestate application on the pharmaceuticals' adsorption behaviour in soil, (ii) batch adsorption tests to evaluate the effect of specific MTE, namely Pb, Ni, Cr, Co, Cu and Zn, on MET and LMT adsorption in soil and amended soil, and (iii) MET, LMT and MTE desorption experiments to assess their potential mobility to groundwater. The knowledge gained from this study is particularly relevant given the growth of the biogas bioeconomy and the use of digestate for land application.

4.2 Materials and Methods

4.2.1 Reagents

Metformin hydrochloride and Lamotrigine (Pharmaceutical secondary standard, certified reference materials) were purchased from Sigma-Aldrich. $CaCl_2.2H_2O$ salt was purchased from Fluka Analytical (ACS reagent, Fisher Scientific). The metal nitrate salts used were $Cu(NO_3)_2.3H_2O$ (GPR RECTAPUR®, VWR), $Zn(NO_3)_2.6H_2O$ (99 % purity metals basis, Alfa Aesar), $Pb(NO_3)_2$ (ACS reagent, Sigma-Aldrich), $Ni(NO_3)_2.6H_2O$ (pro analysis grade, Merck), $Cr(NO_3)_3.9H_2O$ (pure grade, Fischer Scientific LABOSI) and $Co(NO_3)_2.6H_2O$ (Normapur, PROLABO). The stock solutions of metformin (MET), lamotrigine (LMT), and $CaCl_2$ were prepared in ultrapure water (Milli-Q®, Merck); while those of metal nitrates were prepared in acidic medium using ultrapure water and nitric acid. Acetonitrile (HiPerSolv CHROMANORM®), orthophosphoric acid (85 %, HiPerSolv CHROMANORM®), nitric acid (67–69 %, NORMATOM®), HCl and KOH (AVS

TITRINORM®) were purchased from VWR Chemicals. Physico-chemical properties of MET and LMT are provided in Table C1 (Appendix C).

4.2.2 Soil and amended soil samples

Two types of solid matrices were used in this work: a soil and a solid digestate originating from non-source-separated organic fraction of municipal solid waste (OFMSW). The soil was taken from a construction site at the outskirts of Ermesinde, Porto (NW Portugal; 41°12'25.7''N, 8°32'26.7''W). Soil type and texture were determined using the digital database for Portuguese soils INFOSOLO [50] and was classified as a Cambisol deriving from schist metamorphic parent rock. The OFMSW solid fraction digestate (forward referred to as digestate) was sampled at a Portuguese municipal solid waste treatment plant, having anaerobic digestion (AD) reactors to treat the non-source-separated municipal organic waste. The two solids were dried at room temperature until constant mass was reached, homogenized, and passed through a 2 mm sieve. Solids were then characterized for pH using the NF ISO 10390 method for soil quality-pH determination, OM content using the loss on ignition method, elemental composition *via* X-Ray Fluorescence (ED-XRD Spectro-XEPOS, model XEP05). MTE total concentrations were determined *via* high-pressure microwave digestion (ETHOS 1, Milestone Inc.) followed by flame or electrothermal atomic absorption spectroscopy (AAnalysit 200 AA spectrometer system and PinAAcle 900Z AA spectrometer (PerkinElmer)) and MTE fractionation using the BCR sequential extraction method of Rauret et al. (1999) [51], as described in Baldasso et al. (2023) [52]. The soil was also characterized for CEC using the cobalt hexamine chloride ISO method (NF X 31-130), and total carbon, nitrogen, and sulphur contents via CHNS analysis (2400 CHNS Organic Elemental Analyzer 100V (PerkinElmer)). For each sample, the analyses were conducted in triplicate. Details of the methodology used can be found in Baldasso et al. (2023) [52] and Appendix C.

The OFMSW digestate amended soil (forward referred to as amended soil) was prepared by mixing the soil and the digestate at a 14:1 dry weight ratio (corresponding to 9 % of digestate content in soil or 130 ton/ha) to simulate a high digestate application rate [53,54].

4.2.3 Sorption experiments

Sorption experiments were conducted following the batch procedure (OECD No 106) [55] at constant temperature (20 ± 1 °C) in the dark to prevent pharmaceutical compound photodegradation. All experiments were carried out using a solid charge of 200 g/L at the solids' natural pH. All sorption experiments were performed in triplicates, with exception of the adsorption kinetics which were performed in duplicate. Control samples (without solid) were also prepared to check that no pharmaceutical compound or MTE degradation and/or retention onto the vessels occurred throughout experiment duration, for all performed experiments.

4.2.3.1 MET and LMT adsorption kinetic experiments

MET and LMT adsorption kinetic experiments were carried out using soil and amended soil. Each pharmaceutical kinetic experiment was conducted in duplicate, resulting in two samples for each defined contact time. Control samples (no solid) were prepared for each experiment to ensure no compound degradation and/or retention onto the vessel. For the adsorption kinetic experiments, 5 g of dry solid (soil or amended soil) were weighed in polypropylene containers and 24.9 mL of ultrapure water were added. The batch containers were placed on a horizontal shaker for 24 h to allow solid hydration. Then, pharmaceutical (MET or LMT) stock solution was added to each batch container to reach a concentration of $5x10^{-6}$ mol/L in a final volume of 25 mL. Batches were shaken again for varying contact times ranging from 15 min to 21 days. Specifically, the contact times defined for MET were 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 16 h, 24 h, 48 h, 72 h, 96 h, 7 days, and 21 days. While the contact times defined for LMT were 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 16 h, 24 h, 48 h, 72 h, 96 h, 7 days, 10 days, 14 days, 17 days, and 21 days. After batch collection, the suspensions were centrifuged, and the supernatants filtered through a polyamide membrane at 0.22 µm pore size into virgin glass high-performance liquid chromatography (HPLC) sample vials. The filtrates were immediately analysed (no sample storage) by HPLC with UV detector (HPLC-UV) as described in section 4.2.4 Analytical techniques. The final adsorbed amounts of MET and LMT were calculated as the difference between the initial spiked contaminant concentration and the measured concentration in the sample filtrates, divided by the solid charge (ratio of dry solid mass to the solution volume).

4.2.3.2 MET and LMT adsorption experiments in the absence and presence of MTE

For these experiments the pharmaceutical concentration (MET and LMT) was fixed at $5x10^{-6}$ mol/L . The MET and LMT adsorption experiments in the presence of MTE (Zn^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+}, Cr^{3+} or Co^{2+}) on soil and amended soil were conducted for pharmaceutical/metal concentration (mol/L) ratios of 1/1, 1/10 and 1/100. The experiments were conducted singularly for each combination of pharmaceutical compound and MTE (e.g. MET-Zn, MET-Cu, MET-Pb, MET-Ni, MET-Cr, and MET-Co), resulting in 3 experimental conditions for each pharmaceutical-MTE pair. Thus, in each condition, the pharmaceutical concentration (MET and LMT) was $5x10^{-6}$ mol/L, while the MTE concentration varied, specifically set to $5x10^{-6}$, $5x10^{-5}$ and $5x10^{-4}$ mol/L, respectively. All experimental conditions tested were conducted in triplicates, resulting in 108 total samples. In addition, single solute system adsorption experiments were carried out, in triplicates, to evaluate the sorption of MTE, at concentrations of $5x10^{-6}$, $5x10^{-5}$ and $5x10^{-4}$ mol/L, on soil and amended soil in the absence of the pharmaceutical compounds. Control samples (no solids) were added to ensure no compound degradation and/or retention onto the vessel.

These adsorption experiments were carried out similarly to those described in section 4.2.3.1, with 5 g of dry solid (soil or amended soil) suspended in a total volume of 25 mL. After hydration step, batches were shaken for a duration equivalent to the equilibration time determined for the selected pharmaceutical compounds in the kinetic experiments (section 4.2.3.1). After batch collection, the suspensions were centrifuged, and the supernatants filtered through a polyamide membrane at 0.22 µm pore size into virgin glass HPLC sample vials and virgin test tubes. The filtrates were immediately analysed (no sample storage) using HPLC-UV for MET and LMT, and by inductively coupled plasma mass spectrometry (ICP-MS) for the quantification of MTE (described in section 4.2.4 Analytical techniques). The final adsorbed amounts of MET, LMT and MTE were calculated as the difference between the initial spiked contaminant concentrations and the measured concentrations in the sample filtrates, divided by the solid charge (ratio of dry solid mass to the solution volume).

4.2.3.3 Desorption experiments

Contaminant desorption was studied through desorption batch tests, conducted with ultrapure water and 0.01 mol/L $CaCl_2$ solution. 5 g of dry solid were weighed in each polypropylene batch and 25 mL of either ultrapure water or $CaCl_2$ solution were added. The batches were shaken for 48 h to allow possible desorption mechanisms to take place. After that the suspensions were centrifuged, and the supernatants filtered through a polyamide membrane at 0.22 µm pore size. The filtrates were analysed for MET and LMT using HPLC-UV and for MTE concentrations using ICP-MS, following the methods described in section 4.2.4.

Firstly, desorption tests were performed with original soil and amended soil samples to evaluate contaminant release of the solid matrices in natural conditions, without any contaminant spiking. Subsequently, desorption tests were carried out with soil and amended soil samples collected from contaminant adsorption experiments conducted specifically to obtain the aforementioned solid samples, following the procedure described in section 4.2.3.2. These adsorption experiments carried out were MET and LMT adsorption experiments conducted in the presence of MTE at pharmaceutical/metal concentration (mol/L) ratios of 1/1 and 1/100 to consider low and high MTE concentrations ($5x10^{-6}$ and $5x10^{-4}$ mol/L, respectively). Based on the results of MET and LMT adsorption experiments in presence of MTE, it was decided to perform the above mentioned desorption tests only in the presence of Cu^{2+} MTE. The solid samples collected from the adsorption experiments were dried at room temperature in a fume-hood (covered to prevent contamination) until reaching constant weight. Then, they were immediately used in the desorption tests. The solid samples' contaminant adsorbed amounts can be found in Appendix C (Table C6). Briefly, the dry soil samples on average had 1.2 and 2.1 µg/g of MET adsorbed amount, 0.5 and 0.3 µg/g of LMT, and 1.6 and 158 µg/g of Cu^{2+} for the 1/1 and 1/100 pharmaceutical/metal concentration (mol/L) ratios, respectively; while the amended soil samples on average had 3.5 and 4.0 µg/g of MET adsorbed amount, 3.3 and 3.5 µg/g of LMT, and 1.6 and 158 µg/g of Cu^{2+} for the 1/1 and 1/100 pharmaceutical/metal concentration (mol/L) ratio, respectively.

4.2.4 Analytical techniques

The pharmaceutical analysis was carried out via HPLC-UV using a 1260 Infinity II LC System (Agilent Technologies) equipped with a quaternary pump, a 2 mL vial-sampler, and a diode array and multiple wavelength detector. For MET analysis, the analyte was eluted by injection onto a Phenomenex Luna HILIC 200A column (5 µm × 250 × 4.60 mm), with an injection volume of 10 µL, following an isocratic method with 20 min duration. The mobile phase was composed by 75 % acetonitrile and 25 % ultrapure water (Milli-Q 18 MΩ cm^{-1}) containing 0.1 % phosphoric acid. The flow rate was adjusted to 1 mL/min. MET detection was performed in the UV range at 234 nm, wavelength pre-determined via UV-Vis spectrophotometer (UV-Vis Carry-5000 Varian spectrophotometer). For LMT analysis, the analyte was eluted by injection onto a reverse-phase Agilent Zorbax SB-C18 (5 µm × 250 × 3.0 mm), with an injection volume of 20 µL, following a gradient method with 14 min duration. The gradient mobile phase was a mixture of acetonitrile (A) and ultrapure water (Milli-Q 18 MΩ cm^{-1}) containing 0.1 % phosphoric acid (B). The gradient method began with a mobile phase composition %A/%B of 95/5 immediately followed by a linear trend to %A/%B of 70/30 in 10 min. Then the mobile phase composition was set to 0/100 for two minutes, and then back to 95/5 for the last two minutes. The flow rate was adjusted to 0.8 mL/min. LMT detection was performed in the UV range at a wavelength of 264 nm, preliminarily determined by UV-Vis spectrophotometry (UV-Vis Carry-5000 Varian spectrophotometer). The pharmaceutical HPLC methods were optimized through the measurement of the MET and LMT standard solutions. Quantification of MET and LMT was obtained by carrying out external calibration curves with pharmaceutical standard solutions of increasing concentrations ranging from $2\text{x}10^{-7}$ to $5\text{x}10^{-6}$ mol/L, covering the experimental range of interest.

ICP-MS analyses were performed using an Agilent 8900 Triple Quadrupole ICP-MS in He mode. Yttrium was used as internal standard, the mass to charge ratio for each MTE was set and the element isotope that resulted in the least interference and highest abundance was selected (Appendix C, Table C5). This procedure was optimized through the measurement of each metal standard solution. Quantification of MTE was obtained by carrying out external calibration curves (corrected by the internal standard signal) with metal standard solutions of increasing concentrations (between 0.1 to 50 μmol/L).

4.2.5 Adsorption kinetic models

The experimental pharmaceutical adsorption kinetic data obtained for both pharmaceutical compounds were fitted with four different commonly applied sorption kinetic models to better understand the underlying mechanisms of the sorption process: (i) Langmuir kinetics model, (ii) Mathews and Weber model, (iii) modified Intra-Particle Diffusion model (IPD) presented by Wang and Guo (2022), and (iv) Elovich model. In more detail, the Langmuir kinetic model is based on a second-order kinetic mechanism, assuming monolayer adsorption on a finite number of sorption sites [56–58]. The Mathew and Weber model is based on a pseudo-second-order kinetic mechanism, assuming that adsorption occurs through pore diffusion [59]. The IPD model assumes that intra-particle diffusion (within the porous structure of adsorbent particles) is a rate limiting step in the adsorption process [60]. The Elovich model is used to represent chemisorption processes in heterogenous systems, involving chemical reactions between the adsorbate and the adsorbent surface [61]. The models were applied with Origin software using a non-linear regression method to avoid linearization estimation errors. The model fitting was evaluated with the root mean square error (RMSE). The equations used for the models are reported in Table 4.

Table 4: Sorption kinetic models with their integral equation and parameters.

Model	Integral Equation	Parameters
Langmuir kinetics [56–58]	$q_t = \frac{q_e(1 - e^{-k_1 t})}{(1 - f_e e^{-k_1 t})}$	q_e - equilibrium adsorbed amount (μg/g) f_e - batch equilibrium factor (-) k_1 - rate constant (h^{-1})
Mathews and Weber [59]	$q_t = \frac{Co}{ms}\left(1 - e^{-k_s t}\right)$	Co - initial sorbate concentration (μg/L) ms – solid charge (g/L) k_s - rate constant (h^{-1})
Intra-Particle Diffusion [60]	For $0 \leq t \leq t_1$ $q_t = k_1 t^{1/2}$ For $t_i \leq t \leq t_j$ $q_t - q_{ti} = k_j(t - t_i)^{\frac{1}{2}}$	k_j - rate constant ($\mu g/g.h^{-1/2}$) q_{t1} - adsorbed amount equal to 30 % of equilibrium adsorbed amount (μg/g) t_i - time at which $q_t = q_{ti}$ (h)
Elovich [61]	$q_t = \frac{1}{k_d}\, ln(k_a k_d t + 1)$	k_a - adsorption rate constant ($\mu g/g^{-1} \cdot h^{-1}$) k_d - constant related to the extent of surface coverage and activation energy for chemisorption (g/μg)

4.3 Results and Discussion

4.3.1 Soil, digestate and amended soil characterization

The soil and digestate's main physico-chemical properties, including soil texture, are reported in Table 5; while their mineral phases were determined previously in a study carried out to understand MTEs' fate in soil after amendment with digestate [52] and are reported in Table C2 (Appendix C). The soil was a loamy sand soil characterized by a low to moderate OM content and an alkaline pH, higher than that of the digestate's. Mineral phase composition indicated that SiO_2 was the dominant phase in both soil and digestate, followed by Al_2O_3 and Fe_2O_3 in soil, and CaO and Al_2O_3 in digestate. The amended soil, mixture of soil and digestate at a 14:1 dry weight ratio, evidenced an intermediate composition and physicochemical properties, with a pH of 8.50 and an OM content of 5.0 %.

Total MTE concentrations and MTE fractionation results are presented in Table C3 and Figure C1 (Appendix C). Except for Fe (higher in soil) and Co (similar in all solid matrices), total concentrations of MTE were lowest in soil and highest in the digestate, as was expected due to the digestate's origin, while the concentrations in the amended soil were in between. The BCR sequential extraction method used for MTE fractionation allowed to assess the MTE contents in distinct operationally-defined chemical forms: exchangeable, reducible (bound to Fe/Mn oxides), oxidizable (bound to OM) and residual fractions of the solid materials. The results showed that Pb, Cu, Cr and Fe were mainly found in the residual fraction in both soil and amended soil, while Zn was found distributed amongst all fractions. Mn in the soil was found mainly in the reducible fraction, while in the amended soil it was found distributed amongst all fractions. Therefore, the soil contained Pb, Cu, Cr, Fe and Mn mainly in immobile forms and Zn in both readily bioavailable and immobile forms; while the amended soil contained Pb, Cu and Cr mainly in immobile forms and Zn, Fe and Mn in both bioavailable and immobile forms.

To ensure that the endogenous metals contained in the solid matrices would not affect the batch adsorption test results, a desorption batch test using ultrapure water was conducted to measure the quantity of metals released by the solids (Table S4, Appendix C). For both soil and amended soil MET released in water were less than 0.4 % of the total MTE concentrations, resulting in amounts in the order of 10^{-8} to 10^{-10} mol/L. For this reason, this amount was considered negligible compared to the introduced MTE concentrations in adsorption experiments, in the orders of 10^{-6} to 10^{-4} mol/L.

Table 5: Physico-chemical properties of soil, digestate and amended soil.

Parameter	pH	OM (% dw)	CEC (meq/100g)	Sand (%dw)	Silt (%dw)	Clay (%dw)
Soil	9.2	3.4	4.5	78.7	14.8	6.5
Digestate	8.2	24.0	44	-	-	-
Amended soil	8.5	5.0*	7.8*	-	-	-

*OM and CEC for amended soil were calculated from the respective data of soil and digestate, and the applied soil/digestate ratio.

4.3.2 MET and LMT adsorption kinetics onto soil and amended soil

In all control samples (absence of solid matrices) no retention on the vessels' surface and no degradation of the pharmaceuticals was observed.

MET and LMT adsorption kinetic curves are reported in Figure 16, where the adsorbed amounts are reported as a function of contact time. The curves relative to MET and LMT are evidently different due to their differing physico-chemical properties and structures (Table C1, Appendix C). MET curves are characterized by a very sharp adsorption increase during the first 2 h for soil and 16 h for amended soil and tend to quickly stabilize to the equilibrium adsorbed amount of 1.2 µg/g (29 %) and 3.6 µg/g (87 %), respectively. LMT curves tend to increase gradually reaching equilibrium after 2 days for soil and 4 days for amended soil. The equilibrium adsorbed amount resulted in 0.8 µg/g for soil and 2.4 µg/g for amended soil, corresponding to an adsorption of 12 % and 38 %, respectively. It can be inferred that MET adsorption, in terms of adsorbed amount and adsorption kinetics, was favoured compared to that of LMT despite MET's very-high water solubility. This difference in behaviour can be attributed to the chemical charge of the pharmaceuticals at the solids' natural pH value, which was 9.2 for soil and 8.5 for amended soil. At these pH values, MET is present dominantly in a single protonated state (MET^+, amphoteric species) since the pH falls in between its two pKa values of 3.1 and 13.8, whereas LMT is present in neutral form (basic species) since its pKa is of 5.7 [62]. Furthermore, the study of the soil's mineralogy (see Appendix C) indicated that the soil was dominated by kaolinite and to a lesser extent by muscovite (phyllosilicate clay minerals), which are characterized by permanent negative surface charges [63] that can favour the retention of MET^+. MET belongs to the biguanides class and is a small very polar hydrophilic molecule. Therefore, it can be derived that MET interacts mainly through electrostatic interactions with the permanent negatively charged surface sites of the soil clay minerals and localized negative charges of soil OM induced by pH deprotonation of functional groups (e.g. carboxyl and hydroxyl groups).[34,64,65] In comparison, LMT belongs to the phenyltriazine class and is a non-polar hydrophobic molecule. Thus, it can interact through van der Waals forces, as π-π interactions, with aromatic moieties of soil OM, and H-bonding with uncharged surface hydroxyl functional groups present on soil OM and soil minerals [25,27,39]. Moreover, it was found that LMT was positively correlated with soil hydrolytic activity, meaning that as soil OM is broken down and transformed LMT sorption is increased [66]. The different chemical nature and the different interaction mechanisms of the two pharmaceutical compounds contributed to their diverse adsorption behaviour in soil and amended soil [11,67,68].

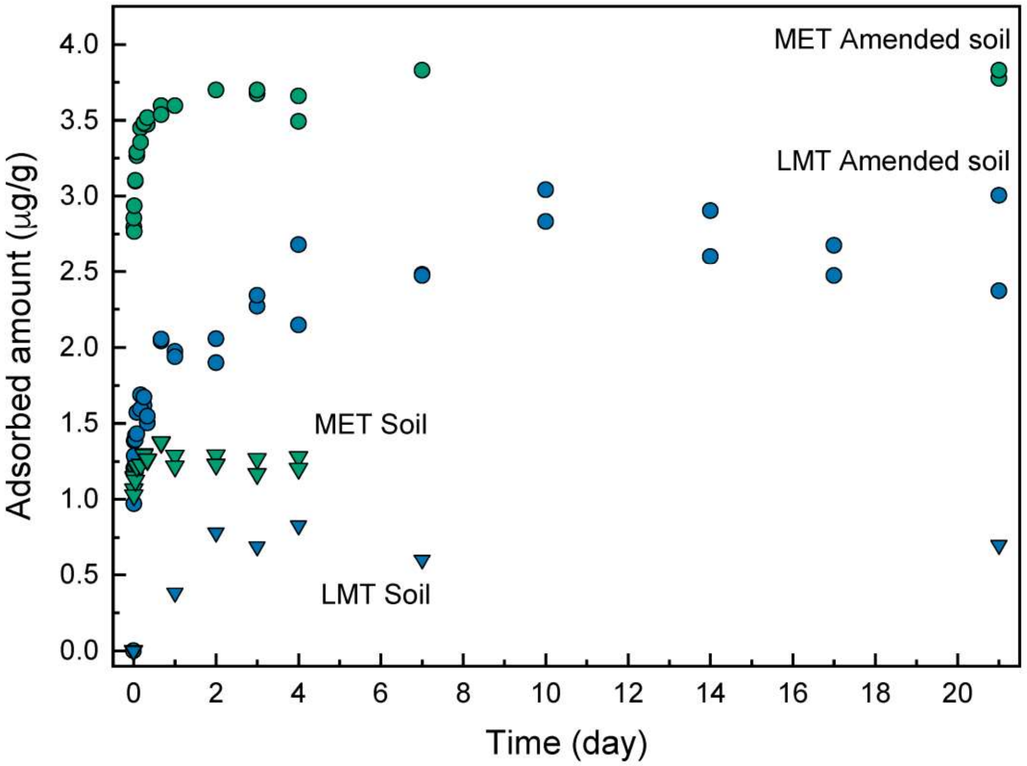

Figure 16: Metformin (MET, green) and lamotrigine (LMT, blue) adsorption kinetic curves on soil (triangles) and digestate-amended soil (circles).

In addition, the amount adsorbed at equilibrium onto digestate-amended soil was 3.1 and 3.4 times greater than that adsorbed onto non-amended soil, for MET and LMT, respectively. This different behaviour can be directly correlated to the addition of digestate which not only increased the soil's OM content from 3 % to 5 % but also significantly increased the CEC (from 4.4 to 7.8 meq/100g), increasing the overall negative surface charge and the potential adsorption surface sites of the soil. This demonstrates the fundamental role of organic amendment, rich in OM, in enhancing the soil's adsorbing capacity for contaminants. For LMT, this agrees with a previous study conducted on the effect of reclaimed wastewater application determining an increase in retardation factor with increasing loadings [67]. LMT adsorption onto soil after digestate amendment was expected to be greater since it is in neutral form and its adsorption is mainly controlled by OM content [66]. For MET, the studies of Briones and Sarmah (2018) [34] showed that sewage sludge biosolid application caused a decrease in MET adsorption as consequence of an increase in the soil's pH (on average from 5.8 to 7.8). However, our study showed that the application of biogas digestate amendment increased MET adsorption without significantly changing the soil's pH, which remained alkaline. Our results can be explained primarily by the increase in the soil's OM content and CEC, which brought an increase in the negative surface charge of the soil and in the available surface adsorption sites. This promoted MET adsorption presumably by electrostatic interactions and H-bonding [36,69]. These results highlight the importance of studying the behaviour of contaminants in diverse soils and amended soils to have a global overview of their fate in systems with different physico-chemical properties.

4.3.3 MET and LMT adsorption kinetic modelling

To further understand the adsorption behaviour, the experimental data were fitted with four different sorption kinetic models (Table 4) as reported in Figure 17. The MET adsorption kinetic data for both soil and amended soil (Figure 17-B and 17-A) had a similar trend to that reported by Briones and Sarmah (2018) [35] for MET adsorption on a natural soil, who determined the IPD model to have the best fit [70]. Similarly, our results showed that the IPD model (RMSE 0.121) gave the best fit for MET adsorption on amended soil, followed by the Langmuir model (RMSE 0.130) (Table 6). Whereas, MET adsorption on soil was best described by the Langmuir model (RMSE 0.047), followed by the Mathew-Weber model (RMSE 0.055). When comparing the experimentally determined equilibrium adsorbed amounts of MET on soil and amended soil (1.27 and 3.69 µg/g) with those modelled, it was found that the Langmuir model (1.28 and 3.58 µg/g) obtained a closer value than the Mathew-Weber model (1.25 µg/g) for soil and the IPD model for amended soil (3.94 µg/g). This suggests that MET adsorption onto the heterogeneous soil surface occurred through binding with mainly one type of surface functional group, allowing to consider it homogeneous. Moreover, it can be inferred that the rate-limiting mechanism of MET adsorption on both soil and amended soil was the adsorption process [60]. Nevertheless, since the Mathew-Weber model and the IPD model had good data fitting, external pore diffusion and intraparticle diffusion have to be considered as potential mechanisms involved in the adsorption process on soil and amended soil, respectively (but not significantly rate-limiting).

The modelled curves for LMT adsorption on soil (Figure 17-D) showed that Mathew-Weber model (RMSE 0.067) and Langmuir kinetic model (RMSE 0.067) had the best goodness of fit, suggesting that the adsorption process on natural soil could be mainly influenced by external pore diffusion and adsorption mechanisms involving mainly one type of surface functional group (homogeneous surface). The Mathew-Weber model and the Langmuir model reached an equilibrium adsorbed amount of LMT equal to 0.77 µg/g, resulting very close to the experimentally determined one (0.76 µg/g). In contrast, the modelled curves for LMT adsorption on amended soil (Figure 17-C) showed that the IPD model had the best goodness of fit (RMSE 0.179), followed by Elovich model (RMSE 0.183). Looking at the equilibrium adsorbed amount of LMT on amended soil, both the Elovich and IPD models well approximated the experimentally determined one (2.67 µg/g), resulting in 2.73 µg/g and 2.76 µg/g, respectively. These results suggest that LMT adsorption on amended soil could have been dominated by sorption mechanisms onto a heterogeneous surface, corroborating with past studies which found that LMT could form electrostatic bonds, as H-bonds, with different functional groups of the non-charged surface sites of solids [25,27]. Furthermore, the results indicated that intra-particle diffusion could have played a rate-limiting role in the adsorption process, oppositely to what was found for MET [60]. This may also explain the gradual increase observed in the corresponding kinetic curve.

In general, the different results obtained between the natural soil and the amended soil could have been due to changes in the soil's physical properties (surface area, aggregate formation, particle size) induced by the application of digestate soil amendment [71].

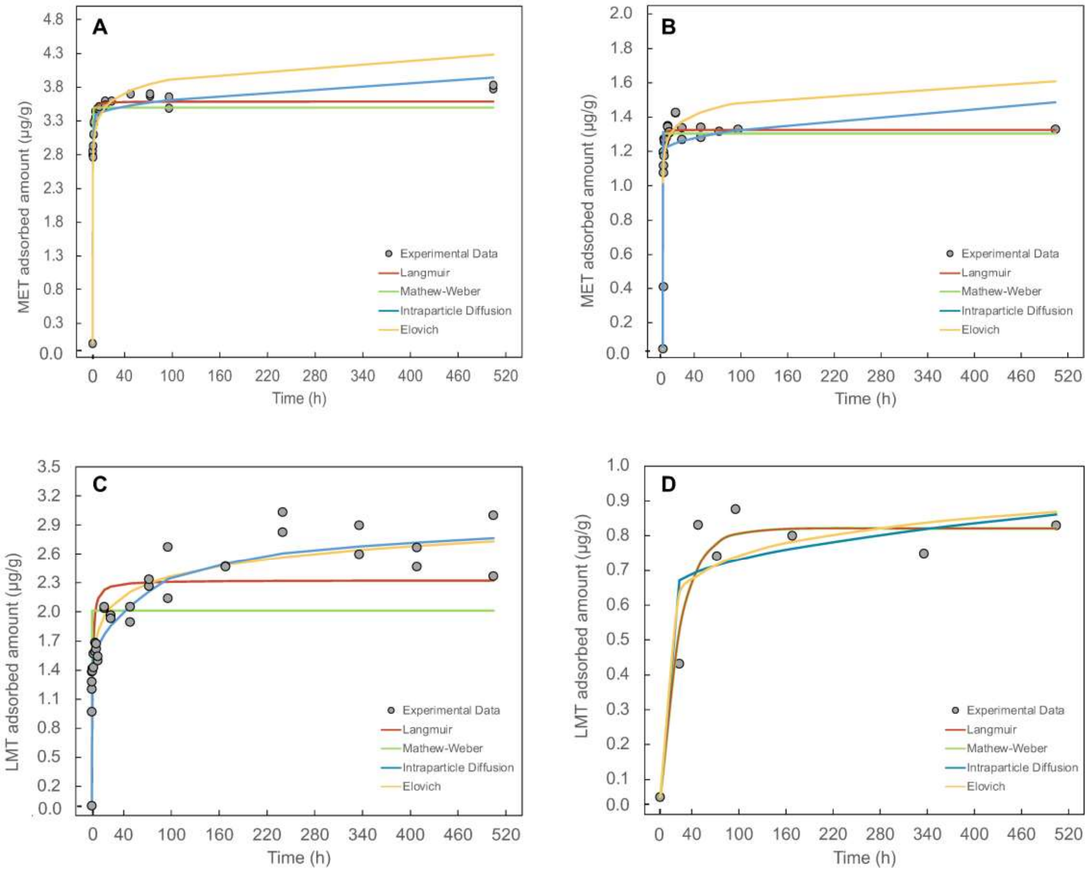

Figure 17: Experimental adsorption data (points) and modelled adsorption kinetics of metformin (MET) (A, B) and lamotrigine (LMT) (C, D) on amended soil and soil, respectively.

Table 6: Adsorption kinetics model parameters for metformin (MET) and lamotrigine (LMT) on amended soil and soil.

Model	MET		LMT	
	Amended soil	Soil	Amended soil	Soil
Langmuir kinetics				
k_1 (h^{-1})	0.004	0.21	0.001	0.04
f_e (-)	1.0	0.99	1.0	0.0
q_e (µg/g)	3.58	1.28	2.33	0.77
RMSE	0.130	0.047	0.362	0.067

Mathews & Weber				
ks (h^{-1})	5	7	106	0.04
q_e (µg/g)	3.50	1.25	2.02	0.77
RMSE	0.214	0.055	0.545	0.067
Elovich				
k_a (µg/g/h)	85523	85500	91	5.3
k_b (g/µg)	4.5	13	4.5	13
q_e (µg/g)	4.28	1.56	2.73	0.82
RMSE	0.221	0.124	0.183	0.106
Intra-Particle Diffusion (Figure C2, Appendix C)				
k_1 (µg/g/$h^{1/2}$)	5.8	1.8	2	0.1
k_2 (µg/g/$h^{1/2}$)	0.3	0.01	0.1	0
k_3 (µg/g/$h^{1/2}$)	0.03	-	0.03	-
q_e (µg/g)	3.94	1.28	2.76	0.78
RMSE	0.121	0.097	0.179	0.080

4.3.4 MET adsorption in presence of MTE

MET adsorption on soil in presence of MTE (Zn^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+}, Cr^{3+} or Co^{2+}) for different pharmaceutical/metal concentration (mol/L) ratios is reported in Figure 18. The pharmaceutical/metal concentration (mol/L) ratios of 1/10 and 1/100 can represent environmentally relevant conditions, in which MTE concentrations are generally greater than that of pharmaceutical compounds. The pH of the suspensions varied between 8.2 and 7.7 according to the MTE present in the system, thus it can be assumed that the final pH did not change the speciation in solution of either contaminant.

The adsorption experiments indicated that MET adsorption in the presence of Pb^{2+}, Ni^{2+}, Cr^{3+}, Co^{2+}, and Zn^{2+} was significantly lower than MET adsorption in the absence of MTE, for both pharmaceutical/metal concentration (mol/L) ratios tested (Figure 18-A). A decrease of 10 % in MET adsorption for the 1/10 ratio for all MTE and of 20 % for the 1/100 ratio was observed. Results also showed a decrease in MET adsorbed amounts with the increase of MTE concentration. In contrast, MET adsorption in the presence of Cu^{2+} resulted in a 9 % and a 69 % increase for the 1/10 and 1/100 ratio, respectively, compared to MET adsorption in the absence of Cu^{2+}. Furthermore, the adsorbed amounts of MTE on soil were similar in the presence and in the absence of MET (Figure 18-B). Overall, these results indicate that there is a possible competition interaction between MET and MTE for adsorption sites to the disadvantage of MET (except in the case of copper). In fact, in solution, MET and MTE are found in their cationic form competing for the negative charged soil surface sites. Also, it can be deduced that the high concentration of free metallic cations in the system, with comparison to the low concentration of MET, led to high concentrations of metallic cations in the particle boundary layer allowing for increased surface interactions. Moreover, the formation of complexes in solution between MET and MTE cannot be ruled out. The results in presence of Cu^{2+} are different compared to those observed for the other MTE. They can be explained by also considering complexation as a possible mechanism of interaction, leading to an increased amount of adsorbed MET on soil. In this case, this could be due to the formation of surface coordination complexes with Cu^{2+}, such as ternary complexes, as was found by previous studies showing the formation of ternary surface complexes on the soil surface involving Cu^{2+} and other pharmaceutical drugs, as antibiotics, leading to a synergistic adsorption behaviour [48,69,72–74].

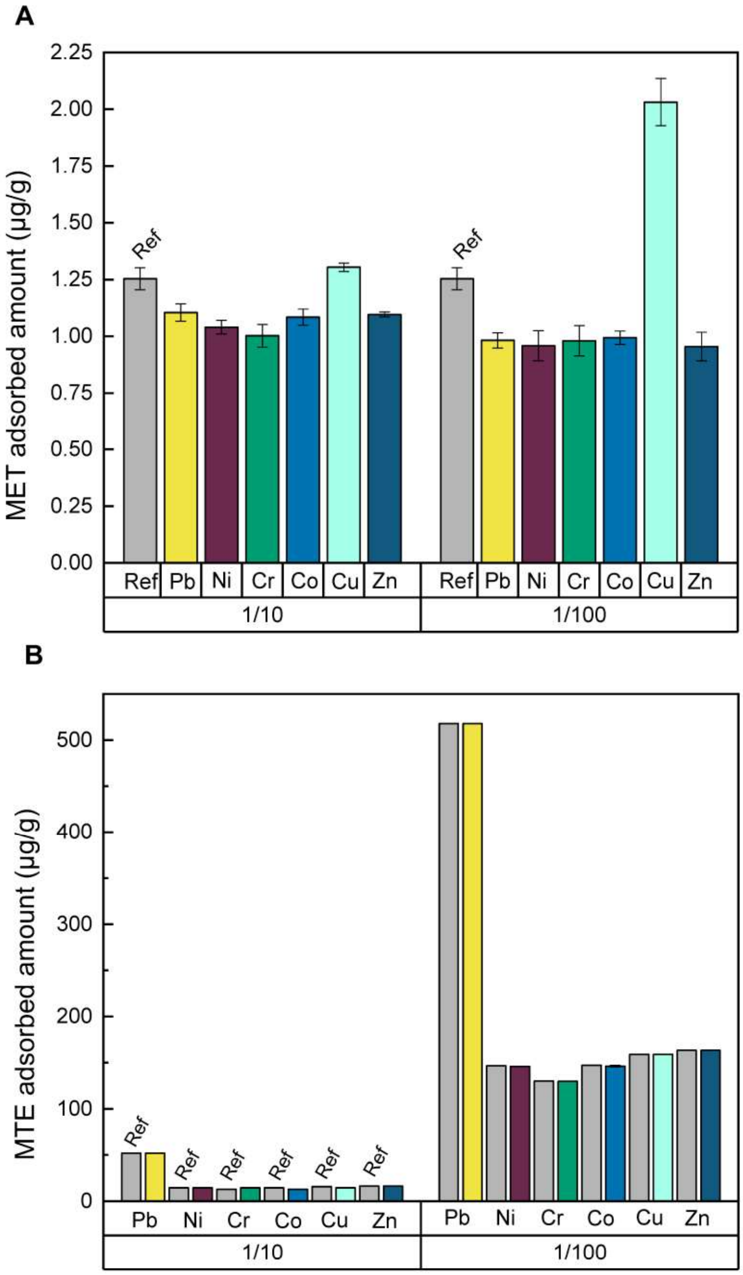

Figure 18: (A) Metformin adsorption on soil in the presence of MTE (mean and standard deviation, n=3) and (B) the respective MTE adsorbed amount, at pharmaceutical/metal concentration (mol/L) ratios of 1/10 and 1/100 (mean and standard deviation, n = 3). These results are compared with the adsorbed amount of metformin and trace metal elements measured in systems without MTE and metformin, respectively (Ref, grey bars). Error bars representing the standard deviation for MTE adsorption are present but not visible at the graph's scale due to the small magnitude of the values.

The results of MET and MTE adsorption experiments on amended soil, for the pharmaceutical/metal concentration (mol/L) ratios of 1/10 and 1/100, are reported in Figure 19. These experiments were performed to understand the effects that digestate could have on the adsorption of MET and MTE when present simultaneously. In the above studied conditions, the pH of the suspensions varied between 8.0 and 7.6.

Overall MET adsorbed amount in the presence of MTE (except Cu^{2+}) was statistically identical to the adsorbed amount in the absence of MTE (3.57 µg/g) whatever the pharmaceutical/metal concentration (mol/L) ratio (Figure 19-A). This indicates that the presence of MTE, even in a large excess, did not influence MET adsorption, which can be explained by an increase of surface sites due to the amendment and thus a decrease of the competition effect. Likewise, MET adsorption in the presence of Cu^{2+}, at both 1/10 and 1/100 pharmaceutical/metal concentration (mol/L) ratios, was significantly higher than MET adsorption in the absence of MTE, resulting in a 5.3 % and 7.0 % increase, respectively. This is in accordance with the possible ternary surface complexation, as in the case of natural soil. The respective MTE adsorbed amounts were again statistically identical in the presence and in the absence of MET (Figure 19-B).

An additional experiment with the pharmaceutical/metal concentration (mol/L) ratio of 1/1 was conducted to evaluate if digestate amendment could increase MET adsorption when present in equal concentration as the MTE. The results showed that MET adsorbed amount was statistically identical to MET adsorbed amount in the absence of MTE, similarly to what was observed for the other pharmaceutical/metal concentration (mol/L) ratios. Furthermore, the results for MTE adsorption can be found in Appendix C (Figure C3).

When comparing the adsorption results on soil with those on amended soil, results show that digestate amendment led to an overall increase in the adsorbed amount of MET, while it did not have any influence on the adsorbed amounts of MTE. In fact, MET adsorption on amended soil, in presence of MTE with a 1/10 and 1/100 ratio, was on average 3.4 and 3.7 times higher than on soil, respectively. These results show how the application of digestate soil amendment could increase MET adsorption capacity of soil in accordance with what has been observed for other biosolids [10,36,67,68], and that the presence of some MTE can change that adsorption.

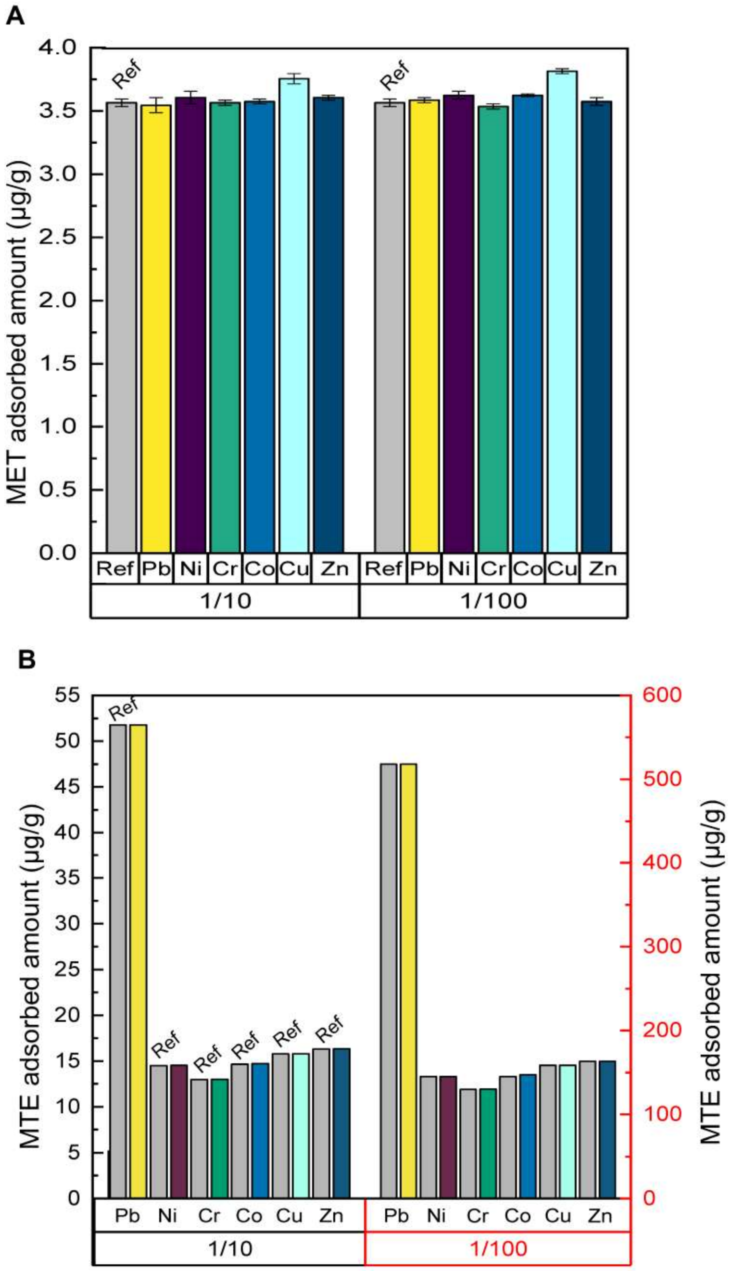

Figure 19: (A) Metformin adsorbed amount on amended soil in the presence of MTE (mean and standard deviation, n = 3) and (B) the respective MTE adsorbed amount at pharmaceutical/metal concentration (mol/L) ratios of 1/10 (left y-axis) and 1/100 (right y-axis) (mean and standard deviation, n = 3). The results are compared with the adsorbed amount of metformin and MTE measured in systems without MTE and metformin, respectively (Ref, grey bars). Error bars representing the standard deviation for MTE adsorption are present but not visible at the graph's scale due to the small magnitude of the values.

4.3.5 LMT adsorption in presence of MTE

For LMT, adsorption experiments in the presence of MTE were conducted initially on amended soil, and only afterwards on soil for the experimental conditions in which a significant effect was observed.

The results of LMT and MTE adsorption experiments on amended soil, for the different pharmaceutical/metal concentration (mol/L) ratios tested, are reported in Figure 20. LMT adsorbed amounts in the presence of Pb^{2+}, Ni^{2+}, Cr^{3+}, Co^{2+}, Zn^{2+} and Cu^{2+} were significantly lower than the adsorbed amount in absence of MTE (2.4 µg/g), for all pharmaceutical/metal concentration (mol/L) ratios tested (Figure 20-A), with a few exceptions for the ratio 1/100, namely for Cu^{2+}. In fact, for the 1/10 ratio, LMT adsorption decreased by around 20 % with Pb^{2+}, Cr^{3+}, Co^{2+} (not statistically significant), Cu^{2+} and Zn^{2+} and only by 12 % with Ni^{2+} (not statistically significant); while, for the 1/100 ratio, it decreased by 39 % with Pb^{2+}, 15 % with Ni^{2+} (not statistically significant), 20 % with Cr^{3+} and 16 % with Co^{2+} (not statistically significant). In contrast with MET results, LMT adsorbed amount varied amongst the different ratios tested, indicating that an increase in MTE concentration had, even if slightly, an influence on LMT adsorption, indicating either possible competition or a possible complex formation. Looking at the results for the system with Pb^{2+}, it is noticeable that LMT adsorption decreased with increasing Pb^{2+} concentrations, indicating a possible competition effect for the adsorption surface sites between the two species. This may be explained by considering Pb's greater affinity to Mn- and Fe-oxides compared to LMT and other MTE, and possible Pb precipitation caused by the formation of Pb-hydroxides and Pb-phosphates on the solid's surface at alkaline pH [75]. While, for the other MTE, having less affinity for oxides and soil mineral phases and greater affinity for soil OM [75–77], the behaviour can be explained taking into consideration possible competition interactions between LMT and MTE for sorption sites on the soil's OM. Moreover, LMT adsorption in the presence of Cu^{2+} and Zn^{2+} increased with higher concentration of the metallic cations (1/100 ratio) suggesting the possible formation of ternary surface complexes [48,72,78,79], as was assumed in the case of MET. Similarly, as was seen for MET, only the condition of Cu^{2+} at a 1/100 ratio showed a greater LMT adsorption compared to the absence of MTE. In fact, LMT adsorption increased by 8 % suggesting possible complexation interactions between the two compounds at high MTE concentration. The respective MTE adsorbed amounts, for the systems with 1/10 and 1/100 pharmaceutical/metal concentration (mol/L) ratios, were identical to the MTE adsorbed amounts in the absence of LMT (Figure 20-B).

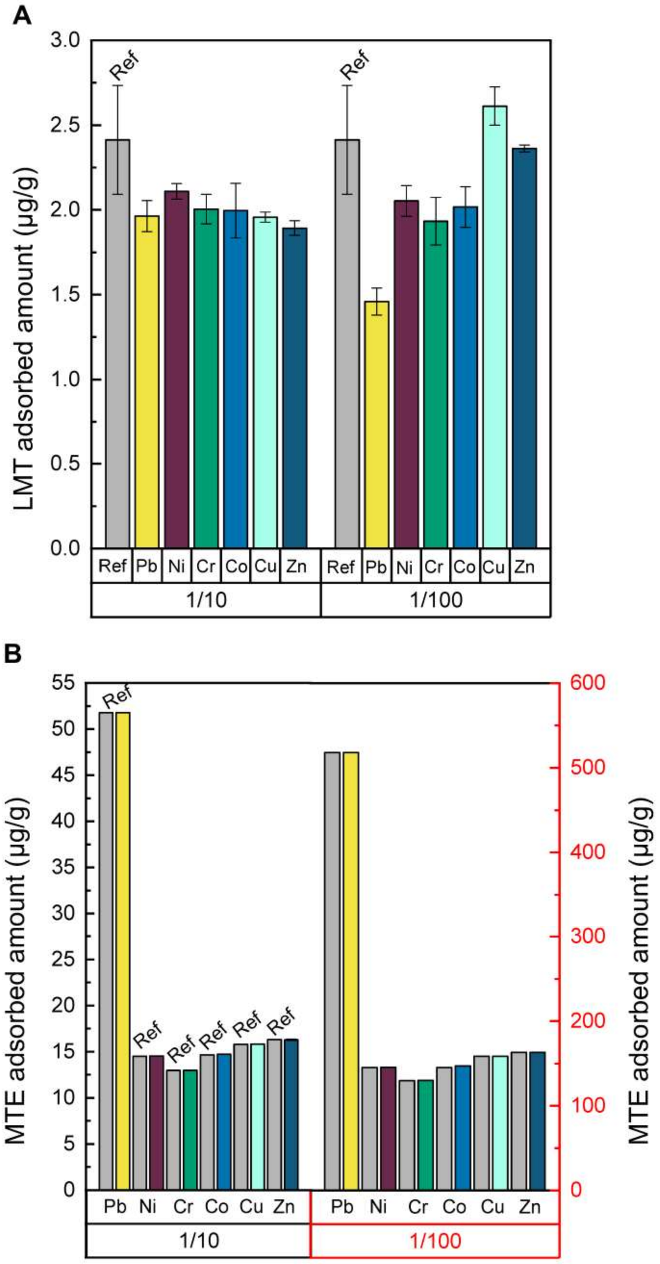

Figure 20: (A) Lamotrigine adsorbed amount on amended soil in the presence of MTE (mean and standard deviation, n=3) and (B) respective MTE adsorption, for pharmaceutical/metal concentration (mol/L) ratios of 1/10 (left y-axis) and 1/100 (right y-axis) (mean and standard deviation, n=3). The results are compared with the adsorbed amount of lamotrigine and MTE measured in systems without MTE and lamotrigine, respectively (Ref, grey bars). Error bars representing the standard deviation for MTE adsorption are present but not visible at the graph's scale due to the small magnitude of the values.

An additional experiment with the pharmaceutical/metal concentration (mol/L) ratio of 1/1 was conducted to evaluate if digestate amendment could increase LMT adsorption when present at equal concentration as the MTE. The results showed that LMT adsorbed amount was significantly lower (20 % decrease) to LMT adsorbed amount in the absence of MTE, similarly to what was observed for the other pharmaceutical/metal concentration (mol/L) ratios. Furthermore, the results for MTE can be found in Appendix C (Figure C4).

Considering the result on amended soil, only one LMT adsorption experiment on soil in the presence of Cu^{2+} at a pharmaceutical/metal concentration (mol/L) ratio of 1/100 was carried out, since that was the only condition for which an increase in LMT adsorption on amended soil was observed. Specifically, the aim was to understand if the amendment had an influence and/or an effect on LMT adsorption in the presence of Cu^{2+}. The results indicate that LMT adsorption on soil in this condition was 0.4 μg/g, almost half of the amount adsorbed on soil in absence of Cu^{2+} (0.78 μg/g). This suggests that Cu^{2+} hindered the adsorption of LMT possibly through competition interaction, which favoured the adsorption of the metallic cation instead of LMT, present in a neutral form at the pH of the experiment. This is opposite to what was found for amended soil and can be explained with the change in soil composition caused by the application of digestate amendment, which brought a rise in OM content increasing available adsorption sites with high affinity to Cu^{2+} [53,80,81]. Furthermore, this was opposite to what was observed for MET, which had an increase in adsorption on soil in the presence of Cu^{2+} at higher concentrations. Overall, this highlights the importance of considering the pharmaceutical compounds' chemical properties in defining their adsorption behaviour on soils.

4.3.6 Pharmaceuticals and MTE desorption

Soil and amended soil desorption experiments, conducted with ultrapure water and 0.01 mol/L $CaCl_2$ solution, resulted in no amount of MET and LMT being detected in the soil solutions. This suggests that the pharmaceutical compounds were either degraded or present at concentrations lower than the instrument's detection limit. The desorption experiments resulted in an overall significant release of MTE from both soil and amended soil, implying a potential risk for MTE mobility under natural rainfall conditions (Appendix C, Figure C5).

The results of specific MET and LMT desorption experiments, conducted on solid samples originating from MET and LMT adsorption experiments in the presence of Cu^{2+} at a pharmaceutical/metal concentration (mol/L) ratio of 1/1 and 1/100, are reported in

Figure 21 (details in Appendix C, Table C6). Cu^{2+} was the only MTE selected for these desorption tests because it was the only MTE for which an increase in MET and LMT adsorbed amount was observed. Moreover, these experimental conditions were selected also to evaluate the pharmaceutical compounds' desorption behaviour in systems characterized by simple adsorption and possible ternary complexation mechanisms.

The results evidence very different desorption behaviours for the two pharmaceutical compounds: MET desorbed amount decreased with increasing MTE concentration, while LMT desorbed amount increased with increasing MTE concentration. These trends were observed for both soil and amended soil. Going into more detail, MET desorbed amount from amended soil (0.80 ± 0.05 µg/g), originating from adsorption experiments with a 1/1 pharmaceutical/metal concentration (mol/L) ratio, resulted slightly higher than that released from soil (0.62 ± 0.07 µg/g). In contrast, MET desorbed amount from amended soil (0.08 ± 0.01 µg/g), originating from adsorption experiments with a 1/100 pharmaceutical/metal concentration (mol/L) ratio, resulted significantly lower than that released from soil (0.38 ± 0.01 µg/g). For LMT, the desorbed amount from amended soil for both pharmaceutical/metal concentration (mol/L) ratios (0.88 ± 0.06 µg/g and 0.99 ± 0.07 µg/g) resulted higher than that released from soil (0.17 ± 0.01 µg/g and 0.21 ± 0.06 µg/g). Considering the desorbed amount of MET and LMT as fraction of their total adsorbed amount on the solids (Figure 22), MET desorption from amended soil resulted being 23 % and 2 % for the solid samples originating from the 1/1 and 1/100 pharmaceutical/metal concentration (mol/L) ratio conditions, respectively, compared to those from soil of 53 % and 18 %. Whereas LMT desorption from amended soil resulted being 28 % and 29 % for the solid samples originating from the 1/1 and 1/100 pharmaceutical/metal concentration (mol/L) ratio condition, respectively, compared to those from soil of 35 % and 63 %. The release of Cu^{2+} resulted negligible (less than 2 % of the Cu^{2+} adsorbed amount) in all tested conditions (Appendix C, Table C6).

Overall, these results highlighted the desorption capacity of pharmaceutical compounds from both soil and amended soil, and the effect of digestate amendment on the desorption behaviour of the compounds. Considering the desorption, expressed as percentage of the adsorbed amount, it was seen that the application of digestate reduced the desorption of both pharmaceutical compounds and thus reduced their mobility compared to the non-amended soil. However, MET and LMT desorption potential observed for soil and digestate-amended soil could lead to contaminant transfer to deeper soil layers and contaminant leaching to groundwater.

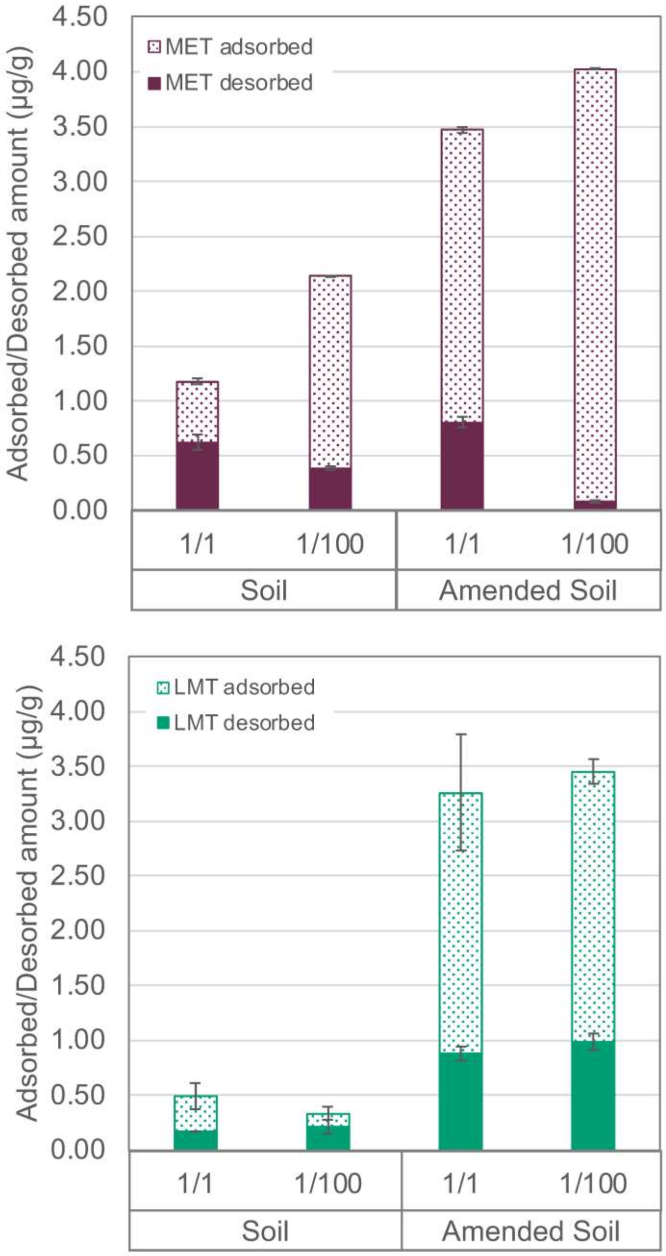

Figure 21: Adsorbed and desorbed amounts of metformin (MET, purple) and lamotrigine (LMT, green) from soil and amended soil samples originating from bi-solute systems with a pharmaceutical/copper ratio of 1/1 and 1/100.

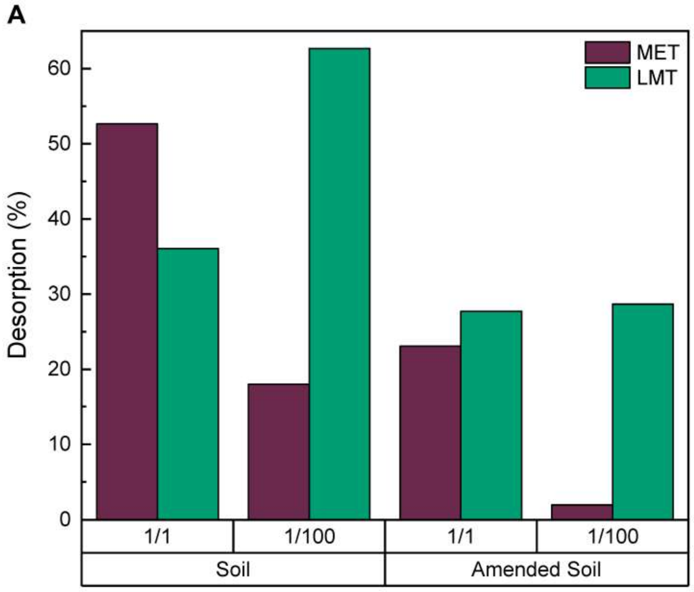

Figure 22: Desorbed amounts of metformin (MET, purple) and lamotrigine (LMT, green) from soil and amended soil samples originating from bi-solute systems with a pharmaceutical/metal concentration (mol/L) ratio of 1/1 and 1/100. Results are reported as percentage of the compound's total adsorbed amount on the solid.

4.4 Conclusions

This study investigated the sorption behaviour of MET and LMT on soil amended with biogas digestate, exploring their behaviour in presence of MTE. Overall, the results revealed increased soil adsorption capacity of pharmaceuticals due to digestate amendment, with MET showing higher affinity than LMT. At the environmentally relevant concentrations examined, 85 % of MET was adsorbed, whereas only 38 % of LMT was adsorbed on amended soil. The pharmaceutical compounds' different chemical nature led to diverse adsorption kinetics behaviour in both soil and amended soil. MET was better characterised by the Langmuir kinetic model, while LMT by the Intraparticle diffusion model and Elovich model. In presence of MTEs (except Cu^{2+}), overall sorption of pharmaceuticals decreased, specifically that of LMT by 12 – 39 %, indicating potential competition for adsorption sites. In the presence of Cu^{2+}, MET and LMT adsorbed amounts on soil and amended soil increased with increasing Cu^{2+} concentrations by 5 – 7 %, suggesting possible complexation mechanisms as ternary surface coordination complexes. The study also determined that digestate application reduced contaminant desorption. Nevertheless the observed desorption potential from soil and amended soil poses a risk of contaminant transfer leading to their leaching to groundwater or uptake by local flora and fauna. For these reasons, it is crucial to assess the concentrations of ECs, as pharmaceuticals, in authorised soil amendments before land application to evaluate the potential risks to ecosystem and human health. Further studies are necessary to evaluate the ecotoxicological effects of these pharmaceutical compounds, with a specific focus on LMT. Additionally, there is a need for in-depth investigations into the interaction mechanisms between MET/LMT and Cu^{2+}.

Associated content

The supporting information for this chapter is reported in Appendix C. This content includes further details on soil, digestate and amended soil characterization; the total metal concentration and fractionation results; the metal desorbed amounts from soil and amended soil; and the sorption kinetic model equations applied in the study.

Author Contributions

Veronica Baldasso: Conceptualization, Methodology, Investigation, Data collection, Formal analysis, Writing – original draft. *Stéphanie Sayen*: Conceptualization, Methodology Writing- review and editing, Supervision. *Carlos A. R. Gomes:* Writing-review and editing, Funding acquisition. *Luigi Frunzo*: Writing- review and editing, Funding acquisition. *C. Marisa R. Almeida*: Writing- review and editing, Funding acquisition. *Emmanuel Guillon*: Conceptualization, Methodology Writing- review and editing, Supervision.

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Chapter 5

Modelling metformin and trace metal sorption and transport in soil amended with digestate

5.1 Introduction

In the last years, digestate, originating from the organic fraction of municipal solid waste (OFMSW), has become a valuable resource not only for the agricultural industry but also for circular bioeconomy and land restoration purposes [1–5]. In fact, digestate is an organic-matter nutrient-rich material with promising agronomic properties, increasing soil's organic matter (OM) content, promoting nutrient cycling and giving support to plant and microbial growth [6–11]. For these reasons, digestate (liquid and solid fractions) has recently been included in the European Union's (EU) regulation on fertilizing materials [12], increasing its value and promoting its use. In the coming years, as a result of governmental zero-waste policies [13] and sustainable development goals [14–17], the production and use of digestate will rise due to an ever increase generation of municipal biowaste as a consequence of population growth. As new digestate-use strategies develop to enhance the circular bioeconomy centred around anaerobic digestion (AD), land application will remain a simple and beneficial approach for land and soil restoration [4,9,14,18–21]. However, this apparent virtuous practice may lead over the years to increased soil pollution due to the presence of contaminants in the digestate itself, which pose a threat to human health, soil ecosystems and groundwater [22–26]. Among the contaminants found in digestate there are trace metals (TM), classified as primary substances [27], pharmaceutical and personal care products (PPCP), and anthropogenic impurities as microplastics [28,29]. Thus, it is of fundamental importance to evaluate and understand contaminants' fate in soil after digestate land application to provide support for decision-making and environmental risk assessment [9,20,30–34].

PPCP are a class of contaminants of emerging concern (EC) that rise great alarm because conventional waste/sludge treatment processes still cannot remove them efficiently [35]. Furthermore, once released into the environment their behaviour and their effects on environmental ecosystems are poorly understood and for some compounds completely unknown [27,30,31,35–39]. One PPCP that has been recently detected in water bodies and in soils around the world is metformin (MET), a widely prescribed drug for type-2 diabetes [40]. In recent years, its occurrence and behaviour in soil, including sorption mechanisms and (bio)degradation pathways, have been investigated to better understand its fate in the environment [41–50]. Sorption is known to be one of the key processes controlling the mobility and fate of contaminants in soils, which can be influenced by the compound's chemical nature, the soil's physico-chemical properties and the presence of other contaminants [51]. Thus, the sorption behaviour of MET may vary between soil types and between different environmental conditions. In fact, the application of soil amendments, as biosolids and digestates, was found to increase soil's MET adsorption capacity and affect the compound's adsorption kinetics [41–43,48,50] (see Chapter 4). Moreover, the sorption of this pharmaceutical compound was found to be greatly affected by the presence of metallic trace elements [30,52] that are found in the digestate as well as in different environmental compartments [53,54]. In fact, TM may form coordination complexes with pharmaceutical compounds and/or compete with them for sorption sites affecting the pharmaceutical compounds' behaviour in the soil system [30,50,55–58]. In general, MET was observed to compete with several TM, such as Pb, Ni, Cr, Co, and Zn, for sorption sites. This competition resulted in a decrease in the soil's adsorption capacity of MET. However, in systems with high Cu concentrations (see Chapter 4), the soil's MET adsorption capacity increased, suggesting the formation of organo-metallic coordination complexes between MET and Cu that enhanced the sorption of the pharmaceutical contaminant. Thus, it is important to also consider the interactions between different TM and pharmaceutical compounds when assessing their fate and behaviour in soils after digestate amendment.

Modelling TM and MET sorption isotherms and their adsorption/desorption kinetic mechanisms is a fundamental tool to assess their behaviour in soils. Over the past decades, numerous studies have aimed to identify the mechanisms influencing the fate of TM in the soil-water environment and apply them to TM sorption and transport modelling [59]. The time-dependent nature of sorption mechanisms of many reactive TM species in soils has been highlighted, emphasizing the necessity to consider sorption kinetics to accurately model their fate in the environment [60–64]. Furthermore, environmental compartments often exhibit the presence of various TM and other contaminants. Competition among TM for available sorption sites on the soil matrix is a frequently observed phenomenon [65–67], which can greatly influence their fate (reaction rates), bioavailability, and mobility in soils [68–71]. There are different models that have been developed to describe sorption mechanisms in soils [59,67,70]. Equilibrium models are based on the assumption of local equilibrium, which considers the contaminant's reaction in soil to be much faster than the transport processes. These models include geochemical models, as ion-exchange and surface complexation, Freundlich and Langmuir isotherms. In contrast to the equilibrium models, there are the dynamic models that consider the time-dependent nature of contaminant retention in soils, such as the first-order kinetic, Freundlich kinetic, irreversible kinetic, and second-order models. These latter approaches can then be incorporated in more complex models, as multireaction and multisite models, which consider the sorbent as a heterogeneous system comprised of several constituents each having multiple sorption sites with specific rates of reaction [59,72–77]. Sorption mechanisms are one of the phenomena that contribute to contaminant fate in soil, while the others can be related to hydrogeological characteristics of the soil environment that define the transport processes occurring in the soil, defined as a porous media. The most applied deterministic mathematical model for soil contaminant transport is the advective-dispersive transport equation (ADE), formulated based on the law of contaminant mass conservation [59,78]. This model takes into account the transport processes due to advection and dispersion mechanisms [79]. Advection is the mechanism that represents the movement of contaminants with groundwater, according to the water seepage velocity of the soil. While dispersion is the mechanism that represents the spreading of contaminants across all flow directions and includes both molecular diffusion and mechanical dispersion. More precisely, molecular diffusion accounts for contaminant concentration gradients that allow molecules to move from an area of higher concentration to an area of lower concentration. Mechanical dispersion accounts for variation in flow velocity at the microscale, as a result of pore size, flow path length and deviation in pore water velocity from the average seepage velocity of the soil, and at the macroscale, due to differences in hydraulic conductivity and increasing probability of soil heterogeneities. The equilibrium and dynamic contaminant sorption models can be integrated in the ADE to properly simulate the fate and transport of contaminants in soils. Other phenomena, such as biological reactions, chemical reactions and interphase mass transfer, may be integrated in the ADE according to physical contaminant transport problem to be modelled. Several studies have integrated complex contaminant sorption models into the ADE to effectively simulate single/competitive TM transport in soils [73,76], and recently few have applied them to simulate contaminant leaching after the application of municipal biosolids or mulch of plant residue on soils [80,81]. These applications demonstrate the potential of simpler empirical models, such as equilibrium or dynamic sorption models, to simulate multi-contaminant behaviour in competitive/non-competitive systems and their versatility in being integrated in transport models.

Current understanding of MET transport in soil and digestate-amended soil is limited. In fact, to our knowledge, only few studies were conducted on MET sorption and mobility in an acidic soil amended with biosolids, originating from wastewater treatment [43]. These studies found that MET transport in soil could be well-described by a transport model including a multisite second-order kinetic model [41–43]. Moreover, to our knowledge, there is currently no study in literature on modelling the competitive sorption behaviour between MET, antidiabetic drug, and TM, and their transport in a digestate-amended soil system. Modelling multi-contaminant transport in soils and gaining more understanding on the effects of digestate amendment is of fundamental importance to help predict contaminants fate in the environment, while reducing expensive and time consuming field testing. Also, the data collected from experiments usually represents singular time frames showing the distribution of contaminants along the soil profile, while modelling can give insights into the dynamic temporal evolution within the soil. This can also be advantageous if it includes possible contaminant interactions, as amendment substances can have more than one type of contaminant. For all these reasons, the key objectives of this study were to (i) investigate the competitive sorption of MET and TM (Pb, Ni, Cr, Co, Zn, and Cu) in an alkaline soil amended with OFMSW digestate using a competitive second-order model, (ii) couple an equilibrium and dynamic sorption model, specifically the Langmuir isotherm and the Langmuir kinetic model, to an ADE utilised for describing transport phenomena in a two-layer digestate-amended soil system, with the soil represented as a saturated porous medium, and (iii) calibrate and validate the integrated contaminant transport model using experimental data originating from previous studies. For the latter two objectives, Cu was chosen as a representative of the different TM considered because it showed a distinct effect on pharmaceutical sorption, making it a case worthy of investigation.

5.2 Materials and Methods

5.2.1 Solids' characterization

Briefly, this study used two types of solid matrices: a natural alkaline soil and a digestate amended soil. The alkaline soil was collected from the subsurface of building plots located in Ermesinde, Porto (Portugal). The digestate amended soil was prepared by mixing (with a 14:1 dry weight ratio) the soil and the digestate, collected from a municipal solid waste treatment plant equipped with an AD unit. The digestate amended soil was prepared to mimic the application and incorporation of digestate into a topsoil *via* tilling. Full details on sampling and digestate-amended soil preparation are described in Chapters 2 and Chapter 3. The solid matrices' main physico-chemical properties are reported in Table 1 (Chapter 3) and in Table 5 (Chapter 4), with the concentrations of TM reported in Figure 3 (Chapter 2) and in Table 2 (Chapter 3), respectively. Moreover, the solid matrices' bulk density (ρ_b), particle density (ρ_p) and water filled porosity (θ) were evaluated and are reported in Table 7. Details of the methods used to characterize the solid matrices can be found in Chapter 2 (section 2.2.4 Analytical determinations) and Appendix A.

Table 7: Physico-chemical properties of digestate-amended soil (AS) and soil (S).

Solid matrix	ρ_b (g/cm^3)	ρ_p (g/cm^3)	θ (-)
AS	0.80	2.22	0.64
S	1.70	2.48	0.39

5.2.2 Experimental data

This work utilized datasets collected in previous laboratory experiments, namely soil column experiments and batch sorption experiments. All these experiments were conducted utilizing the same solid matrices (soil, digestate and digestate-amended soil) and details can be found in the previous chapters.

The initial dataset utilized consisted of sorption data for MET and TM (Pb, Ni, Cr, Co, Cu, and Zn), obtained from adsorption kinetic tests and batch sorption tests performed on soil and soil amended with digestate (see Chapter 4). Specifically, the adsorption kinetic tests yielded time-dependent adsorption data for MET, which was used for the determination of MET adsorption constant rates. Batch sorption tests provided data for equilibrium bi-solute sorption data which was used for the determination of MET and TM competitive sorption model parameters. It is important to mention that MET and TM concentrations were fixed at environmentally relevant concentrations of 5×10^{-6} mol/L and 5×10^{-6} – 5×10^{-4} mol/L, respectively, for all the above mentioned experiments.

The second dataset utilized was derived from the soil mesocosm column experiments conducted, as detailed in Chapter 2 and Chapter 3. These experiments aimed to evaluate TM fate in soil after the application of digestate originating from the non-source separated OFMSW, including in the absence and presence of MET, which was added to the digestate before its amendment on soil. The derived dataset was used to define the physical system, define the model's initial and boundary conditions, and assess the model's performance by comparing simulated results with the experimental data.

5.2.3 Modelling

5.2.3.1 Physical system

The physical system used for multi-contaminant transport modelling is shown in Figure 23. The physical system was taken as an image of the soil system characterizing the soil mesocosm column experiments, described in Chapter 2 and Chapter 3. In detail, the soil system was characterised by two distinct soil layers: a top digestate amended soil layer and a bottom natural soil layer, both considered homogenous. The surface of the digestate amended layer in contact with the atmospheric air comprised the top boundary of the system, and the lower surface of the natural soil layer comprised the bottom boundary. The contact surface between the two layers was defined as the interface boundary. The soil system was saturated, implying constant soil water holding capacity of 100 %. For this reason, the water filled porosity, θ, was considered constant throughout time. This condition reflected the saturated state observed during soil column irrigation in the experimental set-up used to simulate a case of intense rainfall, which could influence contaminants distribution. Irrigation water (200 mL/h) was applied at the top boundary and allowed to exit from the bottom boundary. The irrigation water flow was set equal to the one used in the soil column experiments. The initial conditions of the system were defined by the soil columns' state evaluated prior to the start of the experiments, with each soil layer characterised by specific physico-chemical and hydrological properties [82–88].

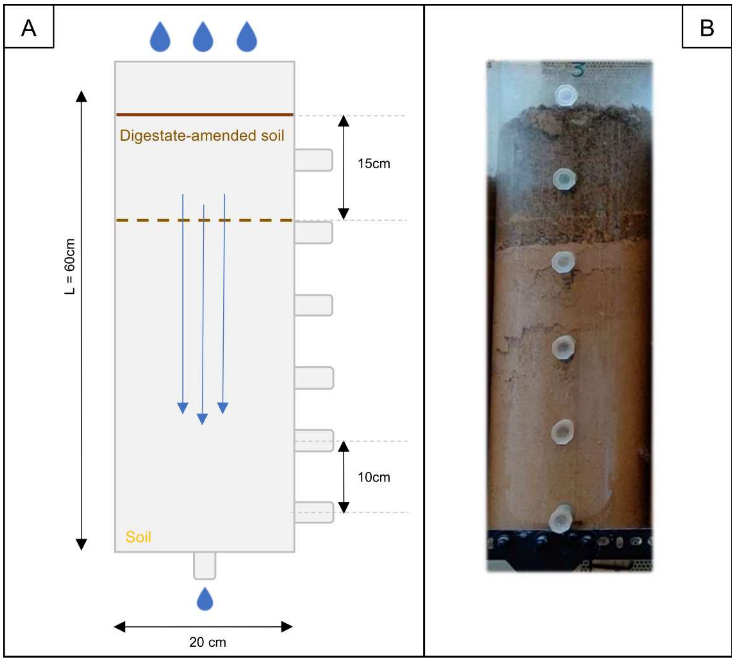


Figure 23: (A) Schematic representation, with key dimensions, of the two-layer soil system considered for contaminant transport modelling. (B) Photograph of the soil column system set-up for the soil mesocosm column experiments (Chapter 2 and Chapter 3).

5.2.3.2 Modelling MET and TM sorption behaviour

The second-order reaction model, commonly known as the Langmuir model, has been used to predict the sorption mechanisms of diverse contaminants in soils [63,65]. This model assumes that sorption is site specific, with the rate of reaction being a function of the contaminant concentration in the soil solution and the available sorption sites [59]. The Langmuir kinetic model can be written as follows:

$$\frac{\partial S}{\partial t} = k_a \theta C (S_{max} - S) - k_d S \tag{1}$$

where S is the contaminant adsorbed on the solid matrix of the soil (M/M), C is the contaminant concentration in soil solution (M/L^3), k_a is the adsorption rate constant (L^3/M/T), k_d is the desorption rate constant (1/T), S_{max} is the maximum adsorption capacity of the soil (M/M), $(S_{max} - S)$ is the amount of contaminant that could still be adsorbed by the solid matrix, and θ is the water filled porosity (-), with L, M and T expressing units of length, mass and time, respectively.

When large times are considered, the reaction is assumed to reach local equilibrium and equation (1) becomes the Langmuir isotherm model [89]:

$$\frac{S}{S_{max}} = \frac{K_L \cdot C}{1 + K_L \cdot C} \tag{2}$$

K_L is the Langmuir constant and can be considered as the ratio between the rate of adsorption and the rate of desorption.

The Langmuir isotherm model can be extended to account for competitive sorption of multiple contaminants in multi-component systems [59,65]. The assumptions at the base of this model are that (i) there is only one set of sorption sites for all competing contaminants and (ii) that the presence of competing contaminants does not affect the sorption affinity of the other contaminants [59,65,66,90]. The competitive Langmuir model (CLM) can be expressed as:

$$S_i = \frac{K_{L,i} \cdot C_i \cdot S_{max}}{1 + \sum_j K_{L,j} \cdot C_j} \tag{3}$$

where the *i* and *j* subscripts refer to the competing contaminants considered in the system.

The time-dependent formulation of the CLM can be expressed as:

$$\frac{\partial S_i}{\partial t} = k_{a,i} \theta C_i \left(S_{max} - \sum_j S_j \right) - k_{d,i} S_i \tag{4}$$

The CLM has been successfully, yet rarely, applied to simulate competitive TM sorption in soils [65,66,90–92]. The CLM, being a second-order model, was chosen for this study for two main reasons: (i) TM sorption on soil is generally well-described by second-order kinetics [59], and (ii) MET time-dependent sorption was found to be well-described by Langmuir kinetics for both soil and digestate amended soil (see Chapter 4). Thus, it was assumed that the sorption of these two types of contaminants, considered in a multi-component system, would continue to follow second-order kinetics.

The Langmuir and CLM model sorption parameters and constant rates were estimated *via* nonlinear fitting of the contaminant sorption data obtained experimentally (see Chapter 4). The goodness-of-fit of the selected models was assessed through the coefficient of determination, R^2, giving a statistical measure of how well the model approximates the experimental data.

It must be stated that further evaluations of contaminant kinetic sorption behaviour were conducted solely for the Cu-MET multi-contaminant system, taken as an example of a pharmaceutical-metal multi-contaminant system. Additionally, this system was chosen because potential interactions between MET and Cu were experimentally observed, as reported in Chapter 4.

5.2.3.3 Modelling MET and Cu transport in soil

The multi-contaminant transport problem for the soil system defined in section 5.2.3.1, was modelled using the well-known one-dimensional ADE [59]. In addition, this multi-contaminant transfer problem was formulated specifically considering two contaminants, namely MET and Cu, with the assumption that they would migrate from the top digestate-amended soil layer to the soil layer beneath it.

The ADEs, incorporating the CLM for sorption, for the two-layer soil system (digestate amended soil and soil) were:

Layer 1
$$\theta_1 \frac{\partial C_i}{\partial t} = \theta_1 D_{1,i} \frac{\partial^2 C_i}{\partial z^2} - q \frac{\partial C_i}{\partial z} - \rho_1 \frac{\partial S_i}{\partial t} \tag{5}$$

Layer 2
$$\theta_2 \frac{\partial C_i}{\partial t} = \theta_2 D_{2,i} \frac{\partial^2 C_i}{\partial z^2} - q \frac{\partial C_i}{\partial z} - \rho_2 \frac{\partial S_i}{\partial t} \tag{6}$$

where i subscript defines the different contaminants, ρ is the bulk density [M/L^3], D is the hydrodynamic dispersion coefficient (L^2/T) and q is the flow rate (L/T), with L, M and T expressing units of length, mass, and time, respectively. Going into more detail, q, defined also as apparent water velocity, was assumed to be defined by Darcy's law, implying it had the same value for all layers [79]. D was defined as the sum of the effective diffusion coefficient, D_W, and the mechanical dispersion, D_M, thus $D = D_W + D_M$. In addition, D_W was equal to the product of the aqueous molecular diffusion coefficient, D_0, and the tortuosity factor, τ, thus $D_W = D_0 \cdot \tau$. While D_M was equal to the product of the seepage velocity ($v = q/\theta$) and the longitudinal dispersivity (α), thus $D_M = v \cdot \alpha$. The sorption term, $\partial S/\partial t$, in equation 5 and 6 was substituted by the CLM (equation 3 and 4).

Two different soil contaminant transport models were assessed and compared: the first one considered the equilibrium CLM described by equation 3, and the second considered the time-dependent CLM described by equation 4. The systems of ADEs for the two different models are the following:

Model with equilibrium CLM

$$R_{1,i}\frac{\partial C_i}{\partial t} = D_{1,i}\frac{\partial^2 C_i}{\partial z^2} - \frac{q}{\theta_1}\frac{\partial C_i}{\partial z} \qquad (7)$$

$$R_{2,i}\frac{\partial C_i}{\partial t} = D_{2,i}\frac{\partial^2 C_i}{\partial z^2} - \frac{q}{\theta_2}\frac{\partial C_i}{\partial z} \qquad (8)$$

$$R_{1,i} = \left(1 + \frac{\rho_1}{\theta_1}\cdot\frac{K_{L1,i}\cdot S_{max,1}}{1+\sum_j K_{L1,j}\cdot C_j}\right) \qquad (9)$$

$$R_{2,i} = \left(1 + \frac{\rho_2}{\theta_2}\cdot\frac{K_{L2,i}\cdot S_{max,2}}{1+\sum_j K_{L2,j}\cdot C_j}\right) \qquad (10)$$

Model with time-dependent CLM

$$\theta_1\frac{\partial C_i}{\partial t} = \theta_1 D_{1,i}\frac{\partial^2 C_i}{\partial z^2} - q\frac{\partial C_i}{\partial z} - \rho_1\left(k_{a1,i}\theta_1 C_i\left(S_{max,1} - \sum\nolimits_j S_j\right) - k_{d1,i}S_i\right) \qquad (11)$$

$$\theta_2\frac{\partial C_i}{\partial t} = \theta_2 D_{2,i}\frac{\partial^2 C_i}{\partial z^2} - q\frac{\partial C_i}{\partial z} - \rho_2\left(k_{a2,i}\theta_2 C_i\left(S_{max,2} - \sum\nolimits_j S_j\right) - k_{d2,i}S_i\right) \qquad (12)$$

To solve the transport problem, the initial conditions, the layer interface condition and the boundary conditions were defined as follows:

Initial conditions

$C_{i,j}(z,0) = \overline{C_{i,1}}$ with $0 \le z \le h$ (13a)

$C_{i,j}(z,0) = \overline{C_{i,2}}$ with $h < z \le L$

$S_{i,j}(z,0) = \overline{S_{i,1}}$ with $0 \le z \le h$ (13b)

$$S_{i,j}(z,0) = \overline{S_{i,2}} \qquad \text{with } h < z \le L$$

Top boundary condition (layer 1)

$$q\, C_{i,1}(0,t) - \theta_1\, D_{1,i} \frac{\partial C_{i,1}(0,t)}{\partial z} = q\, C_{0,i} = 0 \tag{14}$$

Bottom boundary condition (layer 2)

$$q\, C_{i,2}(L,t) - \theta_2\, D_{2,j} \frac{\partial C_{i,2}(L,t)}{\partial z} = q\, C_{L,j} = 0 \tag{15}$$

Layer interface condition (between layer 1 and 2)

$$C_{i,1}(h,t) = C_{i,2}(h,t) \tag{16a}$$

$$q\, C_{i,1}(h,t) - \theta_1\, D_{1,i} \frac{\partial C_{i,1}(h,t)}{\partial z} = q\, C_{i,2}(h_2,t) - \theta_2\, D_{2,i} \frac{\partial C_{i,2}(h,t)}{\partial z} \tag{16b}$$

where *i* and *j* subscripts define the contaminant and the soil layer, respectively. *L* is the depth of the soil system given by the sum of the depth of first and second layer, and *h* is the depth of the layers' interface. The initial conditions (13a and 13b) set constant values for *C* and *S* within the two defined layers at time 0. The choice of boundary conditions was based on the physical problem considered. Boundary conditions (14) and (15), also defined as Robin boundary conditions, were used to represent a zero flux inlet and zero flux outlet condition, respectively, to simulate no contaminant inflow and no contaminant outflow from the system. The interface condition was defined by a continuity condition, simultaneously imposing the continuity of contaminant concentration (16a) and of contaminant flux (16b) [87,88].

Each contaminant transport model defined (equations 7 – 8 and equations 11 – 12) was used to simulate two different modelling scenarios, defined by the soil mesocosm column experiments described in Chapter 2 and Chapter 3, respectively. The first scenario was defined based on the first soil column experiment performed (see Chapter 2) and the second scenario was defined based on the second soil column experiment performed (see Chapter 3).

5.2.3.4 Numerical method

The two ADEs systems (equations 7 – 10 and equations 11 – 12) were solved by applying an upwind method that uses a forward approximation for the contaminant concentration time derivative, a central approximation for the contaminant concentration second-order space derivative and a backward approximation for the contaminant concentration first-order space derivative [93]. The sorption term was approximated by applying a forward Euler scheme. The upwind method was selected based on the assumption that water flow in the system is always positive, in accordance with the reference system where the z-axis is defined as positive downwards. The upwind scheme is reported in Appendix D.

To obtain model simulations of competitive MET and Cu transport in the soil system, the numerical method was implemented in MATLAB (version R2023_b) using the parameters estimated through nonlinear fitting of sorption data. Then, the simulation results were compared with the experimental data collected from the soil mesocosm experiments described in Chapter 2 and Chapter 3.

5.3. Results and discussion

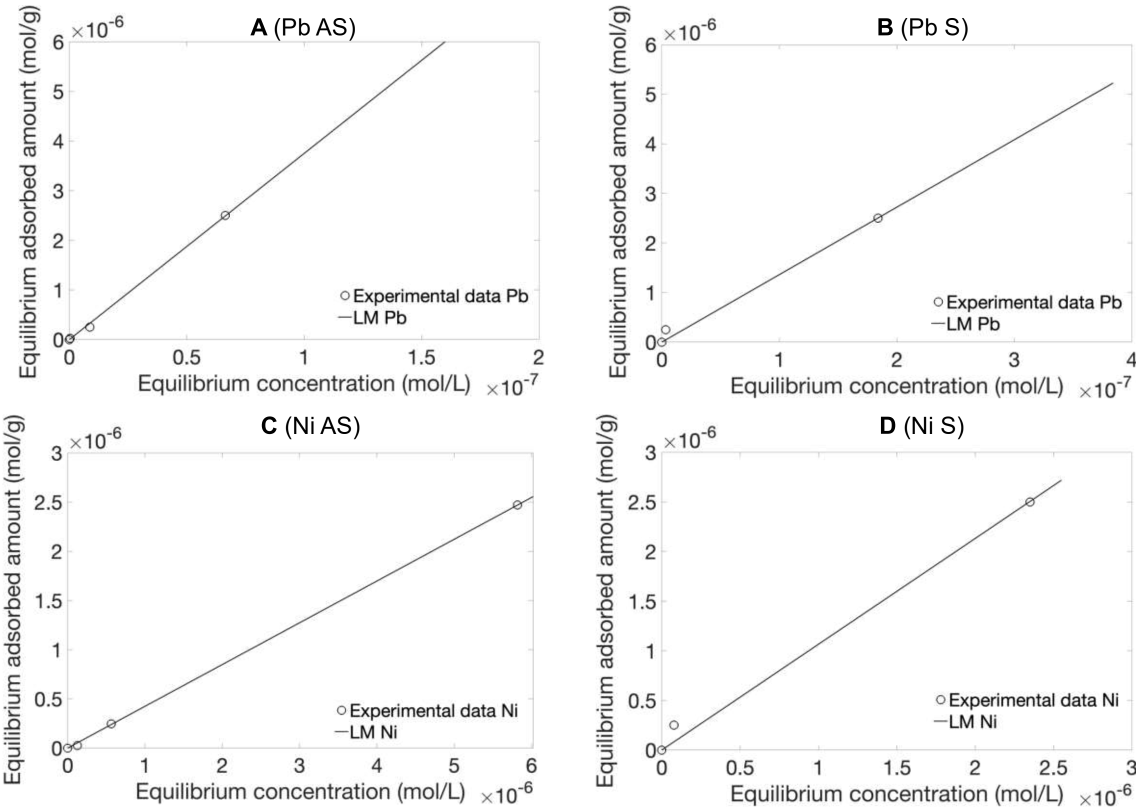
The results are presented sequentially, first for the competitive sorption isotherms (5.3.1), then for the sorption kinetic constant rates (5.3.2), and finally for the contaminant transport model (5.3.3), immediately followed by discussion.

5.3.1 Sorption isotherms

Modelling results of TM sorption isotherms in soil and digestate-amended soil using the Langmuir model (Equation 2) are shown in Figure 24. The Langmuir constants are reported in Table 8, together with the R^2 value. It must be highlighted that the sorption data utilised represents the TM sorption capacity of the soil and digestate-amended soil after an equilibration time of 48 h, simulating the immediate short term interaction between the contaminants and the solid matrices. The TM concentrations considered were selected from an environmentally relevant concentration range of $5x10^{-6}$ – $5x10^{-4}$ mol/L, characterising the sorption isotherms' linear curve trend.

First, the Langmuir model resulted in a high goodness-of-fit for all TM studied (Pb, Cr, Ni, Co, Cu and Zn) in both soil and digestate-amended soil. In fact, the R^2 value of the isotherms ranged between 99.9 – 96.1 % for digestate-amended soil and between 99.0 – 90.1 % for soil. It can be noticed that the model's goodness-of-fit was slightly higher for the isotherms representing TM sorption in digestate-amended soil. Nevertheless, the Langmuir model was able to describe reasonably well the TM sorption in both solid matrices.

The results showed similar TM sorption behaviours between amended soil and natural soil. Overall, the estimated Langmuir constants, $K_{L,TM}$, for the TM in soil and amended soil ranged from 1.33 to 5.67 L/mol, having the same order of magnitude. The estimated maximum adsorbing capacities, S_{max}, ranged from 0.29 to 6.59 mol/g, revealing slightly different affinities between the TM and the solid matrices. Going into detail, Pb and Cr showed greater sorption affinity in digestate-amended soil than natural soil, with a $K_{L,TM}$ of 5.67 and 5.08 L/mol for amended soil compared to 5.23 and 3.81 L/mol for soil, respectively. Pb and Cr isotherms also showed greater maximum sorption capacity S_{max} in digestate-amended soil (6.59 and 1.48 mol/g, respectively) compared to soil (2.60 and 0.77 mol/g, respectively). In addition, Pb resulted being the TM amongst all the studied TM with the greatest sorption affinity for both solid matrices. All the other TM, as Ni, Co, Cu and Zn, showed greater sorption affinity (with increased maximum sorption capacity) in natural soil compared to digestate-amended soil. Amongst these TM, Cu and Zn showed the greatest affinity with $K_{L,TM}$ being 5.21 and 5.02 L/mol, respectively, and S_{max} being 2.44 and 1.13 mol/g, respectively.



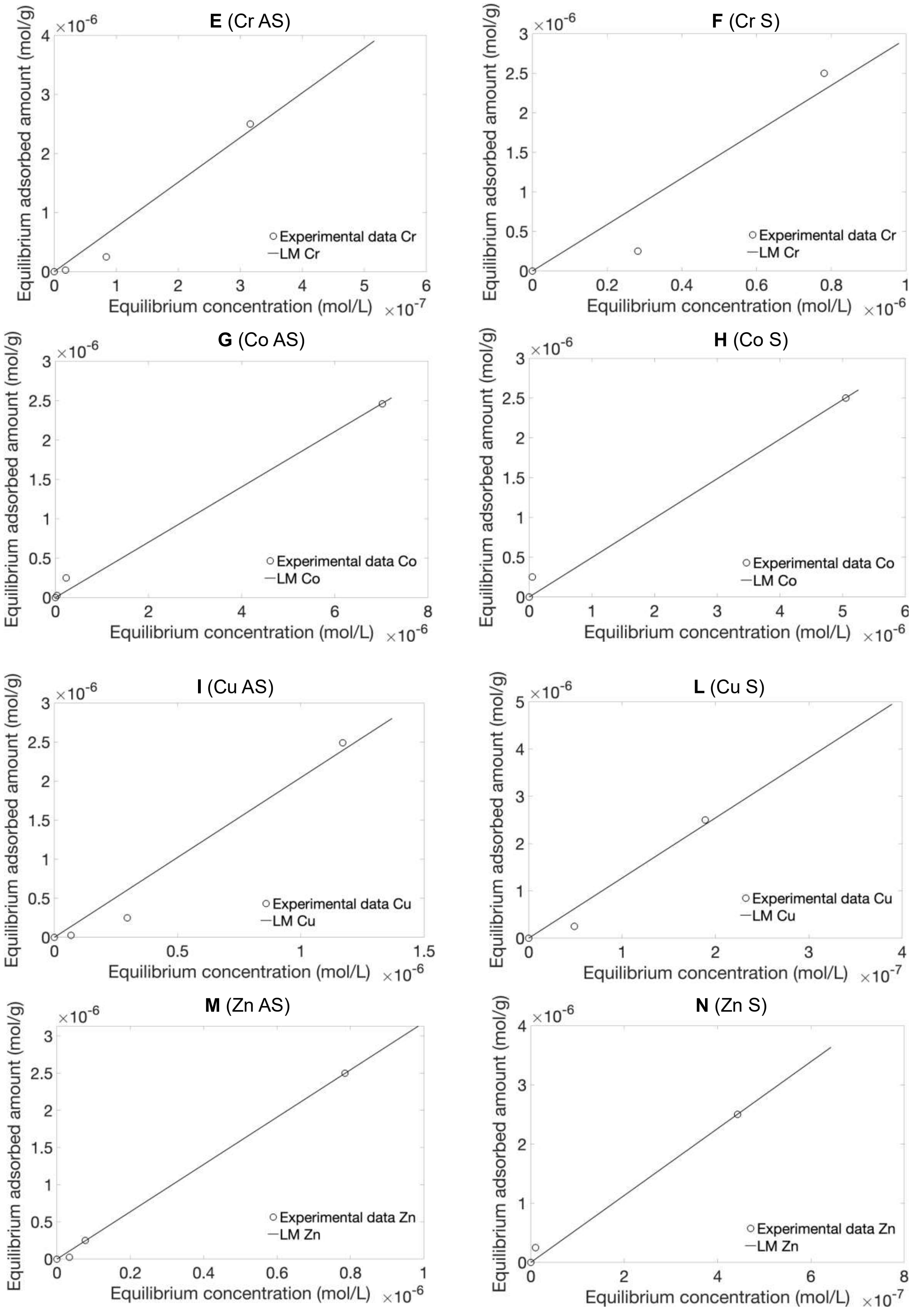


Figure 24: TM Langmuir fitting of sorption isotherms for digestate amended soil (A, C, E, G, I, M) and soil (B, D, F, H, L, N).

Table 8: Langmuir fitting parameters, S_{max} and Langmuir constant $K_{L,TM}$, of the single TM sorption isotherms for digestate-amended soil and soil.

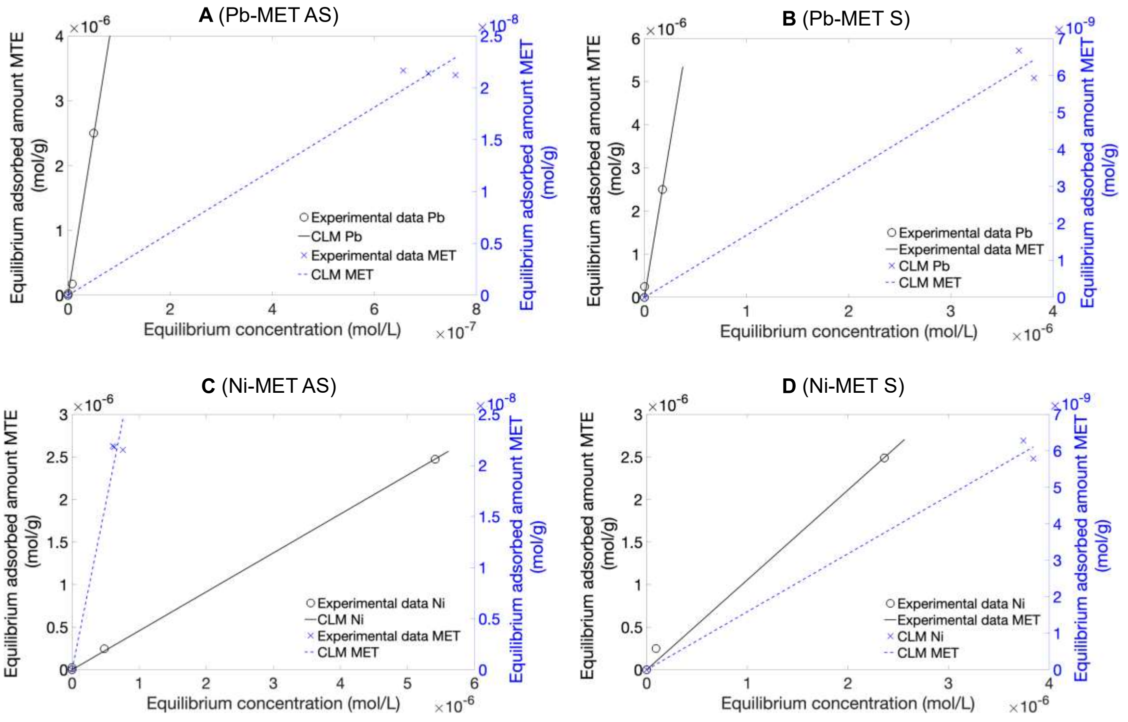
Solid Matrix	TM	S_{max} (mol/g)	$K_{L,TM}$ (L/mol)	R^2 (%)
Amended Soil	Co	0.26	1.33	99.3
	Cr	1.49	5.08	96.1
	Cu	1.01	2.02	96.6
	Ni	0.29	1.46	100
	Pb	6.59	5.67	99.9
	Zn	0.78	3.99	99.8
Soil	Co	0.31	1.58	98.7
	Cr	0.77	3.83	90.1
	Cu	2.44	5.21	96.1
	Ni	0.46	2.31	99.3
	Pb	2.60	5.23	98.9
	Zn	1.13	5.02	99.0

Simulation results of MET and TM competitive sorption isotherms for digestate-amended soil using CLM (Equation 3) are shown in Figure 25. The CLM constants are reported in Table 9, together with the R^2 value. It must be highlighted that the sorption data utilised represents the TM and MET sorption capacity of the soil and digestate-amended soil after an equilibration time of 48 h. Again, the TM concentrations considered were selected from an environmentally relevant concentration range of $5x10^{-6}$ – $5x10^{-4}$ mol/L, characterising the sorption isotherms' linear curve trend. The MET concentration was considered constant at an environmentally relevant concentration of $5x10^{-6}$ mol/L.

The CLM resulted, in general, in a high goodness-of-fit for all TM-MET combinations studied, in both soil and digestate-amended soil, with only a few exceptions. The R^2 value of the CLM ranged between 100 – 98.2 % for digestate-amended soil and between 99.7 – 98.4 % for soil, with the exception of Co-MET in amended soil and Cr-MET in soil with a R^2 of 27 and 77 %, respectively. The results for Co-MET competitive sorption in amended soil and Cr-MET competitive sorption in soil could suggest that Langmuir competitive sorption may not be the appropriate model to describe the competition effect determined in the laboratory experiments between these contaminants. However, given that the CLM well described the competitive sorption of Co-MET in soil and Cr-MET in amended soil, it could be possible that the data utilised was insufficient to define a clear trend, suggesting that more experimental data should be obtained to adequately model these bi-solute systems. For these reasons, the results of these bi-solute systems (Co-MET and Cr-MET) will not be further considered and discussed.

Going into detail, the CLM results revealed that the estimated Langmuir constant for Pb and Zn ($K_{L,TM}$) in amended soil were significantly greater than those for natural soil, indicating that these TM in presence of MET had stronger sorption affinity to digestate-amended soil compared to soil. Pb and Zn $K_{L,TM}$ in amended soil were 14.0 and 4.77 L/mol, while in soil they were 7.47 and 3.11 L/mol, respectively. So, Pb maintained the behaviour observed in the absence of MET, whereas Zn showed a different sorption behaviour in the presence of MET. For Ni and Cu, $K_{L,TM}$ results for soil were greater than those for amended soil indicating that these TM in presence of MET had greater sorption affinity to soil, a feature also observed in the absence of MET. The $K_{L,TM}$ of Ni and Cu in soil were 1.47 and 10.8 L/mol, respectively, and 1.35 and 2.05 mol/L in amended soil, respectively. In addition, the estimated Langmuir constants for MET, $K_{L,MET}$, resulted being one to four orders of magnitude lower than the $K_{L,TM}$ in all multi-contaminant systems studied, indicating that all TM had grater sorption affinity on both soil and digestate-amended soil compared to MET. The $K_{L,TM}$ for amended soil ranged between 0.009 and 0.1 L/mol, with the lowest value found for the Pb-MET system and the highest for the Ni-MET system. The $K_{L,MET}$ for soil ranged between 0.0009 and 0.002 L/mol, with the lowest found also for the system Pb-MET and the highest for the systems Ni/Cu-MET. This indicated that MET in the presence of TM had greater sorption affinity on digestate-amended soil than on soil, implying that digestate-amended soil would be able to adsorb greater quantities of MET compared to soil. Overall, these results were able to well-describe the TM and MET sorption behaviours observed through multi-contaminant batch sorption experiments (see Chapter 4). This suggests that the CLM may be an effective model to describe the competitive behaviour between TM and MET, an inorganic and a microorganic compound, as was determined for other similar multi-component systems [59,95]. Notably, these studies focused on Cu, Pb, Zn, Cd, Cr and Ni TM in combination with antibiotic compounds, such as tetracyclines, within pharmaceutical-metal systems. These studies determined the applicability of both

Freundlich and extended Langmuir isotherm models as robust tools for analysing experimental isotherm data, effectively considering competition and/or complexation mechanisms determined experimentally. Thus, applying the CLM to TM-MET isotherm data stands as a pioneering case study, offering insights into the sorption behaviour of a metal-antidiabetic system, a novel contribution in the field according to the authors knowledge.



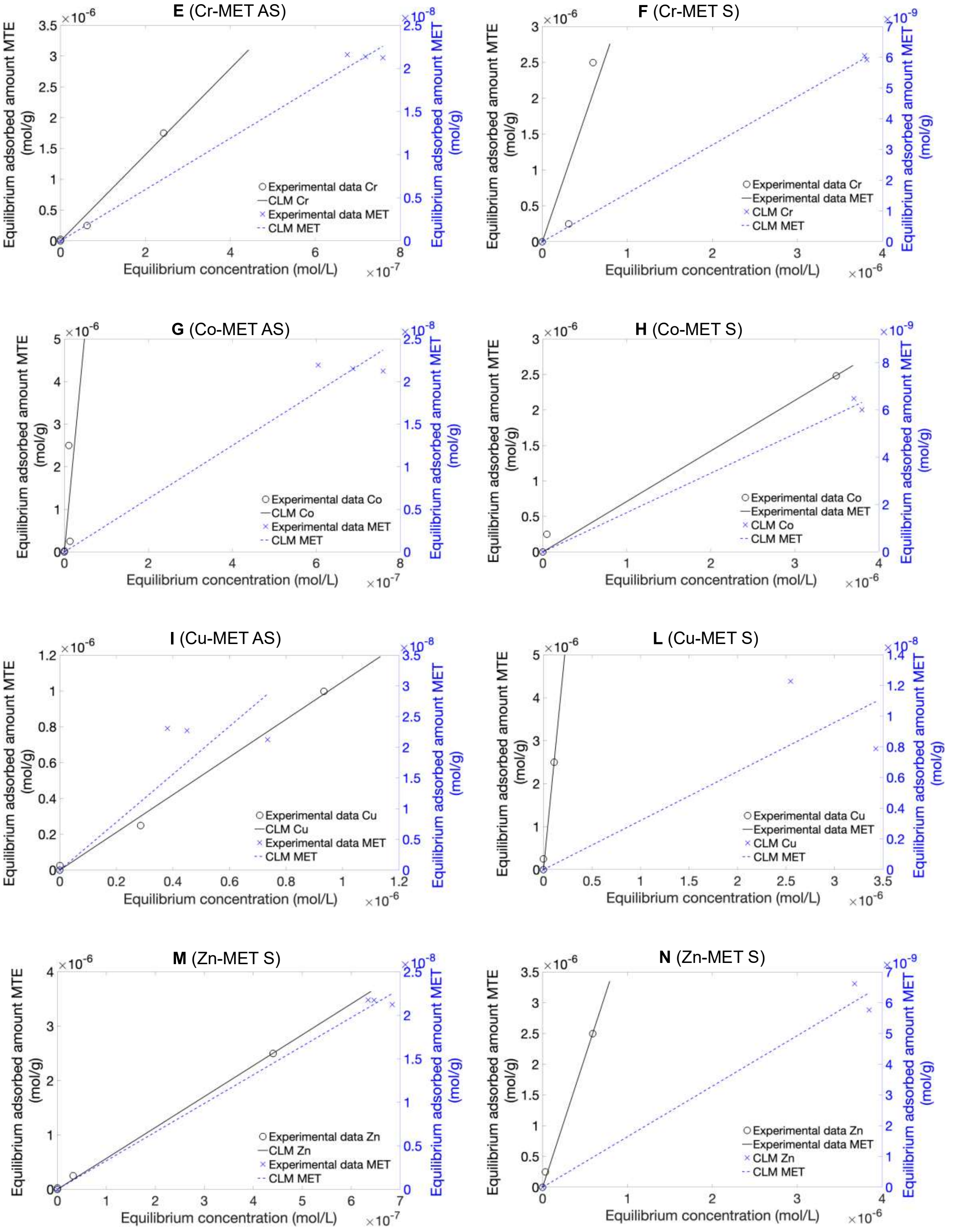


Figure 25: TM and MET Langmuir fitting of competitive sorption isotherms for digestate amended soil (A, C, E, G, I, M) and soil (B, D, F, H, L, N).

Table 9: Langmuir fitting parameters of the competitive sorption isotherms for digestate-amended soil and soil, for TM and MET bi-solute systems.

Solid Matrix	TM	S_{max} (mol/g)	$K_{L,TM}$ (L/mol)	$K_{L,MET}$ (L/mol)	R^2 (%)
Amended Soil	Co	5.12	20.5	0.006	26.6
	Cr	1.32	5.29	0.02	98.2
	Cu	0.51	2.05	0.08	99.4
	Ni	0.34	1.35	0.10	100
	Pb	3.50	14.0	0.009	98.9
	Zn	1.19	4.77	0.03	99.9
Soil	Co	0.60	1.20	0.003	98.8
	Cr	1.19	2.92	0.001	77.0
	Cu	2.10	10.8	0.002	98.4
	Ni	0.72	1.47	0.002	99.4
	Pb	1.90	7.47	0.0009	98.5
	Zn	1.36	3.11	0.001	99.7

Considering the single TM systems and the TM-MET systems, it was noticeable how the presence of MET compound increased the sorption affinity of specific TM on the solid matrices. In particular, this was evident for Pb and Cu. In fact, the $K_{L,TM}$ of Pb for the single solute system was equal to 5.67 L/mol in amended soil and 5.23 L/mol in soil, while for the bi-solute system (Pb-MET) it was equal to 14.0 and 7.47 L/mol, respectively. Similarly, the $K_{L,TM}$ of Cu for the single solute system was equal to 2.02 L/mol in amended soil and 5.21 L/mol in soil, while for the bi-solute system (Cu-MET) it was equal to 2.05 and 10.8 L/mol, respectively. The effect of MET on the sorption behaviour of the other TM, namely Ni and Zn, was varied. The sorption affinity of Ni in the presence of MET slightly decreased for both solid matrices (from 1.46 to 1.35 L/mol for amended soil and from 2.31 to 1.47 L/mol for soil), while the sorption affinity of Zn in the presence of MET decreased for soil (from 5.02 to 3.11 L/mol) and increased for amended soil (from 3.99 to 4.77 L/mol. Overall, the presence of MET had a slight effect on the sorption behaviour of TM in the studied solid matrices, with different TM leading to different behaviours, such as competitive or synergistic interactions. In addition, the increase in Pb and Cu adsorption in presence of MET was particularly pronounced in soils having lower OM content, as evidenced in the increased sorption on soil compared to the digestate-amended soil. Moreover, it is important to note that in a real case scenario various TM coexist simultaneously, giving rise to potential metal-metal interactions. In this regard, it is worth mentioning that the current study did not specifically address the potential interactions between multiple metals, which is an aspect that deserves further consideration. In general, TM can be characterised by different sorption behaviours, and it is essential to acknowledge and understand these complexities for a comprehensive assessment of all contaminant interactions in real environmental contexts.

5.3.2 Sorption kinetics

As mentioned in section 5.2 (Methods), the only bi-solute system considered for sorption kinetic assessment was the Cu-MET system. This specific system was chosen for sorption kinetic evaluation because MET and Cu were the contaminants selected as examples for contaminant transport modelling. In addition, as discussed in Chapter 4, MET adsorption behaviour was influenced in the presence of Cu in bi-solute system, suggesting possible competition and complexation mechanisms.

MET adsorption kinetics for the soil and a digestate-amended soil were found to be best described by the second-order kinetic model or Langmuir model (refer to Chapter 4). Similarly, Cu adsorption kinetics for soil was found to be best described by a second-order kinetic model [76]. The results found in Chapter 4 and in Elbana and Selim [76], were used in this study to estimate the adsorption and desorption constant rates for the time-dependent CLM (equation 4). The kinetic sorption data found for Cu was considered representative of the kinetic behaviour of Cu in the soil column system studied based on the fact that the soil used in the soil column experiments had similar physico-chemical properties of the soil used in Elbana and Selim [76]. In fact, both soils were alkaline with a pH around 9.2 and a cation exchange capacity (CEC) between 4.50 and 4.84 meq/100g, indicating that both soils had a similar ability in holding positively charged ions. Furthermore, it was assumed for simplicity that the adsorption kinetic behaviour of MET and Cu in multi-contaminant systems would be the same as their kinetic behaviour observed in single contaminant systems. Therefore, the contaminants' desorption constant rates in bi-solute systems were estimated using the competitive Langmuir's constant, obtained *via* nonlinear fitting of the CLM as described in section 5.3.1, and the adsorption constant rates derived from literature (Chapter4 and 76). The desorption constant rates were calculated as follows [59]:

$$k_d = k_a / K_L \tag{17}$$

With k_d being the desorption constant rate [1/T], being the adsorption constant rate [L/M/T] and being Langmuir's constant. The adsorption and desorption constant rates of MET and Cu in soil and digestate-amended soil are reported in Table 10.

The estimated results showed that for both amended soil and soil the adsorption constant rates of Cu were significantly greater than the desorption constants rates, while the adsorption constant rates of MET were significantly lower than the desorption constant rates. Overall, these results suggest that (i) Cu was characterised by a faster adsorption process with the tendency to occupy the available adsorption sites having a competitive advantage over MET, and that (ii) MET was characterized by faster desorption indicating the possibility to be easily displaced by other solutes having greater affinity to the adsorbent material. Moreover, these results showed that Cu and MET adsorption constant rates in soil were significantly greater (one or two orders of magnitude) than their respective adsorption rates in digestate-amended soil. Similarly, the desorption rate of MET in soil was three orders of magnitude greater than the one estimated for amended soil, while the desorption constant rate of Cu in amended soil was in the same range (even if slightly greater) than the one estimated for soil. These results can be explained by the physico-chemical changes induced by the addition of digestate to the soil. In fact, as seen in the previous Chapters, the digestate induced an increase in OM content and in CEC, determining as a result an increase in the available sorption sites and matrix complexity. As seen in Chapter 4, this led to an increase in MET adsorbed amount in digestate-amended soil, also in the presence of TM, while TM adsorbed amount resulted similar on both soil and digestate-amended soil. However, given the higher complexity of the digestate-amended soil, the kinetic rates resulted being slower. In addition, it was shown that digestate application reduced contaminant desorption, thus reducing their mobility in the soil system and their risk for transfer to deeper soil layers.

A more complex kinetic model, which simultaneously considers various sorption sites characterised by specific sorption mechanisms, can be developed and applied to improve the accuracy of model predictions. This can be achieved by utilising a multi-site second-order model [77], which considers a specific sorption site for every key sorption mechanism defined in the problem with the kinetic sites characterised by second-order kinetics. To assess the added value of the more complex model, the results should be compared with those of the simpler CLM utilised in this study.

Table 10: Competitive Langmuir model's adsorption (k_a) and desorption (k_d) constant rates for MET and Cu in digestate-amended soil and natural soil.

Solid matrix	Contaminant	k_a (L/mol/h)	k_d (L/mol/h)
Amended soil	Cu	35.0	17.1
	MET	2.03	26.7
Soil	Cu	280	25.8
	MET	100	$5 \cdot 10^4$

5.3.3 MET and Cu transport

The parameters of the soil system considered in this studied and used for contaminant transport modelling are reported in Table 11. The parameters defined were the soil layer's depth, the water filled porosity (θ), the apparent water velocity (q), the tortuosity factor (τ) and the longitudinal dispersivity (α). The water filled porosity, θ, was set equal to the total porosity assuming, for simplicity, no air filled porosity was present at saturation. The total porosity was calculated by subtracting the ratio of ρ_b to ρ_p from 1. The apparent water velocity, q, was calculated as the ratio of the water flow used for irrigation in the soil column experiments and the surface area on which the water flow was applied (see section 5.2.3.1). The tortuosity factor was derived from Millington's formula [79], which calculates the tortuosity factor as the ratio of the water filled porosity elevated to the 7/3 to the water filled porosity at saturation elevated to the 2. However, since the study considered the soil system at saturation, the tortuosity factor became equal to the water filled porosity at saturation elevated to the 1/3. Lastly, the longitudinal dispersivity was derived from Gelhar's diagram [96], which correlates the longitudinal dispersivity to the scale of observation.

It was noticed that the digestate-amended soil layer resulted in higher porosity, lower longitudinal dispersivity and greater tortuosity compared to soil, indicating a potential increase in fluid retention, a higher water velocity and a greater contaminant dispersion. Furthermore, the hydrodynamic dispersion coefficient of the contaminants considered

(D_{MET} and D_{Cu}) resulted being very similar between the digestate-amended soil and the natural soil layers.

Table 11: Properties of the two-layer soil system considered for the transport model (digestate-amended soil – AS and soil – S). The properties reported are the soil layer's depth, the water filled porosity (θ), the apparent water velocity (q), the tortuosity factor (τ), the longitudinal dispersivity (α), and the hydrodynamic dispersion coefficients (D_{MET} and D_{Cu}) for each contaminant considered.

Solid matrix	Depth (cm)	θ (-)	q (cm/h)	τ (-)	α (cm)	D_{MET} [97] (cm^2/h)	D_{Cu} [98,99] (cm^2/h)
AS	15	0.64	0.64	0.86	1.5	1.53	1.51
S	35	0.40	0.64	0.73	3.5	5.67	5.66

The initial conditions defined for the two contaminant transport scenarios are reported in Table 12 and Table 13, respectively. The first scenario was defined based on the first soil column experiment performed (see Chapter 2) and the second scenario was defined based on the second soil column experiment performed (see Chapter 3). The initial Cu soil solution concentrations for the digestate-amended soil layer and the natural soil layer were set equal to the Cu concentrations measured in soil solution after a 48 h (equilibration time) desorption experiment, conducted using the digestate-amended soil and the natural soil, respectively (refer to Chapter 4 and Appendix C). The initial MET soil solution concentration for the digestate-amended soil layer was set equal to the MET concentration measured after a 48 h (equilibration time) desorption experiment, conducted using the digestate-amended soil characterized by a 1/1 MET to Cu ratio with concentrations in the order of 10^{-6} mol/L (see Chapter 4 for experimental details). It must be stated that these experimental conditions well represented the experimental condition of the soil mesocosm column experiments [34], which had a similar MET/Cu ratio with MET and Cu concentrations in the same order of magnitude (refer to Chapter 2 and Chapter 3). The initial MET soil solution concentration in the soil layer was set to zero, representing a MET-free soil as was the case for the soil column experiment. Furthermore, it was assumed that the pool of Cu subject to relatively fast sorption reactions was that comprised of the exchangeable, reducible (bound to Fe/Al oxides), and bound to OM metal fractions. For this reason, only Cu found in these metal fractions was considered for sorption and transport modelling. This assumption also considers that Cu found in the residual fraction, representing the metal species strongly bound to soil minerals, is less susceptible to short term changes (low mobility and availability) [66]. As expected from the data of the soil column experiments, the initial conditions for the two scenarios resulted being very similar, with minor differences in the initial adsorbed amounts of Cu ($S_{0,Cu}$).

Table 12: Initial conditions defined for the first simulation scenario, relating to the first soil column experiment (see Chapter 2), indicating the initial MET concentration in soil solution ($C_{0,MET}$), the initial MET adsorbed amount ($S_{0,MET}$), the initial Cu concentration in soil solution ($C_{0,Cu}$), and the initial Cu adsorbed amount ($S_{0,Cu}$).

Solid matrix	$C_{0,MET}$ (mol/L)	$S_{0,MET}$ (mol/g)	$C_{0,Cu}$ (mol/L)	$S_{0,Cu}$ (mol/g)
AS	$9.7 \cdot 10^{-8}$	$1.6 \cdot 10^{-8}$	$7.2 \cdot 10^{-8}$	$11.6 \cdot 10^{-8}$
S	0	0	$7.4 \cdot 10^{-9}$	$2.4 \cdot 10^{-8}$

Table 13: Initial conditions defined for the second simulation scenario, relating to the second soil column experiment (see Chapter 3), indicating the initial MET concentration in soil solution ($C_{0,MET}$), the initial MET adsorbed amount ($S_{0,MET}$), the initial Cu concentration in soil solution ($C_{0,Cu}$), and the initial Cu adsorbed amount ($S_{0,Cu}$).

Solid matrix	$C_{0,MET}$ (mol/L)	$S_{0,MET}$ (mol/g)	$C_{0,Cu}$ (mol/L)	$S_{0,Cu}$ (mol/g)
AS	$9.7 \cdot 10^{-8}$	$1.6 \cdot 10^{-8}$	$7.2 \cdot 10^{-8}$	$2.9 \cdot 10^{-8}$
S	0	0	$7.4 \cdot 10^{-9}$	$2.0 \cdot 10^{-8}$

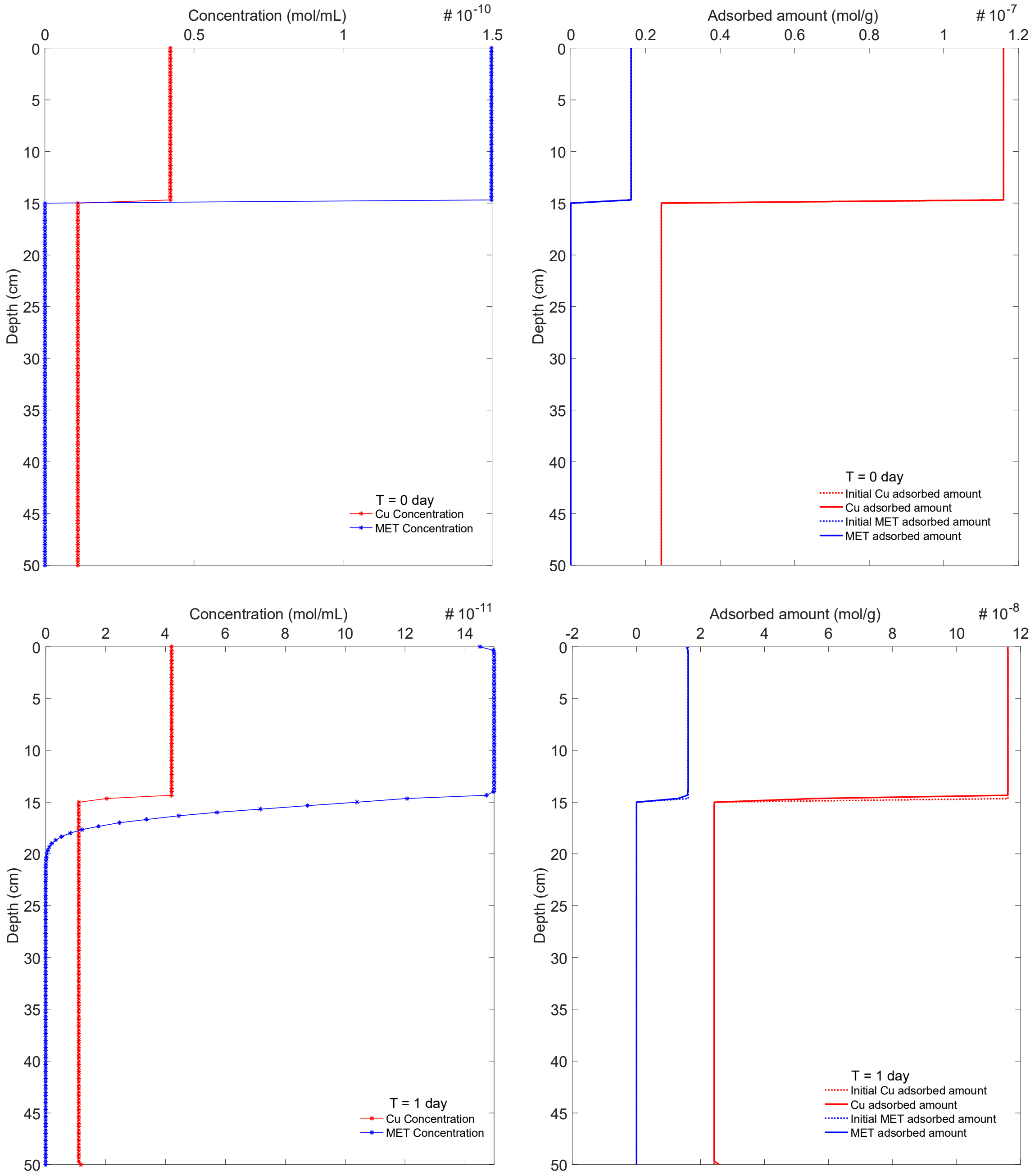
The simulation results for MET and Cu transport in the two-layer soil system, subject to equilibrium CLM sorption, for the first scenario (relative to the first soil column experiment, Chapter 2) and second scenario (relative to the second soil column experiment, Chapter 3) are reported in Figure 28 and Figure 29, respectively. The simulation results for MET and Cu transport in the two-layer soil system, subject to time-dependent CLM sorption, for the first and second scenario are reported in Figure 26 and Figure 27, respectively. All simulated results depict the contaminant concentration in the soil solution and the contaminant adsorbed amount on the solid matrix, along the soil column profile over experiment time (from day 0 to day 28).

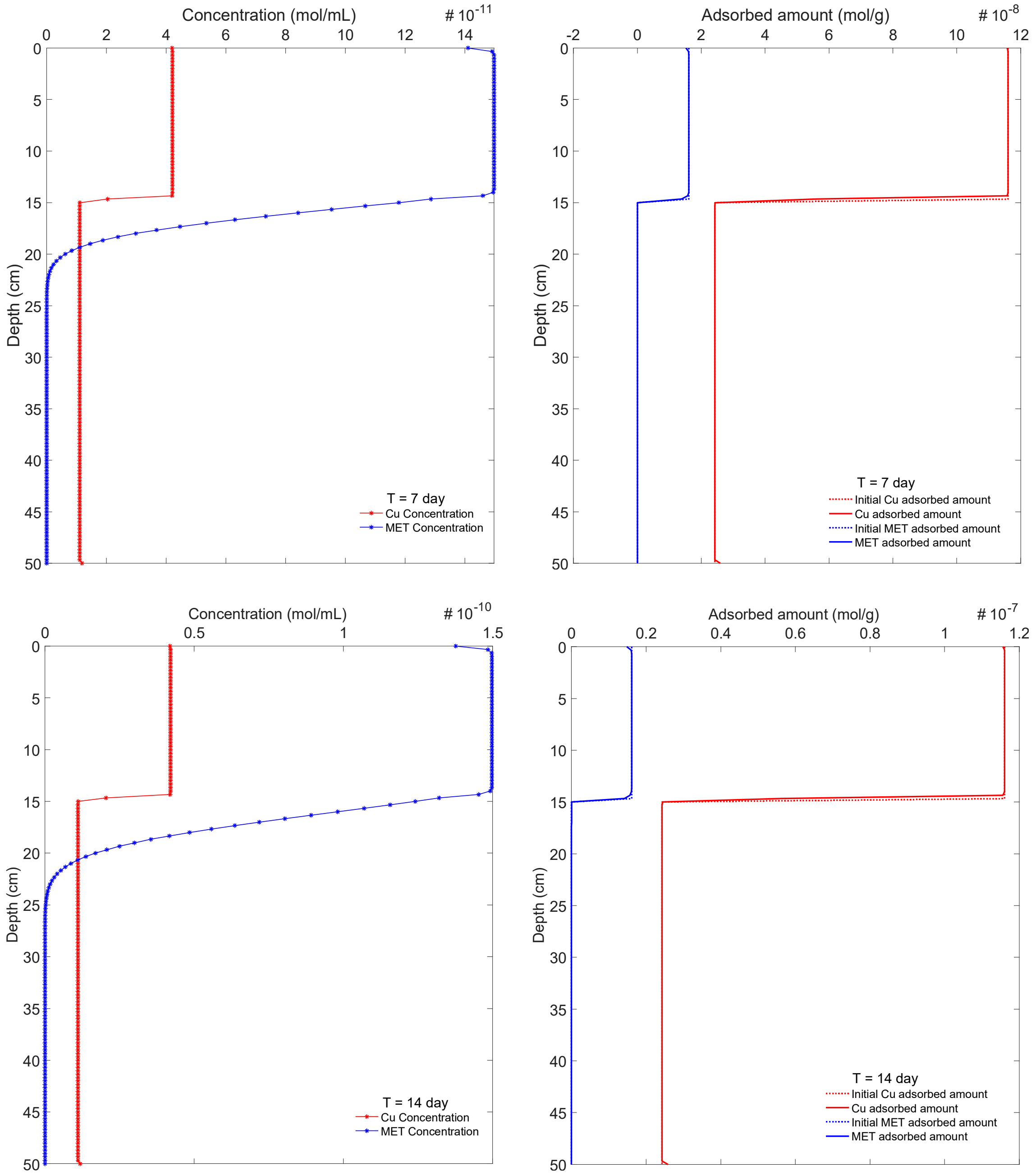
The curves representing contaminant soil solution concentration and contaminant adsorbed amount exhibited similar trends in both simulated scenarios. This observation held true for the results obtained with the model using equilibrium CLM and the model using time-dependent CLM. This similar curve trend is due to the fact that the initial concentrations defined for the two simulation scenarios were not significantly different from each another.

In general, for the results obtained with the model considering equilibrium sorption, MET concentration in soil solution resulted one order of magnitude greater than that of Cu, while MET adsorbed amount resulted one order of magnitude lower than that of Cu, over the whole time of simulation (from day 0 to day 28). In general, MET concentration over time was characterised by a slow advancing front wave curve, while Cu concentration remained constant over time. The MET behaviour can be explained by considering the initial MET adsorbed amount in the soil layer, which was set to 0, establishing a non-equilibrium state. Over time, a small amount of MET would gradually desorb from the amended soil and transfer to the soil layer, attempting to reach equilibrium. Similarly, the behaviour of Cu can be explained by the fact that contaminant sorption was considered at equilibrium, determining that the quantity of Cu released into solution was balanced by the quantity adsorbed onto the solid matrix. In addition, MET and Cu adsorbed amounts along the soil profile did not vary significantly over experimental time, with exception for the adsorbed amounts at the top boundary that slightly decreased from day 1 to day 28 for effect of the uncontaminated water inflow. In fact, due to the tendency of the system to reach an equilibrium state, the inflow of uncontaminated water allows the desorption of the contaminants adsorbed on the top layer, which become free in solution and subject to transport through the soil system. The adsorbed amounts decreased from 1.61×10^{-8} on day 0 to 1.42×10^{-8} mol/g on day 28 for MET and from 1.16×10^{-7} to 1.15×10^{-7} mol/g for Cu for the first scenario, and from 1.61×10^{-8} on day 0 to 1.42×10^{-8} mol/g on day 28 for MET and from 4.57×10^{-8} to 4.54×10^{-8} mol/g for Cu for the second scenario. This effect was also detected on contaminant concentration, which decreased over time from 1.49×10^{-10} on day 0 to 1.32×10^{-10} mol/L on day 28 for MET and from 0.42×10^{-10} to 0.41×10^{-10} mol/L for Cu for the first scenario, and from 1.49×10^{-10} on day 0 to 1.32×10^{-10} mol/L on day 28 for MET and from 1.66×10^{-11} to 1.64×10^{-11} mol/L for Cu for the for the second scenario. Furthermore, a very small increase in Cu adsorbed amount (from 2.42×10^{-8} on day 0 to 2.6×10^{-8} mol/g on day 28 for the first scenario and from 2.05×10^{-8} on day 0 to 2.19×10^{-8} mol/g on day 28 for the second scenario) was detected at the bottom boundary as consequence of the imposed zero total flux boundary condition.

In general, for the results obtained with the model considering time-dependent sorption, MET concentration in soil solution and MET adsorbed amount resulted one order of magnitude greater than that of Cu over the whole time of simulation (from day 0 to day 28). Similarly to what was found for the previous results, MET soil solution concentration over time was characterised by a slow advancing front wave curve, while Cu concentration was characterized by a step curve. The observed trends are most likely due to the initial adsorbed amounts of MET and Cu on the two layers, specifically the MET adsorbed amount set to 0 and the positive Cu adsorbed amount on the soil layer. One main difference between these results and those obtained with the model considering equilibrium sorption, was the variation in contaminant soil solution concentration and contaminant adsorbed amount from day 0 to day 1. While in the case of equilibrium sorption these values maintained the same order of magnitude, in the case of kinetic sorption the values for contaminant concentration decreased by four orders of magnitude (mol/L) and those for adsorbed amounts increased by one order of magnitude (mol/g). This behaviour can be explained by the kinetic behaviour of the contaminants. In fact, MET, characterised by lower adsorption constant rates and higher desorption constant rates, can be easily released into solution due to lower sorption affinity to the surface of the solid matrix and can be subject to greater transport through the soil system. Whereas Cu, characterised by higher adsorption constant rates and lower desorption constant rates, has greater sorption affinity to the solid matrix being less subject to desorption and transport. Furthermore, from day 1 to day 28, the contaminant concentrations slightly decreased at the top boundary from $7.67x10^{-10}$ to $7.52x10^{-10}$ mol/L for MET and from $6.28x10^{-11}$ to $6.22x10^{-11}$ mol/L for Cu for the first scenario and from $7.67x10^{-10}$ to $7.52x10^{-10}$ mol/L for MET and from $3.73x10^{-11}$ to $3.7x10^{-11}$ mol/L for Cu for the second scenario. On the other hand, the contaminants slightly increased in the soil (considering the layer immediately below the interface) from $6.0x10^{-10}$ to $7.2x10^{-10}$ mol/L for MET and from $1.2x10^{-11}$ to $1.24x10^{-11}$ mol/L for Cu for the first scenario and from $6.0x10^{-10}$ to $7.2x10^{-10}$ mol/L for MET and from $1.03x10^{-11}$ to $1.05x10^{-11}$ mol/L for Cu for the second scenario. Over the same time interval, the contaminant adsorbed amounts slightly decreased at the top boundary from $7.86x10^{-7}$ to $7.69x10^{-7}$ mol/g for MET and from $1.73x10^{-7}$ to $1.71x10^{-7}$ mol/g for Cu for the first scenario, and from $7.86x10^{-7}$ to $7.69x10^{-7}$ mol/g for MET and from $1.03x10^{-7}$ to $1.02x10^{-7}$ mol/g for Cu for the second scenario. In soil the contaminant adsorbed amounts increased from $2.43x10^{-10}$ to $2.91x10^{-10}$ mol/g for MET and from $2.64x10^{-8}$ to $2.73x10^{-8}$ mol/g for Cu for the first scenario and from $2.43x10^{-10}$ to $2.91x10^{-10}$ mol/g for MET and from $2.25x10^{-8}$ to $2.30x10^{-}$

^{8} mol/g for Cu for the second scenario. Overall, it can be said that the curves of contaminant concentration and adsorbed amounts described the expected trends.




Concentration (mol/mL)
10-11
0 2 4 6 8 10 12 14
Depth (cm)
0 5 10 15 20 25 30 35 40 45 50
T = 7 day
Cu Concentration
MET Concentration
Adsorbed amount (mol/g)
10-8
-2 0 2 4 6 8 10 12
Depth (cm)
T = 7 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount
Concentration (mol/mL)
10-10
0 0.5 1 1.5
Depth (cm)
T = 14 day
Cu Concentration
MET Concentration
Adsorbed amount (mol/g)
10-7
0 0.2 0.4 0.6 0.8 1 1.2
Depth (cm)
T = 14 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount

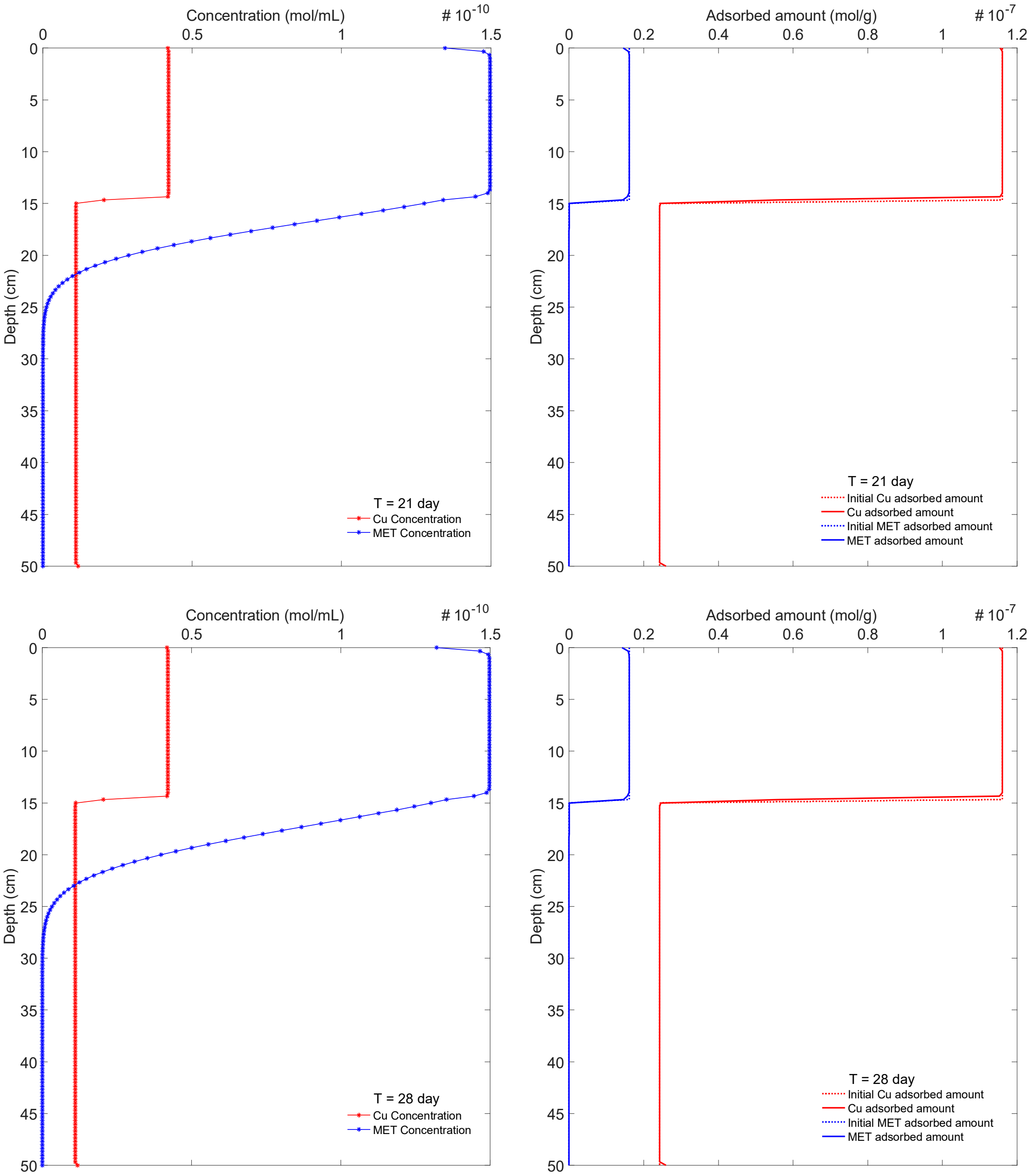
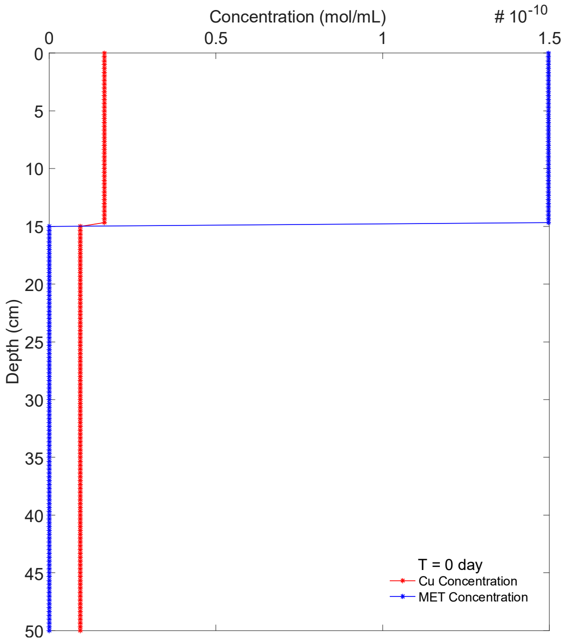
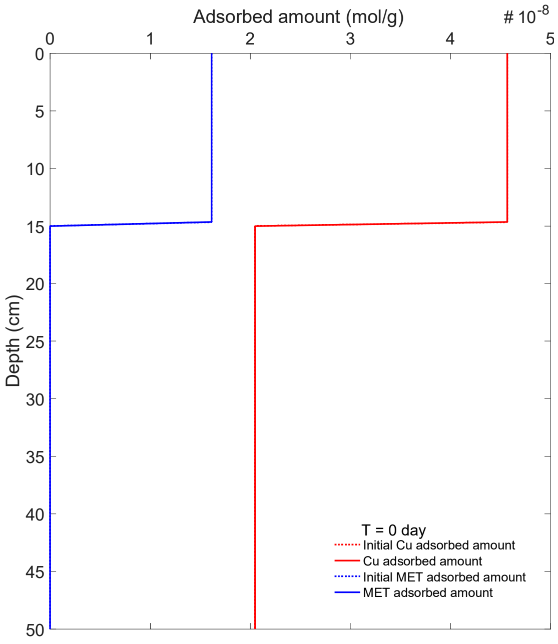
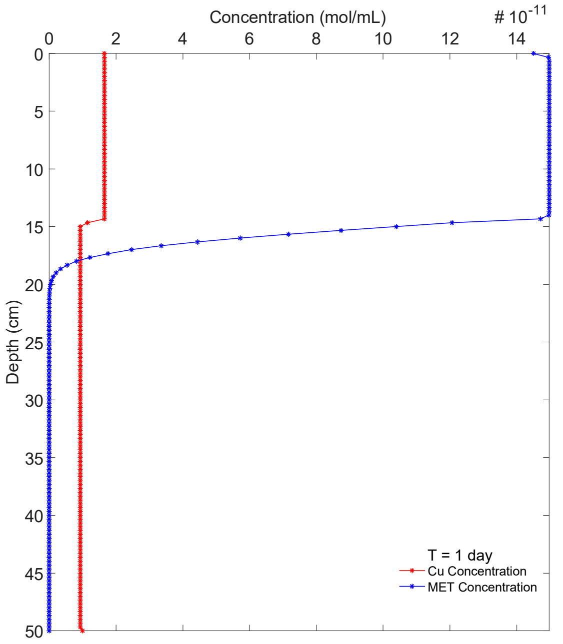
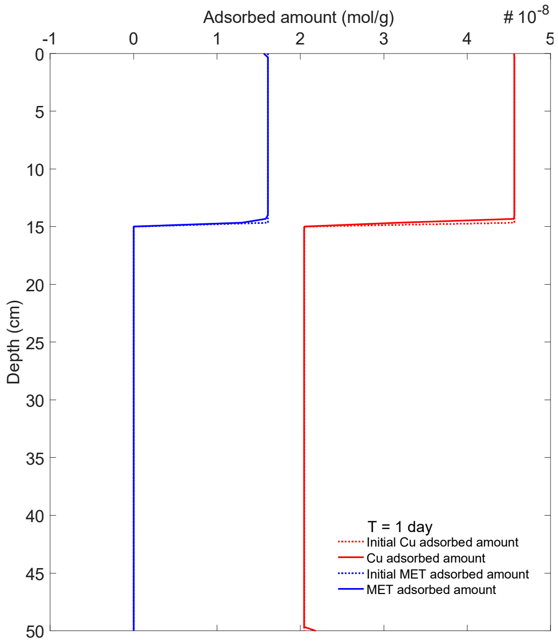


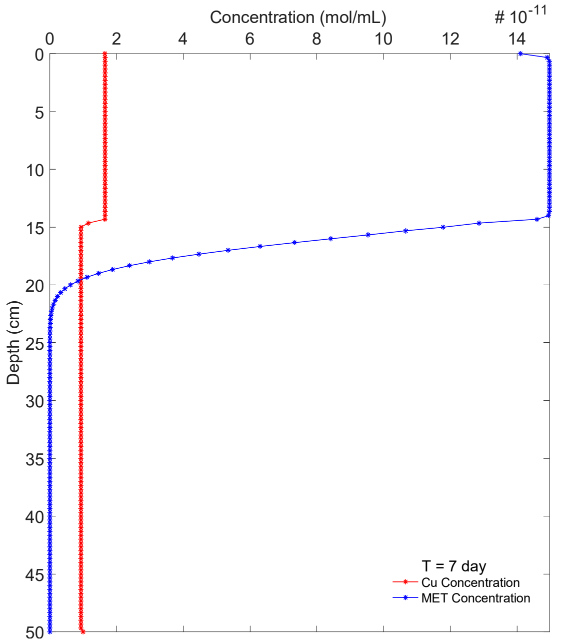
Figure 26: Simulated Cu (red) and MET (blue) concentration in soil solution (line with asterisks) and adsorbed amount on the solid matrix (solid line) throughout time, using the transport model with equilibrium CLM and first experimental conditions (see Chapter 2).

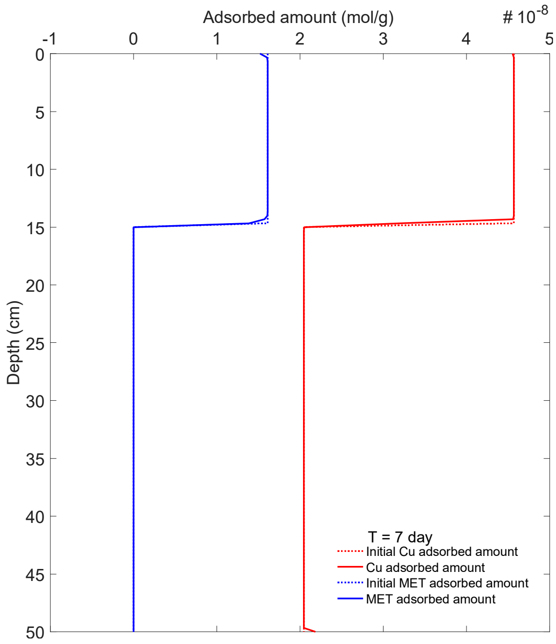

Concentration (mol/mL)
10^-10
0
0.5
1
1.5
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 0 day
Cu Concentration
MET Concentration

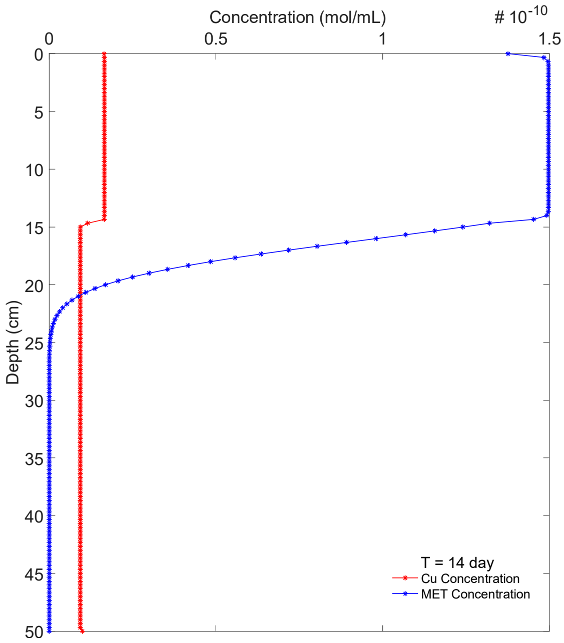

Adsorbed amount (mol/g)
10^-8
0
1
2
3
4
5
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 0 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount

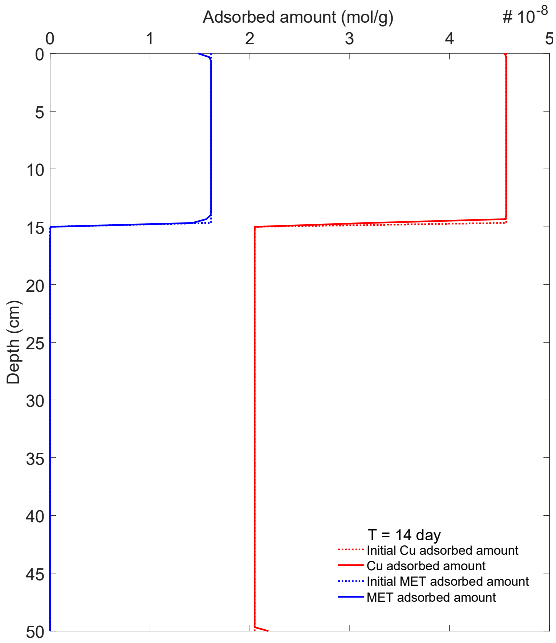

Concentration (mol/mL)
10^-11
0
2
4
6
8
10
12
14
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 1 day
Cu Concentration
MET Concentration


Adsorbed amount (mol/g)
10^-8
-1
0
1
2
3
4
5
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 1 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount


Concentration (mol/mL)
10^{-11}
0 2 4 6 8 10 12 14
Depth (cm)
0 5 10 15 20 25 30 35 40 45 50
T = 7 day
Cu Concentration
MET Concentration


Adsorbed amount (mol/g)
10^{-8}
-1 0 1 2 3 4 5
Depth (cm)
0 5 10 15 20 25 30 35 40 45 50
T = 7 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount


Concentration (mol/mL)
10^{-10}
0 0.5 1 1.5
Depth (cm)
0 5 10 15 20 25 30 35 40 45 50
T = 14 day
Cu Concentration
MET Concentration


Adsorbed amount (mol/g)
10^{-8}
0 1 2 3 4 5
Depth (cm)
0 5 10 15 20 25 30 35 40 45 50
T = 14 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount

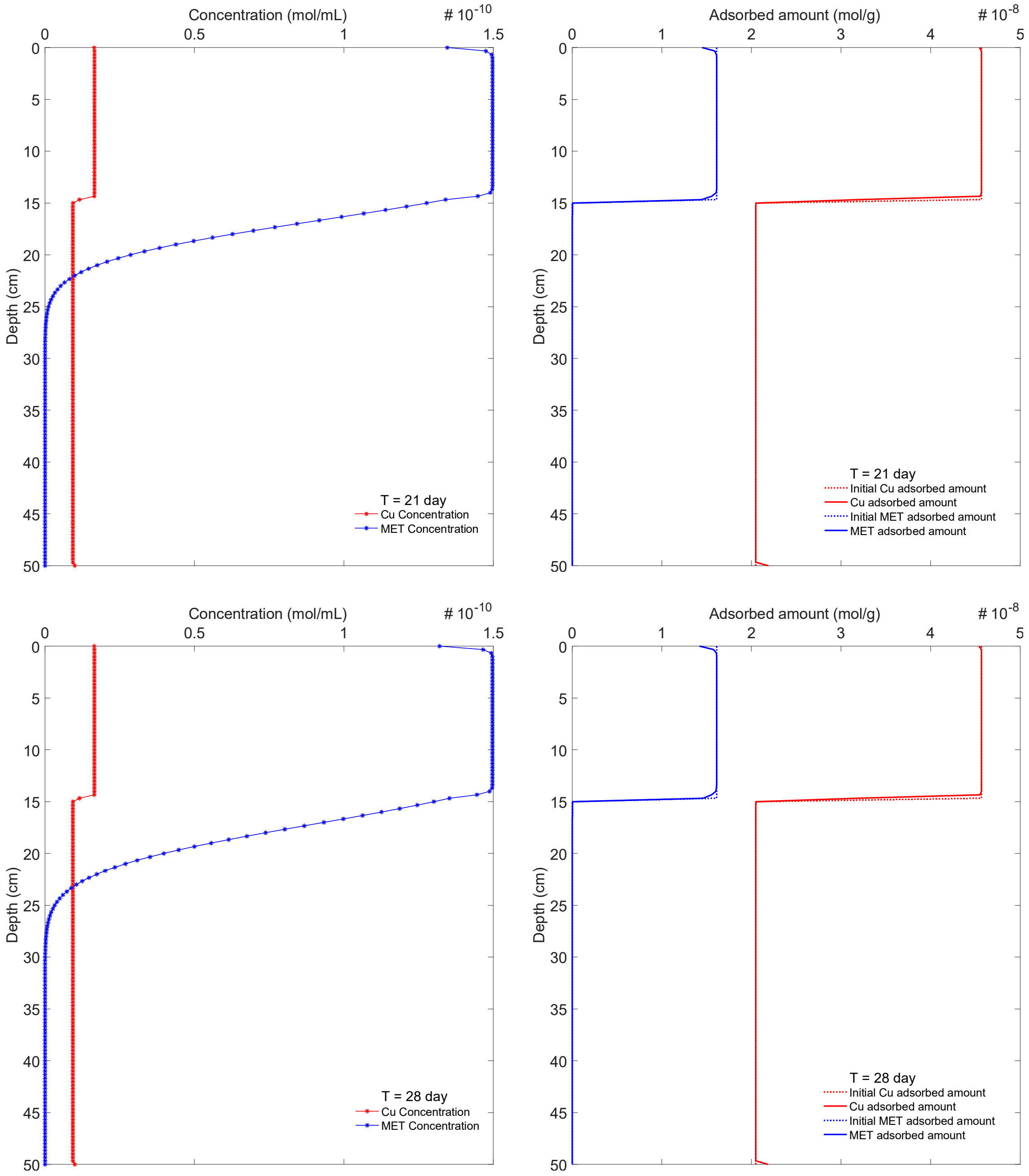
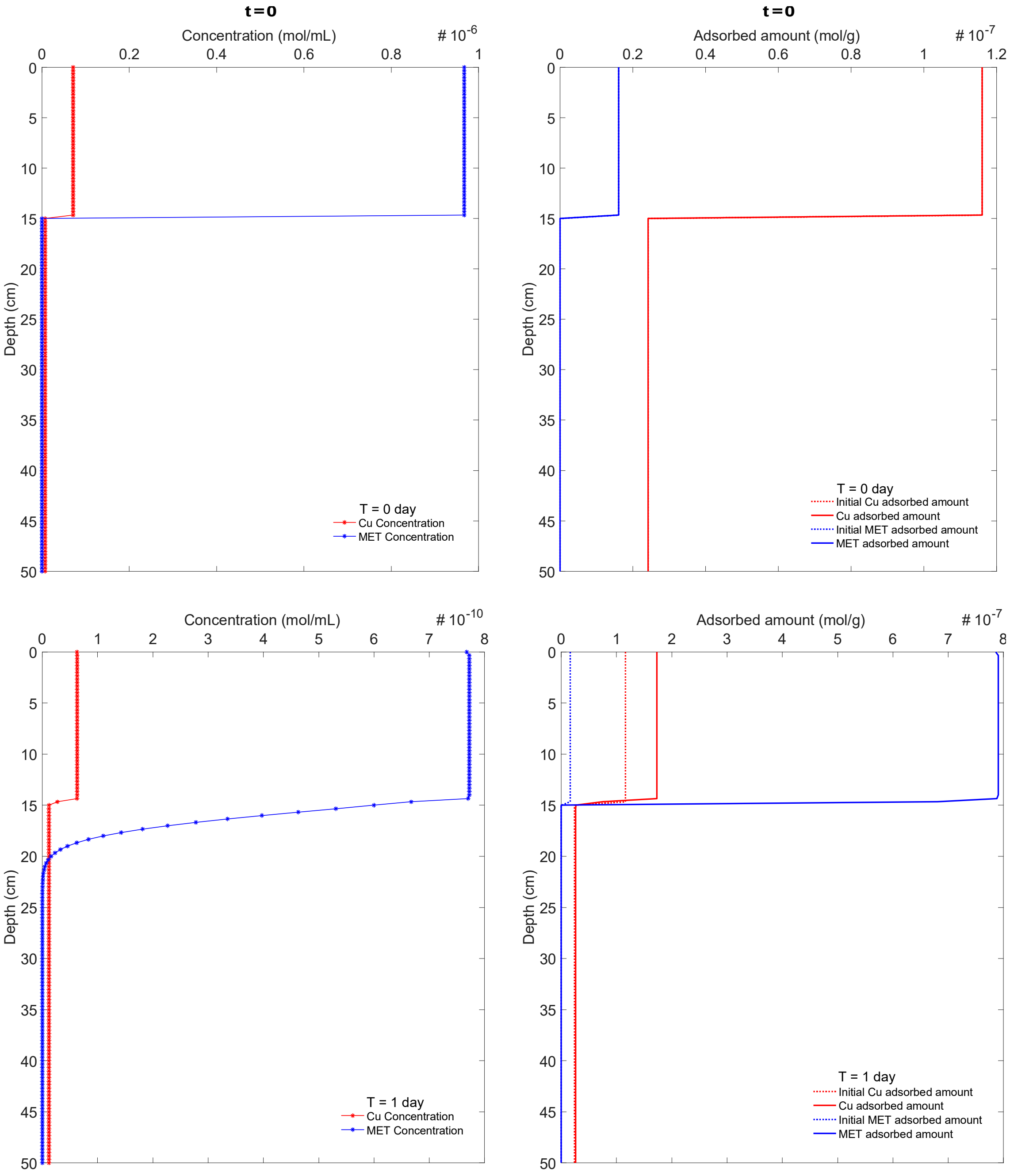
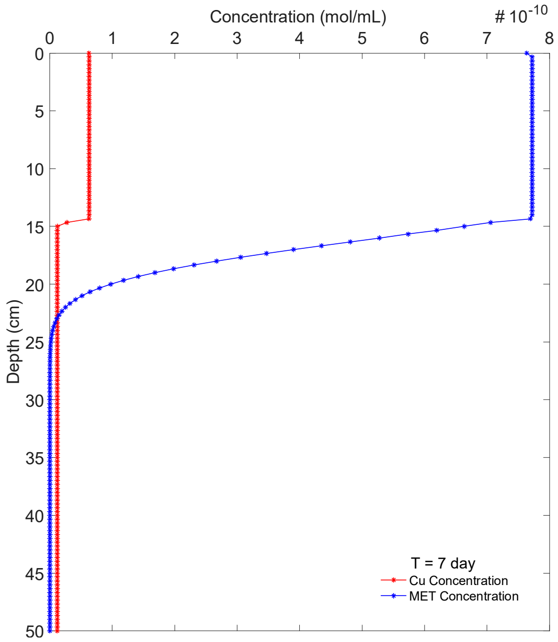
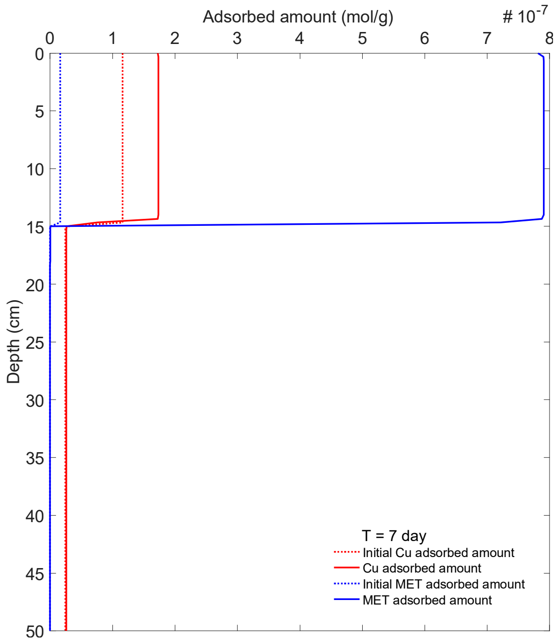
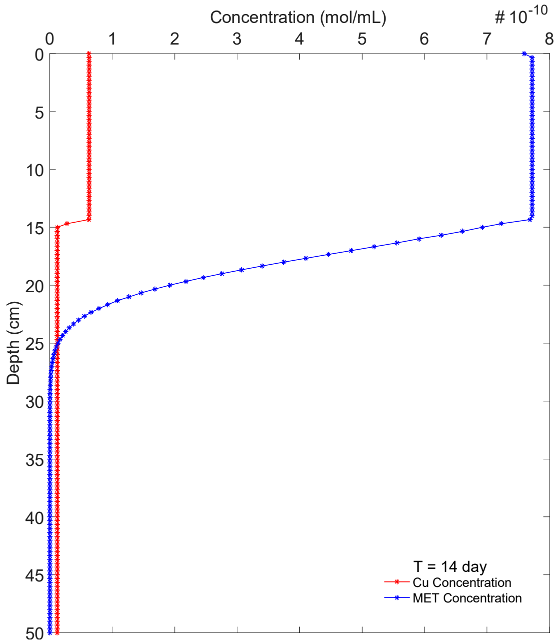


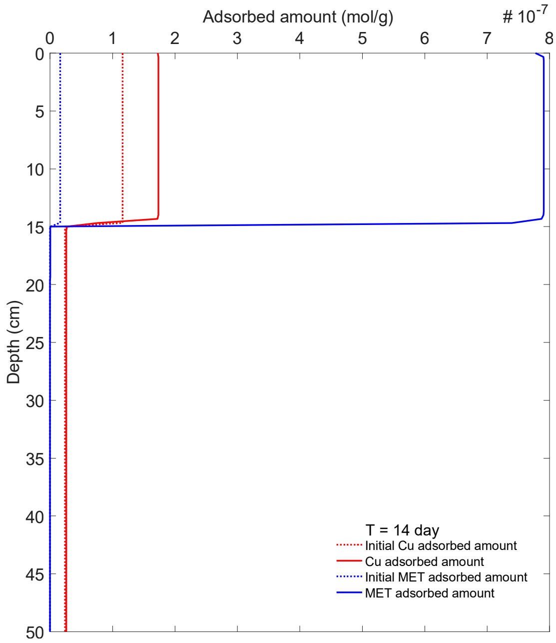
Figure 27: Simulated Cu (red) and MET (blue) concentration in soil solution (line with asterisks) and adsorbed amount on the solid matrix (solid line) throughout time, using the transport model with equilibrium CLM and second experimental conditions (see Chapter 3).


t=0
Concentration (mol/mL)
10-6
0
0.2
0.4
0.6
0.8
1
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 0 day
Cu Concentration
MET Concentration
t=0
Adsorbed amount (mol/g)
10-7
0
0.2
0.4
0.6
0.8
1
1.2
Depth (cm)
T = 0 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount
Concentration (mol/mL)
10-10
0
1
2
3
4
5
6
7
8
Depth (cm)
T = 1 day
Cu Concentration
MET Concentration
Adsorbed amount (mol/g)
10-7
0
1
2
3
4
5
6
7
8
Depth (cm)
T = 1 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount


Concentration (mol/mL)
10^-10
0
1
2
3
4
5
6
7
8
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 7 day
Cu Concentration
MET Concentration


Adsorbed amount (mol/g)
10^-7
0
1
2
3
4
5
6
7
8
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 7 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount


Concentration (mol/mL)
10^-10
0
1
2
3
4
5
6
7
8
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 14 day
Cu Concentration
MET Concentration


Adsorbed amount (mol/g)
10^-7
0
1
2
3
4
5
6
7
8
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 14 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount

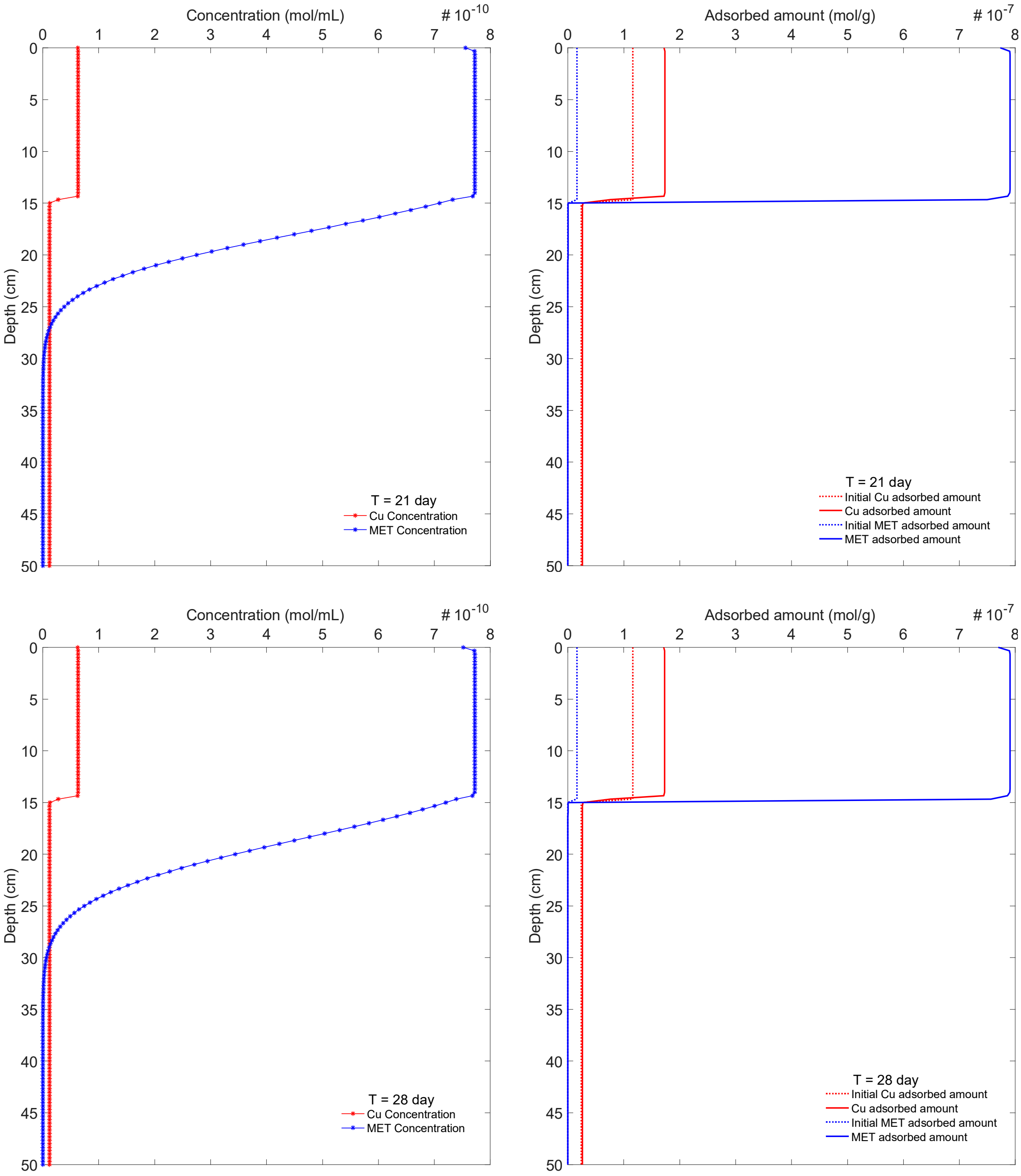
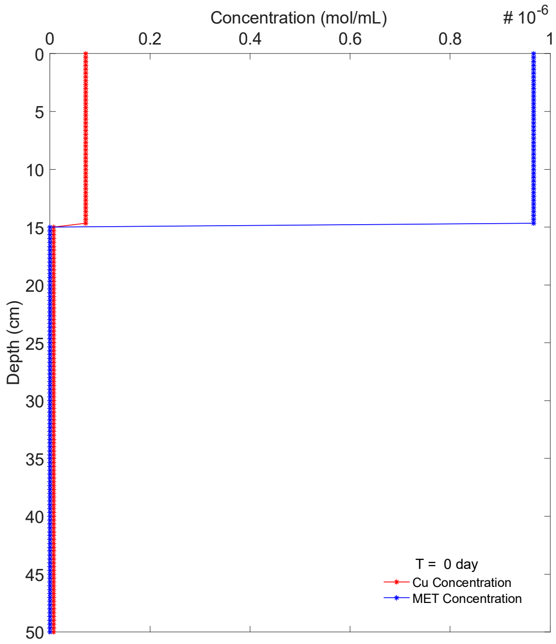
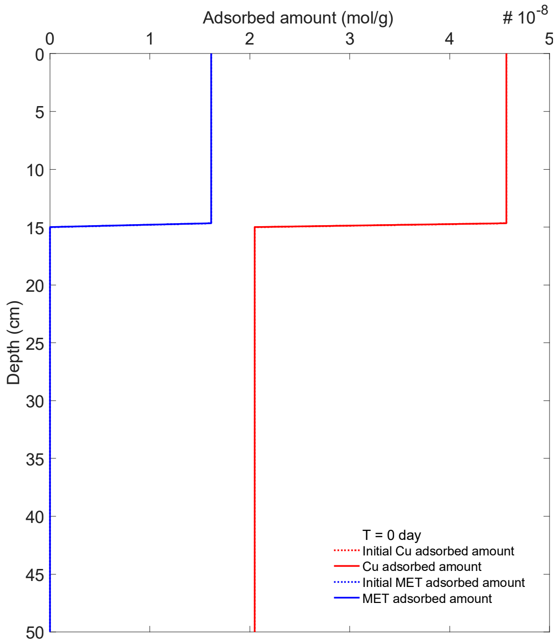
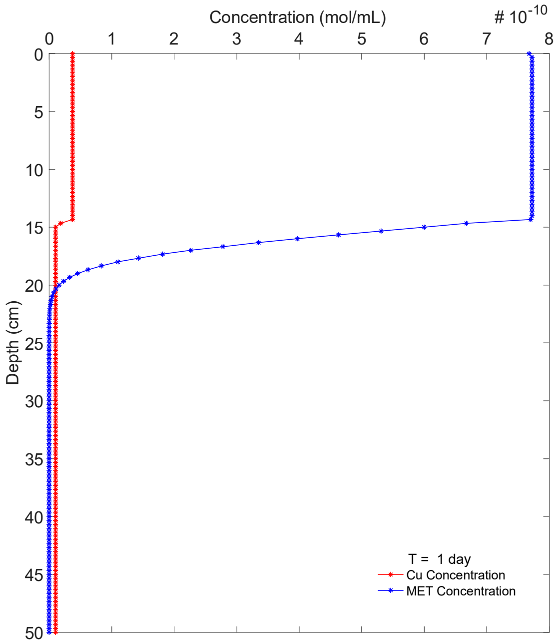
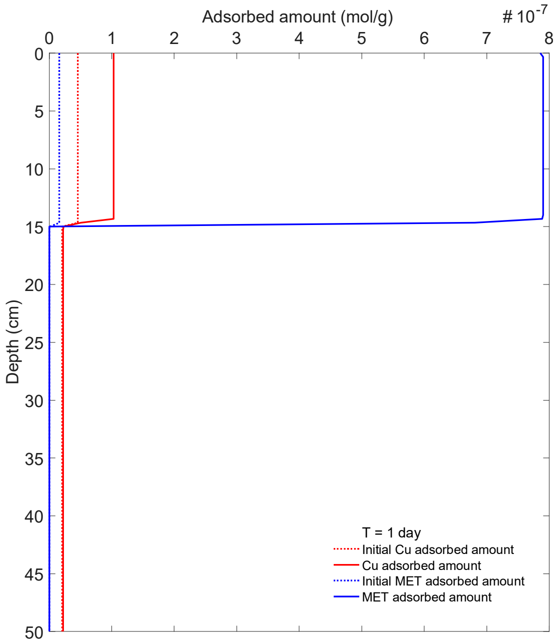


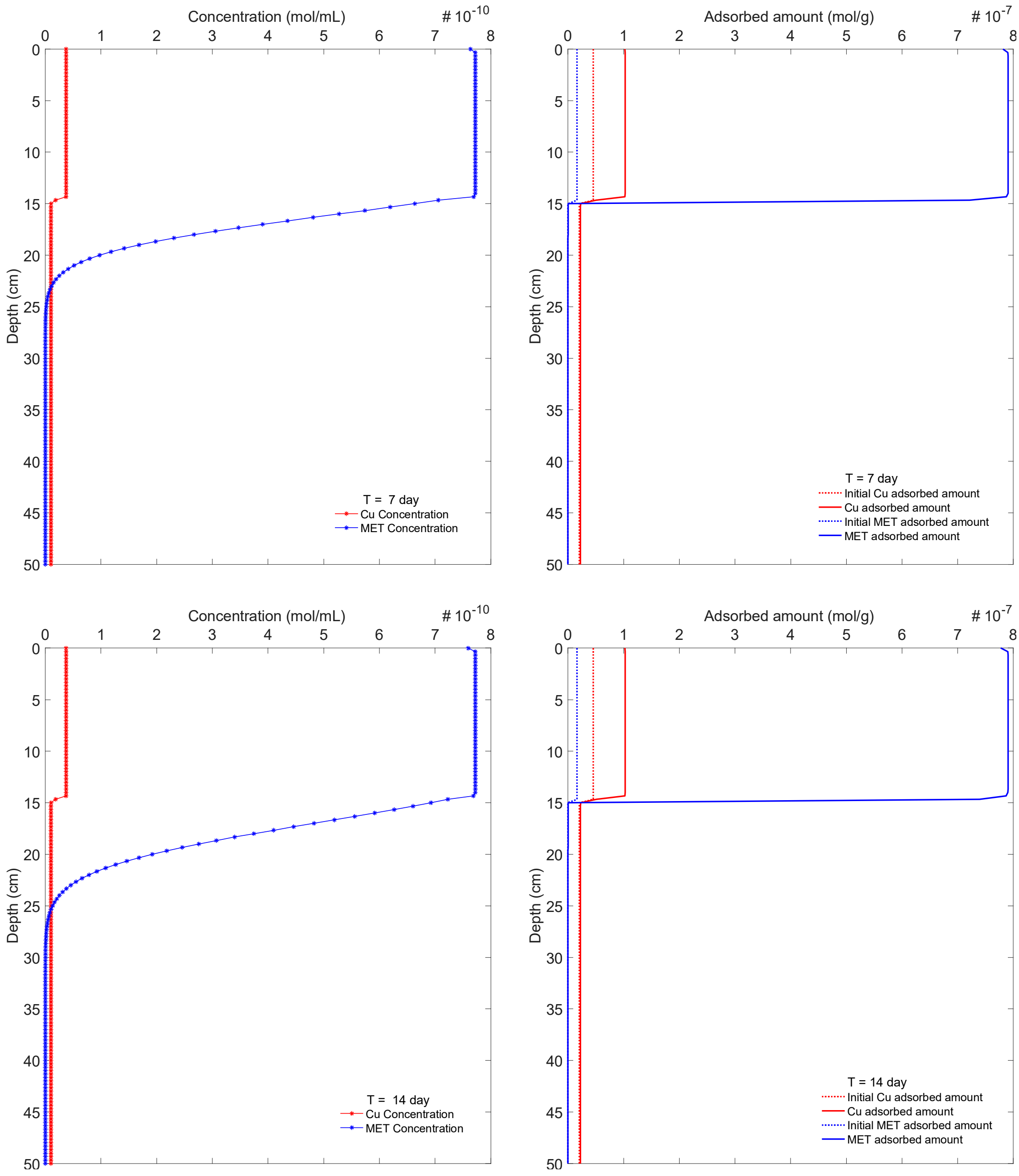
Figure 28: Simulated Cu (red) and MET (blue) concentration in soil solution (line with asterisks) and adsorbed amount on the solid matrix (solid line) throughout time, using the transport model with time-dependent CLM and first experimental conditions (see Chapter 2).


Concentration (mol/mL)
10^{-6}
0
0.2
0.4
0.6
0.8
1
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 0 day
Cu Concentration
MET Concentration


Adsorbed amount (mol/g)
10^{-8}
0
1
2
3
4
5
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 0 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount


Concentration (mol/mL)
10^{-10}
0
1
2
3
4
5
6
7
8
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 1 day
Cu Concentration
MET Concentration


Adsorbed amount (mol/g)
10^{-7}
0
1
2
3
4
5
6
7
8
Depth (cm)
0
5
10
15
20
25
30
35
40
45
50
T = 1 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount


Concentration (mol/mL)
10^-10
0 1 2 3 4 5 6 7 8
Depth (cm)
0 5 10 15 20 25 30 35 40 45 50
T = 7 day
Cu Concentration
MET Concentration
Adsorbed amount (mol/g)
10^-7
0 1 2 3 4 5 6 7 8
Depth (cm)
0 5 10 15 20 25 30 35 40 45 50
T = 7 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount
Concentration (mol/mL)
10^-10
0 1 2 3 4 5 6 7 8
Depth (cm)
0 5 10 15 20 25 30 35 40 45 50
T = 14 day
Cu Concentration
MET Concentration
Adsorbed amount (mol/g)
10^-7
0 1 2 3 4 5 6 7 8
Depth (cm)
0 5 10 15 20 25 30 35 40 45 50
T = 14 day
Initial Cu adsorbed amount
Cu adsorbed amount
Initial MET adsorbed amount
MET adsorbed amount

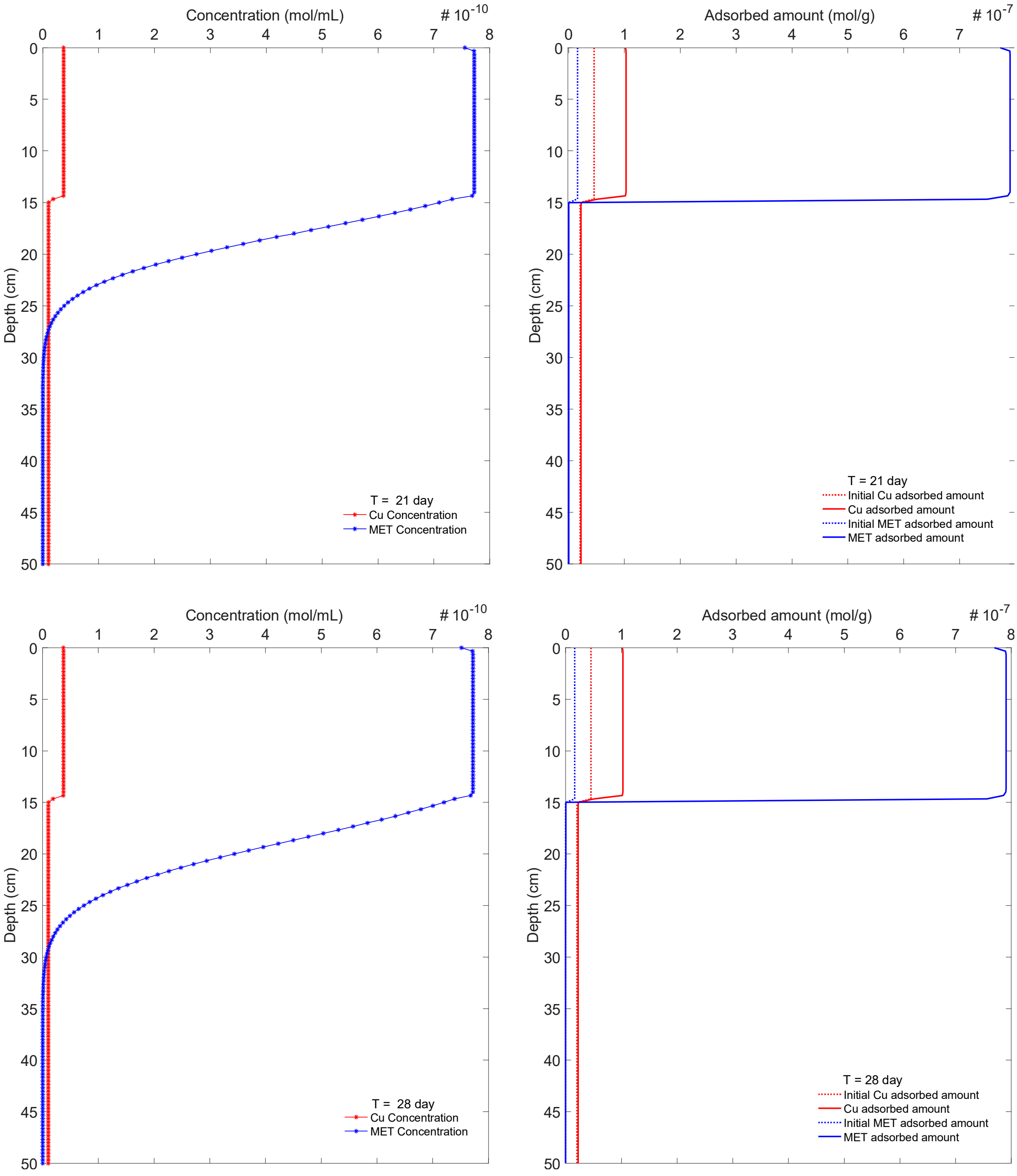


Figure 29: Simulated Cu (red) and MET (blue) concentration in soil solution (line with asterisks) and adsorbed amount on the solid matrix (solid line) throughout time, using the transport model with time-dependent CLM and second experimental conditions (see Chapter 3).

The soil contaminant transport modelling results for the two scenarios defined (first and second soil mesocosm column experiment) were compared against the experimental data obtained from the soil column experiments presented in Chapter 2 and Chapter 3. Figure 30 and Figure 31 show the simulated Cu adsorbed amounts (mol/g), obtained using the model considering equilibrium sorption, for the first and second scenario, respectively. Similarly, Figure 32 and Figure 33 depict simulated Cu adsorbed amounts (mol/g), obtained using the model considering time-dependent sorption, for the first and second scenario, respectively. All simulated values were compared against the experimentally determined Cu adsorbed amounts measured along the soil profile over time. Furthermore, the simulated results were evaluated through the determination coefficient R^2, reported in Table 14.

In the first simulation scenario, relative to the first soil column experiment (Chapter 2), it is evident that the simulated results from the model with equilibrium sorption largely underestimated the values of Cu adsorbed amounts within both the digestate-amended soil layer and the natural soil layer. In comparison, the simulated results from the model with time-dependent sorption better approximated the experimental data in both soil column layers. However, on day 1 there was a slight overestimation for the digestate-amended soil layer and underestimation for the natural soil layer, while on day 7 there was a slight underestimation for both layers. Furthermore, it can be noticed that the third experimental data point, collected on day 7 and day 21, was inaccurately simulated by both models. Additionally, this data point aligns, within its standard deviation, with the data points characterizing the digestate-amended soil layer. This suggests a potential downward shift of the digestate-amended soil layer that occurred during the experiment, impacting the depth of the respective layers. This potential aspect was not considered in the model, which assumed constant layer depths throughout time. Consequently, this data point was excluded when calculating the R^2 coefficient. Overall, the model with time-dependent sorption performed better, with R^2 values ranging from 94 to 99 %, than the model with equilibrium sorption that resulted in R^2 values ranging from 56 to 91 %.

In the second simulation scenario, relative to the second soil column experiment (Chapter 3), the model with equilibrium sorption again largely underestimated the values of Cu adsorbed amounts within the digestate-amended soil layer, while closely-approximated those within the natural soil layer. In comparison, the model with time-dependent sorption accurately estimated the experimental data in both soil column layers, even though on day 14 and day 28 there was a very slight overestimation for a few data points within the soil layer. Overall, the model with time-dependent sorption, with R^2 values ranging from 97 to 99 %, outperformed the model with equilibrium sorption that resulted in R^2 values ranging from 19 to 30 %.

These results indicate that the contaminant transport model including time-dependent sorption mechanisms (kinetics) globally performed better than the model including equilibrium sorption. This confirmed the need to apply time-dependent sorption mechanisms to better predict contaminant behaviour and transport in the soil system [59], especially when considering multi-component systems. This also agrees with what was found in the soil column experiments (Chapter 2 and Chapter 3) which showed a slight downward movement of TM from the digestate amended soil to the soil layer beneath, indicating that the system was not in an equilibrium state. Overall, modelling can be considered an important means for predicting and assessing contaminants' fate in the environment, providing insights into the temporal evolution of the defined system and minimizing expensive and time-consuming experimental/field testing.

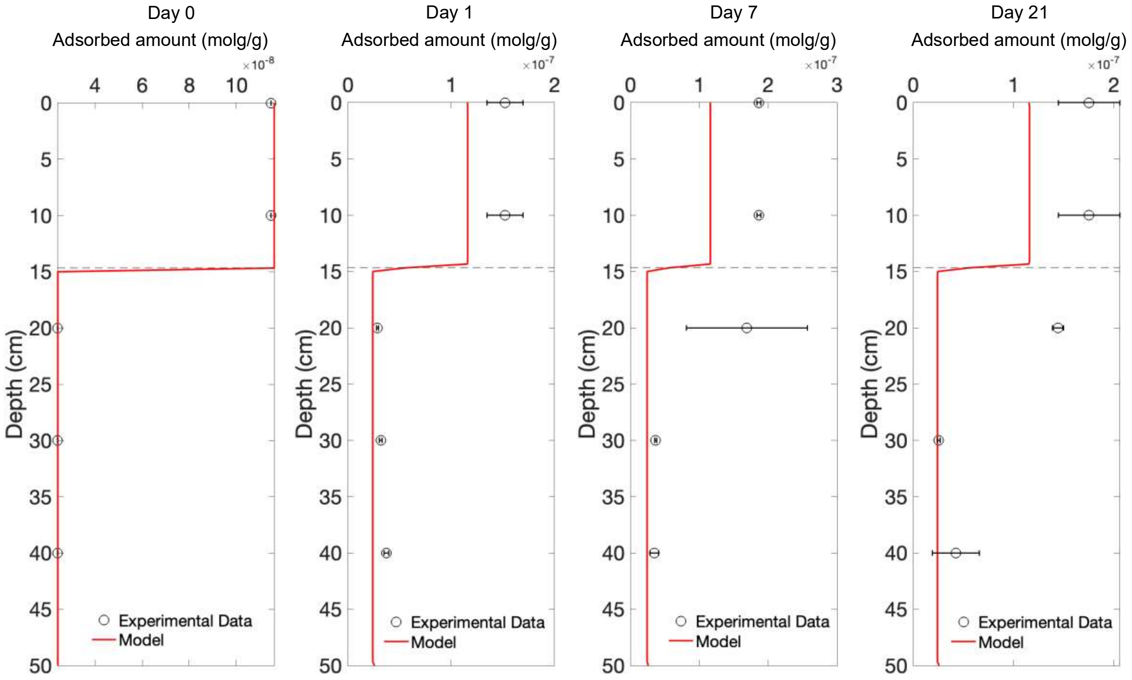


Figure 30: Simulated Cu adsorbed amount for the first experimental conditions (see Chapter 2), using the equilibrium CLM, and experimental data of the first soil mesocosm column experiment (see Chapter 2), along soil depth and throughout time (day 0, 1, 7 and 21).

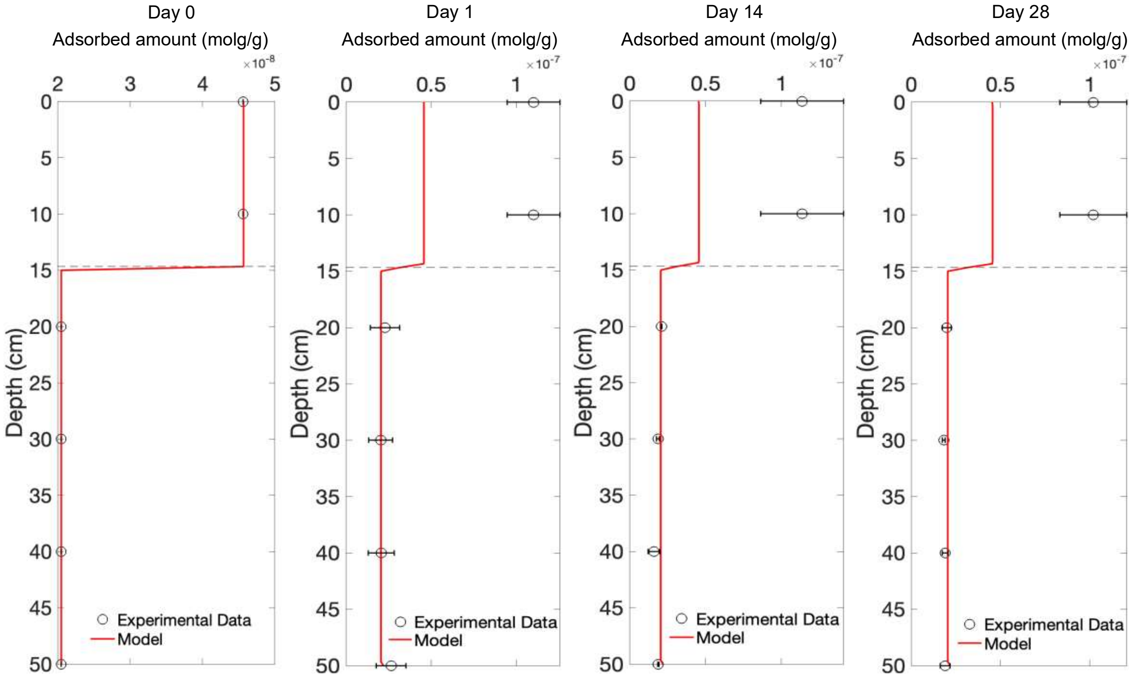


Figure 31: Simulated Cu adsorbed amount for the second experimental condition (see Chapter 3), using the equilibrium CLM, and experimental data of the second soil mesocosm column experiment (see Chapter 3), along soil depth and throughout time (day 0, 1, 14 and 28).

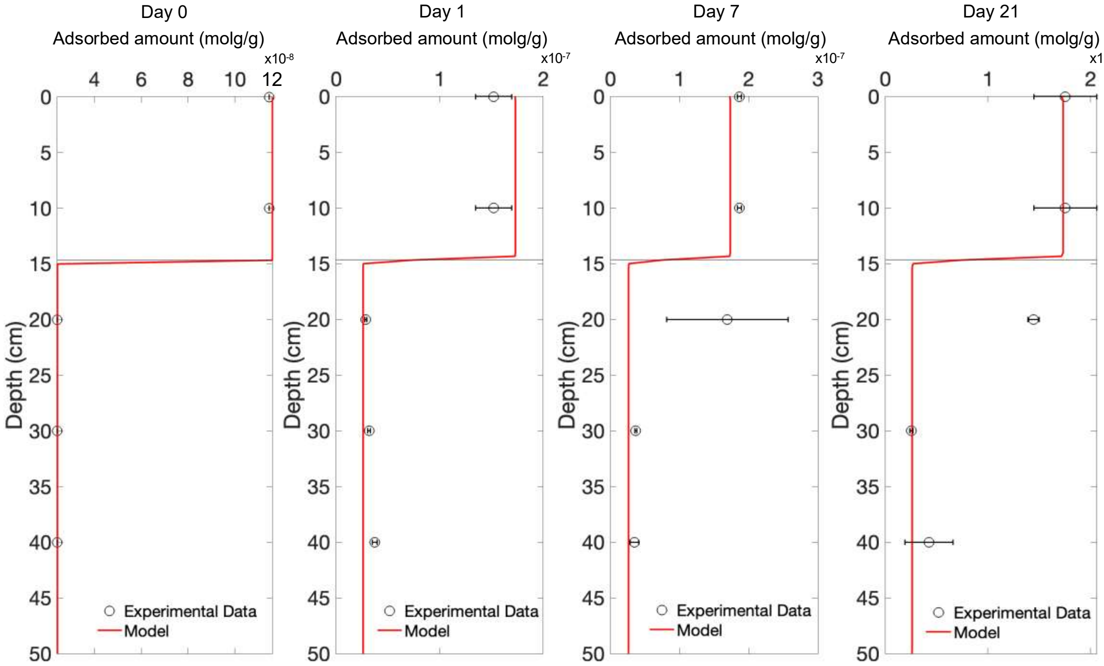


Figure 32: Simulated Cu adsorbed amount for the first experimental condition (see Chapter 2), using the time-dependent CLM, and experimental data of the first soil

mesocosm column experiment (see Chapter 2), along soil depth and throughout time (day 0, 1, 7 and 21).

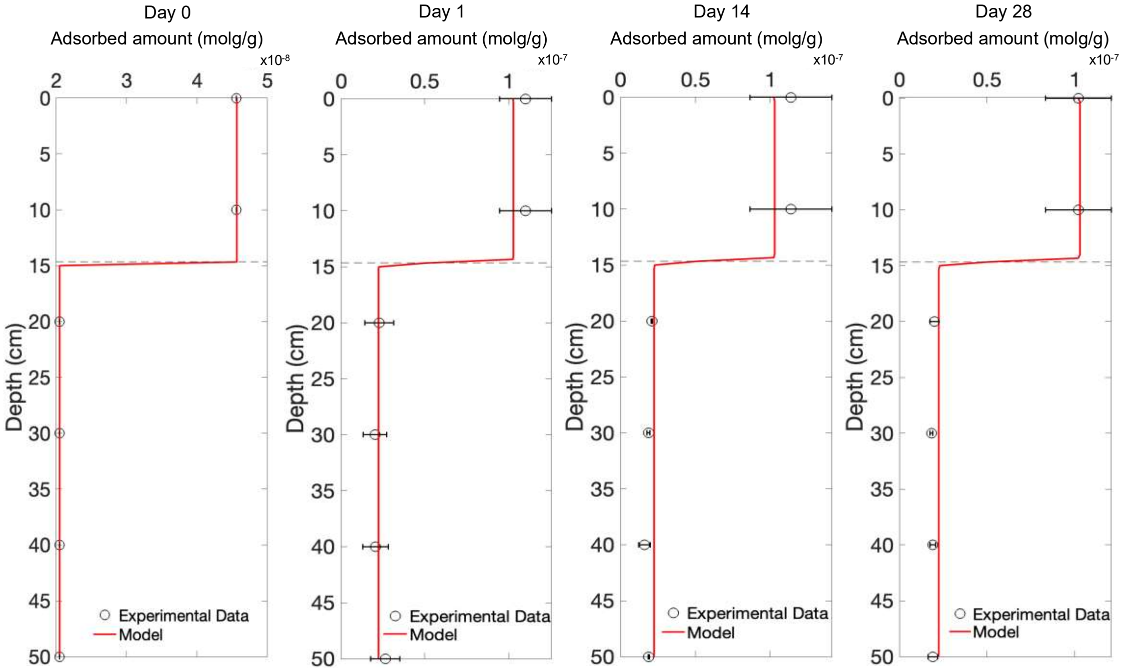


Figure 33: Simulated Cu adsorbed amount for the second experimental condition (see Chapter 3), using the time-dependent CLM, and experimental data of the second soil mesocosm column experiment (see Chapter 3), along soil depth and throughout time (day 0, 1, 14 and 28).

Table 14: Determination coefficient (R^2) for the equilibrium CLM and the time-dependent CLM evaluated at different time instances for each experimental condition simulated.

Model	Simulation	Time instant (day)	R^2 (%)	**R^2 (%)
Equilibrium CLM	1st experimental condition	1	90.6	-
		7	-22.4	56.3
		21	-2.26	63.4
	2nd experimental condition	1	18.8	-
		14	22.7	-
		28	30.3	-
		1	94.1	-

Time-dependent CLM	1st experimental condition	7	17.6	97.7
		21	33.5	98.6
	2nd experimental condition	1	99.0	-
		14	97.4	-
		28	98.5	-

5.4 Conclusions

This study employed the Competitive Langmuir Model (CLM) to assess the competitive sorption behaviour of TM and MET in both digestate-amended soil and natural soil. The transport of Cu, (selected as one of the TM), and MET in soil after digestate application was also simulated using a transport model incorporating the CLM either considering equilibrium sorption or time-dependent sorption. To the authors' knowledge, this is the first study to model MET and TM competitive sorption behaviour and their transport in a digestate-amended soil system. In addition, this is the first study to apply the CLM to describe the competitive sorption behaviour between an antidiabetic drug and TM, given that it has been applied primarily to antibiotics and metal mixtures.

The CLM showed high goodness-of-fit for most TM-MET combinations, with exceptions noted for Co-MET in amended soil and Cr-MET in soil. The latter cases suggested that Langmuir competitive sorption might not be the ideal model for describing the competition effect observed in laboratory experiments for these contaminants. Analysing the CLM results, it was observed that in the presence of MET the estimated Langmuir constants for Pb and Zn in amended soil were significantly greater than those in natural soil, indicating stronger sorption affinity. Conversely, Ni and Cu showed greater sorption affinity to natural soil. Additionally, the Langmuir constants for MET were consistently lower than those for TM, suggesting greater sorption affinity for TM. In addition, this study found that the presence of MET increased the sorption affinity of specific TM, notably Pb and Cu.

The kinetic behaviour of contaminants in the studied soil system, specifically MET and Cu, indicated that MET had lower adsorption rates and higher desorption rates, making it more easily released into soil solution. Cu, with higher adsorption rates and lower desorption rates, demonstrated a faster adsorption process, giving it a competitive sorption advantage over MET.

Comparing contaminant transport simulation results against the experimental data, the model considering time-dependent sorption mechanisms performed better than the model with equilibrium sorption. This affirmed the importance of applying time-dependent sorption mechanisms, especially for multi-component systems, to accurately predict contaminant behaviour and transport in soil systems.

Overall, the findings of this study suggest that the CLM is a valuable tool for describing the competitive behaviour between TM and MET, and further studies should be conducted to verify and deepen these novel insights. In addition, future studies should explore and develop more complex kinetic models, as multi-site and multi-reaction models, to provide a more detailed description of the sorption mechanisms and compare them with the CLM to assess their efficacy.

Associated content

The supporting information for this chapter is reported in Appendix D. This content includes further details on the FDS used to solve the systems of ADEs.

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Chapter 6

Conclusions and future research

In this research work, the distribution and fate of trace metals (TM) in marginal land soil following the application of solid biogas digestate derived from non-source separated organic fraction of municipal solid waste (OFMSW) was thoroughly investigated. The research addressed various aspects including the influence of microorganic contaminants, such as pharmaceutical compounds, on their behaviour in digestate-amended soil, TM and pharmaceutical sorption behaviours, and the effects of OFMSW digestate, containing TM and pharmaceuticals, on soil microbial community.

Through lab-scale soil mesocosm column experiments, the research work revealed a slight variable distribution of TM (Cu, Zn, Pb, Cr) in the soil profile over time, with a slight increase in total TM concentration in the layer of soil directly beneath the digestate-amended soil layer. This was most likely due to the higher quantities of TM found in the exchangeable and reducible metal fractions, leading to greater TM mobility and bioavailability. However, TM in deeper soil layers exhibited limited mobility and redistribution, primarily due to the predominant presence of TM in the residual metal fraction. This suggested that TM were strongly bound to the soil minerals, in particular silicates, supporting their stability and limited mobility potential. Additionally, the study revealed that the presence of pharmaceutical compounds, namely metformin (MET) and lamotrigine (LMT) did not affect TM distribution and mobility within the soil profile over the experimental time. Furthermore, the investigation into the impact of OFMSW digestate amendment on the soil prokaryotic community overall showed an increase in the prokaryotic community's diversity and a rapid stabilization of the community within 14 days, showing an initial positive microbial response to the digestate amendment.

A deeper investigation into the sorption behaviours of TM and pharmaceutical compounds (MET and LMT) in OFMSW digestate-amended soil provided insights into their interaction mechanisms and on the effects that OFMSW digestate had on their sorption behaviour. Soil and digestate-amended soil sorption batch tests (kinetic, adsorption and desorption tests) were conducted for each combination of pharmaceutical compound (MET and LMT) and TM (Zn, Cu, Pb, Ni, Cr, and Co), for the pharmaceutical/metal concentration ratios of 1/1, 1/10 and 1/100. The study found that the applied digestate-amendment enhanced the soil's adsorption capacity for both MET and LMT thanks to an increase in the soil's OM content, while the adsorption of TM remained quite constant. The presence of MET and LMT did not significantly affect the sorption of TM on soil and amended soil, as also observed in the soil column experiments, except for when TM where present in the same concentration as the pharmaceutical compounds. In fact, in this later condition, TM and pharmaceutical compounds were found in competition for the available sorption sites. Contrarily, pharmaceutical compound adsorption, especially for LMT, was found to be significantly decreased by the presence of TM. However, the presence of Cu led to an increase of MET and LMT adsorption possibly due to the formation of ternary surface complexes between the TM, pharmaceuticals and soil surface. The study also highlighted the importance of considering contaminant desorption potential from digestate-amended soil, especially for MET and LMT, which gave indication on low potential contaminant mobility and leaching to groundwater under natural rainfall conditions. Overall, MET and LMT seemed to have had different behaviours in soil and different influence on TM fate. This stresses the difficulty and the intrinsic challenge in generalizing sorption behaviours and environmental fate to other microorganic contaminants.

To further evaluate the experimentally observed behaviour of TM and pharmaceutical compounds (specifically MET) when present simultaneously, the competitive Langmuir model (CLM) was applied to assess their competitive sorption behaviour. The CLM demonstrated good fit for most TM-MET bi-solute systems, revealing variations in contaminant sorption affinity between natural soil and digestate-amended soil. In addition, considering the bi-solute system MET-Cu, selected in this study for contaminant transport modelling, it was found that MET exhibited lower adsorption rates and higher desorption rates compared to Cu, suggesting that Cu had faster adsorption processes and a competitive advantage over MET. Moreover, MET-Cu transport modelling results highlighted the need to include time-dependent sorption mechanisms to accurately predict the behaviour and fate of contaminants in the soil system. The study also suggested the CLM as a valuable preliminary tool for better understanding the competitive behaviour of Cu and MET and their transport in soil, encouraging future investigations into more complex kinetic models, such as multi-site and multi-reaction models, that can simulate TM fate and distribution in soil after digestate amendment.

The findings of this research work indicate the potential of non-source-separated OFMSW digestate as a viable soil amendment for marginal lands. Digestate increases soil organic matter content and improves soil structure, leading to a positive impact on the soil ecosystem and on the abundance of soil microbial communities. Moreover, its contaminant adsorption capacity, coupled with its relatively low desorption potential, indicates that digestate may act as a contaminant sink. However, one cannot exclude contaminant leaching in the long run. For this reason, OFMSW digestate application on marginal land should always be combined with proper monitoring strategies and nature-based remediation solutions, as bioremediation and/or phytoremediation, to safeguard the environment while improving its state.

Thus, the OFMSW digestate may be considered as a promising resource for soil restoration, supporting sustainable soil management. However, this research work emphasizes the importance of continued research on the topic, specifically to evaluate:

i. The effects of different types of digestate and biosolids (originating from different feed materials) on the distribution and fate of TM and pharmaceutical compounds in the soil environment.
ii. The aging effects of OFMSW digestate on the fate and mobility of TM. This may be addressed with long term digestate application experiments studying how the TM behaviour and speciation evolves over time in soils that have been amended with digestate, looking at factors such as organic matter breakdown and physico-chemical transformations occurring in the soil system.
iii. Potential ecotoxicological impacts of inherently contaminated OFMSW digestate on the soil ecosystem, focusing on microbial communities that are key in currently applied bioremediation processes. This may be addressed by ecotoxicological assessments conducted to study the impacts of TM and other present contaminants on these microbial communities. The results of these assessments are crucial for providing guidance to authorities in formulating well-informed policies on the matter.
iv. The differing behaviour of MET/LMT in absence and presence of TM in the soil and digestate-amended soil, specifically addressing the synergistic interaction found between the pharmaceutical compounds and Cu. This may be addressed by molecular experiments, looking at complexation mechanism at a molecular level, and by complexation experiments, looking at the metal-organic complex formation mechanisms, metal-organic complex kinetics, and sorption.
v. A more complex contaminant transport model, including digestate degradation/weathering mechanisms and more complex sorption mechanisms, to enhance our understanding of the role of sorption on contaminant distribution and fate and refine the model's predictive capabilities for these complex soil systems.

In conclusion, this research work is a strong base for future research into the sorption mechanism and potential interactions between TM and pharmaceutical compounds present in digestate-amended soils, and possible effects on microbial communities that can impact the use of bioremediation strategies. The knowledge gathered lays a solid foundation for the development of sustainable soil management practices through the safe use of OFMSW digestate. These potential strategies will help society to achieve the set sustainable development goals, making society more resilient and improving the quality of the environment for future generations.

Appendices

Appendix A

A.1 Soil and OFMSW digestate characterisation

CHNS elemental analysis on soil determined that total carbon content was 0.40% with 0.20% being total organic carbon and 0.20% inorganic carbon, with N and S content below quantification limit. Soil and digestate were characterized for elemental composition via XRF, XRD and FTIR-ATR. These analyses were conducted by the E2Lim laboratory of the University of Limoges (France).

The XRF, XRD and FTIR-ATR analysis showed that the predominant phases in the soil were quartz (SiO_2) and kaolinite, with minor phases of muscovite and calcite (Table A1).

The OFMSW solid digestate characterization done by the supplier, the MSW waste treatment company, gave us additional information: the digestate mass density was equal to 1.14 g/cm^3 and the digestate inert material content was equal to 22 dw% and 41 dw% considering the fraction > 2 mm and > 0.5 mm, respectively. The OFMSW digestate derives from an AD plant treating the non-source-separated organic fraction of municipal solid waste. The AD is a one stage process using the VALORGA digester technology operated in semi-continuous mode. The AD is conducted in mesophilic conditions (around 40 °C). The organic loading rate is around 4 kg/m^3/day, and the hydraulic retention time is between 40 – 45 days. The digester is fed varying hours during 6 days per week according to the incoming quantity of organic waste to be treated maximum and the maximum organic loading rate. The feed material is organic residue deriving from the MSW treatment line placed upstream of the digesters. The feed material is a heterogenous mixture with a dry matter content ranging between 45 – 55 %, a dry volatile matter content around 45 – 60 % and inert material around 30 % (fraction > 0.5 mm). Before being fed into the digester, the triaged organic matter is diluted with treated plant wastewater in order to have a dry matter content between 30 – 35 %. The diluted organic matter is homogenized through mixing and heated to 40 °C.

Table A1:Elemental composition of soil and digestate.

		Al_2O_3	CaO	Fe_2O_3	K_2O	MgO	Na_2O	P_2O_5	SiO_2	SO_3
Soil	(wt%)	23.5	1.3	5.7	2.7	0.3	0.1	0.1	58.1	0.3
Digestate	(wt%)	7.6	9.9	1.5	1.2	3.8	< LQ	1.1	34.9	2.0

Table A2: Calibration quality parameters (Linear fit R^2, and limit of detection (LOD) and limit of quantification (LOQ) obtained through linear calibration) for atomic absorption spectrometry with flame atomisation (F-AAS) or electrothermal atomization (ET-AAS) determinations of different metals in solid samples. Two sample masses (0.25g or 0.50 g) are considered.

	Flame AAS				
		Sample of 0.50 g		Sample of 0.25 g	
Metal	R2	LOD (ug/g)	LOQ (ug/g)	LOD (ug/g)	LOQ (ug/g)
Fe	1.000	3.00	10.0	6.00	20.0
Mn	0.998	1.50	5.00	3.00	10.0
Ni	0.999	3.00	10.0	6.00	20.0
Zn	0.998	0.75	2.50	1.50	5.00
Pb	0.997	7.50	25.0	15.0	50.0
Cu	0.999	4.00	13.3	8.00	26.7
Cr	-	-	-	-	-
Co	-	-	-	-	-

	ET AAS				
		Sample of 0.50 g		Sample of 0.25 g	
Metal	R2	LOD (ug/g)	LOQ (ug/g)	LOD (ug/g)	LOQ (ug/g)
Fe	-	-	-	-	-
Mn	-	-	-	-	-
Ni	-	-	-	-	-
Zn	-	-	-	-	-
Pb	0.999	0.15	0.50	0.30	1.00
Cu	0.999	0.19	0.64	0.38	1.28
Cr	0.999	0.19	0.64	0.38	1.28
Co	0.994	0.28	0.93	0.56	1.87

Microplastics in soil, digestate and amended soil were determined adapting an optimized microplastic extraction protocol for sediments, based on an oxidation treatment and a density separation (1). The samples were assessed in triplicates. Initially, 50 g of each dried sample was weighted and placed in a glass beaker. A first density separation was performed by adding a saturated solution of NaCl to the beaker and gently stirring the sample. The floating layer was collected and passed through a filtered cloth (with mesh 0.03 mm), which was then placed in an oven to dry overnight at 90 °C. All solids on the filter cloth were transferred to a glass beaker for the oxidation treatment with Fe(II) solution and 30% hydrogen peroxide solution. After the oxidation treatment, NaCl was added to the oxidized solution to obtain a saturated NaCl solution for a second density separation. The top layer, in which the microplastics potentially were present, was collected and passed through the filter cloth, which was placed to dry in an oven for 24 h at 90 °C. The dried filter cloth was analysed under a stereomicroscope at 10-20X magnification to identify and count the extracted microplastics. Negative controls were also prepared and analysed to account for any possible microplastic contamination during all experiment steps. The results are reported in Table A3.

Table A3: Microplastics quantification and characterization by type, colour, the dimension range and the total count for soil, digestate and digestate amended soil samples.

Sample	Type	Colours	Dimensions (mm)	Count (N/kg)
Soil 1	Fibre	Black, blue	0.5 - 3.0	140
Soil 2	Fibre	Black, transparent	1.0 - 2.0	120
	Foam	Yellow	1.0 - 2.0	80
Soil 3	Fibre	Black, blue, transparent	0.5 - 2.0	180
Amended soil 1	Fibre	Black, transparent	2.0 - 3.0	40
	Foam	Yellow	0.5 - 3.0	100
	Fragment	Transparent, white, green	0.1 - 1.5	120
	Particle	Transparent	1.0	20
	Film	Transparent, white	0.1 - 0.5	40
Amended soil 2	Fibre	Transparent, black	1.0 - 4.0	160
	Fragment	Red, orange, yellow, green, Blue, white	0.1 - 0.2	200
	Film	White	0.2	20

Amended soil 3	Fibre	Black, blue, transparent	0.5 - 5.0	320
	Fragment	Transparent, orange, blue	0.1 - 1.5	80
	Film	White	0.1 - 1.0	80
Digestate 1	Fibre	Transparent, black, white, red	1.0 - 4.0	380
	Foam	White	0.4	20
	Fragment	Red, yellow, blue, purple	0.1 - 0.5	680
	Particle	Red, yellow, green, blue, pink	0.1 - 0.5	760
	Film	Transparent, green, blue	0.1 - 0.5	60
Digestate 2	Fibre	Transparent, green, blue, black	0.5 - 3.0	220
	Fragment	Yellow, blue, white	0.2 - 1.0	160
	Particle	Pink	0.5	40
	Film	Red, yellow, green, blue	0.1	1400
Digestate 3	Fibre	Red, orange, green, blue, pink, white, transparent	0.4 - 5.0	500
	Foam	Blue	0.5	20
	Fragment	Red, yellow, green, blue, Purple, pink, white, transparent, grey	0.1 - 4.0	940
	Particle	Blue	0.5	20
	Film	Red, yellow, green, blue, pink, white transparent	0.1 - 2.0	1000

A.2 Soil column experiment: Data analyses results

Table A4: Two-way ANOVA and Tukey's post-hoc test (THSH) results for total trace metal concentration variation amongst the experimental conditions (E1-Dig and E1-Dig-Met) and through time. Only statistically significant results are reported with p-value and p-value adjusted < 0.05.

Two-way ANOVA					THSD test				
					Group factor		Comparison terms		p adj.
Metal	Depth	Significant Effect	F - value	p - value	Exp. condition	Time	A	B	
Zn	port 2	Interaction	5.45	0.01		all days	E1-Dig	E1-Dig-Met	-
		Time	29.19	0.00002	E1-Dig		day 1	day 21	0.004
					E1-Dig		day 1	day 90	0.04
					E1-Dig		day 7	day 21	0.004
					E1-Dig		day 7	day 90	0.04
					E1-Dig-Met		day 1	day 35	0.02
					E1-Dig-Met		day 7	day 35	0.04
	port 3	Time	6.79	0.007	-		-	-	-
Cu	port 2	Time	32.84	0.00002	E1-Dig-Met		day 1	day 21	0.009
					E1-Dig-Met		day 1	day 35	0.003
					E1-Dig-Met		day 1	day 90	0.006
					E1-Dig-Met		day 7	day 21	0.046
					E1-Dig-Met		day 7	day 35	0.01
					E1-Dig-Met		day 7	day 90	0.03
Pb	port 2	Exp condition	11.89	0.006		all days	E1-Dig	E1-Dig-Met	-
		Time	31.29	0.00001	E1-Dig		day 1	day 21	0.002
					E1-Dig		day 1	day 35	0.007
					E1-Dig		day 1	day 90	0.008
					E1-Dig		day 7	day 21	0.002
					E1-Dig		day 7	day 35	0.006
					E1-Dig		day 7	day 90	0.008
					E1-Dig-Met		day 1	day 21	0.03
	port 4	Time	13.60	0.0005	E1-Dig		day 1	day 21	0.03
					E1-Dig		day 1	day 35	0.02
					E1-Dig		day 1	day 90	0.01
Cr	port 2	Time	13.39	0.0005	E1-Dig		day 1	day 21	0.002

	E1-Dig	day 1	day 35	0.005
	E1-Dig	day 1	day 90	0.006
	E1-Dig	day 7	day 21	0.01

Table A5: Two-way ANOVA and Tukey's post-hoc test (THSD) results for trace metal fractionation (E = exchangeable, R = Reducible, O&R = Oxidizable-Residual) variation amongst the experimental conditions (E1-Dig and E1-Dig-Met) and through time. Only statistically significant results are reported with p-value and p-value adjusted < 0.05.

Two-way ANOVA						THSD test				
						Group factor		Comparison terms		
Metal	Depth	Fraction	Effect	F - value	p - value	Exp. condition	Time	A	B	p adj.
Zn	port 2	E	Exp condition	8.11	0.016	-	-	-	-	-
			Time	20.62	0.0008	-	-	-	-	-
		O&R	Time	6.98	0.023	E1-Dig-Met	-	day 1	day 21	0.03
						E1-Dig-Met	-	day 1	day 90	0.03
						E1-Dig-Met	-	day 7	day 21	0.047
						E1-Dig-Met	-	day 7	day 90	0.047
	port 3	R	Time	15.29	0.002	-	-	-	-	-
	port 4	E	Exp condition	6.48	0.03	-	-	-	-	-
			Interaction	10.14	0.009	-	-	-	-	-
		R	Time	7.42	0.02	-	-	-	-	-
		O&R	Exp condition	7.87	0.03	-	-	-	-	-
			Time	5.62	0.03	E1 - Dig	-	day 1	day 90	0.005
						E1 - Dig	-	day 7	day 90	0.02
						E1 - Dig	-	day 21	day 90	0.008
			Interaction	7.19	0.02					
Cu	port 1	E	Time	15.158	0.001	E1-Dig-Met	-	day 1	day 21	0.008
						E1-Dig-Met	-	day 7	day 21	0.02
		R	Time	6.106	0.02	E1-Dig-Met	-	day 7	day 90	0.03
						E1-Dig-Met	-	day 21	day 90	0.046

		O&R	Time	5.162	0.03	E1-Dig-Met	-	day 21	day 90	0.04
	port 2	R	Time	15.258	0.001	E1-Dig-Met	-	day 1	day 21	0.03
		O&R	Time	15.769	0.001	E1-Dig-Met	-	day 1	day 21	0.04
Pb	port 1	R	Exp condition	5.351	0.05	-	day 90	E1 - Dig	E1 - Met	0.04
			Time	6.754	0.01	-	-	-	-	-
		O&R	Time	6.621	0.02	-	-	-	-	-
	port 2	E	Time	8.592	0.007	E1 - Dig	-	day 1	day 21	0.008
						E1 - Dig	-	day 1	day 90	0.006
						E1 - Dig	-	day 7	day 21	0.01
						E1 - Dig	-	day 7	day 90	0.009
	port 3	E	Time	5.971	0.02	-	-	-	-	-
		R	Time	7.345	0.01	-	-	-	-	-
		O&R	Time	7.81	0.009	-	-	-	-	-
	port 4	E	Time	4.635	0.04	-	-	-	-	-
		R	Time	16.14	0.001	E1-Dig-Met	-	day 1	day 21	0.02
						E1-Dig-Met	-	day 7	day 21	0.03
						E1-Dig-Met	-	day 21	day 90	0.02
		O&R	Time	16.273	0.001	E1-Dig-Met	-	day 1	day 21	0.03
						E1-Dig-Met	-	day 7	day 21	0.04
						E1-Dig-Met	-	day 21	day 90	0.04
Cr	port 2	E	Time	6.857	0.01	E1 - Dig	-	day 1	day 21	0.04
		R	Time	6.491	0.02	E1 - Dig	-	day 1	day 90	0.01
						E1 - Dig	-	day 7	day 90	0.005
		O&R	Time	4.671	0.04	E1 - Dig	-	day 1	day 90	0.01
						E1 - Dig	-	day 7	day 90	0.004
						E1 - Dig	-	day 21	day 90	0.03

A.3 Supplementary figures

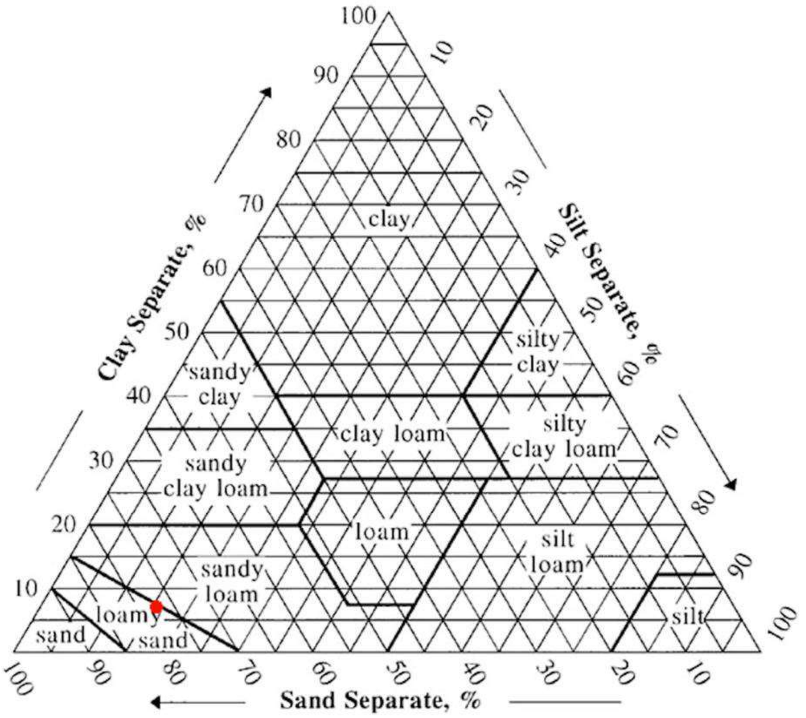

Figure A1: Soil texture diagram identifying the soil texture class. The diagram was constructed using the soil texture calculator presented by USDA Natural Resources Conservation Service (USDA Natural Resources Conservation Service, Soil Survey Staff (2019). Soil Texture Calculator. Natural Resources Conservation Service, United States Department of Agriculture. https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/survey/?cid=nrcs142p2_054167)

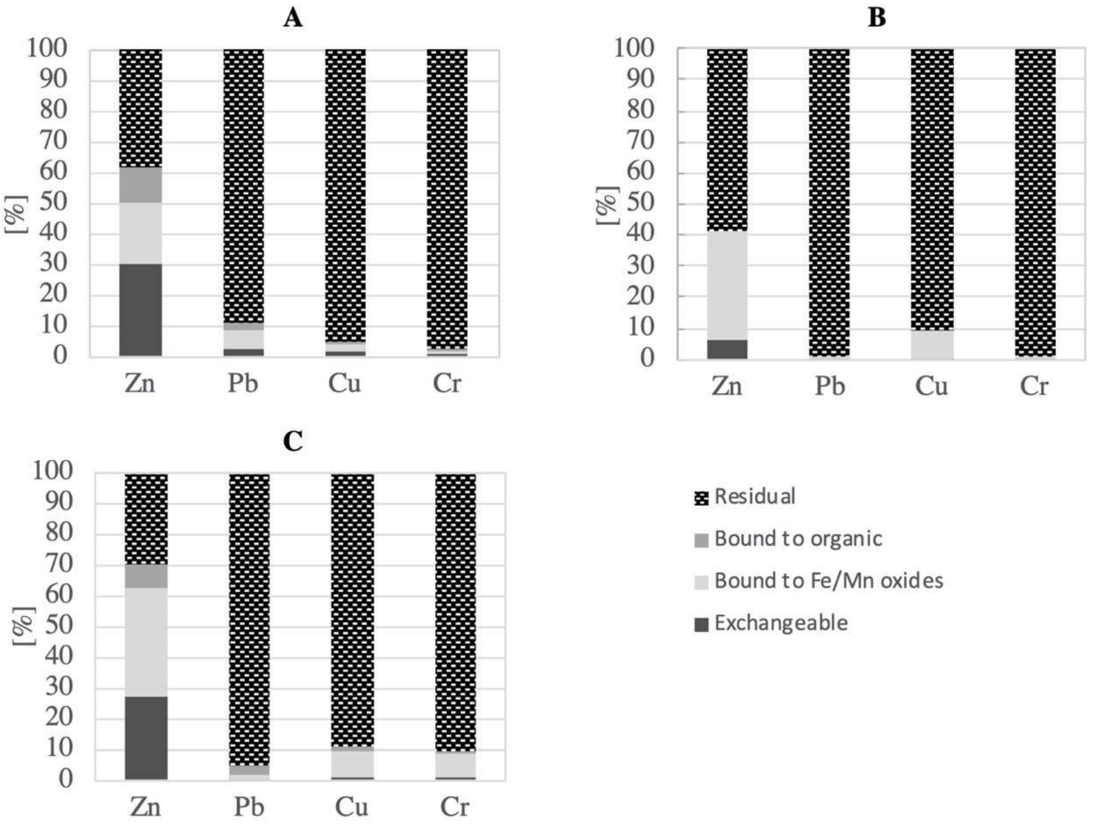

Figure A2: Trace metals fractionation in the initial soil (A), digestate (B) and digestate amended soil (C) (mean ± standard deviation, n=2).

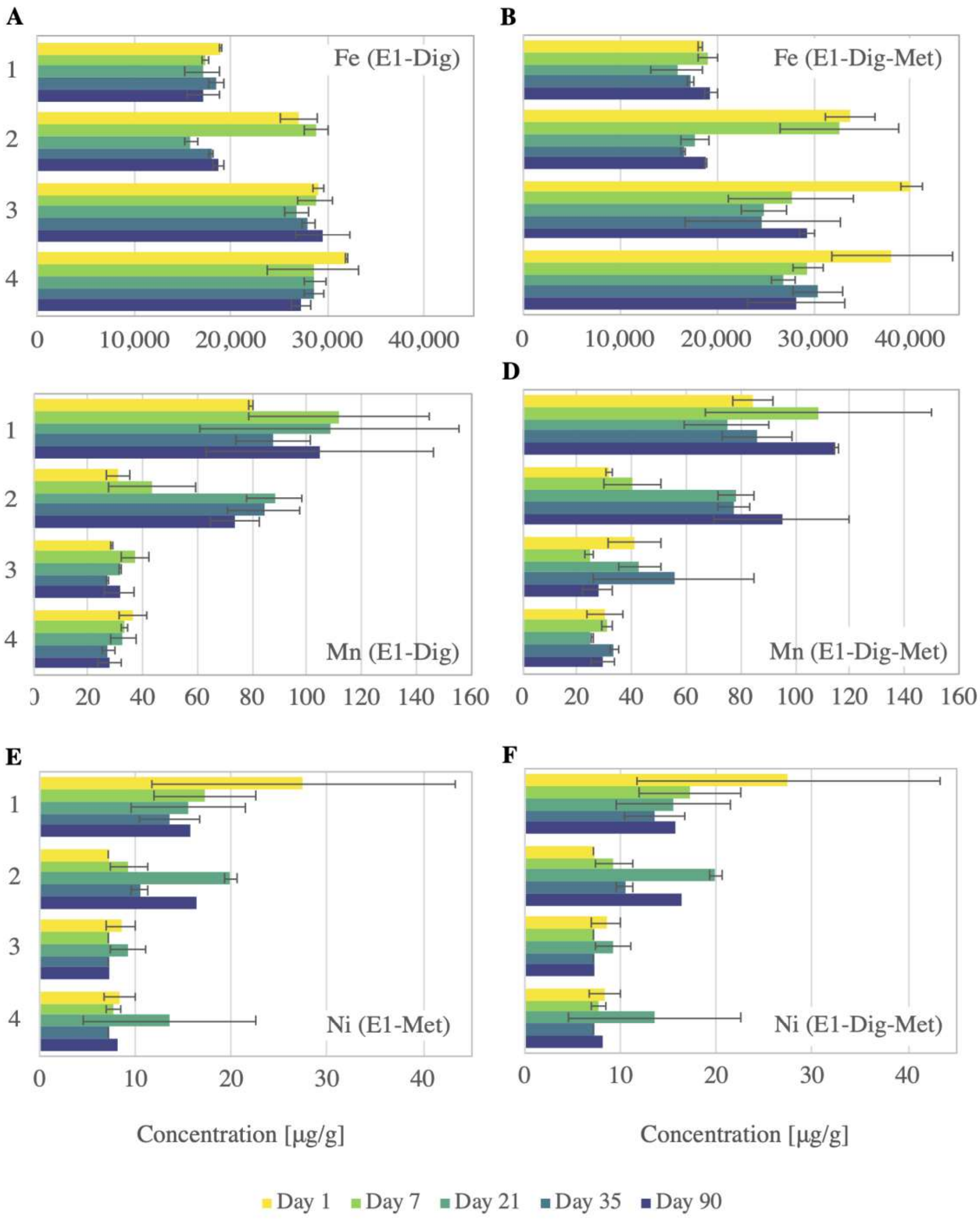

Figure A3: Fe, Mn and Ni total concentrations in the different column layers over time, for the two experimental conditions E1-Dig (soil amended with digestate (A, C, E)) and E1-Dig-Met (soil amended with digestate doped with metformin (B, D, F)) (mean ±standard deviation, n=2).

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Appendix B

B.1 Total trace metal analysis

Table B1: Calibration quality parameters for flame - atomic absorption spectrometry (F-AAS) and electrothermal – atomic absorption spectroscopy (ET-AAS), for two sample masses. Linear fit R^2, limit of detection - LOD and limit of quantification – LOQ.

	Flame AAS				
		Sample of 0.50 g		Sample of 0.25 g	
Metal	R2	LOD (ug/g)	LOQ (ug/g)	LOD (ug/g)	LOQ (ug/g)
Fe	1.000	3.00	10.0	6.00	20.0
Mn	0.998	1.50	5.00	3.00	10.0
Ni	0.999	3.00	10.0	6.00	20.0
Zn	0.998	0.75	2.50	1.50	5.00
Pb	0.997	7.50	25.0	15.0	50.0
Cu	0.999	4.00	13.3	8.00	26.7
	ET AAS				
		Sample of 0.50 g		Sample of 0.25 g	
Metal	R2	LOD (ug/g)	LOQ (ug/g)	LOD (ug/g)	LOQ (ug/g)
Pb	0.999	0.15	0.50	0.30	1.00
Cu	0.999	0.19	0.64	0.38	1.28
Cr	0.999	0.19	0.64	0.38	1.28
Co	0.994	0.28	0.93	0.56	1.87

All statistical analysis were performed with SPSS statistical software (version 28.0.1.0). Below are reported the results of the statistical tests implemented to analyse the datasets of trace metals' total concentration.

Zn total concentration statistical analysis:

a) Evaluating if Zn total concentrations at different depths are significantly different: non-parametric Kruskal-Wallis test coupled with non-parametric pairwise comparison test.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of Total Average [ug/g] is the same across categories of sampling depth.	Independent-Samples Kruskal-Wallis Test	<.001	Reject the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary

Total N	45
Test Statistic	22.401[a]
Degree Of Freedom	4
Asymptotic Sig. (2-sided test)	<.001

a. The test statistic is adjusted for ties.

Pairwise Comparisons of sampling depths

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
3-2	2.778	6.191	.449	.654	1.000
3-4	-5.222	6.191	-.843	.399	1.000
3-5	-6.444	6.191	-1.041	.298	1.000
3-1	26.111	6.191	4.217	<.001	.000
2-4	-2.444	6.191	-.395	.693	1.000
2-5	-3.667	6.191	-.592	.554	1.000
2-1	23.333	6.191	3.769	<.001	.002
4-5	-1.222	6.191	-.197	.844	1.000
4-1	20.889	6.191	3.374	<.001	.007
5-1	19.667	6.191	3.176	.001	.015

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.

Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

b) Evaluating if Zn total concentrations in first soil column layer are significantly affected by time and experimental condition with one-way ANOVA. Pairwise comparison Tukey HSD test was not performed because the main effects were not significant.

Tests of Between-Subjects Effects

Dependent Variable: Total Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	568.425[a]	2	284.213	3.911	.082

Intercept	72480.425	1	72480.425	997.434	<.001
Time	568.425	2	284.213	3.911	.082
Error	436.002	6	72.667		
Total	73484.851	9			
Corrected Total	1004.427	8			

a. R Squared = .566 (Adjusted R Squared = .421)

Tests of Between-Subjects Effects

Dependent Variable: Total Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	320.027[a]	2	160.013	1.403	.316
Intercept	72480.425	1	72480.425	635.421	<.001
Experimental Condition	320.027	2	160.013	1.403	.316
Error	684.400	6	114.067		
Total	73484.851	9			
Corrected Total	1004.427	8			

a. R Squared = .319 (Adjusted R Squared = .091)

c) Evaluating if Zn total concentrations in the second, third, fourth and fifth soil column layers are significantly affected by time and experimental condition with two-way ANOVA coupled with pairwise comparison Tukey HSD test.

Tests of Between-Subjects Effects

Dependent Variable: Total Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	39.369[a]	8	4.921	1.395	.243
Intercept	14430.120	1	14430.120	4090.356	<.001
Condition	25.766	2	12.883	3.652	.039
Time	2.446	2	1.223	.347	.710
Experimental Condition * Time	11.157	4	2.789	.791	.542
Error	95.252	27	3.528		
Total	14564.740	36			
Corrected Total	134.621	35			

a. R Squared = .292 (Adjusted R Squared = .083)

Multiple Comparisons

Dependent Variable: Total Average [ug/g]

Tukey HSD

(I) Condition	(J) Condition	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Digestate	Lamotrigine	1.916*	.767	.048	.015	3.817
	Metformin	1.641	.767	.101	-.260	3.542
Lamotrigine	Digestate	-1.916*	.767	.048	-3.817	-.015
	Metformin	-.275	.767	.932	-2.176	1.625
Metformin	Digestate	-1.641	.767	.101	-3.542	.260

Lamotrigine	.275	.767	.932	-1.625	2.176

Based on observed means.

The error term is Mean Square (Error) = 3.528.

*. The mean difference is significant at the .05 level.

Multiple Comparisons

Dependent Variable: Total Average [ug/g]

Tukey HSD

(I) Time [day]	(J) Time [day]	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	14	-.605	.767	.713	-2.506	1.297
	28	-.480	.767	.807	-2.381	1.421
14	1	.605	.767	.713	-1.297	2.506
	28	.124	.767	.986	-1.777	2.025
28	1	.480	.767	.807	-1.421	2.381
	14	-.124	.767	.986	-2.025	1.777

Based on observed means.

The error term is Mean Square(Error) = 3.528.

Cu statistical analysis:

a) Evaluating if Cu total concentrations at different depths are significantly different: non-parametric Kruskal-Wallis test coupled with non-parametric pairwise comparison test.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of Total Average [ug/g] is the same across categories of sampling depth.	Independent-Samples Kruskal-Wallis Test	<.001	Reject the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary

Total N	45
Test Statistic	21.832[a]
Degree Of Freedom	4
Asymptotic Sig.(2-sided test)	<.001

a. The test statistic is adjusted for ties.

Pairwise Comparisons of sampling depths

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
2-1	24.222	6.191	3.912	<.001	.001
3-5	-2.556	6.191	-.413	.680	1.000
3-4	-4.333	6.191	-.700	.484	1.000
3-1	24.222	6.191	3.912	<.001	.001
2-3	.000	6.191	.000	1.000	1.000

2-5	-2.556	6.191	-.413	.680	1.000
2-4	-4.333	6.191	-.700	.484	1.000
5-4	1.778	6.191	.287	.774	1.000
5-1	21.667	6.191	3.499	<.001	.005
4-1	19.889	6.191	3.212	.001	.013

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.

Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

b) Evaluating if Cu total concentrations in the first soil column layer are significantly affected by time and experimental condition: one-way ANOVA for each factor variable. Pairwise comparison Tukey HSD test was not performed because the main effects were not significant.

Tests of Between-Subjects Effects

Dependent Variable: Total Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	64.724[a]	2	32.362	.686	.539
Intercept	20702.001	1	20702.001	438.884	<.001
Experimental Condition	64.724	2	32.362	.686	.539
Error	283.018	6	47.170		
Total	21049.742	9			
Corrected Total	347.741	8			

a. R Squared = .186 (Adjusted R Squared = -.085)

Multiple Comparisons

Dependent Variable: Total Average [ug/g]

Tukey HSD

(I) Condition	(J) Condition	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Digestate	Lamotrigine	-5.969	5.608	.568	-23.175	11.237
	Metformin	-5.359	5.608	.628	-22.566	11.846
Lamotrigine	Digestate	5.969	5.608	.568	-11.237	23.175
	Metformin	.609	5.608	.994	-16.597	17.815
Metformin	Digestate	5.359	5.608	.628	-11.846	22.566
	Lamotrigine	-.609	5.608	.994	-17.815	16.597

Based on observed means.

The error term is Mean Square(Error) = 47.170.

Tests of Between-Subjects Effects

Dependent Variable: Total Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	37.178[a]	2	18.589	.359	.712
Intercept	20702.001	1	20702.001	399.957	<.001
Time	37.178	2	18.589	.359	.712
Error	310.563	6	51.761		
Total	21049.742	9			

Corrected Total	347.741	8			

a. R Squared = .107 (Adjusted R Squared = -.191)

c) Evaluating if Cu total concentrations in the second, third, fourth and fifth soil column layer are significantly affected by time and experimental condition: one-way ANOVA for each factor variable coupled with pairwise comparison Tukey HSD test.

Tests of Between-Subjects Effects

Dependent Variable: Total Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.145[a]	2	1.072	.259	.773
Intercept	37554.601	1	37554.601	9066.075	<.001
Experimental Condition	2.145	2	1.072	.259	.773
Error	136.697	33	4.142		
Total	37693.442	36			
Corrected Total	138.841	35			

a. R Squared = .015 (Adjusted R Squared = -.044)

Multiple Comparisons

Dependent Variable: Total Average [ug/g]

Tukey HSD

	Std. Error	Sig.	95% Confidence Interval

(I) Condition	(J) Condition	Mean Difference (I-J)			Lower Bound	Upper Bound
Digestate	Lamotrigine	.118	.831	.989	-1.921	2.157
	Metformin	.567	.831	.776	-1.472	2.606
Lamotrigine	Digestate	-.118	.831	.989	-2.157	1.921
	Metformin	.448	.831	.852	-1.590	2.487
Metformin	Digestate	-.567	.831	.776	-2.606	1.472
	Lamotrigine	-.448	.831	.852	-2.487	1.590

Based on observed means.

The error term is Mean Square(Error) = 4.142.

Tests of Between-Subjects Effects

Dependent Variable: Total Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	42.845[a]	2	21.422	7.364	.002
Intercept	37554.601	1	37554.601	12909.827	<.001
Time	42.845	2	21.422	7.364	.002
Error	95.997	33	2.909		
Total	37693.442	36			
Corrected Total	138.841	35			

a. R Squared = .309 (Adjusted R Squared = .267)

Multiple Comparisons

Dependent Variable: Total Average [ug/g]

Tukey HSD

(I) Time [day]	(J) Time [day]	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	14	-.037	.696	.998	-1.746	1.671
	28	2.295*	.696	.006	.587	4.004
14	1	.037	.696	.998	-1.671	1.746
	28	2.332*	.696	.006	.624	4.041
28	1	-2.295*	.696	.006	-4.004	-.587
	14	-2.332*	.696	.006	-4.041	-.624

Based on observed means.

The error term is Mean Square(Error) = 2.909.

*. The mean difference is significant at the .05 level.

Pb statistical analysis:

a) Evaluating if Pb total concentrations at different depths are significantly different: non-parametric Kruskal-Wallis test coupled with non-parametric pairwise comparison test.

Hypothesis Test Summary

Null Hypothesis	Test	Sig.[a,b]	Decision

1	The distribution of Total Average [ug/g] is the same across categories of sample depth.	Independent-Samples Kruskal-Wallis Test	.008	Reject the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary

Total N	45
Test Statistic	13.901[a]
Degree Of Freedom	4
Asymptotic Sig.(2-sided test)	.008

a. The test statistic is adjusted for ties.

Pairwise Comparisons of sample depths

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
5-2	2.333	6.191	.377	.706	1.000
5-4	7.222	6.191	1.166	.243	1.000
5-3	8.889	6.191	1.436	.151	1.000
5-1	21.000	6.191	3.392	<.001	.007
2-4	-4.889	6.191	-.790	.430	1.000
2-3	-6.556	6.191	-1.059	.290	1.000
2-1	18.667	6.191	3.015	.003	.026

4-3	1.667	6.191	.269	.788	1.000
4-1	13.778	6.191	2.225	.026	.261
3-1	12.111	6.191	1.956	.050	.505

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.

Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

b) Evaluating if Pb total concentrations in the first soil column layer are significantly affected by time and experimental condition: one-way ANOVA for each factor variable. Pairwise comparison Tukey HSD test was not performed because the main effects were not significant.

Tests of Between-Subjects Effects

Dependent Variable: Total Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	19.101[a]	2	9.550	.520	.619
Intercept	9254.746	1	9254.746	503.533	<.001
Experimental Condition	19.101	2	9.550	.520	.619
Error	110.278	6	18.380		
Total	9384.124	9			
Corrected Total	129.379	8			

a. R Squared = .148 (Adjusted R Squared = -.136)

Tests of Between-Subjects Effects

Dependent Variable: Total Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	69.073[a]	2	34.537	3.436	.101
Intercept	9254.746	1	9254.746	920.785	<.001
Time	69.073	2	34.537	3.436	.101
Error	60.306	6	10.051		
Total	9384.124	9			
Corrected Total	129.379	8			

a. R Squared = .534 (Adjusted R Squared = .379)

c) Evaluating if Pb total concentrations of second, third, fourth and fifth soil column layer are significantly affected by time and experimental condition: non-parametric Kruskal-Wallis test for each factor variable coupled with non-parametric pairwise comparison test.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of Total Average [ug/g] is the same across categories of Time [day].	Independent-Samples Kruskal-Wallis Test	<.001	Reject the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary

Total N	36

Test Statistic	19.113[a]
Degree Of Freedom	2
Asymptotic Sig.(2-sided test)	<.001

a. The test statistic is adjusted for ties.

Pairwise Comparisons of Time [day]

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
28-14	3.500	4.301	.814	.416	1.000
28-1	17.750	4.301	4.127	<.001	.000
14-1	14.250	4.301	3.313	<.001	.003

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.

Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of Total Average [ug/g] is the same across categories of Condition.	Independent-Samples Kruskal-Wallis Test	.337	Retain the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary

Total N	36
Test Statistic	2.173[a]
Degree Of Freedom	2
Asymptotic Sig.(2-sided test)	.337

a. The test statistic is adjusted for ties.

Pairwise Comparisons of Condition

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
Metformin-Lamotrigine	2.917	4.301	.678	.498	1.000
Metformin-Digestate	6.333	4.301	1.472	.141	.423
Lamotrigine-Digestate	3.417	4.301	.794	.427	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.

Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

B.2 Trace metal fractionation analysis

Below are reported the results of the statistical tests performed to analyse the datasets of trace metals' fractionation.

Zn fractionation statistical analysis:

a) Evaluating if Zn total concentrations at different depths are significantly different: non-parametric Kruskal-Wallis test coupled with non-parametric pairwise comparison test.

Hypothesis Test Summary

ID	Null Hypothesis	Test	Sig.^{a,b}	Decision
1	The distribution of Exchangeable Average [ug/g] is the same across categories of sample depth.	Independent-Samples Kruskal-Wallis Test	<.001	Reject the null hypothesis.
2	The distribution of Bound Fe/Mn Average [ug/g] is the same across categories of sample depth.	Independent-Samples Kruskal-Wallis Test	<.001	Reject the null hypothesis.
3	The distribution of Residual Average [ug/g] is the same across categories of sample depth.	Independent-Samples Kruskal-Wallis Test	.516	Retain the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary

1	Total N	45

	Test Statistic	23.684[a]
	Degree Of Freedom	4
	Asymptotic Sig.(2-sided test)	<.001
2	Total N	45
	Test Statistic	21.999[a]
	Degree Of Freedom	4
	Asymptotic Sig.(2-sided test)	<.001
3	Total N	45
	Test Statistic	3.254[a]
	Degree Of Freedom	4
	Asymptotic Sig.(2-sided test)	.516

a. The test statistic is adjusted for ties.

Pairwise Comparisons of sample depths

ID	Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
1	4-5	-6.111	6.188	-.988	.323	1.000
	4-2	8.222	6.188	1.329	.184	1.000
	4-3	8.778	6.188	1.418	.156	1.000
	4-1	28.278	6.188	4.570	<.001	.000
	5-2	2.111	6.188	.341	.733	1.000
	5-3	2.667	6.188	.431	.667	1.000
	5-1	22.167	6.188	3.582	<.001	.003

	2-3	-.556	6.188	-.090	.928	1.000
	2-1	20.056	6.188	3.241	.001	.012
	3-1	19.500	6.188	3.151	.002	.016
2	4-5	-.444	6.132	-.072	.942	1.000
	4-3	1.111	6.132	.181	.856	1.000
	4-2	3.778	6.132	.616	.538	1.000
	4-1	23.833	6.132	3.887	<.001	.001
	5-3	.667	6.132	.109	.913	1.000
	5-2	3.333	6.132	.544	.587	1.000
	5-1	23.389	6.132	3.814	<.001	.001
	3-2	2.667	6.132	.435	.664	1.000
	3-1	22.722	6.132	3.706	<.001	.002
	2-1	20.056	6.132	3.271	.001	.011
3	3-2	2.778	6.191	.449	.654	1.000
	3-5	-6.667	6.191	-1.077	.282	1.000
	3-4	-8.444	6.191	-1.364	.173	1.000
	3-1	9.333	6.191	1.507	.132	1.000
	2-5	-3.889	6.191	-.628	.530	1.000
	2-4	-5.667	6.191	-.915	.360	1.000
	2-1	6.556	6.191	1.059	.290	1.000
	5-4	1.778	6.191	.287	.774	1.000
	5-1	2.667	6.191	.431	.667	1.000
	4-1	.889	6.191	.144	.886	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.

Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

b) Evaluating if Zn fractionation in the first soil column layer was significantly affected by time and experimental condition: one-way MANOVA for each factor variable coupled with pairwise comparison Tukey HSD test for the main effects that resulted to be significant (p-value <0.05).

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Exchangeable Average [ug/g]	14.075[a]	2	7.037	.131	.879
	Bound Fe/Mn Average [ug/g]	16.134[b]	2	8.067	.537	.610
	Residual Average [ug/g]	590.206[c]	2	295.103	1.114	.388
Intercept	Exchangeable Average [ug/g]	7683.283	1	7683.283	143.319	<.001
	Bound Fe/Mn Average [ug/g]	12301.970	1	12301.970	818.590	<.001
	Residual Average [ug/g]	4991.890	1	4991.890	18.850	.005
Experimental Condition	Exchangeable Average [ug/g]	14.075	2	7.037	.131	.879
	Bound Fe/Mn Average [ug/g]	16.134	2	8.067	.537	.610

	Residual Average [ug/g]	590.206	2	295.103	1.114	.388
Error	Exchangeable Average [ug/g]	321.658	6	53.610		
	Bound Fe/Mn Average [ug/g]	90.169	6	15.028		
	Residual Average [ug/g]	1588.972	6	264.829		
Total	Exchangeable Average [ug/g]	8019.015	9			
	Bound Fe/Mn Average [ug/g]	12408.273	9			
	Residual Average [ug/g]	7171.068	9			
Corrected Total	Exchangeable Average [ug/g]	335.733	8			
	Bound Fe/Mn Average [ug/g]	106.303	8			
	Residual Average [ug/g]	2179.178	8			

a. R Squared = .042 (Adjusted R Squared = -.277)

b. R Squared = .152 (Adjusted R Squared = -.131)

c. R Squared = .271 (Adjusted R Squared = .028)

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.

Corrected Model	Exchangeable Average [ug/g]	298.198[a]	2	149.099	23.834	.001
	Bound Fe/Mn Average [ug/g]	23.065[b]	2	11.533	.831	.480
	Residual Average [ug/g]	1360.106[c]	2	680.053	4.982	.053
Intercept	Exchangeable Average [ug/g]	7683.283	1	7683.283	1228.192	<.001
	Bound Fe/Mn Average [ug/g]	12301.970	1	12301.970	886.757	<.001
	Residual Average [ug/g]	4991.890	1	4991.890	36.567	<.001
Time	Exchangeable Average [ug/g]	298.198	2	149.099	23.834	.001
	Bound Fe/Mn Average [ug/g]	23.065	2	11.533	.831	.480
	Residual Average [ug/g]	1360.106	2	680.053	4.982	.053
Error	Exchangeable Average [ug/g]	37.535	6	6.256		
	Bound Fe/Mn Average [ug/g]	83.238	6	13.873		
	Residual Average [ug/g]	819.072	6	136.512		
Total	Exchangeable Average [ug/g]	8019.015	9			
	Bound Fe/Mn Average [ug/g]	12408.273	9			

	Residual Average [ug/g]	7171.068	9			
Corrected Total	Exchangeable Average [ug/g]	335.733	8			
	Bound Fe/Mn Average [ug/g]	106.303	8			
	Residual Average [ug/g]	2179.178	8			

a. R Squared = .888 (Adjusted R Squared = .851)

b. R Squared = .217 (Adjusted R Squared = -.044)

c. R Squared = .624 (Adjusted R Squared = .499)

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Time [day]	(J) Time [day]	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Exchangeable Average [ug/g]	1	14	-4.858	2.042	.119	-11.124	1.408
		28	-13.892*	2.042	.001	-20.158	-7.626
	14	1	4.858	2.042	.119	-1.408	11.124
		28	-9.034*	2.042	.011	-15.299	-2.768
	28	1	13.892*	2.042	.001	7.626	20.158
		14	9.034*	2.042	.011	2.768	15.299

Based on observed means.

The error term is Mean Square(Error) = 136.512.

*. The mean difference is significant at the .05 level.

c) Evaluating if total concentrations of second, third, fourth and fifth soil column layer are significantly affected by time and experimental condition: two-way MANOVA coupled with pairwise comparison Tukey test when evaluated factors had a significant effect (p-value<0.05).

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Exchangeable Average [ug/g]	18.586[a]	8	2.323	1.632	.162
	Bound Fe/Mn Average [ug/g]	25.188[b]	8	3.149	.997	.461
	Residual Average [ug/g]	160.128[c]	8	20.016	1.307	.282
Intercept	Exchangeable Average [ug/g]	110.454	1	110.454	77.581	<.001
	Bound Fe/Mn Average [ug/g]	82.768	1	82.768	26.209	<.001
	Residual Average [ug/g]	10103.878	1	10103.878	659.986	<.001
Experimental Condition	Exchangeable Average [ug/g]	4.524	2	2.262	1.589	.223
	Bound Fe/Mn Average [ug/g]	4.673	2	2.337	.740	.487
	Residual Average [ug/g]	67.746	2	33.873	2.213	.129
Time	Exchangeable Average [ug/g]	5.535	2	2.768	1.944	.163

	Bound Fe/Mn Average [ug/g]	8.047	2	4.023	1.274	.296
	Residual Average [ug/g]	25.292	2	12.646	.826	.449
Condition * Time	Exchangeable Average [ug/g]	8.527	4	2.132	1.497	.231
	Bound Fe/Mn Average [ug/g]	12.468	4	3.117	.987	.431
	Residual Average [ug/g]	67.090	4	16.772	1.096	.379
Error	Exchangeable Average [ug/g]	38.441	27	1.424		
	Bound Fe/Mn Average [ug/g]	85.265	27	3.158		
	Residual Average [ug/g]	413.349	27	15.309		
Total	Exchangeable Average [ug/g]	167.481	36			
	Bound Fe/Mn Average [ug/g]	193.222	36			
	Residual Average [ug/g]	10677.355	36			
Corrected Total	Exchangeable Average [ug/g]	57.027	35			
	Bound Fe/Mn Average [ug/g]	110.453	35			
	Residual Average [ug/g]	573.477	35			

a. R Squared = .326 (Adjusted R Squared = .126)

b. R Squared = .228 (Adjusted R Squared = -.001)

c. R Squared = .279 (Adjusted R Squared = .066)

Cu fractionation statistical analysis:

a) Evaluating if Cu fractionation at different depths is significantly different: ANOVA with pairwise comparison Tukey HSD test for exchangeable and oxidizable and residual fractions fraction and non-parametric Kruskal-Wallis test coupled with non-parametric pairwise comparison test for reducible fraction.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Exchangeable Average [ug/g]	1	2	.258*	.058	<.001	.092	.423
		3	.193*	.058	.015	.028	.359
		4	.274*	.058	<.001	.108	.440
		5	.259*	.058	<.001	.093	.425
	2	1	-.258*	.058	<.001	-.423	-.092
		3	-.064	.058	.804	-.229	.102
		4	.017	.058	.998	-.149	.183
		5	.002	.058	1.000	-.164	.168
	3	1	-.193*	.058	.015	-.359	-.028
		2	.064	.058	.804	-.102	.229
		4	.081	.058	.638	-.085	.247
		5	.066	.058	.789	-.100	.232
	4	1	-.274*	.058	<.001	-.440	-.108
		2	-.017	.058	.998	-.183	.149
		3	-.081	.058	.638	-.247	.085
		5	-.015	.058	.999	-.181	.151
	5	1	-.259*	.058	<.001	-.425	-.093
		2	-.002	.058	1.000	-.1677	.164
		3	-.066	.058	.789	-.232	.100

		4	.015	.058	.999	-.151	.181
Residual Average [ug/g]	1	2	11.448*	1.742	<.001	6.472	16.425
		3	11.427*	1.742	<.001	6.450	16.404
		4	10.397*	1.742	<.001	5.420	15.374
		5	10.822*	1.742	<.001	5.845	15.798
	2	1	-11.448*	1.742	<.001	-16.425	-6.472
		3	-.0214	1.742	1.000	-4.998	4.955
		4	-1.052	1.742	.974	-6.028	3.925
		5	-.627	1.742	.996	-5.603	4.350
	3	1	-11.427*	1.742	<.001	-16.404	-6.450
		2	.0214	1.742	1.000	-4.955	4.998
		4	-1.030	1.742	.976	-6.007	3.947
		5	-.605	1.742	.997	-5.582	4.371
	4	1	-10.397*	1.742	<.001	-15.374	-5.420
		2	1.052	1.742	.974	-3.925	6.028
		3	1.030	1.742	.976	-3.947	6.007
		5	.425	1.742	.999	-4.552	5.402
	5	1	-10.823*	1.742	<.001	-15.798	-5.845
		2	.627	1.742	.996	-4.350	5.603
		3	.605	1.742	.997	-4.371	5.582
		4	-.425	1.742	.999	-5.402	4.552

Based on observed means.

The error term is Mean Square(Error) = 13.663.

*. The mean difference is significant at the .05 level.

Hypothesis Test Summary

ID	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of Bound Fe/Mn Average [ug/g] is the same across categories of sample depth.	Independent-Samples Kruskal-Wallis Test	<.001	Reject the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary

1	Total N	45
	Test Statistic	27.136[a]
	Degree Of Freedom	4
	Asymptotic Sig.(2-sided test)	<.001

a. The test statistic is adjusted for ties.

Pairwise Comparisons of Sample

ID	Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
1	5-4	1.111	6.191	.179	.858	1.000
	5-3	6.444	6.191	1.041	.298	1.000
	5-2	13.556	6.191	2.189	.029	.286
	5-1	27.778	6.191	4.487	<.001	.000
	4-3	5.333	6.191	.861	.389	1.000
	4-2	12.444	6.191	2.010	.044	.444
	4-1	26.667	6.191	4.307	<.001	.000
	3-2	7.111	6.191	1.149	.251	1.000
	3-1	21.333	6.191	3.446	<.001	.006
	2-1	14.222	6.191	2.297	.022	.216

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .050.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

b) Evaluating if Cu fractionation in the first soil column layer was significantly affected by time and experimental condition: one-way MANOVA for each factor variable coupled with pairwise comparison Tukey HSD test for the main effects that resulted to be significant (p-value <0.05).

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Exchangeable Average [ug/g]	.013[a]	2	.006	.165	.851
	Bound Fe/Mn Average [ug/g]	.272[b]	2	.136	.031	.970
	Residual Average [ug/g]	69.811[c]	2	34.906	.597	.580
Intercept	Exchangeable Average [ug/g]	5.315	1	5.315	139.728	<.001
	Bound Fe/Mn Average [ug/g]	244.982	1	244.982	55.053	<.001
	Residual Average [ug/g]	15857.020	1	15857.020	271.304	<.001
Experimental Condition	Exchangeable Average [ug/g]	.013	2	.006	.165	.851
	Bound Fe/Mn Average [ug/g]	.272	2	.136	.031	.970
	Residual Average [ug/g]	69.811	2	34.906	.597	.580
Error	Exchangeable Average [ug/g]	.228	6	.038		
	Bound Fe/Mn Average [ug/g]	26.700	6	4.450		
	Residual Average [ug/g]	350.684	6	58.447		

Total	Exchangeable Average [ug/g]	5.555	9			
	Bound Fe/Mn Average [ug/g]	271.953	9			
	Residual Average [ug/g]	16277.516	9			
Corrected Total	Exchangeable Average [ug/g]	.241	8			
	Bound Fe/Mn Average [ug/g]	26.971	8			
	Residual Average [ug/g]	420.495	8			

a. R Squared = .052 (Adjusted R Squared = -.264)

b. R Squared = .010 (Adjusted R Squared = -.320)

c. R Squared = .166 (Adjusted R Squared = -.112)

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Exchangeable Average [ug/g]	.093[a]	2	.047	1.897	.230
	Bound Fe/Mn Average [ug/g]	25.246[b]	2	12.623	43.901	<.001
	Residual Average [ug/g]	109.004[c]	2	54.502	1.050	.406
Intercept	Exchangeable Average [ug/g]	5.315	1	5.315	216.144	<.001
	Bound Fe/Mn Average [ug/g]	244.982	1	244.982	852.006	<.001
	Residual Average [ug/g]	15857.020	1	15857.020	305.440	<.001
Time	Exchangeable Average [ug/g]	.093	2	.047	1.897	.230

	Bound Fe/Mn Average [ug/g]	25.246	2	12.623	43.901	<.001
	Residual Average [ug/g]	109.004	2	54.502	1.050	.406
Error	Exchangeable Average [ug/g]	.148	6	.025		
	Bound Fe/Mn Average [ug/g]	1.725	6	.288		
	Residual Average [ug/g]	311.492	6	51.915		
Total	Exchangeable Average [ug/g]	5.555	9			
	Bound Fe/Mn Average [ug/g]	271.953	9			
	Residual Average [ug/g]	16277.516	9			
Corrected Total	Exchangeable Average [ug/g]	.241	8			
	Bound Fe/Mn Average [ug/g]	26.971	8			
	Residual Average [ug/g]	420.495	8			

a. R Squared = .387 (Adjusted R Squared = .183)

b. R Squared = .936 (Adjusted R Squared = .915)

c. R Squared = .259 (Adjusted R Squared = .012)

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Time [day]	(J) Time [day]	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Exchangeable Average [ug/g]	1	14	-.166	.128	.446	-.559	.227
		28	.078	.128	.821	-.315	.471

	14	1	.166	.128	.446	-.227	.559
		28	.244	.128	.217	-.149	.637
	28	1	-.078	.128	.821	-.471	.315
		14	-.244	.128	.217	-.637	.149
Bound Fe/Mn Average [ug/g]	1	14	-3.686*	.438	<.001	-5.029	-2.342
		28	-3.403*	.438	<.001	-4.747	-2.059
	14	1	3.686*	.438	<.001	2.342	5.029
		28	.283	.438	.802	-1.061	1.626
	28	1	3.403*	.438	<.001	2.059	4.747
		14	-.283	.438	.802	-1.626	1.061
Residual Average [ug/g]	1	14	8.524	5.883	.377	-9.527	26.575
		28	4.172	5.883	.767	-13.879	22.223
	14	1	-8.524	5.883	.377	-26.575	9.527
		28	-4.352	5.883	.750	-22.403	13.699
	28	1	-4.172	5.883	.767	-22.223	13.879
		14	4.352	5.883	.750	-13.699	22.403

Based on observed means.

The error term is Mean Square(Error) = 51.915.

*. The mean difference is significant at the .05 level.

c) Evaluating if Cu fractionations in the second, third, fourth and fifth soil column layer are significantly affected by time and experimental condition: two-way MANOVA coupled with pairwise comparison Tukey HSD test, when evaluated factor variables had a significant effect (p-value<0.05).

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Exchangeable Average [ug/g]	.104[a]	8	.013	1.183	.345

	Bound Fe/Mn Average [ug/g]	.289[b]	8	.036	1.686	.148
	Residual Average [ug/g]	77.626[c]	8	9.703	4.730	.001
Intercept	Exchangeable Average [ug/g]	9.822	1	9.822	891.775	<.001
	Bound Fe/Mn Average [ug/g]	24.481	1	24.481	1141.959	<.001
	Residual Average [ug/g]	34487.558	1	34487.558	16810.620	<.001
Experimental Condition	Exchangeable Average [ug/g]	.047	2	.023	2.116	.140
	Bound Fe/Mn Average [ug/g]	.042	2	.021	.991	.384
	Residual Average [ug/g]	1.588	2	.794	.387	.683
Time	Exchangeable Average [ug/g]	.022	2	.011	.996	.383
	Bound Fe/Mn Average [ug/g]	.143	2	.071	3.332	.051
	Residual Average [ug/g]	36.755	2	18.377	8.958	.001
Condition * Time	Exchangeable Average [ug/g]	.036	4	.009	.810	.530
	Bound Fe/Mn Average [ug/g]	.104	4	.026	1.211	.329
	Residual Average [ug/g]	39.283	4	9.821	4.787	.005
Error	Exchangeable Average [ug/g]	.297	27	.011		
	Bound Fe/Mn Average [ug/g]	.579	27	.021		
	Residual Average [ug/g]	55.391	27	2.052		
Total	Exchangeable Average [ug/g]	10.224	36			

	Bound Fe/Mn Average [ug/g]	25.349	36			
	Residual Average [ug/g]	34620.576	36			
Corrected Total	Exchangeable Average [ug/g]	.402	35			
	Bound Fe/Mn Average [ug/g]	.868	35			
	Residual Average [ug/g]	133.018	35			

a. R Squared = .260 (Adjusted R Squared = .040)

b. R Squared = .333 (Adjusted R Squared = .136)

c. R Squared = .584 (Adjusted R Squared = .460)

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Time [day]	(J) Time [day]	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Exchangeable Average [ug/g]	1	14	-.009	.043	.975	-.116	.097
		28	.047	.043	.523	-.059	.153
	14	1	.009	.043	.975	-.097	.115
		28	.056	.043	.399	-.049	.163
	28	1	-.047	.043	.523	-.153	.059
		14	-.056	.043	.399	-.162	.049
Bound Fe/Mn Average [ug/g]	1	14	.067	.059	.510	-.081	.215
		28	.157*	.059	.041	.006	.302
	14	1	-.067	.059	.510	-.215	.081
		28	.087	.059	.329	-.061	.235

	28	1	-.154*	.059	.041	-.302	-.006
		14	-.087	.0597	.329	-.235	.061
Residual Average [ug/g]	1	14	-.095	.585	.986	-1.545	1.355
		28	2.094*	.585	.004	.645	3.545
	14	1	.095	.585	.986	-1.355	1.545
		28	2.189*	.585	.002	.739	3.639
	28	1	-2.094*	.585	.004	-3.544	-.645
		14	-2.189*	.585	.002	-3.639	-.739

Based on observed means.

The error term is Mean Square (Error) = 2.052.

*. The mean difference is significant at the .05 level.

Pb fractionation statistical analysis:

a) Evaluating if Pb fractionation at different depths are significantly different: ANOVA for exchangeable fraction and non-parametric Kruskal-Wallis test for the other fractions. Pairwise comparison tests were performed for the main effects that resulted being significant (p-value < 0.05).

Tests of Between-Subjects Effects

Dependent Variable: Exchangeable Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.042[a]	4	.011	.131	.970
Intercept	7.458	1	7.458	91.793	<.001
Sample depth	.042	4	.011	.131	.970
Error	3.250	40	.081		
Total	10.750	45			
Corrected Total	3.292	44			

a. R Squared = .013 (Adjusted R Squared = -.086)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of Bound Fe/Mn Average [ug/g] is the same across categories of sample depth.	Independent-Samples Kruskal-Wallis Test	<.001	Reject the null hypothesis.
2	The distribution of Residual Average [ug/g] is the same across categories of sample depth.	Independent-Samples Kruskal-Wallis Test	.018	Reject the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary

1	Total N	45
	Test Statistic	21.399[a]
	Degree Of Freedom	4
	Asymptotic Sig.(2-sided test)	<.001
2	Total N	45
	Test Statistic	11.972[a]
	Degree Of Freedom	4
	Asymptotic Sig.(2-sided test)	.018

a. The test statistic is adjusted for ties.

Pairwise Comparisons of Sample – Bound to Fe/Mn fraction sample depths

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
5-3	2.333	6.191	.377	.706	1.000

5-2	2.667	6.191	.431	.667	1.000
5-4	2.778	6.191	.449	.654	1.000
5-1	24.444	6.191	3.948	<.001	.001
3-2	.333	6.191	.054	.957	1.000
3-4	-.444	6.191	-.072	.943	1.000
3-1	22.111	6.191	3.571	<.001	.004
2-4	-.111	6.191	-.018	.986	1.000
2-1	21.778	6.191	3.517	<.001	.004
4-1	21.667	6.191	3.499	<.001	.005

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Pairwise Comparisons of Sample – Residual fraction sample depths

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
1-5	-11.222	6.191	-1.813	.070	.699
1-2	-13.111	6.191	-2.118	.034	.342
1-4	-17.333	6.191	-2.800	.005	.051
1-3	-19.444	6.191	-3.141	.002	.017
5-2	1.889	6.191	.305	.760	1.000
5-4	6.111	6.191	.987	.324	1.000
5-3	8.222	6.191	1.328	.184	1.000
2-4	-4.222	6.191	-.682	.495	1.000
2-3	-6.333	6.191	-1.023	.306	1.000
4-3	2.111	6.191	.341	.733	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

b) Evaluating if Pb reducible fraction was significantly affected by time and experimental condition: one-way ANOVA for each factor variable coupled with pairwise comparison Tukey HSD test for the main effects that resulted to be significant (p-value <0.05).

Tests of Between-Subjects Effects – Sampling depth 1

Dependent Variable: Bound Fe/Mn Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	23.594[a]	2	11.797	.245	.790
Intercept	2589.004	1	2589.004	53.728	<.001
Condition	23.594	2	11.797	.245	.790
Error	289.125	6	48.188		
Total	2901.722	9			
Corrected Total	312.719	8			

a. R Squared = .075 (Adjusted R Squared = -.233)

Tests of Between-Subjects Effects – Sampling depth 1

Dependent Variable: Bound Fe/Mn Average [ug/g]

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	236.397[a]	2	118.199	9.292	.015
Intercept	2589.004	1	2589.004	203.534	<.001
Time	236.397	2	118.199	9.292	.015
Error	76.322	6	12.720		
Total	2901.722	9			

Corrected Total	312.719	8			

a. R Squared = .756 (Adjusted R Squared = .675)

Multiple Comparisons – Sampling depth 1

Dependent Variable: Bound Fe/Mn Average [ug/g]

Tukey HSD

(I) Time [day]	(J) Time [day]	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	14	.257	2.912	.996	-8.678	9.192
	28	-10.741*	2.912	.024	-19.676	-1.806
14	1	-.257	2.912	.996	-9.192	8.678
	28	-10.998*	2.912	.022	-19.933	-2.063
28	1	10.741*	2.912	.024	1.806	19.676
	14	10.998*	2.912	.022	2.063	19.933

Based on observed means.
The error term is Mean Square(Error) = 12.720.

*. The mean difference is significant at the .05 level.

Hypothesis Test Summary – Experimental condition – Sampling depths 2, 3, 4 and 5

	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of Bound Fe/Mn Average [ug/g] is the same across categories of Condition.	Independent-Samples Kruskal-Wallis Test	.308	Retain the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary – Time - Sampling depths 2, 3, 4 and 5

Total N	36
Test Statistic	2.353[a]
Degree Of Freedom	2
Asymptotic Sig.(2-sided test)	.308

a. The test statistic is adjusted for ties.

Hypothesis Test Summary - Sampling depths 2, 3, 4 and 5

	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of Bound Fe/Mn Average [ug/g] is the same across categories of Time [day].	Independent-Samples Kruskal-Wallis Test	<.001	Reject the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Independent-Samples Kruskal-Wallis Test Summary - Sampling depths 2, 3, 4 and 5

Total N	36
Test Statistic	24.164[a]
Degree Of Freedom	2
Asymptotic Sig.(2-sided test)	<.001

a. The test statistic is adjusted for ties.

Pairwise Comparisons of Time [day] - Sampling depths 2, 3, 4 and 5

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
14-1	4.583	4.301	1.066	.287	.860

14-28	-20.167	4.301	-4.689	<.001	.000
1-28	-15.583	4.301	-3.623	<.001	.001

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

c) Evaluating if Pb exchangeable and oxidizable and residual fractions are significantly affected by time and experimental condition: two-way MANOVA coupled with pairwise comparison Tukey test when evaluated factors had a significant effect (p-value<0.05).

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Exchangeable Average [ug/g]	1.779[a]	8	.222	5.291	<.001
	Residual Average [ug/g]	935.121[b]	8	116.890	3.392	.005
Intercept	Exchangeable Average [ug/g]	7.458	1	7.458	177.434	<.001
	Residual Average [ug/g]	20759.903	1	20759.903	602.437	<.001
Experimental Condition	Exchangeable Average [ug/g]	.112	2	.056	1.338	.275
	Residual Average [ug/g]	130.309	2	65.154	1.891	.166
Time	Exchangeable Average [ug/g]	1.323	2	.661	15.735	<.001
	Residual Average [ug/g]	689.116	2	344.558	9.999	<.001
Condition * Time	Exchangeable Average [ug/g]	.344	4	.086	2.046	.109
	Residual Average [ug/g]	115.696	4	28.924	.839	.509

Error	Exchangeable Average [ug/g]	1.513	36	.042		
	Residual Average [ug/g]	1240.556	36	34.460		
Total	Exchangeable Average [ug/g]	10.750	45			
	Residual Average [ug/g]	22935.580	45			
Corrected Total	Exchangeable Average [ug/g]	3.292	44			
	Residual Average [ug/g]	2175.677	44			

a. R Squared = .540 (Adjusted R Squared = .438)

b. R Squared = .430 (Adjusted R Squared = .303)

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Time [day]	(J) Time [day]	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Exchangeable Average [ug/g]	1	14	-.005	.075	.998	-.1879	.178
		28	-.366*	.075	<.001	-.549	-.183
	14	1	.005	.075	.998	-.178	.188
		28	-.361*	.075	<.001	-.544	-.178
	28	1	.366*	. 075	<.001	.183	.549
		14	.361*	. 075	<.001	.178	.544
Residual Average [ug/g]	1	14	4.887	2.144	.072	-.352	10.127
		28	9.584*	2.144	<.001	4.346	14.824
	14	1	-4.888	2.144	.072	-10.127	.352
		28	4.697	2.144	.086	-.542	9.937

28	1	-9.585*	2.144	<.001	-14.824	-4.346
	14	-4.697	2.144	.086	-9.937	.542

Based on observed means.
The error term is Mean Square(Error) = 34.460.

*. The mean difference is significant at the .05 level.

B.3 Soil prokaryotic community analysis

B.3.1 Amplification details

DNA was reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. The first PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit according to manufacturer suggestions: 0.3 µM of each PCR primer (forward primer 515F-Y and reverse primer 806rB) and 12.5 ng of template DNA in a total volume of 25 µL. The PCR conditions of 16S region amplification involved a 3 min denaturation at 95 °C, followed by 30 cycles of 98 °C for 20 s, 64 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. The second PCR reactions added indexes and sequencing adapters to both ends of the amplified V4 region according to manufacturer's recommendations. Negative PCR controls were included for all amplification procedures.

Table B2: DNA quantification of all samples initially considered in the study. Limit of Quantification (LOQ) for the Qubit dsDNA HS Assay Kit equal to 0.1 ng/µl.

Sample	Sampling Time (day)	DNA Concentration (ng/µl)	DNA Concentration - after evaporation (ng/µl)
Initial soil 1	0	<LOQ	<LOQ
Initial soil 2	0	<LOQ	<LOQ
Initial soil 3	0	<LOQ	<LOQ
Initial soil 5	0	3.32	-
Initial soil 6	0	4.42	-
Initial soil 7	0	3.72	-
Initial mixture 1	0	34.8	-
Initial mixture 2	0	55.0	36
Initial mixture 3	0	47.70	47.9
Initial digestate 1	0	0.33	48.1

Initial digestate 2	0	11.40	7.15
Initial digestate 3	0	29.20	11.1
Column 1 - port 1	1	57	-
Column 2 - port 1	1	3.72	-
Column 3 - port 1	1	6.19	-
Column 4 - port 1	1	30.6	-
Column 5 - port 1	1	2.79	-
Column 6 - port 1	1	2.97	-
Column 7 - port 1	1	0.32	-
Column 8 - port 1	1	15.3	-
Column 9 - port 1	1	6.17	-
Column 1 - port 1	14	53.00	29.8
Column 1 - port 2	14	<LOQ	<LOQ
Column 1 - port 4	14	<LOQ	0.062
Column 2 - port 1	14	29.20	-
Column 2 - port 2	14	<LOQ	<LOQ
Column 2 - port 4	14	<LOQ	<LOQ
Column 3 - port 1	14	40.90	29.6
Column 3 - port 2	14	<LOQ	<LOQ
Column 3 - port 4	14	<LOQ	<LOQ
Column 4 - port 1	14	29.90	40.3
Column 4 - port 2	14	<LOQ	0.069
Column 4 - port 4	14	<LOQ	<LOQ
Column 5 - port 1	14	46.60	35.3
Column 5 - port 2	14	<LOQ	9.98
Column 5 - port 4	14	<LOQ	<LOQ
Column 6 - port 1	14	32.80	51
Column 6 - port 2	14	<LOQ	<LOQ
Column 6 - port 4	14	<LOQ	<LOQ
Column 7 - port 1	14	33.90	30.7
Column 7 - port 2	14	<LOQ	<LOQ
Column 7 - port 4	14	<LOQ	<LOQ
Column 8 - port 1	14	37.50	32.4
Column 8 - port 2	14	<LOQ	<LOQ
Column 8 - port 4	14	<LOQ	<LOQ
Column 9 - port 1	14	28.40	40.5
Column 9 - port 2	14	<LOQ	0.145
Column 9 - port 4	14	<LOQ	<LOQ

Column 1 - port 1	28	41.70	32.1
Column 1 - port 2	28	<LOQ	0.901
Column 1 - port 4	28	<LOQ	<LOQ
Column 2 - port 1	28	29.90	25.3
Column 2 - port 2	28	<LOQ	<LOQ
Column 2 - port 4	28	<LOQ	<LOQ
Column 3 - port 1	28	20.70	30.2
Column 3 - port 2	28	<LOQ	<LOQ
Column 3 - port 4	28	<LOQ	<LOQ
Column 4 - port 1	28	22.70	21
Column 4 - port 2	28	<LOQ	<LOQ
Column 4 - port 4	28	<LOQ	<LOQ
Column 5 - port 1	28	32.00	33.3
Column 5 - port 2	28	<LOQ	<LOQ
Column 5 - port 4	28	<LOQ	<LOQ
Column 6 - port 1	28	11.80	25.4
Column 6 - port 2	28	<LOQ	<LOQ
Column 6 - port 4	28	<LOQ	<LOQ
Column 7 - port 1	28	20.70	11.4
Column 7 - port 2	28	<LOQ	<LOQ
Column 7 - port 4	28	<LOQ	<LOQ
Column 8 - port 1	28	30.30	20.5
Column 8 - port 2	28	<LOQ	<LOQ
Column 8 - port 4	28	<LOQ	<LOQ
Column 9 - port 1	28	31.10	-
Column 9 - port 2	28	<LOQ	0.149
Column 9 - port 4	28	<LOQ	<LOQ

B.3.2 Bioinformatic analysis

Table B3: Read counts throughout the upstream workflow in the prokaryotic community analysis.

Sample	Input	Filtered	Denoised F	Denoised R	Merged	No-chimeras	Reads (%)
Digestate1	77443	56966	55253	55595	53498	53311	68.8
Digestate2	49192	38592	36882	37219	35514	35427	72
Digestate3	94492	70677	68347	68799	66148	65834	69.7
Mixture1	76650	59651	56605	57272	53623	49459	64.5

Mixture2	98347	79730	76075	76958	72519	66489	67.6
Mixture3	136989	103423	100401	101233	97124	85908	62.7
C1Day1	80420	59632	57329	57823	55167	53856	67
C2Day1	101038	70505	68352	68662	66117	64251	63.6
C3Day1	73981	54988	52845	53448	50780	49430	66.8
C4Day1	127834	91168	88285	88937	85693	83067	65
C5Day1	104101	74487	72241	72626	69843	67951	65.3
C6Day1	129944	88912	86266	86681	83270	80306	61.8
C7Day1	116253	84701	82539	83204	79436	59729	51.4
C8Day1	101285	72974	70088	70798	67591	65634	64.8
C9Day1	107074	77908	74625	75337	71754	69248	64.7
C1Day14	44848	40219	39137	39252	37645	37291	83.1
C2Day14	56599	45268	43701	44094	41924	41694	73.7
C3Day14	106861	83946	80978	81365	78488	77612	72.6
C4Day14	82769	65427	62744	63191	60750	60157	72.7
C5Day14	100703	78405	74648	75435	72250	66815	66.3
C6Day14	50938	39040	36592	37180	34840	33382	65.5
C7Day14	78077	59033	56217	56815	54276	50376	64.5
C8Day14	79100	61233	58528	59115	56539	52815	66.8
C9Day14	35181	26754	25281	25625	24108	23182	65.9
C1Day28	80570	59877	56987	57434	54559	51231	63.6
C2Day28	211092	167231	161740	163009	157225	141030	66.8
C3Day28	71536	54724	51917	52473	49818	46576	65.1
C4Day28	99606	73166	69810	70471	67081	61896	62.1
C5Day28	80743	59033	55634	56209	53270	48362	59.9
C6Day28	145748	104818	101406	102221	98474	89247	61.2
C7Day28	58263	43316	41083	41590	39412	37202	63.9
C8Day28	50958	37645	35532	35957	34072	32136	63.1
C9Day28	39197	28702	27164	27515	26035	25103	64

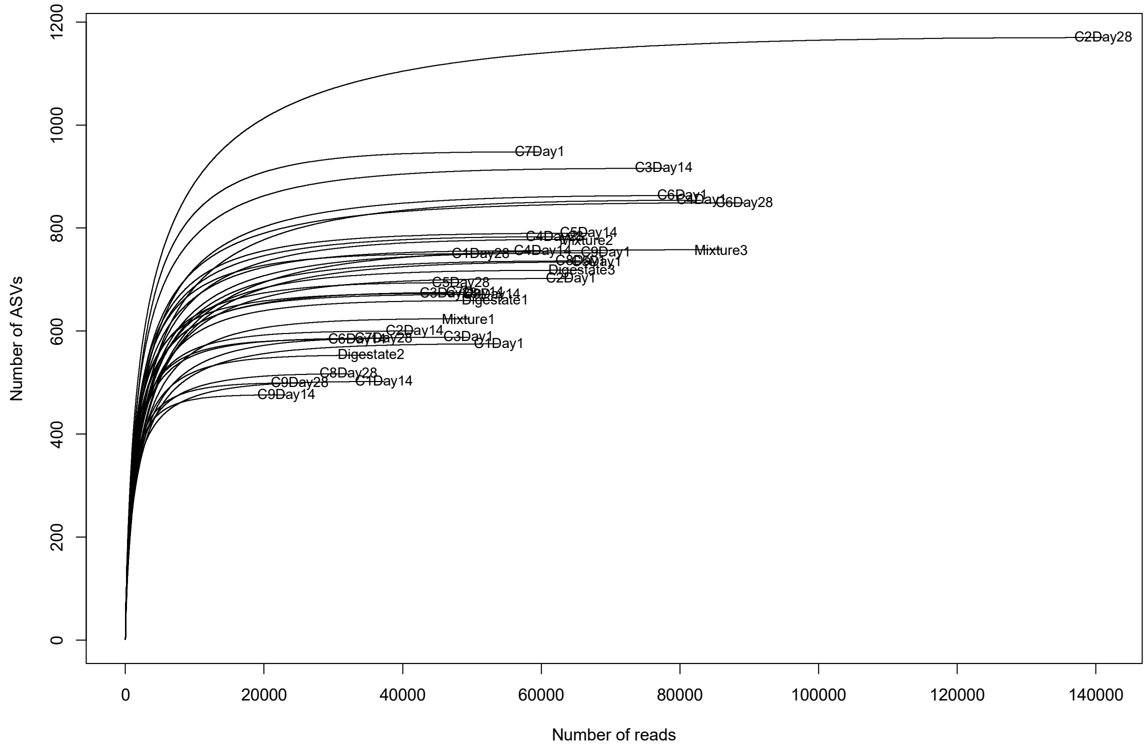


Figure B1: Rarefaction curves of the initial substrate samples and the samples from the first soil column layers for the 16S rRNA gene dataset.

Below are reported the statistical analyses carried out for alpha-diversity of the samples' prokaryotic community. Non-parametric Kruskal-Wallis test was conducted to assess the effects of experimental condition and time factors on the Shannon and inverse Simpson alpha-diversity indices. A non-parametric Wilcoxon pairwise comparison test was conducted to assess which groups within the factors were significantly different.

Table B4: Kruskall-Wallis non-parametric test on Shannon, Inverse Simpson and Chao indices evaluating experimental condition and time factors.

Test	*Shannon index by experimental condition*
statistic Kruskal-Wallis chi-squared	9.33
parameter	4
p.value	0.0533
method	Kruskal-Wallis rank sum test

Test	*Inverse Simpson index by experimental condition*
statistic Kruskal-Wallis chi-squared	1.46
parameter	4
p.value	0.834
method	Kruskal-Wallis rank sum test
Test	*Chao index by experimental condition*
statistic Kruskal-Wallis chi-squared	12.1
parameter	4
p.value	0.0168
method	Kruskal-Wallis rank sum test
Test	*Shannon index by time*
statistic	21.77
parameter	3
p.value	0.00007
method	Kruskal-Wallis rank sum test
Test	*Inverse Simpson index by time*
statistic	24.49
parameter	3
p.value	0.00002
method	Kruskal-Wallis rank sum test
Test	*Chao index by time*
statistic	1.86
parameter	3
p.value	0.602
method	Kruskal-Wallis rank sum test

Table B5: Wilcoxon rank sum test pairwise comparison for Shannon and Inverse Simpson indices between experimental condition and time factor levels.

Wilcoxon rank sum test pairwise comparison: Shannon index and experimental condition				
Experimental condition	Dig	Lmt	Met	Digestate
Lmt	0.931	-	-	-
Met	0.931	0.931	-	-
Digestate	0.127	0.127	0.470	-
Amended Soil	0.091	0.091	0.121	0.571
Wilcoxon rank sum test pairwise comparison: Inverse Simpson index and experimental condition				
Experimental condition	E-Dig	E-Lmt	E-Met	Digestate
E-Lmt	0.483	-	-	-
E-Met	0.741	1.0	-	-
Digestate	0.030	0.127	0.208	-
Amended Soil	0.030	0.030	0.045	0.167

Wilcoxon rank sum test pairwise comparison: Shannon index and time			
Time (day)	0	1	14
1	0.327	-	-
14	0.00074	0.00074	-
28	0.00074	0.00074	0.489
Wilcoxon rank sum test pairwise comparison: Inverse Simpson index and time			
Time (day)	0	1	14
1	0.04316		

14	0.0006	0.00025	-
28	0.0006	0.00025	0.93143

Below are reported the statistical analysis carried out for the beta-diversity assessed for the prokaryotic community characterization. PERMANOVA (NMDS method with Bray-Curtis distance) coupled with permanova pairwise comparison test was conducted to evaluate the effects of experimental condition and time factors on the beta diversity of the samples.

Table B6: PERMANOVA and pairwise comparison test results for the beta diversity of the prokaryotic communities.

Parameters	Df	SS	R2	F	Pr (>F)
Main effects and interaction term					
Time (day)	2	1.28	0.27	5.07	0.001
Experimental condition	4	1.96	0.41	15.5	0.001
Time*Experimental condition	4	0.19	0.04	0.77	0.669
Residual	22	1.39	0.29	NA	NA
Total	32	4.82	1.000	NA	NA
Main effects					
Time (day)	2	1.28	0.27	5.26	0.001
Experimental condition	4	1.96	0.41	16.1	0.001
Residual	26	1.58	0.33	NA	NA
Total	32	4.82	1.000	NA	NA
Time single main effect					
Time (day)	3	2.93	0.61	14.9	0.001
Residual	29	1.89	0.39	NA	NA

Total	32	4.82	1.00	NA	NA
Experimental condition single main effect					
Experimental condition	4	1.28	0.27	2.53	0.012
Residual	28	3.54	0.73	NA	NA
Total	32	4.82	1.000	NA	NA

Pairwise comparison by time levels

Pairs	Df	SS	F.Model	R2	p.value	p.adjusted
0 vs 1	1	0.19	3.45	0.21	0.001	0.006
0 vs 14	1	1.39	22.4	0.63	0.001	0.006
0 vs 28	1	1.34	17.7	0.58	0.001	0.006
1 vs 14	1	1.46	26.0	0.62	0.001	0.006
1 vs 28	1	1.40	20.7	0.56	0.001	0.006
14 vs 28	1	0.07	0.86	0.96	0.385	1.000

Below are reported abundance results for the composition of the microbial community.

Table B7**:** Abundance and percental abundance of ASVs for the different domains characterizing the soil microbial community.

Kingdom	Abundance	Abundance (%)
All	1916704	100
Bacteria	1911560	99.7
Archeae	2744	0.14
Unclassified	2400	0.13

Table B8**:** Abundance and percental abundance of ASVs of the archaea groups characterizing the archaea domain within the soil microbial community.

	Digestate		Amended soil	
	Abundance	Abundance (%)	Abundance	Abundance (%)
Archeae domain	125	100	544	100
Methanobacteriaceae	65	52	204	37.5
Methanomicrobiaceae	39	31.2	219	40.3
Nitrososphaeraceae	21	16.8	121	22.2

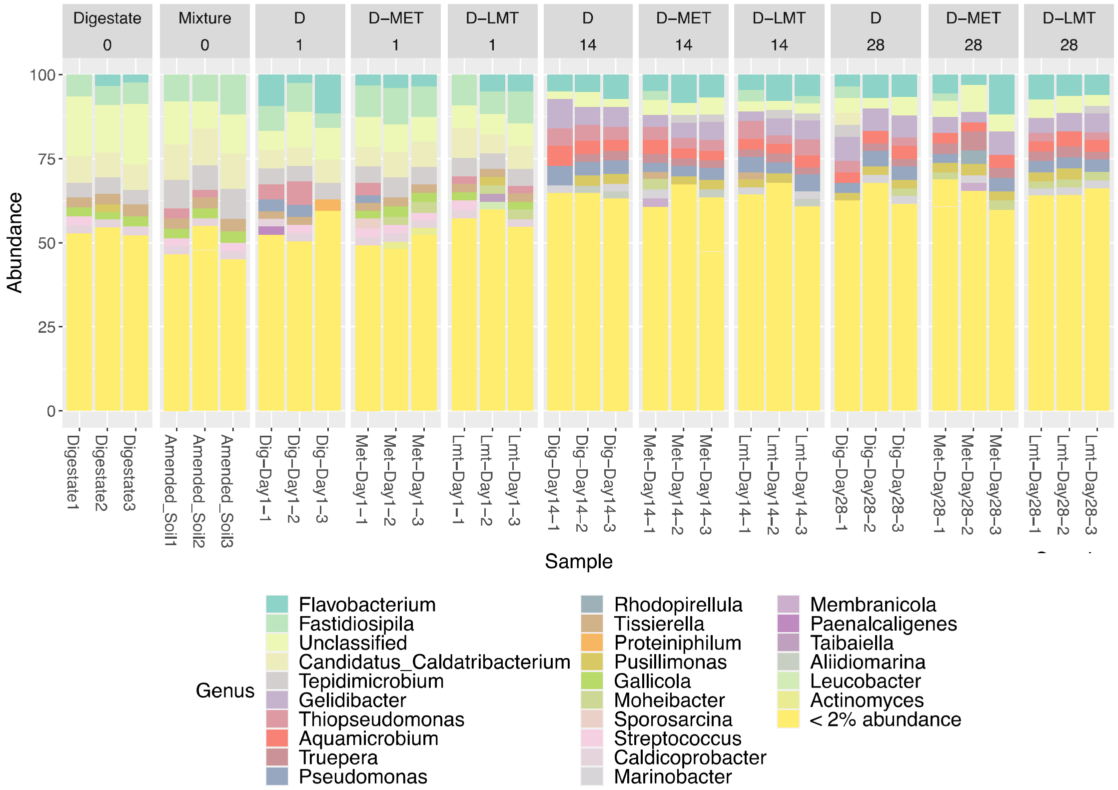


Figure B2: Taxonomic profiles at the genus level of the bacterial community of digestate, amended soil and samples taken from the first soil column layer over time.

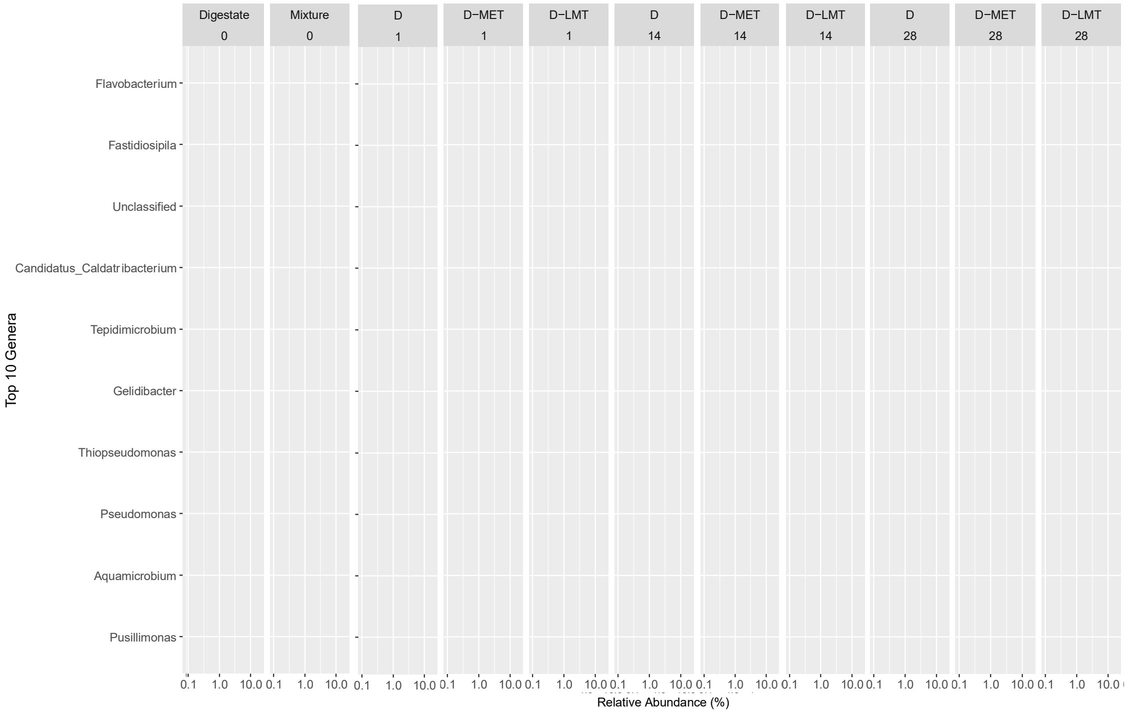
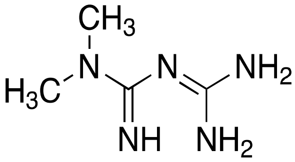
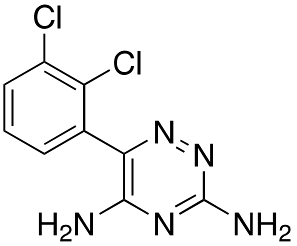


Figure B3: Taxonomic profiles of the top 10 genera of the bacterial communities of initial digestate, initial amended soil and samples taken from the first soil column layer over time (day 1, day 14 and day 28) for the different experimental conditions (without (D) or with spiking of lamotrigine (D-LMT) or metformin (D-MET), expressed in terms of relative abundance (%).

Appendix C

C.1 Soil, digestate and amended soil characterization

Table C1: Physico-chemical properties of metformin (MET) and lamotrigine (LMT).

	Metformin (MET)	Lamotrigine (LMT)
Molecular Structure		
Molecular weight (g/mol)	165.62	256.09
pKa [1,2]	3.1 and 13.8	5.7
Water solubility (g/L)	350 (at 20 °C)	0.17 (at 25 °C)

[1] Hernández et al., 2015

[2] Williams, 2013

Methodology of soil and digestate characterization

Organic matter content was evaluated via Loss on Ignition (LOI) method, heating 2 g dry solid sample in a muffle furnace at 500°C for 4 h. Subsequently, the difference between the initial mass and the dried mass was calculated. Inorganic carbon content was measured by CHNS analysis after samples had been treated with 10 % hydrochloric acid (at 50 °C for 24h), while total organic carbon (TOC) content was measured as the difference between the TC and IC. The soil and digestate elemental composition (Table S2) was obtained by X-Ray Fluorescence using ED-XRD SPECTRO-XEPOS (model XEP05), on a grinded soil sample and SD samples pre-treated at 550 °C, respectively.

Table C2: Composition of mineral fraction of soil and digestate (determined as described in Baldasso et al (3)). Adapted from Baldasso et al. (3).

	Soil	Digestate
	(wt%)	(wt%)
Al_2O_3	23.5	7.6
CaO	1.3	9.9
Fe_2O_3	5.7	1.5
K_2O	2.7	1.2
MgO	0.3	3.8
Na_2O	0.1	< LOQ
P_2O_5	0.1	1.1
SiO_2	58.1	34.9
SO_3	0.3	2

Table C3: Total trace metals contents in soil and amended soil (mean ± std, n=3) (determined as described in Baldasso et al (3)).

Sample	Soil	Digestate	Amended Soil
Metal	Total C (µg/g)	Total C (µg/g)	Total C (µg/g)
Zn	18 ± 2	291 ± 30	113 ± 20
Pb	8.98 ± 0.08	142 ± 30	69 ± 2
Cu	34 ± 2	103 ± 70	69 ± 9
Cr	24 ± 10	35.3 ± 0.4	23 ± 5
Co	6 ± 2	5.6 ± 0.3	6.8 ± 0.7
Ni	11 ± 2	20 ± 4	15.5 ± 0.3
Fe	$(27 \pm 3)\cdot10^3$	$(8 \pm 2)\cdot10^3$	$(15 \pm 2)\cdot10^3$
Mn	46 ± 20	188 ± 50	90 ± 5

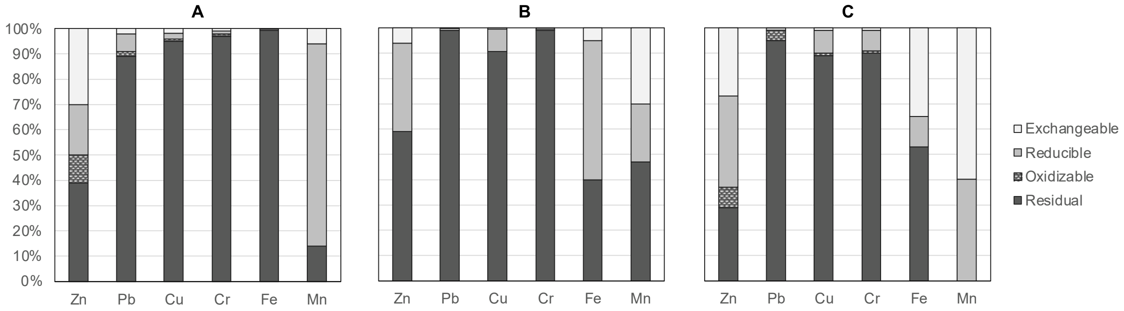


Figure C1: Trace metal fractionation in soil (A), digestate (B) and amended soil (C) (determined as described in Baldasso et al (3)).

Table C4: Released amounts of trace metals from soil, digestate and amended soil to ultrapure water as percentage of initial total trace metal content.

Metal	Sample	Released amount (%)
Pb	Soil	$(1.2 \pm 0.7)\cdot 10^{-3}$
	Digestate	0.07 ± 0.04
	Amended Soil	0.004 ± 0.003
Ni	Soil	$(3.81 \pm 0.02)\cdot 10^{-3}$
	Digestate	3.5 ± 0.1
	Amended Soil	0.094 ± 0.008
Cr	Soil	0.018 ± 0.002
	Digestate	0.47 ± 0.05
	Amended Soil	$(1.45 \pm 0.09)\cdot 10^{-2}$
Co	Soil	0.2 ± 0.1
	Digestate	14 ± 2
	Amended Soil	0.39 ± 0.05
Cu	Soil	0.007 ± 0.002
	Digestate	1.0 ± 0.3
	Amended Soil	$(5.1 \pm 0.6)\cdot 10^{-2}$
Zn	Soil	0.1 ± 0.2
	Digestate	0.18 ± 0.07
	Amended Soil	$(9.6 \pm 0.5)\cdot 10^{-3}$

C.2 Sorption kinetic models

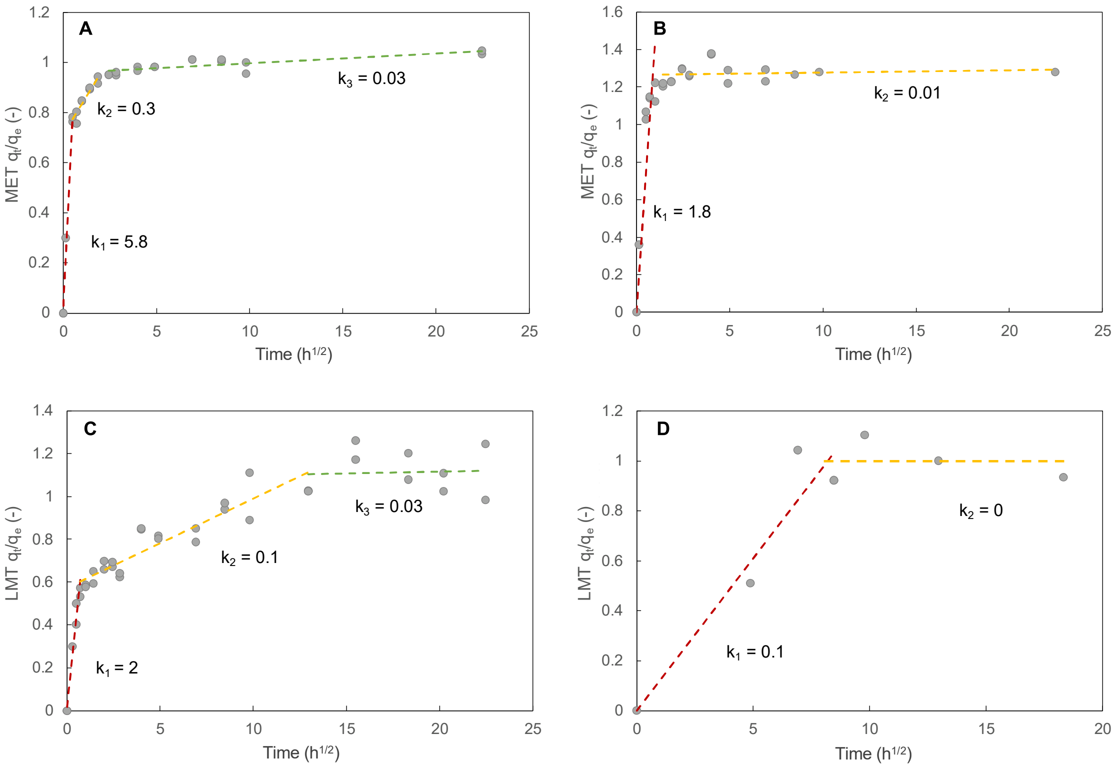


Figure C2: Intraparticle diffusion model for metformin (MET; A and B) and lamotrigine (LMT; C and D) adsorption onto amended soil and soil, respectively. The plots show the multi-linearity trend with k_1, k_2, k_3 (μg/g/h$^{1/2}$) being the model constant rates.

Note: The data are plotted as the ratio of the adsorbed amount (q_t) and the equilibrium adsorbed amount (q_e) against the square root of time.

C.3 Adsorption experiments

For MET adsorption experiments (Appendix - C, Figure C3), in the condition of a 1/1 ratio, MTE adsorbed amounts were significantly lower than their respective reference adsorbed amounts in absence of MET, for all metals (-72% for Ni^{2+} and Co^{2+}, -75% for Cr^{3+}, -69% for Cu^{2+} and -68% for Zn^{2+}) with exception for Pb^{2+} that had an adsorbed amount equal to its reference (5.2 µg/g). This may be explained by considering Pb's greater affinity to Mn- and Fe-oxides compared to the other MTE and Pb precipitation caused by the formation of Pb-hydroxides and Pb-phosphates at alkaline pH (4,5). While, for the other MTE, having less affinity for oxides and soil mineral phases and greater affinity for soil organic matter (4,6), the behaviour can be explained taking into consideration possible competitive interactions between MET and MTE for soil's organic matter sorption sites, resulting in the prevailing adsorption of MET.

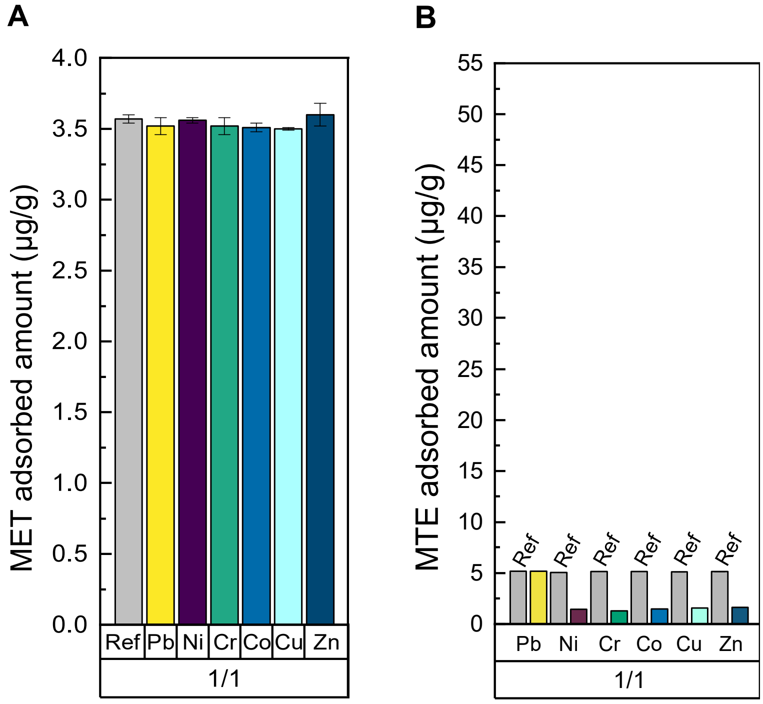


Figure C3: (A) Metformin (MET) adsorbed amount on amended soil in the presence of MTE (mean and standard deviation, n=3) and (B) the respective MTE adsorbed amount at pharmaceutical/metal ratios of 1/1 (mean and standard deviation, n=3). The results are compared with the adsorbed amount of metformin and MTE measured in systems without MTE and metformin, respectively (Reference, gray bars).

For LMT adsorption experiments (Appendix - C, Figure C4), with a 1/1 pharmaceutical/metal ratio, LMT adsorbed amounts in presence of all MTE were significantly lower (decrease of about 20 %) than the adsorbed amount in absence of MTE (2.4 µg/g). Furthermore, MTE adsorbed amounts were lower than in the absence of LMT, for all metals (-72% for Ni^{2+} and Co^{2+}, -75% for Cr^{3+} and -69% for Cu^{2+} and Zn^{2+}) with exception of Pb^{2+}, as observed for MET. As discussed above, this result may be due to Pb^{2+} greater affinity to Mn- and Fe-oxides compared to the other MTE considered and its possible precipitation in alkaline environments.

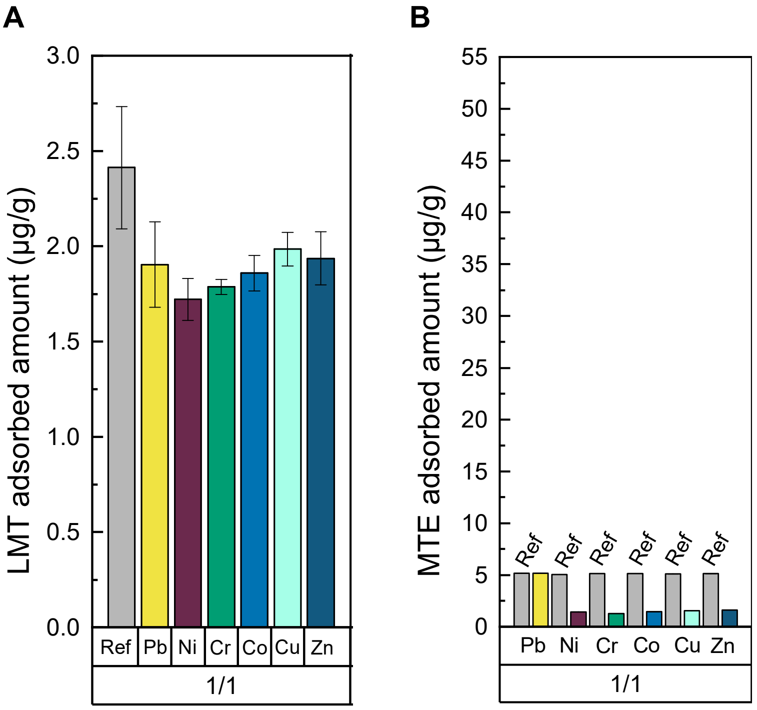


Figure C4: (A) Lamotrigine (LMT) adsorbed amount on amended soil in the presence of MTE (mean and standard deviation, n=3) and (B) the respective MTE adsorbed amount at pharmaceutical/metal ratios of 1/1 (mean and standard deviation, n=3). The results are compared with the adsorbed amount of lamotrigine and MTE measured in systems without MTE and lamotrigine, respectively (Reference, gray bars).

C.4 ICP-MS analysis

Table C5: MTE isotope selected for ICP-MS analysis that resulted in the least interference and highest abundance.

MTE	Y	Pb	Ni	Cr	Co	Cu	Zn
MTE Isotope	89	208	60	52	59	63	66

C.5 Desorption experiments

The results for MTE desorption from soil and amended soil are reported in Figure C5 (Appendix - C). The graph compares the desorbed MTE amounts in ultrapure water with those in 0.01 mol/L $CaCl_2$ solution which was used for rainfall simulation. MTE release from soil and amended soil was greater when applying the 0.01 mol/L $CaCl_2$ solution for all MTE analysed with exception of Cr^{3+}, implying a potential risk of MTE mobility under natural rainfall conditions. MTE release from soil was low for Pb^{2+}, Ni^{2+} and Co^{2+}, while it was higher for Cr^{3+}, Cu^{2+} and Zn^{2+}. In addition, MTE release from amended soil was significant for all MTE considered with exception of Pb^{2+}. This can be correlated to metal fractionation results, which showed an increase in MTE less immobile forms, as exchangeable in amended soil due to digestate application, with exception for Pb^{2+} that increased in the immobile residual fraction (Figure C1). Furthermore, no amount of MET and LMT was detected in the analysed solution, meaning that the compounds were either degraded or present at concentrations lower than the instrument's detection limit.

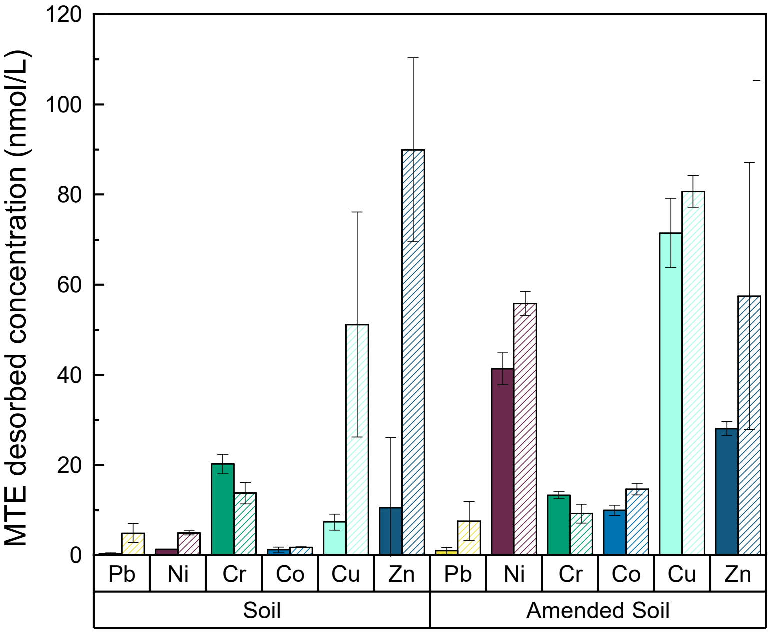


Figure C5: Desorbed amount of MTE from soil and amended soil using ultrapure water (solid colour bars) and 0.01 mol/L $CaCl_2$ solution (striped colour bars).

Table C6: Adsorbed and desorbed amounts and desorption percentages of metformin (MET), lamotrigine (LMT) and copper (Cu2+) for the different experimental conditions tested (mean ± std, n=3).

Condition		MET in presence of Cu^{2+}		
		MET adsorbed amounts (µg/g)	MET desorbed amounts (µg/g)	Desorbed amounts (%)
Soil	1/1	1.18 ± 0.03	0.62 ± 0.07	53 ± 7
	1/100	2.14 ± 0.01	0.38 ± 0.01	18.0 ± 0.6
Amended soil	1/1	3.47 ± 0.03	0.80 ± 0.05	23 ± 1
	1/100	4.02 ± 0.01	0.08 ± 0.01	2.0 ± 0.3
Condition		LMT in presence of Cu^{2+}		
		LMT adsorbed amounts (µg/g)	LMT desorbed amount s(µg/g)	Desorbed amounts (%)
Soil	1/1	0.5 ± 0.1	0.17 ± 0.00	35 ± 9
	1/100	0.34 ± 0.06	0.21 ± 0.06	63 ± 9
Amended soil	1/1	3.3 ± 0.5	0.88 ± 0.06	28 ± 6
	1/100	3.5 ± 0.1	0.99 ± 0.07	29 ± 2
Condition		Cu^{2+} in presence of MET or LMT		
		Cu adsorbed amounts (µg/g)	Cu desorbed amounts (µg/g)	Desorbed amounts (%)
Soil - MET	1/1	1.579 ± 0.003	0.010 ± 0.003	0.6 ± 0.2
	1/100	158.818 ± 0.002	0.023 ± 0.005	0.015 ± 0.003
Soil - LMT	1/1	1.583 ± 0.002	0.02 ± 0.01	1.5 ± 0.6
	1/100	158.841 ± 0.004	0.020 ± 0.007	0.012 ± 0.004
Amended soil - MET	1/1	1.545 ± 0.003	0.016 ± 0.001	1.03 ± 0.06
	1/100	158.40 ± 0.04	0.120 ± 0.009	0.08 ± 0.01
Amended soil - LMT	1/1	1.550 ± 0.001	0.021 ± 0.007	1.3 ± 0.4
	1/100	158.50 ± 0.02	0.092 ± 0.009	0.06 ± 0.01

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Appendix D

D.1 Numerical Method

The upwind scheme formulation, considering the competitive Langmuir model (Chapter 5, equation 2 and 3) for the sorption term, is reported below for an arbitrary soil layer. The subscript *i* defines the space level, and *j* and *j+1* define the known and unknown time levels. is the time step and is the step size along the vertical direction of flow.

The upwind scheme formulation for the ADEs system for two contaminants including the equilibrium competitive Langmuir adsorption term in a generic soil layer is:

$$R_{1,i}\left(\frac{C_{1,i}^{j+1}-C_{1,i}^{j}}{\Delta t}\right)=D\left(\frac{C_{1,i+1}^{j}-2\,C_{1,i}^{j}+C_{1,i-1}^{j}}{\Delta z^2}\right)-\frac{q}{\theta}\left(\frac{C_{1,i}^{j}-C_{1,i-1}^{j}}{\Delta z}\right)$$

$$R_{2,i}\left(\frac{C_{2,i}^{j+1}-C_{2,i}^{j}}{\Delta t}\right)=D\left(\frac{C_{2,i+1}^{j}-2\,C_{2,i}^{j}+C_{2,i-1}^{j}}{\Delta z^2}\right)-\frac{q}{\theta}\left(\frac{C_{2,i}^{j}-C_{2,i-1}^{j}}{\Delta z}\right)$$

$$\left(\frac{S_{1,i}^{j+1}-S_{1,i}^{j}}{\Delta t}\right)=\frac{K_{L,1}\cdot C_{1,i}\cdot S_{max}}{1+K_{L,1}\cdot C_{1,i}+K_{L,2}\cdot C_{2,i}}$$

$$\left(\frac{S_{2,i}^{j+1}-S_{2,i}^{j}}{\Delta t}\right)=\frac{K_{L,2}\cdot C_{2,i}\cdot S_{max}}{1+K_{L,1}\cdot C_{1,i}+K_{L,2}\cdot C_{2,i}}$$

Similarly, the upwind formulation for the ADEs system for two contaminants including the time-dependent competitive Langmuir adsorption term in a generic soil layer is:

$$\left(\frac{C_{1,i}^{j+1}-C_{1,i}^{j}}{\Delta t}\right)=D\left(\frac{C_{1,i+1}^{j}-2\,C_{1,i}^{j}+C_{1,i-1}^{j}}{\Delta z^2}\right)-\frac{q}{\theta}\left(\frac{C_{1,i}^{j}-C_{1,i-1}^{j}}{\Delta z}\right)$$
$$-\frac{\rho}{\theta}\left(k_{a,1}\frac{\theta}{\rho}C_{1,i}^{j}\left(S_{max}-S_{1,i}^{j}-S_{2,i}^{j}\right)-k_{d,1}S_{1,i}^{j}\right)$$

$$\left(\frac{C_{2,i}^{j+1} - C_{2,i}^{j}}{\Delta t}\right) = D\left(\frac{C_{2,i+1}^{j} - 2\,C_{2,i}^{j} + C_{2,i-1}^{j}}{\Delta z^2}\right) - \frac{q}{\theta}\left(\frac{C_{2,i}^{j} - C_{2,i-1}^{j}}{\Delta z}\right)$$
$$- \frac{\rho}{\theta}\left(k_{a,2}\frac{\theta}{\rho}C_{i}^{j}\left(S_{max} - S_{1,i}^{j} - S_{2,i}^{j}\right) - k_{d,2}S_{2,i}^{j}\right)$$

$$\left(\frac{S_{1,i}^{j+1} - S_{1,i}^{j}}{\Delta t}\right) = k_{a,1}\frac{\theta}{\rho}C_{1,i}^{j}\left(S_{max} - S_{1,i}^{j} - S_{2,i}^{j}\right) - k_{d,1}S_{1,i}^{j}$$

$$\left(\frac{S_{2,i}^{j+1} - S_{2,i}^{j}}{\Delta t}\right) = k_{a,2}\frac{\theta}{\rho}C_{2,i}^{j}\left(S_{max} - S_{1,i}^{j} - S_{2,i}^{j}\right) - k_{d,2}S_{2,i}^{j}$$

Appendix E

This appendix presents the report on the collaboration carried out between the PhD candidate, Veronica Baldasso, and Tratolixo, a Portuguese municipal solid waste management and treatment company, industrial partner of the M2ex project.

The company secondment had a 1 month duration, starting on 02/02/2021 and ending on 02/03/2021. The secondment was conducted in a hybrid format due to Covid-19 pandemic. The activities carried out during the collaboration were weekly online meetings between the PhD candidate and the Tratolixo managers (plant manager and process control manager), and a three-day site visit to the company's waste treatment plant.

Going more into detail, the first meeting was an introductory meeting to meet Tratolixo's plant manager, Ricardo Castro, and process control manager, Maria João Alves, and plan the secondment activities. The following meetings were conducted to learn and understand the waste treatment plant operations and all the process units installed in the treatment plant. The meetings gave the possibility to interact directly with the managers and discuss in detail the operations and management of the plant, specifically the anaerobic digestion and the composting unit. During the secondment, also operational documents and guidelines were shared to learn the plant's processing units' specifics. The secondment was concluded with a complete site visit to Tratolixo waste treatment plant located in Abrunheira. The visit included an in-depth presentation about the company and the company's plant site, a tour of the entire treatment plant, including the analytical laboratories, and in-line sample collection for the planned academic research experiments.

The collaboration with Tratolixo continued throughout the entire first year of PhD research by sharing experimental data and laboratory results to observe possible time-dependent variations digestate characterization and compare different laboratory analysis techniques.

The collaboration with Tratolixo was of fundamental importance for the present research work for it enabled to have access to digestate originating from the organic fraction of municipal solid waste needed to conduct all the experiments throughout the PhD research project.

Appendix F

This appendix presents the report on the collaboration carried out between the PhD candidate, Veronica Baldasso, and C&F Energy Società Agricola, an Italian organic waste management and treatment company focused on anaerobic digestion, industrial partner of the M2ex project.

The company secondment had a 1 month duration, starting on 01/10/2023 and ending on 31/10/2021.

During the company secondment, regular meetings were carried out between the PhD candidate and major stakeholders of C&F Energy Società Agricola to get to know the company's line of work and expertise, and to learn the operational units and processes installed in the company's waste treatment plants. The secondment was mainly focused on designing an experimental plan for a long-term field-scale experiment to study the effects of long-term land application of digestate on soil quality and trace metal fate in the soil system.

The experiment proposal type-up is reported below.

LONG TERM DIGESTATE FIELD APPLICATION TO EVALUATE THE EFFECTS ON SOIL QUALITY, TRACE METAL AGING AND TRANSPORT THROUGH THE SOIL SYSTEM

Collaborators:

Veronica Baldasso
PhD student
FCUP and CIIMAR, Porto, Portugal
UNINA, Naples, Italy

C&F Energy Società Agricola S.r.l
Angri, Salerno, Italy

1. Introduction

The utilization of digestate as an organic soil amendment has gained prominence in sustainable agriculture, especially since it has been incorporated in the list of approved soil amendments of the EU (ref). While short-term effects have been investigated at laboratory scale through soil column and pot experiments, there is a critical knowledge gap regarding the long-term impacts of digestate application on soil quality and the fate and transport of contaminants within the soil after its application at field level. Thus, it is critical to have access to field data not only to validate lab findings but also to gain deeper insights on the mechanism at play in a real-case scenario of digestate application to land. In this context, the proposed experiment aims to address this gap, providing long term field data that are crucial to complete the knowledge around the use of digestate as soil amendment and give more insights to develop more sustainable agricultural practices. The proposed experiment will be conducted in collaboration with C&F Energy Società Agricola, waste treatment company specialized in anaerobic digestion processes, who will give access to land and supply the digestate that will be used in the experiments. Through this collaboration, the researchers will be able to perform field experiments gathering all the data of interest while the company will explore a different and competitive strategy to re-utilize their digestate, potentially opening up new market opportunities.

2. Aims and Objectives

The aims and the objectives of the experiment proposal are the following:

Evaluate changes in soil physico-chemical properties, including structure, texture, pH, cation exchange capacity, organic matter content, nutrient levels, trace metals concentration, other contaminant concentrations) over an extended period of time after digestate application.

Investigate the impact of long-term digestate application on the soil microbial community, evaluating any shifts in compositions.

Investigate trace metal aging in digestate amended soil throughout the experiment duration by analysing their concentration and speciation, assessing their change in bio/availability and mobility in defined soil horizons over time.

Evaluate the mechanisms that govern trace metal behaviour in long-term digestate amended soil and assess their transport in the soil system, looking as well at their leaching potential to groundwater.

Conduct a risk assessment on the use of digestate as soil amendment using all the data gathered from the experiment and evaluate the feasibility of bring the digestate to the market.

3. Methodology

The proposed experiment will be conducted over an extended period of time, involving controlled digestate applications to specifically designated field plots. During the experiment duration, soil samples will be collected at regular intervals of time to assess the changes in the soil physico-chemical properties and trace metal behaviours by using numerous analytical techniques.

The proposed experimental plan is the following:

- Experiment duration: 1 to 2 years (long-term)
- Number of land plots: will be defined based on the number of experimental conditions chosen, following a factorial experimental design strategy (to be discussed more in detail, e.g. experimental conditions may differ based on the number of digestate applications, making sure that the final digestate loading rate is equal or they may differ on the digestate loading rate).

  It is important to consider at least 3 plots of land for every experimental condition to have statistically significant results.

  The plots of land should be 1 m^2 to have enough space to gather all necessary samples without generating soil perturbation.

  The plots of land for each experimental condition should be randomly allocated (for statistical significance) in the given space.
- Complete physico-chemical characterization of the digestate selected for the experiment and the soil of the land where the experiment is going to be set-up.
- Soil sampling will be conducted every month through core drilling (small enough to cause no soil perturbations and big enough to obtain samples for all required analysis).

  The samples will be divided by soil horizons using, subsampled using sterile and clean soil tools, placed in appropriate vessels (samples for microbiology will be placed in sterile containers) and stored immediately at -20° until further processing. See methodology for details.
- The weather forecast will be recorded, noting down at least temperature and rainfall.
- Soil properties, specifically moisture, water content, pH and cation exchange capacity will be taken every week using portable soil probes.

The proposed methodology for the experimental plan is the following:

- Classical soil characterization analysis, evaluating water content, pH, cation exchange capacity, organic matter content, and elemental composition.
- Physical characterization using microscopy techniques to evaluate the soil's and the digestate's structure and texture throughout the experiment duration.
- Analysis of trace metal content using acid digestion followed by atomic absorption spectroscopy or ICP-MS, and analysis of trace metal fractionation using the BCR fractionation method followed by atomic absorption spectroscopy or ICP-MS.
- Data analysis, including statistical data analysis centred on ANOVA based methods and correlation analysis, to uncover influential factors affecting trace metal behaviour in the soil system under investigation.
- Microbial community analysis performed through DNA extraction, followed by rRNA sequencing and bioinformatic data analysis. (Optional. Recommended if data on soil microbial community through time is needed.)

It is important to consider that changes can be made to the proposed experimental plan and methodology based on modifications to the defined aims and objectives, land/space availability, and available laboratory equipment.

4. Expected outcome and impact

This research aims to contribute valuable information for the involved company as well as for policymakers and environmental scientists. Anticipated outcomes include new knowledge and further understanding on long-term effects of digestate application on soil quality, trace metal aging, and trace metal transport mechanisms. The findings will help the company develop new strategies for sustainable digestate use, helping them become more sustainable.

5. Conclusions

By addressing the long-term implications of digestate application on soil, this experiment strived to advance the scientific understanding of the complex interactions within the soil system at a field scale. The knowledge gained will not only contribute to scientific findings but will also provide practical insights to support environmentally conscious and sustainable land restoration practices.