Transformation of contaminant metals in agricultural soil amended with digested sludge over time due to repeated plant growth

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The work presented in **Chapter 2** has been appeared in Publication 1. The work presented in **Chapter 3** is developed from the “Materials and Methods” section of Publications 2 and 3. The work presented in **Chapters 4 and 5** has been appeared in Publication 2. The work presented in **Chapter 6** has been appeared in Publication 3.

1. Jianting Feng*, Ian T. Burke, Xiaohui Chen, Douglas I. Stewart. 2023. Assessing metal contamination and speciation in sewage sludge: implications for soil application and environmental risk. *Reviews in Environmental Science and Bio/Technology*. 22: 1037-1058. https://doi.org/10.1007/s11157-023-09675-y.
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3. Jianting Feng*, Ian T. Burke, Felipe E. Sepulveda Olea, Xiaohui Chen, Douglas I. Stewart. 2025. Distribution and speciation of Cu and Zn near spring barley (*Hordeum vulgare*) roots in digested sewage sludge-amended soil. *Journal of Environmental Management* (under review).

In publications 1-3, Jianting Feng carried out the project conceptualisation, literature review, experimental methodology design, data collection, formal analysis, and writing-original draft. Ian T. Burke, Felipe E. Sepulveda Olea, Xiaohui Chen and Douglas I. Stewart contributed to the writing-review and editing of the publications and provided assistance with XAS data collection.

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Various interesting definitions on ‘‘PhD’’ can be found, such as “Patiently Hoping for a Degree”, “Permanent Head Damage”, and “Poor, Helpless Desperation”. Although they are just jokes for fun, they reflect a fact that it is not easy to pursue a PhD degree. There are lots of problems, difficulties, and challenges to be solved during the PhD journey. Personally, I cannot get rid of these difficulties without the limitless help and support of my supervisors. Therefore, I am grateful for my supervisors from the bottom of my heart. First, I would like to thank my primary supervisor, Professor Doug Stewart. I have been so impressed by his wisdom, enthusiasm, knowledge, and expertise. I have learned and grown a lot thanks to his guidance. I can still remember many of his wise words such as “tell an interesting and scientific story”, “say it once, say it right”. His constant encouragement gives me a lot of confidence on the completion of my PhD project with the highest quality. I would also like to thank my co-supervisor, Professor Ian Burke. His expertise and knowledge have significantly contributed to the direction of the laboratory experiments design and the project completion. Without his immense input I cannot succeeded in applying for the access to the beamtime at Diamond Light Source which is a very important part of this project. I am also very grateful for my co-supervisor, Dr Xiaohui Chen, for his great support and helpful suggestions.

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Abstract

The agricultural use of sewage sludge is a beneficial way for recycling the abundant plant nutrients and organic matter contained within it. Unfortunately, sewage sludge also contains modest levels of contaminant metals. The sludge application can lead to the gradual accumulation of contaminant metals in the agricultural soil, potentially causing environmental and health risks. Therefore, current regulations require the monitoring of total soil metal concentrations following sludge application to prevent introducing excessive metals to the soil. However, these controls are being increasingly considered unduly conservative as they fail to account for the transformation of metal behaviour and fate in sludge-soil/sludge-soil-plant systems on the risk assessments. To improve the agricultural use of sewage sludge and better assess the associated risk, it is necessary to comprehensively understand the transformation behaviour of metals in the systems. To achieve this aim, four key issues are identified and addressed in this project through a systematic literature review and a series of pot trials.

(1) Based on the most recently published data, it is estimated that the annual global production of sewage sludge may rise from ~53 million tons dry solids currently to ~160 million tons if global wastewater were to be treated to a similar level as in the 27 European Union countries/UK. Industrial wastewater, domestic wastewater and urban runoff are the three main sources of contaminant metals in sewage sludge. Conventional treatment processes generally result in the partitioning of over 70% of metals from wastewater into primary and secondary sludges. Typically, the order of metal concentrations in sewage sludge is Zn > Cu > Cr ≈ Pb ≈ Ni > Cd. The proportion of these metals that are easily mobilised is highest for Zn and Ni, followed by Cd and Cu, then Pb and Cr. The sludge application to agricultural land will lead to elevated metal concentrations, and potentially to short-term changes to the dominant metal species in the soil. However, the speciation of sludge-associated metals will change over time due to interactions with plant roots and soil minerals and as organic matter is mineralised by rhizo-microbiome.

(2) Substantial proportions of Cu and Zn can become incorporated into the sludge matrix after the amending of sewage sludge with soluble metal salts. Surfaces of original digested sludge are close to the saturation limit when the added ratio of metals is within 2% ($m_{\text{metals}}/m_{\text{dry sludge solids}}$). In the metal-

amended sludges, Cu is predominantly distributed in the oxidizable fraction when the added ratio is within 2%, which is similar to the original digested sludge (indicated by BCR data). There is no dominant speciation for Zn in the original digested sludge, but it is mainly distributed in the exchangeable fraction after initially adding Zn to the original digested sludge at a ratio of 0.4%. The proportion of exchangeable Zn fraction can reach over 90% when the added ratio is 5% or 8%.

(3) Cu and Zn in the original digested sludge are primarily in metal sulphide phases formed during anaerobic digestion. When the amended sludge is mixed with the soil, about 40% of Cu(I)-S phases and all Zn(II)-S phases in the amended sludge are converted to other phases (mainly Cu(I)-O and outer sphere Zn(II)-O phases). Further transformations occur over time, and with plant growth. After 18 weeks of plant growth, about 60% of the Cu added as Cu(I)-S phases is converted to other phases, with an increase in organo-Cu(II) phases. As a result, Cu and Zn extractability in the sludge-amended soil decrease with time and plant growth. Over 18 weeks, the proportions of Cu and Zn in the exchangeable fraction decrease from 36% and 70%, respectively, in the freshly amended soil, to 28% and 59% without plant growth, and to 24% and 53% with plant growth.

(4) In the digested sludge-amended soil, spring barley absorbs Cu only from the immediate vicinity of the roots (<< 1mm), but Zn is taken up from further afield (> 1mm). In the rhizosphere Cu is predominately present as Cu(I) oxides or as Cu(II) absorbed/bonded to phosphate, whereas Zn is present as Zn(II) in inner-sphere complexes with metal oxide surfaces, Zn(II) sulphides or Zn(II) bonded to/incorporated into carbonates. Cu taken-up by spring barley roots is largely sequestered in the root epidermis and/or cortex predominately in the coordination environments similar to those seen in the rhizosphere. Only a small proportion of the Cu is translocated into the vascular tissue (where it is in the same two bonding environments). Zn taken-up by spring barley roots is present as Zn(II) sulphides, Zn(II) absorbed to/incorporated into carbonates, or Zn(II)-organic complexes. Zn is readily translocated from roots to shoots. Better understanding of these differences in the mobility and uptake of Cu and Zn in sludge-amended agricultural soil could be used to undertake element specific risk assessments.

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List of Abbreviations

AAS	Atomic absorption spectrophotometer
AD	Anaerobic digestion
ANOVA	Analysis of variance
AS	Amended soil
ASL	Amended sludge
BCR	European Community Bureau of Reference
BOD	Biochemical oxygen demand
BSE	Backscattered electrons
BS_1R	Bulk soil from the first round
BS_2R	Bulk soil from the second round
BS_3R	Bulk soil from the third round
cm	Centimetre
CS_1R	Control soil from the first round
CS_2R	Control soil from the second round
CS_3R	Control soil from the third round
Cu-HA	Cu-humic acid complex
EDS	Energy dispersive spectrometer
EPMA	Electron probe microanalysis
EU-27	27 European Union Countries
h	Hour
K	Potassium
kg	Kilogram
LCF	Linear combination fitting
min	Minute
ml	Milliliter
Mt	Million tons
N	Nitrogen
NP	Nanoparticles
OM	Organic matter
OS	Original soil
OSL	Original sludge

P	Phosphorus
PS	Primary sludge
SEM	Scanning electron microscopy
SES	Secondary sludge
SS	Sewage sludge
SSM	Safe sludge matrix
WDS	Wavelength dispersive spectrometers
WWTPs	Wastewater treatment plants
(μ)XANES	(microfocus) X-ray absorption near edge structure spectra
XAS	X-ray absorption spectroscopy
XRF	X-ray fluorescence
yr	Year
Zn^{2+} (aq)	Aqueous Zn^{2+}
Zn-HFO	Zn-hydrous ferric oxide
Zn-O_{IS}	Inner sphere binding to metal oxide surfaces
Zn-O_{OS}	Outer sphere binding to metal oxide complexes
μm	micrometre
℃	Celsius
%	Percentage

Chapter 1: Introduction

1.1 Research background

Sewage sludge (SS) is a by-product of wastewater treatment (Janaszek et al., 2024). Many studies have revealed that this material contains valuable plant nutrients such as nitrogen (*N*), phosphorus (*P*), potassium (*K*), and organic matter (OM) (Eid et al., 2017; Latare et al., 2014; Manga et al., 2022; Martinez et al., 2003; Singh and Agrawal, 2007; Ye et al., 2022). It can be therefore applied to agricultural land to replace soil organic matter otherwise lost during tillage and to substitute mineral fertilisers (producing *N*-fertiliser is extremely energy intensive, and *P*-fertiliser is obtained from mineral reserves that are becoming scarce and are located in geo-politically sensitive regions) (Abreu et al., 2017; Lasheen and Ammar, 2009; Luo and Christie, 1998). Benefiting from its agricultural application, the pressure of disposing such a huge volume of SS can also be decreased (Iticescu et al., 2021). Unfortunately, SS also contains various contaminants, such as organic pollutants, pathogens, contaminant metals and other emerging contaminants (Kesari et al., 2021; Ye et al., 2022). Out of them, contaminant metals have become the primary obstacle for the agricultural application of SS. Typically, the metal concentrations in SS are higher than in the agricultural soil (Achkir et al., 2023; Cardoso et al., 2018; Karvelas et al., 2003; Rovira et al., 2011). As a result, the sludge application to agricultural soils will predictably elevate metal levels in the soil and those metals are likely to reside for long periods due to their resistance to biochemical degradation (Hasnine et al., 2017; Iloms et al., 2020; Kesari et al., 2021; Priya et al., 2023). Once excessive metals accumulation in the soil, they can threaten public health through contact, entry into the food chain and other ways (Nogueira et al., 2013; Parvin et al., 2022; Zhang et al., 2019; Zhang et al., 2024). In addition, the gradual accumulation of contaminant metals in the soil may lead to serious environmental problems, such as the changes in soil’s physico-chemical and biological properties and the deterioration of soil function, as well as the groundwater contamination caused by metals transport (Briffa et al., 2020; Lamastra et al., 2018; Obbard, 2001; Ullah et al., 2022).

Over the past decades, the potential secondary contamination of agricultural soil caused by sludge application has attracted the attention worldwide (particularly metal-related concerns) (Buta et al., 2021; Meng et al., 2016; Saha et al., 2017; Suanon et al., 2016; Tytla and Widziewicz-Rzońca, 2021). To ensure human health and environmental safety, many countries have produced relevant regulations on guiding SS utilisation in agriculture (Christodoulou and Stamatelatou, 2016; DEFRA, 2018; EPA, 2022). These regulations generally allow using SS in farming only if metal concentrations in SS do not exceed the permissible levels and require careful monitoring of bulk soil metal concentrations after SS use to ensure metal levels remain below the prescribed limits (DEFRA, 2018; Tytla, 2019). It is interesting that the “Safe Sludge Matrix (SSM)” regulation does not consider the impacts of contaminant metals during sludge application. Because it primarily focuses on mitigating microbiological risks to food safety, assuming that the existing sludge regulations sufficiently manage the metal-related concerns (SSM is included in the “Sludge Regulation”) (ADAS, 2001). Undoubtedly, these controls are sufficient to avoid introducing excessive metals to agricultural land. However, they will also restrict the application of SS as organic fertilisers at a time when there is a pressing need to increase the sustainability of agriculture (particularly barren soils), increase soil organic matter content, and reduce the dependence on mineral fertilisers (Feng et al., 2023). Also, where the monitoring is less effective, it is still possible to lead to the problem of excessive metals accumulating in the soil from the use of sludge.

Furthermore, these regulations have been increasingly viewed as simplistic and overly conservative, as they do not recognize that the risk posed by the metals is largely dependent on their chemical forms in the system rather than total metal concentrations (Parvin et al., 2022; Saffari et al., 2020; Tiberg et al., 2021; Uddin et al., 2024; Yang et al., 2013). For example, the metals present as exchangeable ions adsorbed to mineral surfaces are bio-accumulated by plants far more readily than those tightly bound to the mineral particles (Fernández-Ondoño et al., 2017; Nemati et al., 2009;

Sharma et al., 2017). The contaminant metals that are tightly bound to minerals generally do not cause any risk, as this fraction of metals has been proved to be highly stable to environmental conditions and not bioavailable for plants uptake (Lu et al., 2016; Sungur et al., 2021). Therefore, currently, there is a consensus that although the total metal concentration is a useful proxy for preventing the undue metal accumulation in agricultural soils (Fernández-Calviño et al., 2017; Zhou et al., 2019), it is poorly indicative of the information on what percentage of sludge-borne metals are bioavailable, and therefore are accessible to plants and can potentially cause environmental problems and health risks (Fadiran et al., 2014; Tytla, 2019; Tytla et al., 2016). To more reasonably evaluate the risk posed by the metals from sludge use in agriculture, apart from the monitoring of total metal concentrations, it is more important to obtain the information on metal speciation in the system following sludge application.

It should be noted that the chemical forms of contaminant metals in the sludge-amended soil are not constant (Zhang et al., 2017). They can evolve over time due to the alteration of soil properties and environmental conditions, as shown in Figure 1.1 (Seshadri et al., 2015). Not only will the organic matter with which the metal is initially associated be mineralised (metabolised by microorganism in the rhizosphere and eventually converted to CO_2), but organic chemicals exuded by the plants can complex metals and microbial metabolism will transform metal speciation (De Conti et al., 2018; Seshadri et al., 2015). Besides, the interactions between metals and other contaminants in SS can also influence the behaviour and fate of metals. For example, microplastics in SS can adsorb Pb, forming microplastic-Pb complexes that will change the bioavailability of metals (Campanale et al., 2020; Chang et al., 2024). The breakdown of organic pollutants also affects the speciation of metals (Feng and Ni, 2024). Undoubtedly, all these interactions can influence the associations that metals develop with the mineral particles over time. As a result, the contaminant metals in the sludge-amended soil may be transformed into more bioavailable forms (mobilisation) or sequestered in unreactive minerals (immobilisation), leading to the increase or decrease of the metal-associated risk within the system. Therefore, to better assess the long-term environmental impacts of sludge application in agriculture and control the potential risk associated with the sludge-introduced metals, in addition to gaining the instant information of metal speciation in the system after sludge application, the understanding of transformation pattern of metal speciation in the system is also needed. However, to date it remains largely unknown that in the sludge-soil/sludge-soil-plant system how these contaminant metals behaviour and fate will evolve over time and/or with repeated plant growth. This is becoming the obstacle for the agricultural utilisation of SS.

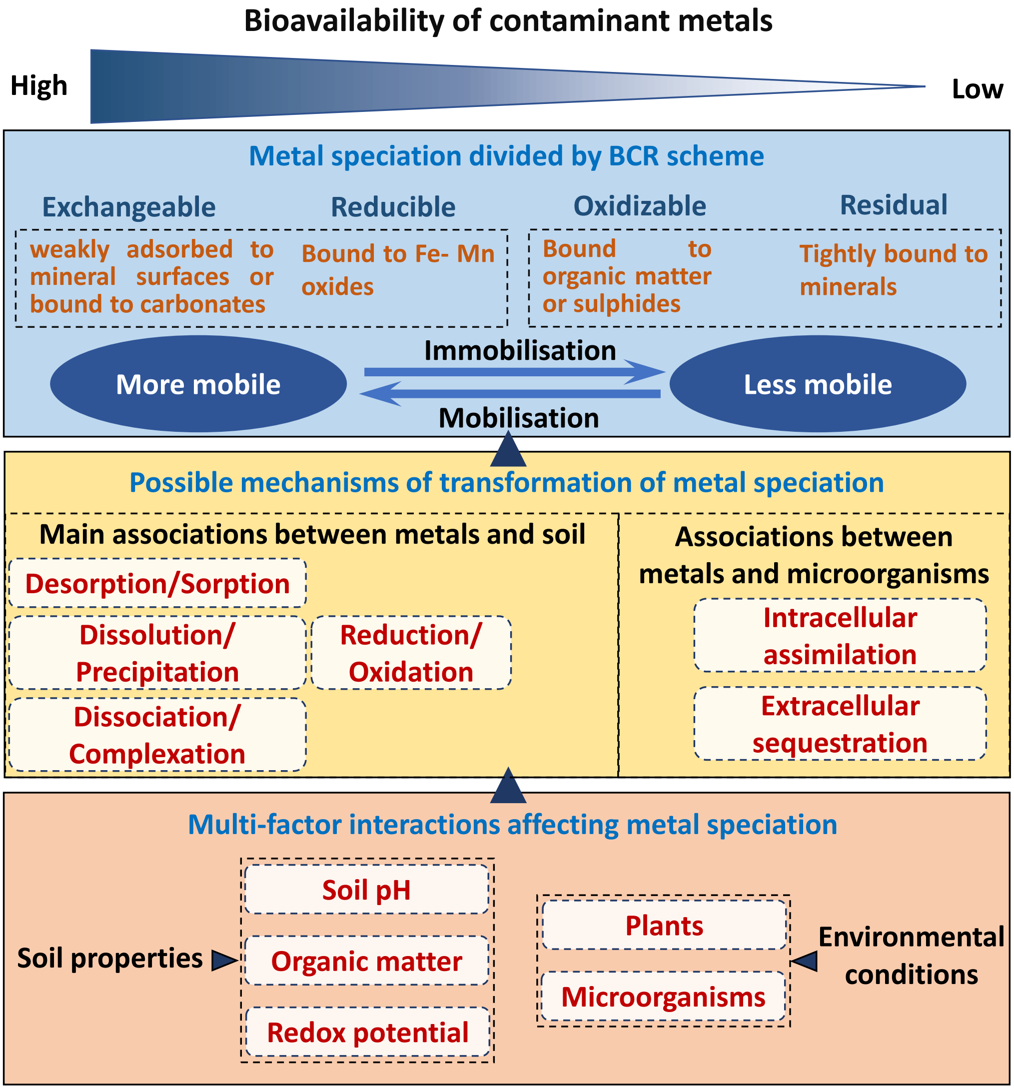

Figure 1.1 Factors and possible mechanisms for the transformation of metal speciation in the sludge-soil-plant system.

In a sludge-soil-plant system, the interactions between contaminant metals and plants are also of great importance for controlling the risk (Alturiqi et al., 2020). In the system, the plant metal uptake is one of the major pathways by which sludge-introduced metals enter the food chain (Soriano-Disla et al., 2013). The amount of contaminant metals transferred from the sludge-amended soil to the plant is very crucial for assessing the associated risks and environmental regulations. Therefore, it is required to investigate the behaviour of plant metal uptake when sewage sludge is applied to agricultural land. To investigate the metal uptake by plants in the sludge-amended soil, it is essential to obtain the knowledge of processes by which metals are transferred to the plant roots, including the transformation processes of metals that take place in the vicinity of plant roots (rhizosphere) (Haldar and Sengupta, 2015; Krishnamurti et al., 1996). To date, few studies attempt to explore the behaviour and fate of contaminant metals in the rhizosphere within the sludge-soil-plant system due to the difficulty in physically separating rhizosphere soil from plant roots and the very narrow region of greatest interest. Knowledge of the metal behaviour and fate in close proximity to plant roots in the sludge-amended soil needs to be expanded for better safety assessments.

To address the knowledge gaps described above, a series of laboratory experiments are conducted to study the transformation behaviour of contaminant metals in the agricultural soil amended with digested sludge from multiple perspectives (see Figure 1.2). The findings of this project will contribute to the enhanced understanding of metals behaviour and fate in the complex sludge-soil/sludge-soil-plant system, informing better risk assessments for sludge use in agricultural land.

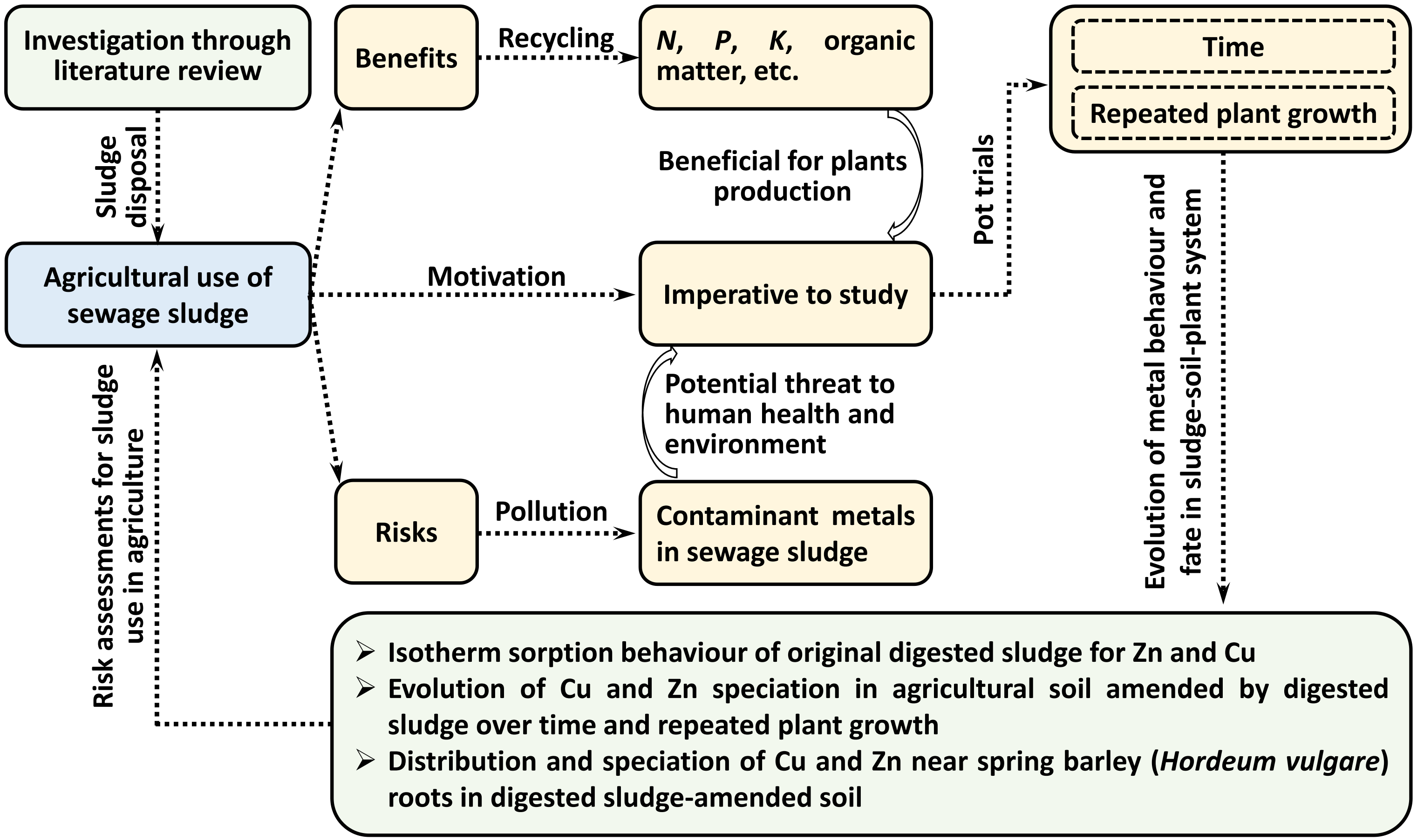

Figure 1.2 Overall research background and motivation of this project.

1.2 Research question statement

As stated above, the agricultural utilisation of SS is widely recognised as a beneficial way to recycle the valuable nutrients and organic matter present in SS (Harder et al., 2019; Kominko et al., 2019). Unfortunately, it may be limited due to the presence of contaminant metals. It is therefore required to assess the risk potentially posed by the metals when SS is applied to agricultural soil. Many studies have indicated that the chemical forms of contaminant metals can significantly control the risk. As a result, lots of studies have attempted to investigate the speciation pattern of contaminant metals in the agricultural soil following sludge application. It should be stressed that most studies have only determined the speciation of contaminant metals in a relatively short period after applying SS to agricultural soil (Malinowska, 2017; Parvin et al., 2022; Wu et al., 2006). In essence, their aim is to address the question "what is the immediate changes in the speciation of contaminant metals in the agricultural soil due to sludge application" but shed little light on the transformation of metal speciation in the longer term. Over time the geochemical and microbiological processes in the sludge-soil/sludge-soil-plant system may transform the chemical forms of contaminant metals from that observed in the short-term studies. Currently, it is still unclear whether these changes will increase the bioavailability of metals or sequester them in the unreactive minerals. In addition, in a sludge-soil-plant system, the interaction between metals and plants is an important factor affecting metals availability to plants and plant safety assessments. More studies are therefore needed to investigate the behaviour and fate of contaminant metals near plant roots in the sludge-amended soil for informing better risk assessments related to the agricultural use of SS. Based on this general context, the following important research questions are identified to be addressed in this project.

Question 1: What are the typical concentrations and chemical forms of contaminant metals in SS? It is essential to comprehensively review the information on concentration and speciation patterns of contaminant metals in SS based on the published literature. The systematic review can provide guidance in the determination of focus metals for this project.

Question 2: What is the isotherm sorption behaviour of original digested sludge for contaminant metals? To experimentally monitor the transformation of contaminant metals in the sludge-soil/sludge-soil-plant system, it is necessary to elevate soil metal concentrations by applying an appropriate sludge to the soil. To determine the appropriate amended sludge for use in soil amendment experiments, there is a need for investigating the isotherm sorption capacity of original digested sludge for metals and the speciation pattern of metals in the amended sludges.

Question 3: How to quantitatively analyse the transformation of metal speciation and what are the evolution patterns of metal speciation in a sludge-amended soil over time and with repeated plant growth? Most studies only use sequentially chemical extractions to quantitatively obtain the information of operationally defined metal speciation in the system. However, this technique has many shortcomings and cannot provide direct *in-situ* information on metal species. Thus, a complementary technique to determine metal speciation is necessary. Also, the transformation of metal speciation in the sludge-soil/sludge-soil-plant system over time and with repeated plant growth is still poorly understood, which needs more studies to fill this knowledge gap.

Question 4: What are the concentration distribution and speciation pattern of contaminant metals in close proximity to plant roots in the digested sludge-amended soil? The proximity of contact between soil and plant roots is a crucial factor influencing the interactions between plants and metals. The knowledge of variations in metal concentrations at several distances from plant root surfaces is important for evaluating the metal dynamics in the sludge-soil-plant system. In addition, the interface between soil and plant roots can result in significant changes of soil micro-environments, further affecting metal behaviour and fate within the system. Therefore, it is necessary to understand the transformation behaviour of contaminant metals in the vicinity of plant roots in the digested sewage sludge-amended soil.

1.3 Research aim and objectives

This project aims to understand how metal behaviour and fate in the sludge-soil/sludge-soil-plant system evolve over time and with repeated plant growth and inform better risk assessments for sludge application to agricultural soils. Out of those contaminant metals present in SS, Cu and Zn are the focus metals in this project because (1) they are generally the most two abundant metals in sewage sludge; (2) Zn has a high mobility and Cu has a moderate mobility; (3) they are essential micronutrients for plants (Juknys et al., 2012; Medas et al., 2019; Shahid et al., 2014); (4) a widespread concern is reported on their environmental risks as contaminant metals when they accumulate in the agricultural soil (Luo and Christie, 1998). Through the investigations of this project, the following objectives are expected to be achieved.

Objective 1: To comprehensively understand the chemical forms of various contaminant metals in SS, and the impacts of SS application on metal concentrations and speciation in the agricultural soil.

Objective 2: To determine the sorption capacity of original digested sludge for Cu and Zn and identify the appropriate metal-amended sludge for use in the soil amendment experiments.

Objective 3a: To quantify the impacts of contact time and repeated plant growth on the transformation of operationally defined Cu and Zn speciation in the digested sludge-amended soil and identify the accumulative characteristics of Cu and Zn concentrations in the plants.

Objective 3b: To quantify the impacts of contact time and repeated plant growth on the transformation of bonding environments and oxidation state of Cu and Zn in the digested sludge-amended soil.

Objective 4a: To identify the effect of root zone on Cu and Zn uptake in the digested sludge-amended soil.

Objective 4b: To identify the *in-situ* spatial distribution and speciation pattern of Cu and Zn near plant roots in the digested sludge-amended soil.

1.4 Thesis structure

This thesis contains 8 chapters (see Figure 1.3). Chapter 1 provides the research background of this project. Chapter 2 conducts the extensive literature review on understanding metal concentration and speciation patterns in SS as well as the impacts of sludge application on the metal behaviour and fate in the agricultural soil (**objective 1**). Chapter 3 introduces the full details of the methodology used in the three pot trials and isotherm sorption experiments of sewage sludge. Chapter 4 investigates the basic characteristics of original materials used and the isotherm sorption behaviour of original digested sludge for Cu and Zn. Through this investigation, the appropriate metal-amended sludge can be determined for use in the soil amendment experiments (**objective 2**). Chapter 5 attempts to determine the transformation pattern of Zn and Cu speciation in the digested sludge-amended soil over time and with repeated plant growth (**objective 3a and 3b**). Chapter 6 aims to use a combined study to explore the distribution and speciation patterns of Cu and Zn in the vicinity of plant roots in the digested sludge-amended soil (**objective 4a and 4b**). It is expected that the interactions between plants and contaminant metals in the sludge-soil-plant system can be elucidated. Chapter 7 provides more in-depth discussion on Cu and Zn behaviour and fate in the sludge-soil/sludge-soil-plant system. Chapter 8 summarises the major work and important findings presented in this project and presents some recommendations for future work.

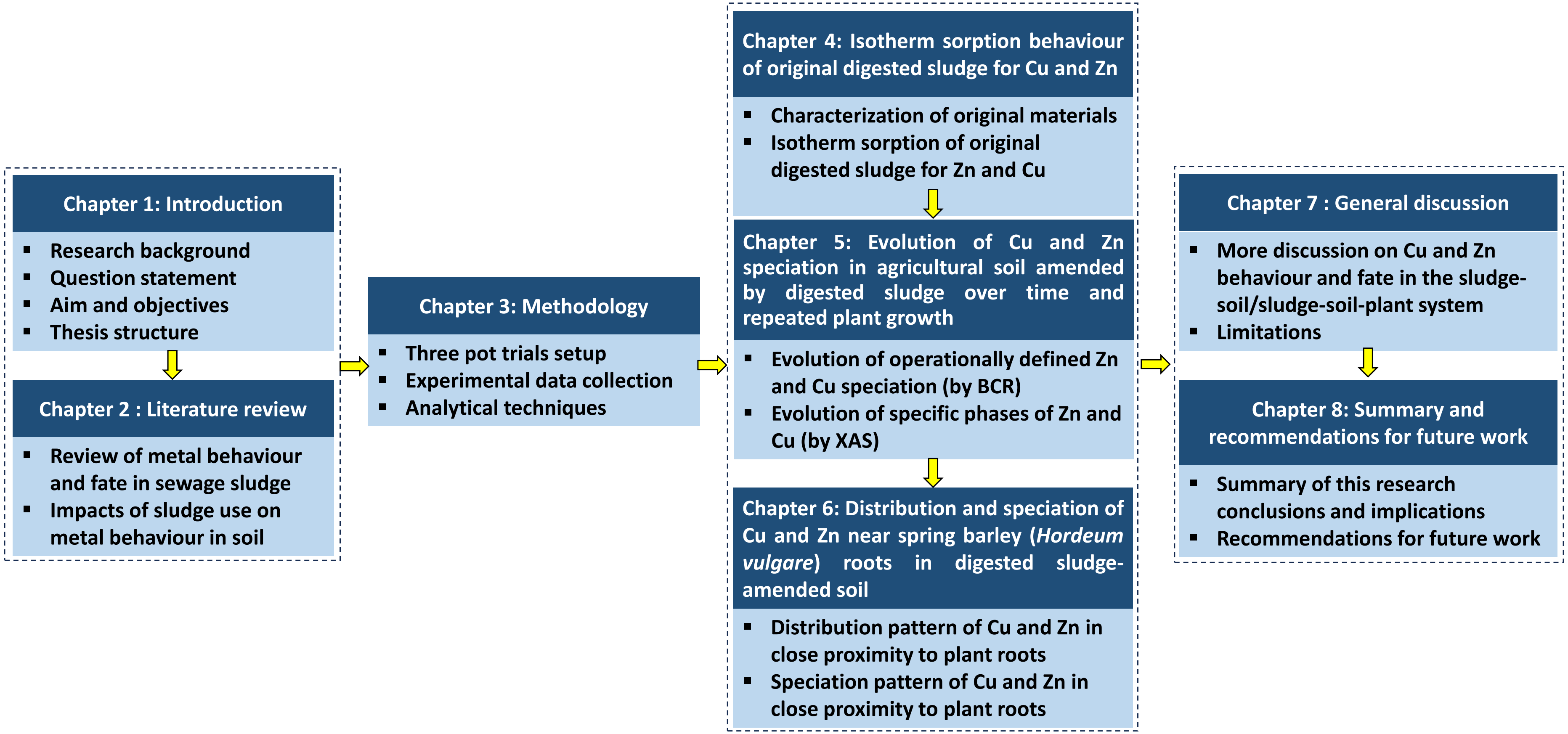

Figure 1.3 Thesis structure.

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Chapter 2: Literature review

2.1 Introduction

Sewage sludge (SS) is the by-product of industrial and/or municipal wastewater treatment and can be generally divided into primary sludge (PS) and secondary sludge (SES) depending on its production process (Kumar et al., 2017; Malinowska, 2017; Pöykiö et al., 2018; Wei and Liu, 2005; Woo et al., 2023; Zoghlami et al., 2020). It is generally composed of water, organic matter (OM) and inorganic components that are often referred to as “biosolids” (Elgarahy et al., 2024; Fijalkowski et al., 2017; Usman et al., 2012; Yuan and Dai, 2016). The total amount of SS produced in the world is increasing year-on-year due to population growth, increased urbanization (which tends to increase the proportion of wastewater treated centrally), and continuous improvements in the level of wastewater treatment (Nahar and Hossen, 2021; Turek et al., 2019; Tytla, 2019, 2020).

Currently, there is no agreement in the literature on the total global production of SS, therefore this must be estimated from the most recently published statistics. As shown in Figure 2.1, the total annual production of SS (reported as dry solids; Mt: million tons) is 4.96 Mt in US, 0.11 Mt in Mexico, 0.15 Mt in Colombia, 2.5 Mt in Brazil, 4.36 Mt in India, 0.41 Mt in Australia, 2.2 Mt in Japan, 11.2 Mt in China, and 1.25 Mt in the UK. The annual cumulative production of dry SS in the 27 European Union Countries (EU-27) is 9.25 Mt. In addition, the amount of SS produced per capita (kg/yr, dry solids) can be calculated based on current populations: EU-27 (19 kg/yr), UK (17 kg/yr), Japan (16 kg/yr), Australia (15 kg/yr), US (14 kg/yr), Brazil (11 kg/yr), China (7 kg/yr), India (3 kg/yr), Colombia (3 kg/yr) and Mexico (0.8 kg/yr). By assuming that neighbouring regions have the same production rates, and using the United Nations (2024) world population statistics, it is estimated that ~53 Mt/yr of SS dry solids is currently produced globally (assumptions: Canada = US; central America = Mexico; South America = the average of Colombia and Brazil; non-EU European countries = UK; other Asian countries = the average of China and India; sludge production in Africa is ignored). If the wastewater produced around the globe were, in future, to be treated to a similar level as in EU-27/UK (*i.e.* assuming an average per capita production rate of 18 kg/yr for the current world population) then ~160 Mt/yr SS dry solids would be generated globally. Furthermore, SS usually contains 0.4-7% dry solids suspended in the water and typical combined PS and SES contains about 3% solids by weight (National Research Council, 1996; Turovskiy and Mathai, 2006; US Army, 1987); so the current production has a volume of about 1.6 billion m^3, and this could potentially increase to about 4.8 billion m^3. Thus, globally, safe and effective disposal of SS is a major problem, which will become particularly problematic in Asian countries due to their large and growing populations, and potential improvements in wastewater treatment standards (Iticescu et al., 2018; Janaszek et al., 2024; Kosobucki, 2024; Seyedi et al., 2021; Teoh and Li, 2020).

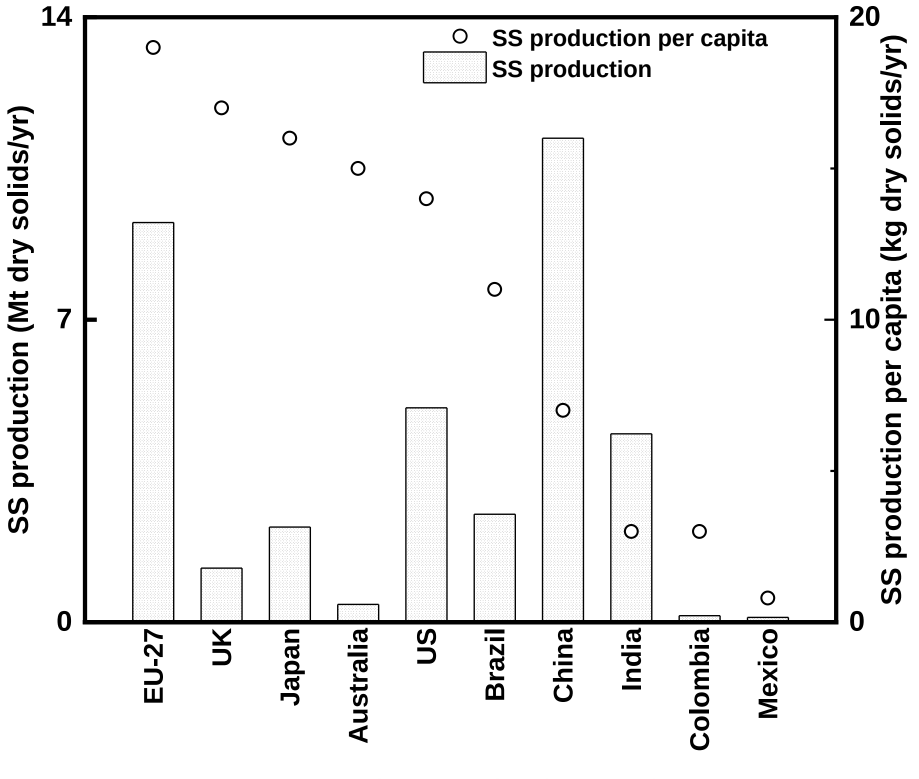

Figure 2.1 Annual sewage sludge production and yearly sewage sludge production per capita in selected countries and regions (Australian Water Association, 2020; Castellanos-Rozo et al., 2020; Environmental Protection Agency, 2022; Eurostat, 2022; Leichman, 2017; Liu et al., 2022; Shiota et al., 2015; Singh et al., 2020; Spinosa, 2007).

Numerous studies have shown that SS typically contains over 50% OM by dry weight (Carabassa et al., 2018; Delibacak et al., 2020; Kominko et al., 2017; Saruhan et al., 2010; Zuo et al., 2019). It also contains macronutrients (N, P, K, Ca, Mg, etc.) and micronutrients (Mn, Fe, etc.) that are important for plant growth (Eid et al., 2017; Kolodziej et al., 2023; Latare et al., 2014; Zoghlami et al., 2018). Thus, where local regulations permit, SS is frequently applied to agricultural soils as an organic fertiliser (Bozkurt et al., 2020; Hechmi et al., 2021; Jamil et al., 2006; Lamastra et al., 2018; Nogueira et al., 2013). One advantage of agricultural use is the avoidance of the cost of fully dewatering SS, as treated SS is typically applied either as a liquid suspension at 3-6% dry solids by injection or as dewatered cake at 15-35% dry solids on the surface (DEFRA, 2018; EPA, 2000; Saxlund, 2022; US Army, 1987).

Unfortunately, SS, particularly that from primary settlement, also contains organic pollutants, pathogens, contaminant metals and other emerging contaminants such as microplastics and pharmaceuticals (Barraoui et al., 2021; Camargo et al., 2016; Centofanti et al., 2016; Nahar and Hossen, 2021; Neumann et al., 2016). Therefore, regulators often require further treatment of SS such as anaerobic digestion (AD), composting, and thermal treatment (see Figure 2.2) to improve sludge properties, degrade organic pollutants, and eliminate some pathogens before it is applied to agriculture (Buta et al., 2021; Camargo et al., 2016; Romdhana et al., 2009; Wang et al., 2005; Wluka et al., 2021). However, it should be noted that these treatment processes are designed to decrease the amount of organic substances and eliminate pathogenic microorganism, and have minimal effect on the contaminant metals removal (Cantinho et al., 2015; Cuprys et al., 2021; Karvelas et al., 2003; Koul et al., 2022; Milik et al., 2017). Hence, SS will still contain the contaminant metals after treatment (Cantinho et al., 2015). As those metals are not biodegradable, SS application to agriculture can lead to a gradual accumulation of metals within the agricultural soils (Camargo et al., 2016; Cantinho et al., 2015; Charlton et al., 2016; Hasnine et al., 2017; Liu et al., 2007; Tytla and Widziewicz-Rzońca, 2021). Potentially, these metals can be incorporated into the food chain or be washed into aquifers, which would cause serious environmental and health risks (Liang et al., 2011; Nyashanu et al., 2023; Valchev et al., 2024).

Over the past decades, the potential for secondary contamination of agricultural soil by metals in sewage sludge has attracted global attention (Buta et al., 2021; Harrison et al., 1999; Li et al., 2014; Meng et al., 2016; Saha et al., 2017; Suanon et al., 2016; Tytla and Widziewicz-Rzońca, 2021). In order to ensure crop quality and safety, soil ecological health, groundwater safety and reduce the potential for metals toxicity, maximum levels of metals in agricultural soil have been set in some countries (Ahmad et al., 2021; Hasnine et al., 2017; Ministry of the Environment, 2007). For example, in the UK, maximum permissible metal concentrations in the agricultural soil are regulated based on their toxicity (see Table 2.1); for some elements (Cu, Ni and Zn), the maximum concentrations are also pH dependant. Thus, it is necessary to monitor the metal concentrations in soils that receive SS to ensure that they remain below prescribed limits after sludge application.

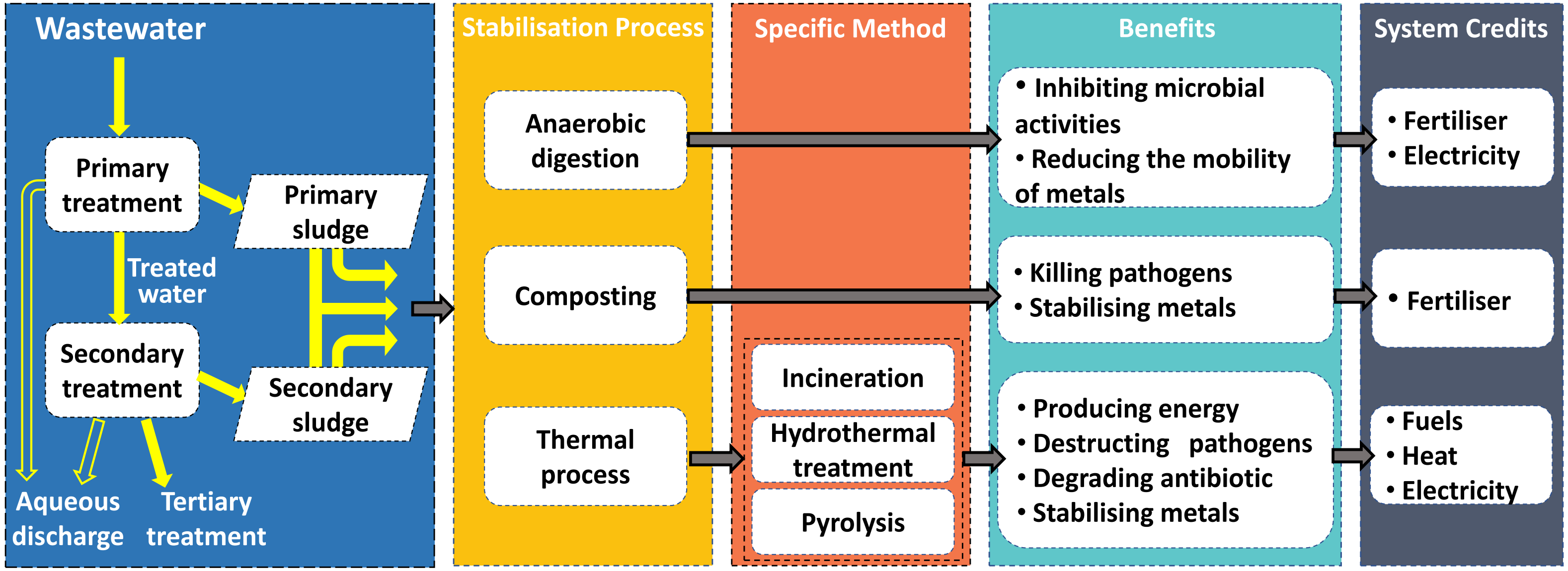

Figure 2.2 Overview of sewage sludge treatment developed from Tarpani et al. (2020) and Teoh and Li (2020).

Table 2.1 UK limits on potentially toxic metals in the soil to be used for arable farming (DEFRA, 2018).

Metals	Maximum metals concentration in soil (mg/kg dry solids)			
	pH (5-5.5)	pH (5.5-6)	pH (6-7)	pH (>7)
Cd	3	3	3	3
Cr	400	400	400	400
Cu	80	100	135	200
Ni	50	60	75	110
Pb	300	300	300	300
Zn	200	200	200	300

However, at a time when there is a pressing need to increase the sustainability of agriculture by reducing its dependence on mineral fertilisers and preventing the depletion of soil OM, these regulations will also eventually restrict the application of SS as fertiliser for agriculture. Moreover, prescribing regulatory limits on metals in agricultural soil is simplistic, as it does not recognise that the potential risks posed by metals to plants and wider environment are both controlled by metal speciation (Ailincăi et al., 2012; Alborés et al., 2000; Chen et al., 2008; Cocarta et al., 2017; Jakubus, 2020; Rudd et al., 1988; Sumalatha et al., 2019; Yan et al., 2020). Therefore, there is a need for more comprehensive information on the chemical forms of various metals in SS and how that speciation evolves upon SS introduction to the soil. This knowledge is crucial in establishing appropriate safe thresholds for metal concentrations in SS that will be applied to agricultural soil, as well as refining the safe limits for the accumulation of metals in the soil.

This chapter will, therefore, primarily provide a comprehensive review of the current knowledge of contaminant metals in SS and the impacts of its application on metals behaviour in the soil. It will also provide new insights and perspectives arising from this synthesis of global data and published research. Specifically, (1) the current global amount of SS and the potential for future change in that quantity are definitively estimated; (2) the typical contaminants in SS are introduced; (3) the sources of contaminant metals and their partitioning from wastewater to PS and SES are presented by using a synthesis of published data; (4) the concentration and speciation of metals in PS and SES are reported; (5) the impacts of sludge application on the concentrations and chemical forms of metals in the soil are discussed by collating and comparing the data for the operationally defined speciation of metals in agricultural soil, SS and sludge-amended soil.

2.2 Typical contaminants in sewage sludge

SS contains various contaminants that may pose risks to the environment and human health (Blaszczyk et al., 2024; Fijalkowski et al., 2017). The types of contaminants in SS are affected by wastewater treatment processes and input sources (Marttinen et al., 2003; Surampalli et al., 1998; Ye et al., 2022). Typically, contaminants in SS include organic pollutants, pathogens, contaminant metals and other emerging contaminants.

2.2.1 Organic pollutants

Numerous organic pollutants (such as personal care products and polycyclic aromatic hydrocarbons) have been reported in wastewater (Fijalkowski et al., 2017). After wastewater treatment, these organic pollutants can accumulate in SS due to their lipophilicity and incomplete degradation (Wluka et al., 2021). It has been reported that >300 organic pollutants that may pose environmental risks have been detected in SS (Cai et al., 2007; Drescher-Kaden et al., 1992). The concentration of organic pollutants in SS varies greatly (from µg/kg to mg/kg), largely depending on their levels in the influent wastewater (Cai et al., 2007; Fijalkowski et al., 2017). Before being applied to agricultural land, SS is generally required to undergo treatment through processes like anaerobic digestion, aerobic digestion, or composting (Hoang et al., 2022). During SS treatment, the breakdown of organic substances will lead to a significant decrease in the concentrations of organic pollutants. Generally, anaerobic digestion reduces organic pollutant levels in SS by 20-40% (Smith, 2009). A higher removal rate may be achieved by aerobic digestion and composting because aerobic conditions promote metabolic pathways that degrade a wider variety of organic substrates. Additionally, aerobic conditions enhance the degradation efficiency of organic pollutants as oxygen can accelerate the breakdown of organic substances (SKIPSHAPIRO, 2024).

Complete removal of organic pollutants in SS cannot be easily achieved by conventional wastewater treatment. As a result, a proportion of them may be transferred into agricultural soils through sludge application. These organic pollutants in SS applied to soils have been suggested to have limited risks (WCA, 2019). Because once sludge is applied to agricultural soil, processes like adsorption, degradation, volatilization, runoff, and leaching can reduce the availability of organic pollutants for plant uptake (Pocurull, 2009). However, it should be stressed that if they accumulate to toxic levels in organisms, they will have harmful effects such as inhibiting plant growth.

2.2.2 Pathogenic organisms

Wastewater entering sewage treatment plants contains diverse pathogenic organisms such as bacteria, viruses, fungi, and parasites (Gamze et al., 2023; Suarez et al., 2022). Therefore, it is not surprising that a wide range of pathogens (such as Salmonella and Clostridium) can be detected in untreated SS (Bouchaala et al., 2024). The presence of these pathogens in untreated SS may pose serious risks if improperly applied to agricultural soils. Fortunately, the vast majority of pathogenic and parasitic organisms can be eliminated by appropriate sludge treatment. Generally, more than 90% of pathogens can be eliminated after conventional sludge treatment and almost 100% pathogen-free SS can be achieved by the enhanced sludge treatment (Water UK, 2010). For example, thermal hydrolysis, which precedes anaerobic digestion at many UK sludge treatment plants, typically involves heating the sludge to between 140-170°C under pressure (Mukawa et al., 2022; SAVE THE WATER, 2023). This will kill many pathogenic bacteria.

2.2.3 Contaminant metals

Contaminant metals in SS mainly originate from urban runoff, industrial wastewater and domestic wastewater (Feng et al., 2023). The common metals in SS include both non-essential elements (such as Cd, Ni, and Pb) and essential trace elements (such as Cr, and Cu and Zn; Udom et al., 2004). Small amounts of non-essential elements can be toxic. Essential trace elements are beneficial for plants production, but only within specific limits. Typical metal concentrations in UK sludges are 1 mg/kg Cd, 46 mg/kg Cr, 176 mg/kg Cu, 19 mg/kg Ni, 77 mg/kg Pb, and 432 mg/kg Zn (European Commission, 2018). Similar concentrations are also found in sludges from other EU countries. In low and middle-income countries, metal concentrations can be 4-5 times higher (Saha et al., 2018; Yang et al., 2014). As metal concentrations in SS are usually higher than in the agricultural soil and they are not biodegradable, long-term repeated sludge application will inevitably lead to the gradual accumulation of metals in the soil (Achkir et al., 2023; Feng et al., 2023). To control the risk associated with sludge-borne metals, many regulations require the monitoring of total metal concentrations in the soil following sludge application (Christodoulou and Stamatelatou, 2016; DEFRA, 2018; EPA, 2022).

2.2.4 Emerging contaminants

Emerging contaminants in SS, such as microplastics and pharmaceuticals, are an increasing concern (Hassan et al., 2023; Jiang et al., 2024). Microplastics primarily originate from personal care products, synthetic textiles, crumb rubber, and the breakdown of larger plastics (OSPAR Commission, 2024;Talukdar et al., 2024). Their presence in SS can pose risks to ecosystems when applied to agricultural land. Pharmaceuticals typically enter wastewater through human excretion and improper disposal (Samal et al., 2022). Due to incomplete removal during wastewater treatment, these emerging contaminants can accumulate in later SS (Fernández-López et al., 2016). They may pose challenges to the conventional sludge treatment and raise concerns about the safety of using treated SS in agriculture (RACO, 2024).

2.3 Sources of metals and their pathways to sewage sludge

Of those contaminants in SS, contaminant metals represent the primary obstacle to the agricultural use of SS due to their toxicity and persistence. At present, most regulations governing the agricultural use of SS are based on total metal concentrations. It is likely because organic pollutants and pathogens can be largely eliminated during sludge treatment. The long-term environmental and health impacts of contaminant metals, along with their regulatory importance, make them a key focus of this project. Therefore, this work has focussed on contaminant metals, so the remaining discussion will also concentrate on these.

2.3.1 Sources

The main sources of contaminant metals in SS are domestic wastewater, industrial wastewater, and urban runoff (see Figure 2.3).

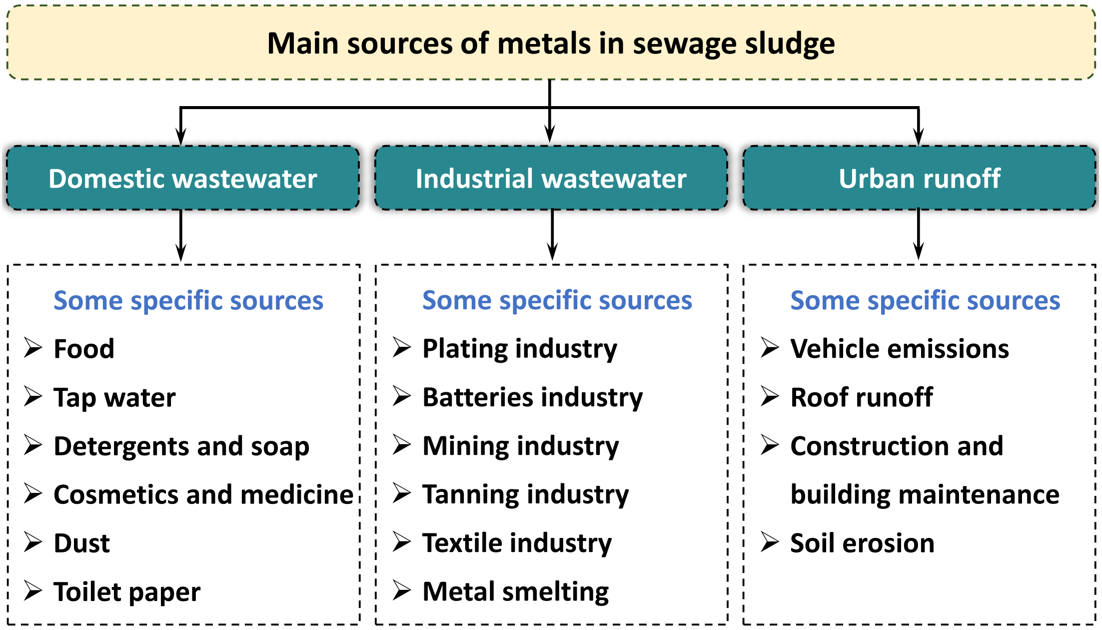

Figure 2.3 Main sources of metals in sewage sludge (Chen, 2019; Cheng et al., 2022; Chirila et al., 2014; Cornelius et al., 1978; Davis III and Jacknow, 1975; Kesari et al., 2021; Moriyama et al., 1989; Spiegel et al., 1985; Zhang et al., 2017).

2.3.1.1 Domestic wastewater

Domestic wastewater always contains metals, which contributes to metals content of SS (Czerwinska et al., 2023; Drozdova et al., 2019; Razak et al., 2016; Yen, K. W. et al., 2024). These metals come from various sources such as food, tap water, faeces, detergents and cosmetics (Kalinowska et al., 2020). Specifically, copper plumbing is a major source of Cu in domestic wastewater and older plumbing is a source of Pb, cleaning of stainless steel cookware is a source of Ni and Cr, human faeces are a source Zn, Cd and Ni, and household agents (such as medicated shampoos and washing powders) accounted for most of Zn and a substantial proportion of Cd, Cr, and Pb (Aonghusa and Gray, 2002; Comber and Gunn, 1996; Drozdova et al., 2019; Kamerud et al., 2013). In domestic wastewater, a high proportion of metals are in a freely dissolved, bioavailable form. For example, Isozaki et al. (2006) found that the fraction of Zn, Cu, and Ni in domestic wastewater that was dissolved ranged from 12-47%, 24-61%, and 16-60%, respectively.

It has been reported that the UK domestic wastewater contains 42 mg Cu, 24 mg Zn, 1 mg Pb, 0.7 mg Ni, 0.5 mg Cr and 0.1 mg Cd per person per day (Comber and Gunn, 1996). These figures are comparable with more recent data (14 mg Cu, 13 mg Zn, 0.7 mg Ni, 0.05 mg Cr, 0.03 mg Pb, 0.02 mg Cd per person per day) from Sweden (Sorme and Lagerkvist, 2002). A 2009 meta-study of European data (about 50% from Germany and about 33% from Sweden) also observed that Cu and Zn were the major metals in domestic wastewater (34 mg Zn, 7 mg Cu, 3 mg Pb, 2 mg Ni, 2 mg Cr and 0.1 mg Cd per person per day), but Zn load exceeded Cu load (Meinzinger and Oldenburg, 2009); this pattern is also shown by data from Japan (Chino et al., 1991). The figures above indicate that domestic inputs of Zn and Cu are major contributors to the overall levels of metals reaching wastewater treatment plants (WWTPs). This is attributed to the extensive use of household agents (laundry detergents, shampoos, and skin care products, etc.), pigments and fungicides, which contain high levels of Zn and/or Cu. Particularly, it should be stressed that these household products are still widely used in daily life, so it will be very difficult to reduce these metals derived from this source in the short-term.

2.3.1.2 Industrial wastewater

Industry can discharge large volumes of wastewater to sewer; it can lead to high levels of contaminant metals being sent to WWTPs if the treatment prior to discharge is not mandated. Industrial activities such as metal plating, battery manufacture and recycling, mining, and leather tanning, are all noted for high metal content of untreated discharges (Barakat, 2011; Guo et al., 2024; Iloms et al., 2020; Qasem et al., 2021). In Lithuania (where presumably there is relatively consistent regulation of industrial discharges to sewer), the level of metals in SS increases with increased industrial development in the catchment area of the WWTP (Praspaliauskas and Pedišius, 2017). In industrial effluent, large amounts of metals are also soluble. For example, high concentrations of soluble Zn and Ni can be generated from the plating industry (Mokhter et al., 2018; Water Technology, 2018). Soluble metals are highly bioavailable and are thus more likely to cause harm. Therefore, industrial wastewaters discharged to sewer are often an important source of metals in SS (Chen, 2019; Qasem et al., 2021).

The metal content of industrial wastewater produced by different industrial activities varies greatly, leading to a large variation in the total concentration of various metals in SS (Islam et al., 2017; Khan et al., 2023). For example, the sludge produced by tanneries usually contains high concentration of Cr because chromium sulphate is an important component of tanning powder (Genawi et al., 2020; Islam et al., 2017). High levels of Cr, Cu, Zn, and Ni can be detected in SS produced by metal-plating wastewater because these metals are frequently used as electroplated coating (Świnder and Lejwoda, 2021). Venkateswaran et al. (2007) reported that 3566 mg/kg Cr, 5996 mg/kg Cu, 966 mg/kg Ni and 6865 mg/kg Zn are detected in SS produced from electroplating industries located in Chennai, India.

Fortunately, many countries have recognised the issues associated with industrial effluents and have successively regulated to minimise discharge to sewer and prevent environmental release (Sylwan and Thorin, 2021). Industry is also adopting a range of clean technologies to treat the wastewater prior to discharge to WWTPs; as a result, the levels of metals emitted to sewer by industry is declining (Cantinho et al., 2015). Sorme and Lagerkvist (2002) observed that from 1973 to 1999 there has been a significant reduction in the concentrations of Cu and Pb in SS produced at three WWTPs in Stockholm, Sweden, which is predominantly attributed to a drastic decrease in industrial discharges and various pre-treatments of wastewater.

2.3.1.3 Urban runoff

The contribution of urban runoff to contaminant metals in SS has received far less attention in the literature than domestic and industrial wastewater. However, metals originating from vehicles, construction activities, residential activities and atmospheric deposition can accumulate on impervious surfaces during dry periods (Shajib et al., 2019). These metals can be washed off by rainfall and conveyed to sewer, contributing to metals content in SS (Wijeyawardana et al., 2022). Comber and Gunn (1994) reported that runoff sources account for an important proportion of metals in Bracknell sewage sludge, UK (Cd, 28%; Cr, 13%; Cu, 2%; Ni, 5%; Pb, 44%; Zn, 9%). Of those contributors to metals in urban runoff, vehicular emissions are widely considered to be the largest contributor (Muller et al., 2020). Traffic-related exhausts emissions are reported to account for 35% of metals in road dust in Beijing, China (Men et al., 2018). However, unlike industrial and domestic wastewater, where metals are in soluble and colloidal forms, those in urban runoff are largely associated with particulates (Adedeji and Olayinka, 2013). In Beijing, 65-93% of metals in runoff from trafficked areas were associated with particulates and about 50% of metals from roof runoff are in the form of particulates (Shajib et al., 2019). Moreover, continuous urban expansion is increasing the area where metal-rich dusts accumulate. Combined with climate change induced intensification of precipitation patterns (*i.e.* more frequent long dry periods followed by high rainfall events), it is predictable that an increase in the impacts of urban runoff to wastewater would occur in future (Adedeji and Olayinka, 2013).

2.3.2 Wastewater treatment and metals partitioning from wastewater to sewage sludge

2.3.2.1 Wastewater treatment

After preliminary treatment to remove large objects and grit, wastewater undergoes primary treatment in settling tanks to separate the majority (typically 50-70%) of the suspended solids from the liquid phase (Jasim and Aziz, 2020; Nemerow, 2006; Pachpute, 2022; Pescod, 1992). Both heavy solids that settle to the bottom and lighter solids that float to the surface are removed (generating PS); the remaining liquid is either discharged (where only primary treatment is undertaken) or passed to the secondary treatment (Britannica, 2022; Das and Basu, 2010; Department of Environmental Protection, 2018; Environmental Protection Agency, 1998; National Research Council, 1996; Zaharia, 2017). Secondary treatment aims to digest and remove the soluble OM, nutrients and organic pollutants from aqueous phases using either a suspended (e.g. activated sludge; most common method) or attached (e.g. trickling filters) growth processes (Di Marcantonio et al., 2024; Roman et al., 2022; Wang, 2021). The resulting biological flocs or biofilms are then settled (generating SES), leaving the liquid substantially free of solids and with greatly reduced pollutant concentrations (Guo et al., 2017; Mannina and Viviani, 2009; National Research Council, 1996; Perez et al., 2006; Zita and Hermansson, 1994). Sometimes the liquid from secondary treatment requires further “polishing” (tertiary treatment) to remove any remaining suspended solids and biochemical oxygen demand (BOD) from the effluent, or to remove nutrients to meet discharge consents (American Water Chemicals, 2022; Blair et al., 2019; EERC, 2022). However, the sludge volume produced by tertiary treatment is small in comparison to the volume of PS and SES (Safe Drinking Water Foundation, 2022).

2.3.2.2 Partitioning of metals from sewage to sludge

The proportion of contaminant metals in wastewater that are transferred to SS during wastewater treatment depends on several factors, such as whether the metals are dissolved, or associated with colloidal organics, organics solids, or particulates, and on the number of stages of wastewater treatment undertaken (Azizi et al., 2016; Benettayeb et al., 2023; Oliveira Ada et al., 2007; Rodrigo Sanz et al., 2021). Generally, more than 70% of metals in wastewater can be transferred to sludges during conventional wastewater treatment (Aganga et al., 2005; Lake et al., 1984; Thornton et al., 2001). However, as early as the late 1970's, removal of 89-96% of the metals in the influent wastewater (89% for Cd; 92% for Pb; 94% for Zn; 96% for Cu) was being achieved by primary sedimentation and activated sludge treatment at a then newly constructed WWTP in Oxford, UK (Lester et al., 1979; Stoveland et al., 1979). Additionally, in comparison with typical European domestic wastewater (assuming 200 L/person/day water usage and the typical metal loads of domestic wastewater), metal concentrations in the effluents from three WWTPs (primarily receiving domestic wastewaters) in the Canary Islands, Spain would indicate more than 95% metal removal (Comber and Gunn, 1996; Meinzinger and Oldenburg, 2009; Rodrigo Sanz et al., 2021). Therefore, it is possible that a far higher proportion of contaminant metals in wastewater can be transferred to SS when there is effective treatment than is generally reported.

The transfer of metals from raw sewage to PS is a physical process that mainly depends on the presence of metals in insoluble, settleable (associated with particulate matter) or possibly settleable forms (Collivignarelli et al., 2022; Poblete et al., 2022; Pöykiö et al., 2019). Therefore, the metal species and the efficiency of suspended solids removal are the dominant factors controlling metals partitioning at this stage (Kempton et al., 1987; Ziolko et al., 2011). The characteristics of each metal (such as valence and chemical affinity) also play an important role in this process, leading to the variation of metal behaviour. For example, it is reported that 24% of Ni in raw sewage, 40% of Cd and Cr are transferred, and over 50% of Zn and Pb are transferred to PS (Thornton et al., 2001; Zheng and Zhang, 2011).

Metal transfer from primary effluent to SES is controlled by biological processes (Benettayeb et al., 2022; Iyare et al., 2020; Pöykiö et al., 2018). Although both activated sludge and trickling filter methods are used for SES, the mechanisms of metals transfer to sludge are similar (Gardner et al., 2013). During secondary treatment, most metals are either adsorbed to, or absorbed by the new biomass and eventually are collected in the biologically settled sludge (Chipasa, 2003). Metals can sorb to biomass by several mechanisms (Benettayeb, Ghosh, et al., 2022; Geng et al., 2020; Sylwan and Thorin, 2021). These include entrapment of insoluble particles with the biomass, adsorption to bacterial walls and extracellular polymers (by ion exchange, complexation, and inorganic micro-precipitation reactions), and uptake by bacteria (metabolic pathways that have evolved for other substrates can transport contaminant metals into bacteria where they bio-accumulate). Obviously, metals partitioning to SES will be highly dependent upon the uptake of metals by the biomass and the separation of the biomass (Thornton et al., 2001).

2.4 Concentrations and speciation of metals in sewage sludge

2.4.1 Metal concentrations in sewage sludge

The metal concentrations in SS vary from country to country, which may be attributed to the wastewater source, the chosen treatment technologies, and the relevant national regulations on wastewater management (An-nori et al., 2022; Davis et al., 2009; Duan and Feng, 2022; Yakamercan and Aygün, 2021). However, the average concentrations of metals in SS from each region generally decrease in the order Zn > Cu > Cr ≈ Pb ≈ Ni > Cd (see Table 2.2), a pattern that has been reported in many other sources (Schiptsova et al., 2020; Tytla et al., 2016; Xu et al., 2012; Zorpas et al., 2008). Metal concentrations in European and Japanese sludges are generally lower than those in sludges from other countries. However, despite regional differences, all the values reported in Table 2.2 are within the EU-27 and US guidelines for metals in SS to be applied to the agricultural land. It may appear that the guideline values are high, but the sludge is typically applied to the soil at low rates, ensuring the sludge application will not cause a dramatic increase of metals in the soil (EPA, 2022; Farmers Weekly, 2022; Hudcová et al., 2019; Morera et al., 2001; Petersen, 2003).

Data from seven WWTPs (5 in Europe, 2 in China) show that individual contaminant metals have a similar concentration in PS and SES (see Table 2.3; in all cases the activated sludge method was used for secondary treatment). While these data necessarily come from a small sample of WWTPs, which potentially increases the influence of specific local features, the relative concentrations of metals generally follow the same pattern as that in Table 2.2. Interestingly, this equal partitioning of metals between PS and SES was found with wastewater from a wide range of sources. However, other workers have reported a different partitioning of metals between PS and SES. For example, Pöykiö et al. (2018) reported that metal concentrations in SES are all much higher than in PS. While Ribeiro et al. (2010) reported that the metal concentrations in SES are all lower than in PS. These different results strongly suggest that the partitioning of metals between PS and SES is controlled by how PS is separated effectively from the treated wastewater after primary treatment (the sludge settling conditions), and thus the amount of metals associated with the suspended solids that are transferred with the aqueous phase for secondary treatment (Chipasa, 2003; Ziolko et al., 2011).

While the dataset report in Table 2.3 is small, it indicates that the co-treatment of industrial wastewater at a WWTP results in the higher concentrations of some contaminant metals in SS, than treating domestic wastewater alone or in combination with urban runoff. Enhancement of the metal concentrations is not uniform at a particular WWTP, presumably because it will depend on the industries in the catchment area, but it should be noted that the mostly highly contaminated sludges in Table 2.3 came from a WWTP in the most industrialized region of Poland (Tytla, 2019). In this dataset, the difference between metal concentrations in SS from WWTPs that co-treat domestic wastewater with urban runoff and those that treat predominantly domestic wastewater, is small. This may indicate that the increase in SS metal concentration from urban run-off is small or may reflect under-reporting of urban run-off in some studies.

Table 2.2 Typical metal concentrations in sewage sludge from different countries and limits of these metals for sewage sludge use in agriculture of EU-27 and US (mg/kg dry solids).

Metals	US [a]	Brazil [b]	EU [c]	UK [c]	South Africa [d]	Egypt [e]	India [f]	China [g]	Japan [h]	EPA legislation	EU-27 legislation [i]
Cd	2.7	40	1.1	1	1.6	3.1	5.7	3.9	1.0	85	0.7-40
Cr	83.6	542	44.4	46.2	76.9	30.2	n.d.	259	44.4	n.d.	70-1500
Cu	568.9	1180	239.6	175.6	418.3	288	325	499	356	4300	75-1750
Ni	53.1	288	26.3	19.4	39.8	112	225	166.9	44.2	420	25-400
Pb	79.8	313	41.9	77.1	76.6	46	470	112.2	19.8	840	45-1200
Zn	1014.3	1772	674.9	432.1	1291.8	1012	1812	2088	663	7500	200-4000

[a] (Hundal et al., 2014), data from the US EPA’s targeted national sewage sludge survey; [b] (Marchioretto, 2003), data from a WWTP treating mixed influent from the Metropolitan Region of São Paulo, Barueri; [c] (European Commision, 2018), data for the UK and 17 EU countries: Bulgaria, Croatia, Cyprus, Czechia, Estonia, Finland, France, Germany, Hungary, Italy, Lithuania, Luxembourg, Netherlands, Portugal, Slovakia, Spain, Sweden; [d] (Shamuyarira and Gumbo, 2014), sludge from 5 WWTPs in Limpopo Province, South Africa; [e] (Elqassas et al., 2022), sludge from Quesna WWTP in Menofyia Governorate, Egypt; [f] (Saha et al., 2018), sludge from 9 different WWTPs in West Bengal, India; [g] (Yang, J et al., 2014), sludge from 107 WWTPs in 48 cities across China; [h] (Chen et al., 2021), 120 sludge samples from 32 WWTPs in Japan; [i] the legislation limits are summarised from individual EU countries legislation. n.d.-no data.

Table 2.3 Average metal concentrations in primary sludge and secondary sludge by principal wastewater source (mg/kg dry solids).

	Metal concentrations in PS and SES													
	Predominantly domestic wastewater				Predominantly domestic and urban runoff wastewater				Domestic and industrial wastewater				Predominantly industrial wastewater	
Metals	PS [a1]	SES [a1]	PS [a2]	SES [a2]	PS [b]	SES [b]	PS [c]	SES [c]	PS [d]	SES [d]	PS [e]	SES [e]	PS [f]	SES [f]
Cd	0.5	0.5	0.5	0.4	1.58	1.04	1	1	n.d.	n.d.	n.d.	n.d.	2.7	3.0
Cr	55.8	58.5	56.2	34.8	26.6	17.8	17	32	64.7	60	n.d.	n.d.	57.3	54.8
Cu	143.7	150.7	248.8	301.4	151	143	100	91	58	67.5	408.8	497.7	123.6	138.3
Ni	20.7	20.6	16.9	16.9	18	13.9	37	31	52.6	49.9	n.d.	n.d.	55.0	62.0
Pb	137.1	124.7	82.5	78.7	94.3	60.2	28	16	35.3	28.1	91.8	109.8	123.5	137.7
Zn	469.1	469	442.4	464.2	418	326	350	440	710.9	595.6	740.8	810.9	1429.5	1641

[a1, a2] two different WWTPs (Gianico et al., 2013); [b] (Solís et al., 2002); [c] (Karvelas et al., 2003); [d] (Wang et al., 2006); [e] (Li et al., 2015); [f] (Tytla, 2019); n.d.-no data.

Sometimes the primary/secondary/combined sludge(s) undergoes further treatment before agricultural use (Kor-Bicakci and Eskicioglu, 2019). One of the most common methods is anaerobic digestion (Dauknys et al., 2016; Hanum et al., 2019). Generally, the anaerobic digestion of sludge can increase metal concentrations in the final digested sludge. For example, Chipasa (2003) reported that the average concentration of metals (Cd, Cu, Pb and Zn) in digested sludges were 1.7-2.0 times higher than those in the undigested sludges. Although this seems to be the worse-case range, as linear regression analysis of Chipasa’s data (see Figure 2.4) suggests the contaminant metal concentrations in anaerobically digested sludge are typically 1.4 times higher than in the undigested sludge. The increase in metal concentrations is principally attributable to the decrease in the mass of volatile solids during digestion (Karvelas et al., 2003; Tytla, 2019). Typically, there are 70-75% volatile solids in the feed sludge and 40-60% of them are digested, corresponding to 1.4-1.7 times increase in metal concentrations (Wisconsin DNR, 1992). Additionally, soluble metal complexes can be transferred to the digester in the water phase of the thickened sludge. These can then be reductively precipitated in the digester as metal sulphides, contributing further to the increase of metals content in the digested sludge (Miller Alonso Camargo-Valero, *pers comm*.).

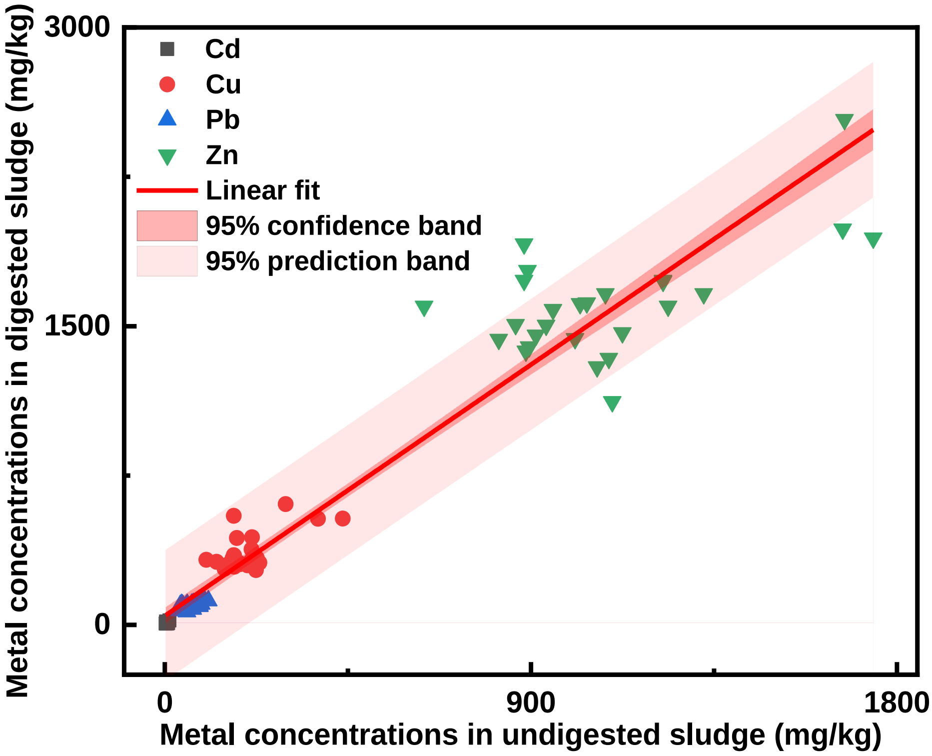

Figure 2.4 Linear fit results of four metals concentration in anaerobically digested sludge versus that in undigested sludge. The data is from Chipasa (2003).

2.4.2 Metal speciation in sewage sludge

The European Community Bureau of Reference (BCR) sequential extraction is widely used to determine the operationally defined speciation of metals (Alan and Kara, 2019; Chwastowska and Sterlinska, 2000; Cid et al., 2001; Larner et al., 2006; Li et al., 2010; Sungur et al., 2014b; Ure et al., 1993; Yao, 2008; Zimmerman and Weindorf, 2010). This extraction procedure was originally developed for soils and sediments (Nemati et al., 2009), but it has subsequently been adopted for a variety of matrices, including SS, due to the simplicity of its operational procedures and the repeatability of the metal species results (Jasińska, 2018; Tong et al., 2020). It initially divides metal speciation into three fractions: exchangeable/acid-extractable, *i.e.* weakly adsorbed to mineral surfaces or bound to carbonates; reducible, *i.e.* bound to Fe-Mn oxides; oxidizable, *i.e.* bound to OM and/or sulphides (Tokalioğlu et al., 2006; Tong et al., 2020). The residual fraction was added into the revised BCR scheme in 1999 (Rauret et al., 1999). There are potential drawbacks with BCR extraction such as the incomplete selectivity of reagents, incomplete dissolution of target phases, re-adsorption and re-precipitation of metals (Horváth et al., 2010; Jasińska, 2018; Larner et al., 2006; Nevidomskaya et al., 2021). However, this straight-forward chemical extractions can provide useful information about the associations of metals with particular geochemical phases, and thus provide insight into processes that may mobilise metals from a material, and the conditions under which a material may present an environmental risk (Dabrowska, 2016; Nevidomskaya et al., 2021).

Studies that have used BCR scheme on SS suggest that the proportion of most metals in each operationally defined fraction is generally similar in PS and SES (see Figure 2.5). Similar findings are also reported by Oake et al. (1984) and Mehrotra et al. (2008). However, the speciation pattern of each metal in sludges is different. In both PS and SES, Cu is principally in the oxidizable fraction, which is usually attributed to the affinity of OM for Cu and high stability of Cu-OM complexes (Gu et al., 2019; Jakubus and Czekała, 2001; Lasheen and Ammar, 2009; Walter et al., 2006; Zufiaurre et al., 1998). Zn and Ni are distributed in between exchangeable, reducible, oxidizable, and residual fractions. The presence of a proportion of these metals in the exchangeable fraction suggests that a proportion of them may be readily solubilised, implying their higher mobility in SS (Katana et al., 2013; Sowunmi et al., 2020). Cr and Pb in both PS and SES are predominantly distributed in between oxidizable and residual fractions, showing their strong ability to form complexes with OM or become incorporated into residual phases thereby reducing their mobility (Narwal et al., 2008). In general, Pb and Cr are less of a concern because of their lower mobility and lower concentrations in SS. Cd is primarily distributed in between reducible, oxidizable, and residual fractions, although a small proportion of Cd is often found in the exchangeable fraction. Despite a small amount of exchangeable Cd in SS, this may still be a concern due to its hazards to environment and human health even at lower concentrations (Burke et al., 2016; Genchi et al., 2020; Hocaoglu-Ozyigit and Genc, 2020).

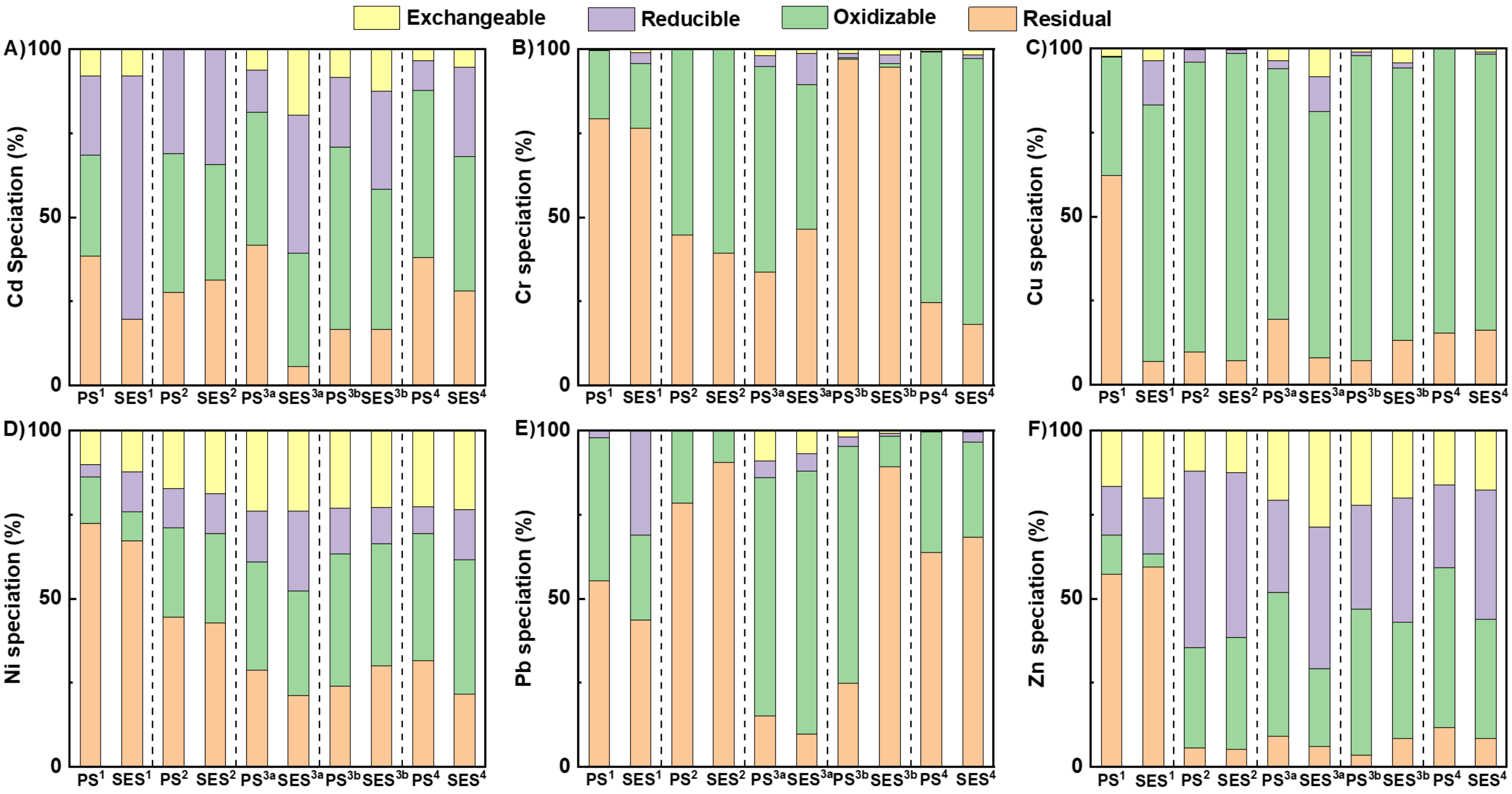

Figure 2.5 The speciation pattern of metals in PS and SES. [1] data from (Solís et al., 2002); [2] data from (Tytla, 2019); [3a, 3b] data from two different WWTPs (Tytla et al., 2016); [4] data from (Alvarez et al., 2002).

2.5 Impacts of sewage sludge application on metal concentration and speciation in soil

2.5.1 Impacts of sludge application on metal concentration in soil

The metal concentrations in SS are generally greater than the natural background, so its agricultural application inevitably increases the metal levels in the soil (Garcia-Delgado et al., 2002; Gibbs et al., 2006; Liu and Sun, 2013; Mcgrath and Cegarra, 1992). Historically, there have been cases where long-term sludge application has resulted in metal accumulation in the soil becoming problematic (Alloway and Jackson, 1991). For example, Chumbley and Unwin (1982) measured metal concentrations in the UK agricultural soils with a history of sludge application during a period when metals disposal to sewer was less regulated, and showed that Cd and Pb concentrations in that soil (26.2 and 496 mg/kg, respectively) were much higher than the current regulatory limits in the UK. Also, the concentration ranges of Cd, Ni, and Cr in soils from Northeast Spain have increased by approximately 15-20% due to 10 years of sludge application (Jordan-Vidal et al., 2020). Despite these reservations, metal concentrations in agricultural soils generally remain below statutory limits even after repeated sludge applications (Jordan-Vidal et al., 2020; Obbard, 2001; Topcuoğlu, 2014), although this situation cannot continue indefinitely without a mechanism for removing metals from the soil (Antonkiewicz et al., 2022; Natal-da-Luz et al., 2012). Therefore, careful monitoring of metals content of the agricultural soil is a necessity if SS is to be applied without causing permanent damage to the agricultural land.

Most metals introduced by SS tend to be retained in surface soils (around 0-20 cm), especially in the short-term (Camobreco et al., 1996; Campos et al., 2019; Canet et al., 1997; Jordan-Vidal et al., 2020; Richards et al., 1998; Sloan et al., 1998). However, high-mobility metals have the potential to be transported deeper into the soil after long-term SS application. For example, Legret et al. (1988) applied very heavily contaminated sludge to soil over an eight-year period and found that metals that persisted in the readily exchangeable fraction of the soil migrated to a depth of 60-80 cm, whereas metals associated primarily with the oxidizable fraction migrated to a depth of 40-60 cm. Thus, the speciation of metals when they are introduced with SS, and any transformations in speciation that may occur over time in the soil will determine whether metal mobility is a potential long-term issue.

2.5.2 Impacts of sludge application on metal speciation in soil

Studies have shown that SS application to soils can cause the redistribution of sludge-borne and soil-borne metals in the sludge-soil mixtures to different operationally defined phases (Fadiran et al., 2014; Rosazlin et al., 2007; Shrivastava and Banerjee, 2004). This redistribution process of metals is controlled by many factors, such as the chemical forms of each metal in sludges and soils, the dosage of sludge application/the amount of metals introduced to soil via sludge (Garcia-Delgado et al., 2002). It has been widely reported that the operationally defined speciation of contaminant metals (determined by sequential extractions) is different in untreated soil and SS, and that metal speciation in sludge-soil mixtures is intermediate between that of the two components (Fadiran et al., 2014; Rosazlin et al., 2007). However, according to the currently published literature, it is still unclear whether the resulting metal distribution between the operationally defined phases is simply the result of mixing, or whether there are changes in metal speciation upon mixing. Furthermore, there is very little information available on whether the operationally defined speciation of metals in SS amended soil evolves over time due to plant growth and rhizo-microbiome respiration.

Logically, changes in the operationally defined speciation of Zn and Cu are easiest to discern in SS-amended soil, because these contaminant metals have the highest concentration in SS. Therefore, studies where operationally defined speciation of Zn and Cu has been determined using the sequential extraction scheme have been collated (see Figure 2.6). In untreated agricultural soil, more than 50% of Cu and Zn are in the residual fraction, suggesting that the majority of these metals in soils are not bioavailable for crops uptake (Kotoky et al., 2015). However, Cu in SS is predominantly associated with the oxidizable fraction, and Zn in SS is distributed in between exchangeable, reducible, and oxidizable fractions (note: data from soil treated with different types of SS, including PS, SES, and anaerobically digested SS, have been averaged in Figure 2.6).

Where soil is treated with an amount SS of typical agricultural use, the speciation pattern of Cu and Zn in the treated soil is similar to that in untreated soil, with most present in the residual fraction (soils were analysed 1-month, 2-month, 1-year, and 2-year after treatment). This is not surprising, as the amount of the metals added with a typical agricultural application of SS is relatively small compared with the background values in the agricultural soil (only minor increases in total concentrations of Cu and Zn were reported in these studies). In two heavily treated soils (where the Cu and Zn introduced by addition of 20% SS accounted for approximately 70% of the metals in the final mixtures) the Zn and Cu speciation patterns were intermediate between the untreated soil and the SS after 50 days, but more similar to that in SS (Brazauskiene et al., 2008). However, this study is insufficient to determine whether the final speciation patterns can be explained solely by mixing. Similarly, Garcia-Delgado et al. (2002) applied SS at about 10-20 times the typical agricultural rates, which produced a final Cu content that was 2-3 times greater than that in the untreated soil, and again found that the Cu speciation in the mixture was more similar to that in the SS than the untreated soil for up to a year after treatment.

So, as would be anticipated, current studies clearly show that the sludge application can increase the amount of bioavailable metals in a soil (at least in the short-term), and long-term application can lead to the accumulation of metals in soils. Therefore, it is essential to regularly monitor the bioavailable metals content when repeatedly applying sludge to the agricultural soil to control those risks. However, current studies are insufficient to show the changes of metal speciation over time due to crop growth and microbial respiration in the rhizosphere (which will mineralise OM), and thus this topic needs further research (Shrivastava and Banerjee, 2004; Zaragüeta et al., 2021).

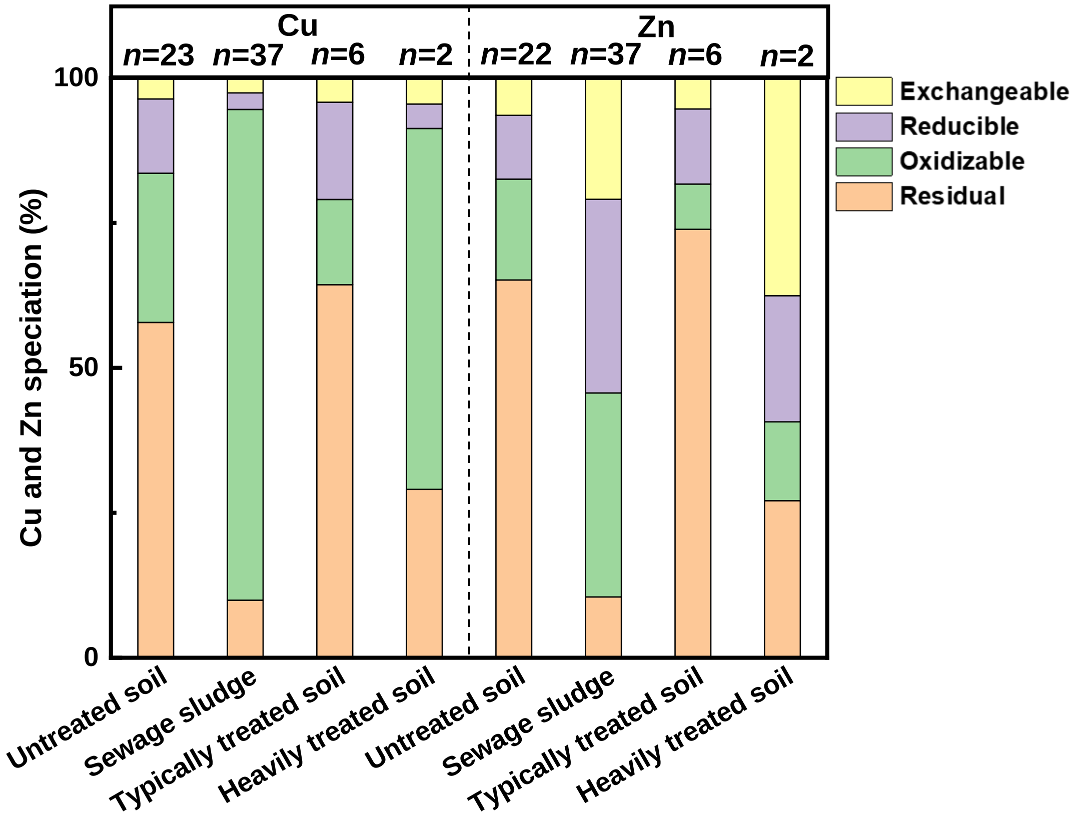

Figure 2.6 Average Zn and Cu speciation in untreated soil, sewage sludge and sludge-amended soil based on BCR scheme. Data for untreated soil from (Alan and Kara, 2019; Brazauskiene et al., 2008; Doelsch et al., 2006; Horváth et al., 2010; Rahman and Schoenau, 2022; Sahito et al., 2015; Sekhar et al., 2002; Sungur et al., 2014a; Topcuoğlu, 2014; Vollprecht et al., 2020; Yang et al., 2013). Data for sewage sludge from (Alvarez et al., 2002; Chen et al., 2008; Dabrowska, 2016; Dabrowska, 2012; Fuentes et al., 2004; Solís et al., 2002; Tytla, 2019, 2020; Tytla et al., 2023; Tytla et al., 2016; Zdeb et al., 2020). Data for typically treated soil from (Doelsch et al., 2006; Illeraa et al., 2000; Jin et al., 2017; Morera et al., 2001). Data for heavily treated soil from (Brazauskiene et al., 2008).

2.6 Problems on metal bioavailability in sludge-soil system

Long-term field studies in the UK have shown that SS has land productivity benefits, such as increased nutrient availability, but it also increases contaminant metal concentrations in the soil (Gibbs et al., 2006; Mantovi et al., 2005; McGrath, 1984). Importantly, it can increase the proportion of the contaminant metals that are bioavailable relative to untreated control soils (Kidd et al., 2007). The long-term field studies after cessation of sludge application have also shown that crop uptake of Zn and Cd in a given season is proportional to the soil concentrations, and that a proportion of the Zn and Cd in the soil remains bioavailable over more than 20 years (Mcgrath and Cegarra 1992; McGrath et al., 2000). More recent studies investigating metal speciation in the sludge-soil mixtures have typically been conducted over relatively short periods following sludge application, typically a few days (Malinowska, 2017; Mehrotra et al., 2008; Parvin et al., 2022; Qiao et al., 2003; Shrivastava and Banerjee, 2004; Wu et al., 2006; Zufiaurre et al., 1998). In essence, they only focus on the question “what is the instant changes in the bioavailability of contaminant metals in agricultural soils over sludge application”, but little attention has been paid to the metal bioavailability in the longer term. It is now becoming apparent that the chemical forms of metals in the environment are not constant, but instead evolves over time before achieving a new equilibrium (Garcia-Delgado et al., 2007; Prica et al., 2010; Scheckel et al., 2010; Wang and Li, 2012).

Soil composition, pH value, redox potential, and OM content will be the principal factors that determine the equilibrium of metal speciation that will be achieved in the long-term (Khadhar et al., 2020; Orhue and Frank, 2011; Zhang, 2015; Zhang et al., 2017). Applying SS to the agricultural land will potentially change the pH value, redox potential, and soil OM content and create disequilibria in the speciation of contaminant metals originally associated with the soil and of those introduced by SS, and these may take time to reach a new equilibrium. Soil pH is widely recognised as the major factor governing metal speciation in the soil (Gabler, 1997; Kicińska et al., 2021; Oburger et al., 2020; Orhue and Frank, 2011; Xu et al., 2020). Generally, the proportion of metals in the readily mobile fractions tends to increase at lower pH and reduce at higher pH values (Aigberua, 2018; Kicińska et al., 2021; Olaniran et al., 2013; Sherene, 2010). For example, Ullrich et al. (1999) observed that the average proportion of exchangeable metals (Cd, Pb and Zn) continuously reduced with the increasing soil pH. In addition, the OM in soil is relatively labile and is likely to be mineralised on longer timescales, potentially releasing any OM-associated metals (Caracciolo and Terenzi, 2021; De Conti et al., 2018; Natal-da-Luz et al., 2012). Local climate, weather, farming practices and irrigation conditions are further vector for longer geochemical changes (Kelderman and Osman, 2007; Parvin et al., 2022; Rieuwerts et al., 2015; Skarpa et al., 2011), as will be plant growth as plants can exude organic chemicals that can complex metals (Caracciolo and Terenzi, 2021; De Conti et al., 2018; Gan et al., 2020).

Therefore, over time geochemical and microbiological processes in the soil may transform the chemical forms of contaminant metals from that observed in short-term studies. At this stage, it is unclear whether these changes will increase the bioavailability of contaminant metals or sequestered them in unreactive minerals. Typical sludge application rates are likely to have a small and transient impact on the soil geochemistry and cause only a small increase in contaminant metal concentrations, so there are no land-use changes. The speciation of contaminant metals within the soil before SS application may be a guide to the long-term “equilibrium” speciation. If so, it may mean that the risks posed by metals introduced by SS application are overestimated by short-term studies and could result in overly conservative restrictions on SS use in agriculture. Potentially, if the metals speciation in treated soil evolves towards that in the untreated soil, it may mean that the risks posed by metals introduced by agricultural application of SS are currently overestimated, and result in unnecessary restrictions on SS use in agriculture. Therefore, to better apply SS to agricultural soils and efficiently control the risk, more studies are required to understand the transformation of metals behaviour and fate in the agricultural soil amended by SS.

2.7 Conclusion

This chapter provides a synthesis of global data on current sewage sludge production rates, estimates future sewage sludge production rates, and comprehensively reviews the literature on contaminant metals in sewage sludge and the impacts of sewage sludge application on metals behaviour in the soil. As a result, this chapter provides the following relevant new insights and perspectives.

- It is definitively estimated that currently about 53 Mt/yr dry sewage sludge is produced globally by wastewater treatment plants. This would increase to about 160 Mt/yr if the wastewater produced around the globe were treated to a similar level as in EU-27/UK.

- Organic pollutants, pathogens, contaminant metals and other emerging contaminants can be detected in sewage sludge. Effective sludge treatment can significantly reduce the concentrations of organic pollutants and pathogens. However, treatment has minimal impacts on metal concentrations.

- The three sources of metals in sewage sludge are domestic wastewater, industrial wastewater, and urban runoff. Typically, co-treatment of industrial wastewater results in higher contaminant metal concentrations in sewage sludge, than treating predominantly domestic wastewater with or without urban runoff. A large proportion of the metals in domestic and industrial wastewater are present in the soluble forms while metals in urban runoff are largely associated with particulates.

- In general, over 70% of metals in wastewater are transferred to primary sludge and secondary sludge. The partitioning of contaminant metals from wastewater to primary sludge is a physical process that is highly dependent on the metals being in forms, or associated with inorganic or organic particles that are settleable. While the partitioning of metals from primary effluent to secondary sludge is a biological process which strongly depends on the uptake of metals by the biomass and the separation of the biomass.

- Typically, the contaminant metal concentrations in sewage sludge decrease in the order of Zn > Cu > Cr ≈ Pb ≈ Ni > Cd. Anaerobic digestion of sewage sludge results in higher metal concentrations in the final sludge due to the loss of volatile solids and the reductive precipitation of soluble metal complexes transferred to the digester in water in the thickened sludge as metal sulphides.

- Zn and Ni are distributed in exchangeable, reducible, oxidizable, and residual fractions of sewage sludge, and are therefore relatively mobile. Cu is predominantly distributed in the oxidizable fraction and Cd is principally distributed in between reducible, oxidizable, and residual fractions. Their mobility is moderate. The mobility of Pb and Cr is generally low because they are predominantly distributed between the oxidizable and residual fractions of sewage sludge.

- The higher the metal content introduced via sludge, the more the pattern of metal speciation in the sludge-soil mixtures tends to be the pattern of metal speciation in the adopted sludge. Additionally, the metals speciation in the sludge-soil system will evolve with time due to the changes in soil properties and/or environmental conditions.

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Chapter 3: Methodology

3.1 Introduction

Through a systematic and comprehensive literature review in Chapter 2, the aim and objectives of this project were determined (see Section 1.3). A series of laboratory experiments were then designed and conducted to achieve the project aim and a wide range of objectives. In specific, (1) an investigation into sorption behaviours of digested sewage sludge (SS) for Cu and Zn was conducted to determine the appropriate amended sludge for later use in soil amendment experiments (Chapter 4); (2) “pot trial 1” was designed for investigating the transformation of Cu and Zn speciation in agricultural soil amended with digested sludge over time and with repeated plant growth (Chapter 5); (3) a combined study consisting of pot trials 2 and 3 was designed for exploring the distribution and speciation of Cu and Zn near plant roots in digested sludge-amended soil (Chapter 6). All the pot trials were carried out in a laboratory setting, using either a plant growth chamber or a LMS Cooled Incubator, to facilitate easy control of plant growing environments. The plant growth performance and transformations of metal behaviour in sludge-soil-plant systems can be therefore monitored experimentally. This chapter will introduce the details of methodology of these experiments.

3.2 Methodology for investigating isotherm sorption behaviour of original digested sludge for Cu and Zn

To discern the transformation of contaminant metals in the sludge-soil/sludge-soil-plant system, it is necessary to produce a sludge-amended soil for plant growth experiments. The metal concentrations in the sludge-amended soil are needed to be elevated through one dose application of amended sludge for experimental monitoring of metal behaviour. To determine the appropriate amended sludge for use in the later soil amendment experiments, there is a need for understanding the isotherm sorption behaviour of original digested sludge for metals (Cu and Zn).

3.2.1 Collection and preparation of materials

3.2.1.1 Collection of original digested sludge and agricultural soil

Original digested sludge was collected from Esholt Wastewater Treatment Works, a large wastewater treatment plant in West Yorkshire, UK, serving approximately 760,000 people (Edina, 2024). It was a secondary treatment sludge that had undergone thermal hydrolysis at a temperature of 165°C and a pressure of 6 bar for a period of 30 min prior to anaerobic digestion. The original agricultural soil was collected from a working arable field at Spen Farm, Tadcaster, UK (Lat. 53.8699, Long. -1.3290) on 24th August 2021 when the field contained a mature plant of maize (taken at 8 randomly selected locations between the plants). The field has been annually ploughed and cropped with conventional management since 1995 (Berdeni et al., 2021). The general fertiliser inputs to this field are detailed in Holden et al. (2019). The agricultural soil is a Calcaric Endoleptic Cambisol, with a silt loam texture containing stone fragments derived from the underlying dolomitic limestone (Cadeby formation) (Guest et al., 2022; Humphries et al., 2023). The original agricultural soil and original digested sludge were stored at 4°C in the refrigerator for future use. Sub-samples of the original agricultural soil and digested sewage sludge were air-dried, disaggregated by the Retsch RS200 Disc Mill (see Figure 3.1), and sieved by a 106-μm sieve for characterization prior to any experiments.

Figure 3.1 Retsch RS200 Disc Mill.

3.2.1.2 Preparation of metal-amended sludges

The analytical grade $ZnCl_2$ and $CuCl_2 \cdot 2H_2O$ (solids) were added to the original digested sludge (dry solids content: 45.6 g/l) at six different ratios (0.4%, 0.8%, 1%, 2%, 5% and 8%; $m_{\text{metal}}/m_{\text{dry sludge solids}}$). Based on a preliminary investigation, all the metal-amended sludges were shaken for 24 hours in an INFORS HT Multitron Pro incubator (see Figure 3.2A), ensuring sufficient metal sorption by original digested sludge. Then the metal-amended sludges were centrifuged at 3000g for 20min (Eppendorf Centrifuge 5810, see Figure 3.2B), before aqueous phase was decanted off and the solids were air-dried, disaggregated, and sieved. The total Zn and Cu concentrations and their chemical forms in the metal-amended sludges were determined by aqua regia digestion and BCR extractions, respectively.

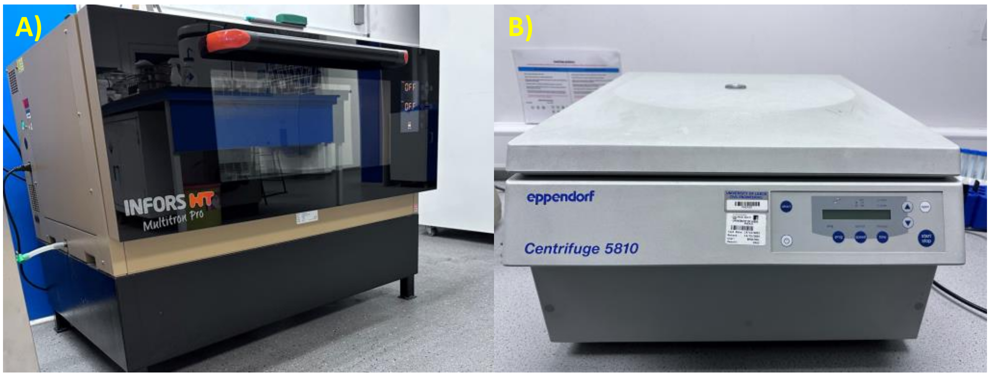

Figure 3.2 A) INFORS HT Multitron Pro incubator shaker and B) Eppendorf centrifuge 5810.

3.2.2 Analytical methods of sludge sorption experiments

3.2.2.1 Characterization of original digested sludge and agricultural soil

The pH was measured by the 1:2.5 solid/water (*w*/*v*) suspension method using a calibrated pH meter (see Figure 3.3A, HQ40d Digital multi meter kit; Jordan-Vidal et al., 2020). The organic matter content was measured with the loss on ignition in the Carbolite AAF 1100 furnace (550°C for 3h), as shown in Figure 3.3B (Gerenfes et al., 2022). The total Kjeldahl nitrogen and total phosphorus content were determined using the total nitrogen kit (Hach APC338) and total phosphorous kit (Hach APC350) using a Hach AP3900 laboratory robot, as shown in Figure 3.3C (AlHaddad, 2022).

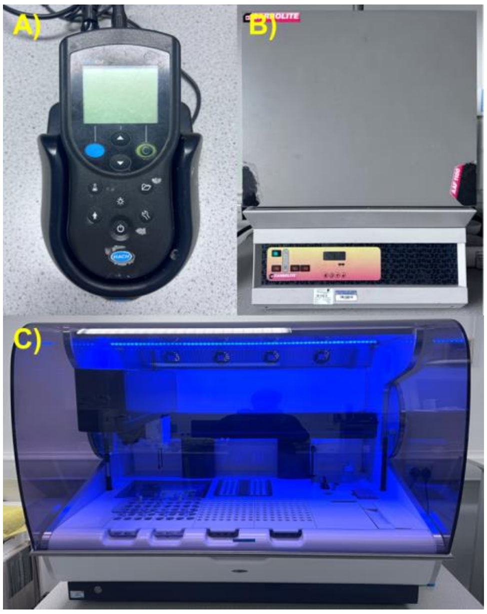

Figure 3.3 A) Hach HQ40d multiple meter; B) Carbolite AAF 1100 furnace; C) Hach AP3900 laboratory robot.

3.2.2.2 Aqua regia digestion procedure

Total metal concentrations in soils and sludges were determined by aqua regia digestion on triplicate samples (Turek et al., 2019). Metal concentrations in the digested solution were then analysed by the atomic absorption spectrophotometer (AAS, VARIAN 240 FS, see Figure 3.4A; optimum working ranges: 0.03-10 μg/ml for Cu at a wavelength of 324.7 nm and 0.01-2 μg/mL for Zn at a wavelength of 213.9 nm).

Specifically, ~0.2 g of dried sludge/soil sample was weighed into a conical flask and a 10-ml aqua regia solution was added. The flask was heated on a hot plate (Cole-Parmer HP-200D-XL-C; see Figure 3.4B) to achieve effervescence and then for a further 30 min. After cooling, the contents of the conical flask were transferred to a 100-ml volumetric flask. The distilled water was added to a volume of 100 ml. The suspension was filtered with a 0.45-μm syringe filter, and the solution was analyzed by AAS. In addition, to ensure comparability with other laboratories, total Zn and Cu concentrations in a certified reference material (LSKD-2, typical lake sediments from various locations within the Canadian Shield, provided by CCRMP, CANMET Mining and Mineral Sciences Laboratories) were determined by this aqua regia digestion procedure.

Figure 3.4 A) Atomic absorption spectrophotometer and B) Cole-Parmer HP-200D-XL-C hot plate.

3.2.2.3 BCR sequential extraction procedure

The operationally defined metal speciation in sludges/soils were determined by a modified BCR scheme (Yang et al., 2018). The extractions were conducted on 0.5g of dry samples in 50-ml polypropylene centrifuge tubes. Specific steps were shown in Table 3.1. BCR extraction analyses were conducted on triplicate samples and the mass-balance on the recovery of contaminant metals was calculated (the amount of metals in the four fractions divided by the amount obtained by total digestion in aqua regia).

Table 3.1 BCR sequential extraction adopted for the determination of operationally defined metal speciation.

Step	Fraction	Extractant	Shaking time	Temperature
F1	Exchangeable	20 ml of 0.11 M CH_3COOH (pH=5)	16 h	Room Temperature
F2	Reducible	20 ml of 0.5 M $NH_2OH{\cdot}HCl$ (pH=2)	16 h	Room Temperature
F3	Oxidizable	5 ml of 30% H_2O_2 (pH=2)	1 h	Room Temperature
		/	1 h	85 ± 2°C
		a further aliquot of 5 ml of 30% H_2O_2 (pH=2)	1 h	85 ± 2°C
		25 ml of 1.0 M CH_3COONH_4 (pH=2.0)	16 h	Room Temperature
F4*	Residual	10 ml of aqua regia	0.5 h (after effervescence)	Heating on a hot plate

* the residue from step F3 is analysed as the same procedure used in the analysis of total metal concentrations to obtain the content of residual fraction. All extracted solution needs to pass through a 0.45-μm syringe filter and then AAS is used to detect Zn and Cu concentrations.

3.2.2.4 Scanning electron microscopy analysis

To prepare for microscopy, the selected sludge samples were mounted in epoxy resin blocks (EpoThin 2, a glycidyl ether epoxy resin), before being ground and polished with diamond pastes using water-free oil-based lubricant. Before scanning electron microscopy analysis (SEM), the selected sludge samples were coated with 15-20 nm carbon by thermal evaporation. Then the Tescan Vega3 XM SEM at Leeds Electron Microscopy and Spectroscopy Centre, University of Leeds, was operated (beam energy 20 keV, working distance 15 mm). This SEM is equipped with an Oxford Instruments X-max 150 SDD EDS using Aztec software. The SEM images of samples surface were obtained using back-scattered electron detector (BSE). The elemental mapping was performed at a resolution of 2 μm. Based on the elemental maps, the spots of interests were selected and analysed by using the equipped energy dispersive spectrometer (EDS) to obtain the chemical composition.

3.2.2.5 X-ray absorption near edge strucure analysis

The bonding environments and oxidation state of Cu and Zn in the original digested sludge and the amended sludge adopted for soil amendment experiments were analysed using the bulk X-ray absorption near edge strucure (XANES) technique. The XANES spectra were collected at Cu and Zn K-edges (8979 and 9659 eV respectively) on beamline I18 at the Diamond Light Source operating at 3 GeV with a typical current of 200 mA, using a nitrogen cooled Si(111) double crystal monochromator and focussing optics. A pair of plane mirrors was used to reduce the harmonic content of the beam and the Kirkpatrick-Baez mirrors were used to produce either a focused or unfocused beam (0.005 mm or 0.1 mm diameter respectively at the sample) for microfocus or bulk sample analysis as required.

For standards prepared as pressed pellets, K-edge spectra were collected in transmission mode using ionisation chamber detectors. For samples and solutions, data were collected in fluorescence mode using a 4 element 1 mm thick sensor Vortex Si Drifts detector with cube pre-amps. All data collection was performed at room temperature (~22°C) within a He-filled bag (to lower the attentuation of low energy fluorescence X-rays). For bulk XANES analysis, multiple scans (at least three scans) were averaged to improve the signal to noise ratio using Athena version 0.9.26 (Ravel and Newville, 2005). All XANES spectra were normalised in Athena over the full data range and plotted from approximately -30 eV to +30 eV relative to the edge position. Spectra were corrected for any drift in E_0 using the data collected from the metal foil standards. Linear combination fitting (LCF) was preformed in Athena using the full range of available standards to determine the most likely combinations of standards to best fit the sample. In LCF analysis, the number of reference standards used was limited to a maximum of 3 to reduce the degree of freedom present and all fits were forced by the software to produce results that summed to 100%. LCF analysis typically produced results of metal speciation with an uncertainity of ±4%. Additionally, it is noted that LCF analysis often cannot identify the exact phases present in environment samples as many phases can share similar molecular coordination environments. Nevertheless, it can provide valuable information on the valence states and dominant elemental bonding environments present.

3.3 Methodology for investigating evolution of Cu and Zn speciation in agricultural soil amended by digested sludge over time and repeated plant growth

3.3.1 Seeds germination

The seedlings of spring barley (*Hordeum vulgare*) were used in all the three pot trials. The spring barley seeds were purchased from Cot5wold Seeds Ltd and germinated between two filter papers soaked with half-strength Murashige and Skoog nutrient solution (2.2 g/l, pH=7.0) on petri dishes (5 seeds per dish). The petri dishes were sealed with the parafilm and kept in the dark at room temperature for 48h to allow seeds germination (dark conditions favour the germination of spring barley seeds, see Figure 3.5). The spring barley seedings were then transplanted to the “planted pots” in the pot trials as required.

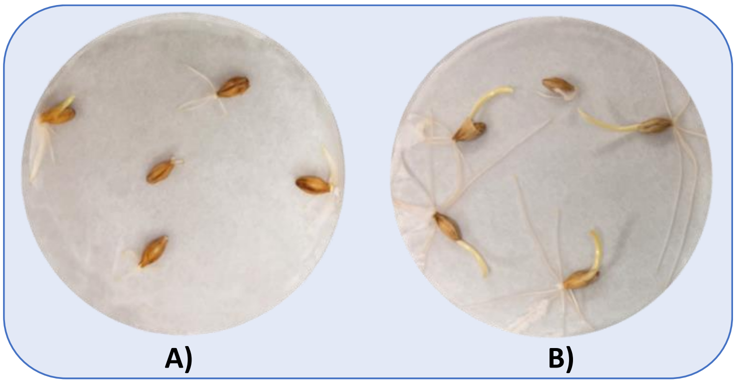

Figure 3.5 Germination of spring barley seeds for 48 h in A) light conditions and B) dark conditions.

3.3.2 Preparation of sludge-amended soil for pot trial 1

Based on the results of sludge sorption experiments, the original digested sludge was amended to a final concentration of ~20,000 mg/kg Cu and Zn. The amended sludge was then added to the original agricultural soil at a ratio of 5 wt% by dry weight. The amended sludge and soil were mixed for ~5 hr in a Hobart mixer (model: A200) and allowed to stand for 21 days. This produced a sludge-amended soil containing ~1000 mg/kg Zn and Cu, where ~90% of the Cu and Zn were introduced with the amended sludge.

3.3.3 Pot trial 1 establishment

In the pot trial 1, the plug-trays containing square prismatic “pots” were used (top surface: 5 cm × 5 cm; bottom surface: 3.5 cm × 3.5 cm; height: 4.5 cm). Each pot was equipped with two layers of needle-punched polypropylene geotextile (supplied by Spudulica) at the bottom to avoid soil loss from drainage holes. Each pot was filled with 95 g prepared sludge-amended soil as described above in Section 3.3.2 (initial moisture content: 15.3%). Spring barley seedlings were transplanted to the “planted pots” by placing them in a prepared hole on the soil surface and then the loose surrounding soil was used to cover the plant seedlings (approximately 0.5-1 cm deep; 1 seedling per pot in the first growth round).

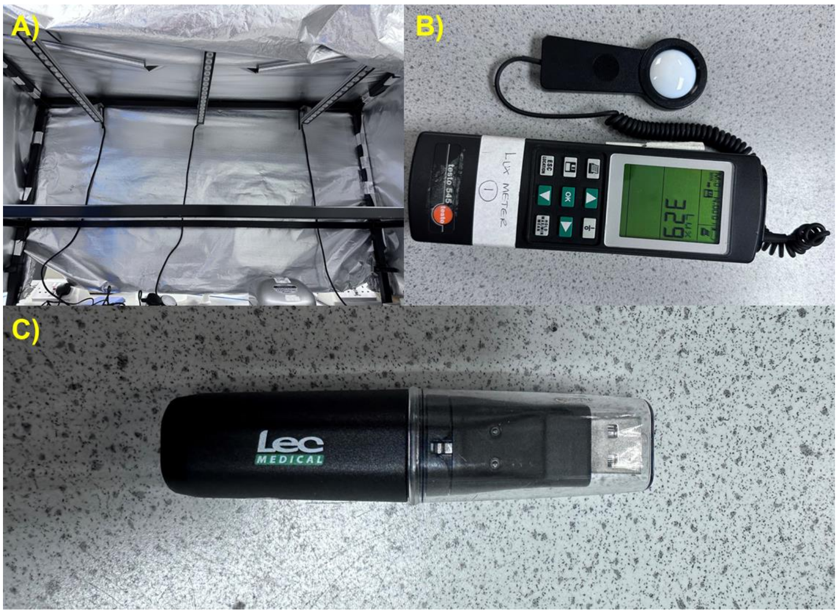

Figure 3.6 A) Growth chamber; B) Testo 545 lux meter; C) Auto-logger.

The planted pots (48 pots) and controls (12 pots) were placed to a laboratory growth chamber (see Figure 3.6A) for 6 weeks. The growth chamber consisted of a steel frame covered with Mylar foil and 3 LED growth lamps (54W LED growth lamps, model: HY-55cm-18*3W-RB, UK) mounted at the top of the frame, powered with timer switches on a 12-h on/off cycle. The lamps delivered about 18-26 μmol/m^2/s of photosynthetically active radiation in the 400-700 nm range, measured at the level of soil surfaces using the Testo 545 lux meter (see Figure 3.6B). During plant growth phase, the moisture content of sludge-amended soil was maintained at 35% (about 70-80% of the field capacity) by regular irrigation with distilled water (Berdeni et al., 2021). The temperature inside the growth chamber was monitored in real-time using the temperature auto-logger (see Figure 3.6C).

After the completion of the first growth round, all the plants were harvested. The soils recovered from the planted pots (bulk soil) and controls (control soil) were collected separately, and each was mixed and sampled. The remainder of the bulk and control soils were used separately for planted pots and controls in second and third rounds. The second and third rounds followed the same procedures, except two spring barley seedlings were planted in each planted pot (38 planted pots and 10 controls in the second round; 32 planted pots and 8 controls in the third round).

3.3.4 Sampling protocols of pot trial 1

3.3.4.1 Plants

The plant height (from soil surface to the tip of plant flag leaf) was recorded every 7 days during growth period. After six weeks, the plants were harvested. The plant root system was carefully removed from the planted pots. All the plant roots were then put into a 1-L beaker containing 600-700 ml distilled water, which was placed in an ultrasonic bath for 30 min to dislodge any soil particles from the plant roots. The washing procedure was repeated 10 times (see Figure 3.7). The plant shoots were also washed with distilled water. The washed plants were oven-dried at 105°C for 30 min and then at 60°C for a further 72 h (see Figure 3.8A and B). The dry weight biomass of plant roots and shoots were measured separately. The plant shoots and roots were ground in a Retsch CryoMill (see Figure 3.8C and D) and stored at 4°C in the refrigerator for future analysis.

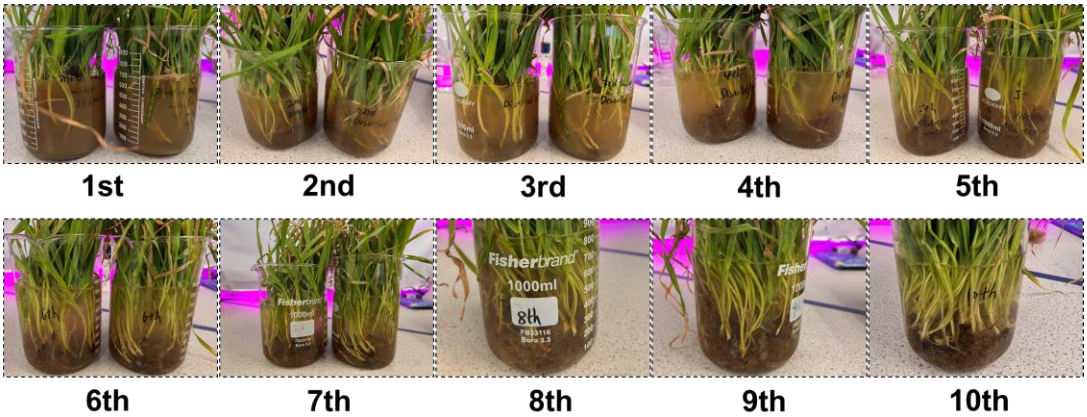

Figure 3.7 Washing procedure for plant roots.

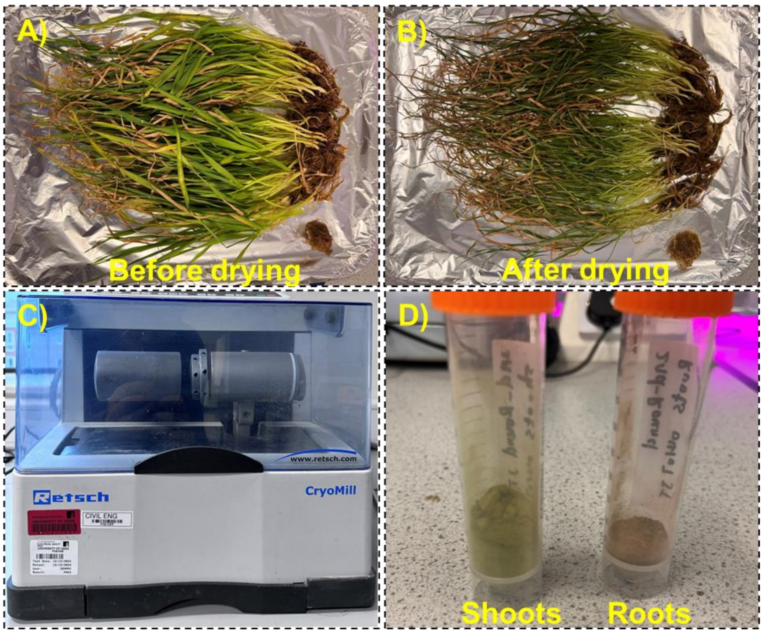

Figure 3.8 Harvested plants A) before and B) after drying procedure; C) Retsch CryoMill; D) ground plant shoots and roots.

3.3.4.2 Sludges and soils

The entire volume of soil suspension obtained from the sonification of plant roots in each growth round was evaporated in an oven at 60°C. This temperature has been suggested to have minimal effect on the chemical forms of metals (Zufiaurre et al., 1998). Any root fragments in the root-bound soil were removed and the remaining root-bound soil was prepared for aqua regia digestion and BCR extraction analysis (the root-bound soil was not retained from the first growth round due to a laboratory accident).

Samples of original agricultural soil, original digested sludge, bulk soil and control soil from the three rounds were each divided into two parts. One part was prepared for BCR sequential extractions and aqua regia digestion. The other part was freeze-dried, ground to a fine powder, and prepared as 8-mm pressed pellets held in Kapton™ tape for XANES analysis (see Figure 3.9A). The metal-amended sludge (approximately 20,000 mg/kg Cu and Zn) and the sludge-amended soil (prior to pot trial 1) were each divided into three parts. Two parts were treated as described above. The last part was dried by solvent displacement and then embedded in the 30-mm polished epoxy resin blocks (EpoThin 2, a glycidyl ether epoxy resin; see Figure 3.9B) to allow

SEM-EDS and microfocus X-ray fluorescence (µXRF) analysis. Each resin-embedded sample block was ground against silicon grinding papers of decreasing grit (P600 and P1200) in the SAPHIR 330 grinder (see Figure 3.9C) before polishing with 3-, 1- and ¼- *µ*m water-free oil-based diamond paste (see Figure 3.9D) in the BUEHLER AutoMet 250 polisher (see Figure 3.9E).

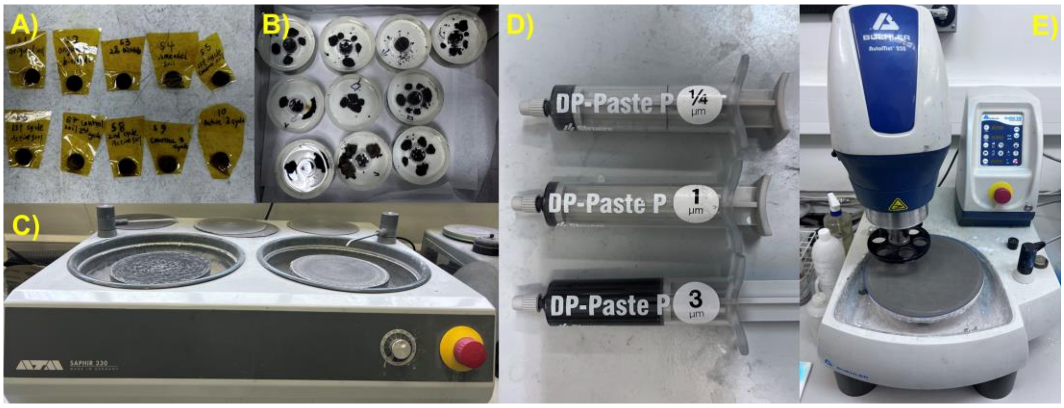

Figure 3.9 Prepared samples for A) XAS and B) SEM-EDS and µXRF analyses; C) Saphir 330 grinder; D) Diamond paste and E) BUEHLER AutoMet 250 polisher.

3.3.5 Analytical methods of pot trial 1

3.3.5.1 Aqua regia digestion analysis

Total metal concentrations in soils, sludges and plants were determined by aqua regia digestion. For soils and sludges, the digestion procedures have been introduced in Section 3.2.2.2. It should be noted that for plants, to avoid excessive effervescence (plant matter reacts quickly with aqua regia), plant roots/shoots (~0.2 g) were first digested in hydrochloric acid (5 ml) at room temperature, and then evaporated to a dry residue. On cooling, the residue underwent aqua regia digestion as described above.

3.3.5.2 Sequential extractions analysis

The operationally defined speciation of Cu and Zn in sludges/soils was determined by the modified BCR scheme described in Section 3.2.2.3. Specific steps of BCR extractions can be seen in Table 3.1.

3.3.5.3 SEM-EDS analysis

To investigate the elemental distribution in the amended sludge and amended soil, the samples were carbon-coated after polishing treatment and received the SEM-EDS analysis. The operating conditions of SEM-EDS have been detailed in Section 3.2.2.4.

3.3.5.4 XAS and µXRF analysis

To determine the bonding environments and oxidation state of Cu and Zn in the sludges/soils, the sludges/soils further recevied the analysis of XANES. The operating conditions of XANES were shown in Section 3.2.2.5.

3.3.5.5 Statistical analysis

Before conducting statistical analysis, the data were assessed for normality and homogeneity of variance. Repeated measures analysis of variance (ANOVA) was used to detect significant differences in plant height and total Cu or Zn concentrations in between the amended soil and control/bulk soil at a significant level of $p<0.05$ with SPSS version 26.0. Post hoc tests were adopted following ANOVA (LSD). The Student’s *t*-test was used to detect significant differences in total Cu or Zn concentrations between the bulk soil and root-bound soil (Kidd et al, 2007).

3.4 Methodology for investigating distribution and speciation of Cu and Zn near spring barley (*Hordeum vulgare*) roots in digested sludge-amended soil

A combined study including two pot trials was used for exploring the transformation of Cu and Zn behaviour and fate in close proximity to plant roots in the agricultural soil amended with digested sludge. A rhizo-pot system was used in the pot trial 2 and a plug-tray system was employed in the pot trial 3. In the subsequent description of experimental details, the terms, "rhizo-pot experiment" and "plug-tray experiment", were used separately to clearly represent each individual pot trial.

3.4.1 Collection and preparation of experimental materials

3.4.1.1 Collection of digested sludge and agricultural soil

For the plug-tray experiment, the sludge-amended soil used for plant growth was collected from the remaining amended soil of pot trial 1. For the rhizo-pot experiment, the sludge-amended soil was freshly prepared. The original digested sludge was collected from Esholt Wastewater Treatment Works, Leeds, UK, on 1st March 2023. The pH of this fresh digested sludge was 7.8 and its dry solids content was 53.3 g/l. The digested sludge contained about 180 mg Cu and 600 mg Zn per kg dry sludge solids. The agricultural soil was collected from the remaining unamended soil of pot trial 1. The description of this agricultural soil has been detailed in Section 3.2.1. The soil and digested sludge were stored at 4°C prior to any experiments.

3.4.1.2 Preparation of sludge-amended soil for rhizo-pot and plug-tray experiments

Two sludge-amended soils were used in this combined study. In the rhizo-pot experiment, to monitor the effect of root zone on Cu and Zn uptake, a sludge-amended soil with about 5000 mg/kg Cu and Zn was prepared. This concentration represents an agricultural soil heavily contaminated by sludge-introduced metals (it was chosen so that metal concentrations could be readily determined by aqua regia digestion and ensure that most of the metals in the sludge-amended soil were introduced with the digested sludge). A previous investigation had determined that linear sorption behaviour of digested sludge was largely maintained up to 2 wt% of Cu and Zn (Feng et al., 2024). Therefore, in this experiment the freshly collected original digested sludge was amended to a final concentration of ~20,000 mg/kg Cu and Zn, before the sludge was added to the soil to produce a final ratio of 25% by dry weight. The amended sludge and soil were mixed for ~10 hr in a Hobart mixer (model: A200) and then allowed to stand for 21 days.

The sludge-amended soil used in the plug-tray experiment contained ~1000 mg/kg of both Cu and Zn. This concentration represents an agricultural soil moderately contaminated by sludge-introduced metals (it was chosen so that most of the metals in the sludge-amended soil were introduced with the sludge, and so that metal bonding environments could be determined by μXANES analysis). This sludge-amended soil was prepared for a preceding study (pot trial 1), where it had undergone three rounds of spring barley growth. It was also a mixture of the amended sludge and agricultural soil as described above, but it contained 5% amended sludge by dry weight.

3.4.2 Plant growth chamber

All plant growth experiments were undertaken in the LMS Cooled Incubator (see Figure 3.10). Three LED growth lamps (Knightsbridge UCLED9CW, 230V, 9W, LED 4000K-538 nm) were mounted above the plants, on a 12-h on/off cycle. The lamps delivered about 99-107 and 43-48 μmol/m^2/s of photosynthetically active radiation for the rhizo-pot experiment and the plug-tray experiment, respectively (measured at the level of rhizo-pot and plug-tray surfaces). A day/night temperature regime of 26°C/24°C was chosen to maximize the plant growth (GHEDirect 2024). The growth conditions matched the experiment of pot trial 1.

Figure 3.10 LMS Cooled Incubators A) used in the rhizo-pot experiment; B) used in the plug-tray experiment.

3.4.3 Pot trial 2 - rhizo-pot growth experiment

3.4.3.1 Rhizo-pot system establishment

A rhizo-pot system similar to that described by Kissoon et al. (2010) was used in this experiment. The diagram of the rhizo-pot system was shown in Figure 3.11. It consisted of an upper plant compartment containing perlite and a lower soil compartment separated by a 32-μm nylon mesh (the size of this nylon mesh was chosen based on the results of a preliminary investigation). During plant growth phase, a horizontal root mat forms above the mesh. The nylon mesh physically separates the plant roots from the lower soil compartment but allow other substances such as water, nutrients and root exudates to exchange between the two compartments (Chen et al., 2021; Gao et al., 2014; McNickle and Godoy, 2020; Oburger et al., 2014). The lower compartment was loaded with 25g sludge-amended soil containing ~5000 mg/kg Zn and Cu (moisture content of 32.0%, slightly compacted to a height of 20 mm). The moisture content of the sludge-amended soil was adjusted to 35% (about 70-80% of the field capacity) and maintained at this level during growth phase by regular irrigation with distilled water. Three seedlings of spring barley were placed in the upper compartment. 4.5 g of saturated perlite (soaked in distilled water for 1 h) was used to cover the plant seedlings. The upper compartment was then placed over the sludge-amended soil surface on the lower compartment (planted rhizo-pot). Control rhizo-pot was prepared in the same way as the "planted rhizo-pot", but with no plant seedlings. Both planted and control rhizo-pots were placed in a LMS Cooled Incubator (see Figure 3.10A).

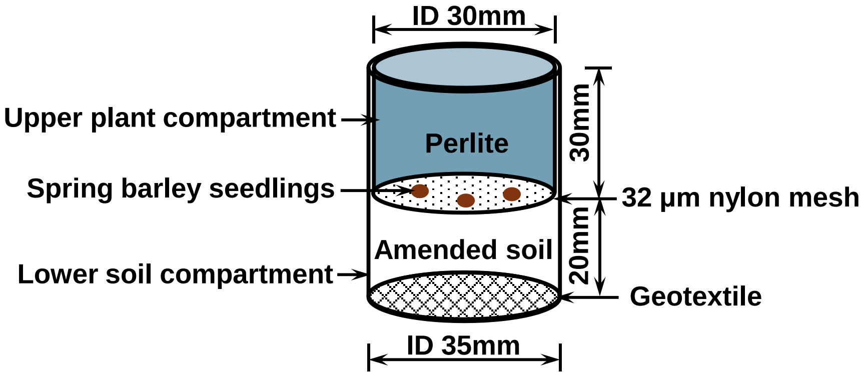

Figure 3.11 Diagram of rhizo-pot system.

A total of 45 planted and 15 control rhizo-pots were prepared for this experiment, which was conducted over five rounds of plant growth, with a 6-week growth period in each round. Upon the completion of each growth round, all the plants were harvested. After the first, third and fifth rounds, 5 planted and 3 control rhizo-pots were sacrificially sampled respectively. In addition, after each round, the nylon meshes were replaced in all remaining rhizo-pots and three more spring barley seedlings were grown in fresh perlite.

3.4.3.2 Sampling protocols of rhizo-pot experiment

Plants: plant height (from perlite surface to the tip of plant flag leaf) was recorded every 7 days during growth period. After each six weeks growth round, the plant roots system was carefully removed from the upper compartment, washed and oven-dried (washing and drying procedures see Section 3.3.4.1). Dry weight biomass of the plant roots and shoots were measured separately. The plant shoots and roots were then ground in a Retsch CryoMill and stored at 4°C for further analysis.

Soils: for the first, third and fifth rounds, the sludge-amended soil in the sampled lower compartments was excavated in six sublayers (0-2, 2-4, 4-7, 7-10, 10-15 and 15-20 mm distance from the nylon mesh) by “T-shaped” samplers (see Figure 3.12). The soil from each sublayer of each pot was thoroughly mixed separately, air-dried, disaggregated, and sieved with a 106-µm sieve for aqua regia digestion analysis.

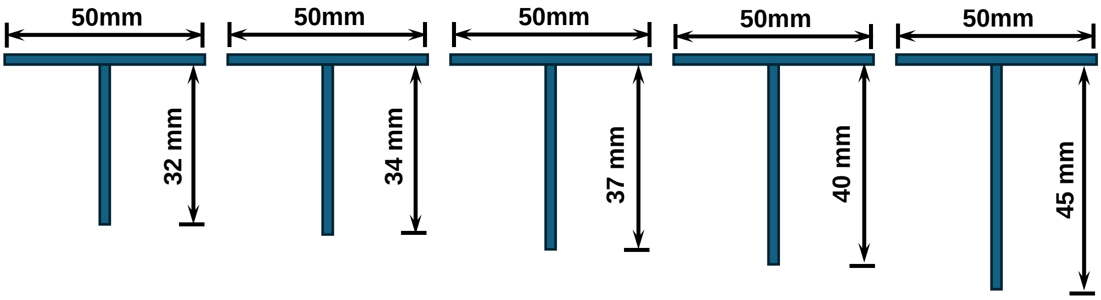

Figure 3.12 Diagram of T-shaped samplers.

3.4.3.3 Total metal concentrations data acquisition

Total metal concentrations in soils, sludges and plants were determined by aqua regia digestion (full details see Section 3.2.2.2). Statistical analysis of Cu and Zn concentrations in the sublayers of planted and control soil was performed by one-way ANOVA at a 5% significance level using SPSS version 26.0. Post hoc tests were adopted following ANOVA (LSD). The Student's *t*-test was used to determine the significant level wherever required. Also, the data were assessed for normality and homogeneity of variance before conducting statistical analysis.

3.4.4 Pot trial 3 - plug-tray growth experiment

3.4.4.1 Plug-tray system establishment

Plug-trays containing square prismatic "pots" (top surface: 2 cm × 2 cm; bottom surface: 1 cm × 1 cm; height: 3 cm) were used for this experiment (see Figure 3.13). The drainage hole at the pot bottom was covered with two pieces of needle-punched polypropylene geotextile (supplied by Spudulica) to avoid soil loss. A close-fitting lining of aluminium mesh (supplied by ISOPON) was then inserted as the permeable sample holder. Each pot was filled with 7.5g sludge-amended soil (~1000 mg/kg Cu and Zn) with an initial moisture content of 11.6%. One spring barley seedling was planted in each planted pot. The moisture content of the sludge-amended soil was adjusted to 35% and maintained at this moisture content during growth phase. The plants were allowed to grow for a period of 6 weeks, then the plant shoots were harvested, and the planted pots were prepared for later analysis.

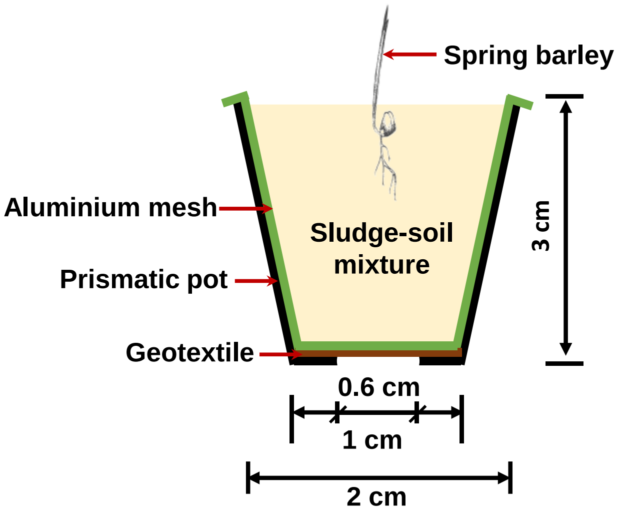

Figure 3.13 Diagram of plug-tray system.

3.4.4.2 Resin impregnation of the soil and roots

The sludge-amended soil containing the plant roots was transferred to the transparent polypropylene plastic cylindrical containers by carefully lifting the aluminium mesh holder from the plug-tray. A fluid displacive drying and the resin-impregnating procedure, developed from EMS (2024), was used to preserve the rootlet zone within the sludge-amended soil pots with minimal physical disturbance. This consisted of (1) Dehydration: immersion in a graded series of ethanol solutions (50%, 70%, 95% and 100%) was used for removal of aqueous pore fluids. Each step lasted one hour, and the 100% ethanol exchange was carried out twice; (2) Resin impregnation: a mixture of ethanol and fresh Spurr Resin in different proportions (2:1, 1:1, 1:3 and 100% fresh resin) was prepared and used successively for immersing samples (see Table 3.2 for resin formulations; Spurr resin is a cycloaliphatic epoxy resin). Each step lasted for 24 hours, except for the step with 1:1 mixture, which lasted 48 hours. The 100% resin step was repeated three times to completely remove the residual ethanol; (3) Curing: the final resin-saturated samples were transferred to an oven for 12 hours at 70°C; (4) Cutting: the cured resin blocks were removed from plastic containers, and replicate samples were sectioned either vertically or horizontally, creating a set of blocks containing both transverse or longitudinal sections exposing the root-soil microfabric; (5) Grinding and polishing: the surface of each cut resin block was ground against silicon grinding papers of decreasing grit (P600 and P1200) before polishing with the 3-, 1- and ¼- μm water-free oil-based diamond paste (polished samples see Figure 3.14).

Before the plug-tray growth experiment, a sample of the sludge-amended soil was prepared for determining the initial bonding environments of Cu and Zn (for comparison with the metal bonding environments in the rhizosphere and within the root structures). This sludge-amended soil was dried by solvent displacement and then embedded in the 30-mm epoxy resin blocks (EpoThin 2, a glycidyl ether epoxy resin). The blocks were also ground and polished.

Table 3.2 Formulation of Spurr Resin.

Ingredients	Amount / g
ERL 4221	10
Diglycidyl Ether of Polypropylene Glycol (DER)	8
Nonenyl Succinic Anhydride (NSA)	25
Dimethylaminoethanol (DMAE) *	0.3

* the catalyst (DMAE) should be added last, after gently mixing the three other components.

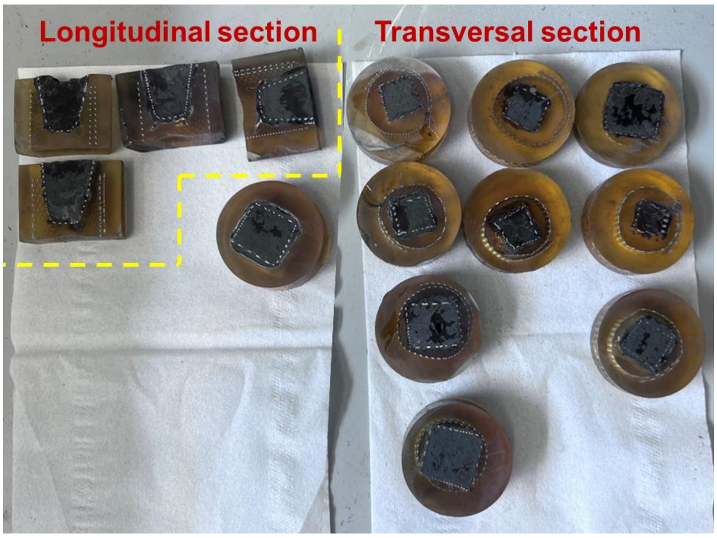

Figure 3.14 Longitudinal and transversal sections of polished samples.

3.4.4.3 SEM-EDS data acquisition

Polished samples were carbon-coated and observed with a SEM-EDS, as an initial assessment of the root structures and metal distribution patterns. The operating conditions of SEM-EDS were detailed in Section 3.2.2.4.

3.4.4.4 EPMA data acquisition

Elemental mapping of the carbon-coated samples was performed on a JEOL JXA-8230 Electron Probe Microanalyzer operating at 20 KeV incident energy. This instrument captures secondary and backscattered electron images. It is equipped with an Energy Dispersive Spectrometer (EDS) and 5 Wavelength Dispersive Spectrometers (WDS), allowing the simultaneous analysis of different elements and the generation of distribution maps for each element. The spatial resolution of the elemental maps is about 1 μm.

3.4.4.5 μXRF and μXANES data acquisition

The μXRF element maps and μXANES spectra of samples were collected on beamline I18 at the Diamond Light Source. The specific operating conditions of beamline I18 were detailed in Section 3.2.2.5. For the prepared samples, approximately 0.5×0.5 mm multielment microfocus XRF spectra were collected. These where then processed by the beamline softeware in real time to produce elemental maps (Al, Si, Ca, Cu and Zn) which were then used to choose points of interests for μXANES spectra collection. Only single μXANES spectra was collected (~10 min) from any one spot within the samples and the sample stage automatically moved to expose an unaffected part of the sample before subsequent scans. The collected μXANES spectra were normalised in Athena 0.9.26 over the full data range and were then corrected for any drift in E_0 using the data collected from the metal foil standards (Ravel and Newville, 2005). LCF was further performed in Athena using the selected standards to determine the most likely combinations of standards to best fit the sample spectra. The number of reference standards permitted in each fit was also limited to 3 to reduce the degree of freedom.

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Chapter 4: Isotherm sorption behaviour of original digested sludge for Cu and Zn

4.1 Introduction

The aim of this project is to investigate the transformation of metal behaviour and fate in the agricultural soil amended with digested sewage sludge (SS) over time due to plant growth. To experimentally monitor the transformation behaviour of contaminant metals within the sludge-soil/sludge-soil-plant system, there is a need to elevate metal levels in the system through the addition of an appropriate sludge. The amending of original digested sludge with metals is a widely used technique in studies as it can increase the rate of metals build-up in the sludge-amended soil without the need to apply excessive sludge amounts to the soil (Barraoui et al., 2021; Kandpal et al., 2004; Smith, 1996). Additionally, this amending process can ensure that the transformation of contaminant metals in the system is principally due to the transformation of sludge-associated metals (Feng et al., 2024). Therefore, prior to pot trials, it is essential to determine the appropriate amended sludge for use in the soil amendment experiments. To achieve this objective, isotherm sorption behaviours of original digested sludge for contaminant metals are needed to be investigated first. It is the primary work of this chapter. The work from this chapter will provide guidance for producing the sludge-amended soil for use in the subsequent pot trials. The characterization of original digested sludge and agricultural soil used in this project was also provided in this chapter. It will allow a comparison between the characteristics of original digested sludge and agricultural soil, serving as the basis for later soil amendment experiments.

4.2 Materials and methods

The materials and methods used in this sorption experiment have been introduced in the Methodology of Chapter 3. Full details of this experimental methodology can be seen in section 3.2.

4.3 Results

4.3.1 Quality control of aqua regia digestion

The results obtained from the aqua regia digestion on the certified reference material indicated a good agreement with the provisional reference values (see Table 4.1). Furthermore, the precision of this aqua regia digestion procedure for Cu and Zn was <7% (relative standard deviation of three measurements on samples), and its accuracy ranged from 95% to 105% (percent recovery from certified materials) (Feng et al., 2024). These results indicated that the procedure of this aqua regia digestion meets the criteria for the satisfactory precision (⩽20%) and accuracy (80-120%) in an aqua regia digestion (Chen and Ma, 2001).

Table 4.1 Comparison between total Zn and Cu concentrations in a certified reference material obtained by aqua regia digestion and provisional values.

Metals	Values of total metal concentrations using aqua regia digestion (mg/kg)	Provisional values (mg/kg)	Recovery ratio (%)
Cu	36 ± 0	37	97
Zn	208 ± 5	209	99

Values are expressed as mean ± standard deviation (*n*=3). The full data are available in Table A.1.

4.3.2 Physico-chemical analysis of original digested sludge and original agricultural soil

The average pH values for the original digested sludge and agricultural soil were 7.9 and 7.5, respectively (see Table 4.2). The organic matter of original digested sludge was about 51% whereas that of the agricultural soil was about 7%. The concentrations of total Kjeldahl nitrogen and total phosphorus in the original digested sludge were about 75,500 mg/kg and 30,200 mg/kg whereas their soil concentrations were about 2900 mg/kg and 700 mg/kg, respectively. Cu and Zn concentrations in the original digested sludge were about 300 mg/kg and 620 mg/kg and those in the agricultural soil were about 80 mg/kg and 110 mg/kg. Both Cu and Zn concentrations in the agricultural soil and original digested sewage sludge are within the regulated limits of the UK (regulations of agricultural soil: the use of sewage sludge is limited by the maximum allowable metal concentrations in the agricultural soil) and US guidelines (regulations on the use of SS in agriculture), respectively (DEFRA, 2018; EPA, 2022).

Table 4.2 Characteristics of original digested sludge and agricultural soil.

Parameters	Original digested sludge *	Agricultural soil *	Maximum permissible metal concentrations in UK soil (DEFRA, 2018)				US Limits of metals in sewage sludge (EPA, 2022)
			pH (5-5.5)	pH (5.5-6)	pH (6-7)	pH (>7)	EPA legislation
pH	7.9 ± 0.0	7.5 ± 0.0	-	-	-	-	-
Organic matter (%)	51.1 ± 0.4	6.6 ± 0.1	-	-	-	-	-
Total Kjeldahl nitrogen (mg/kg)	75525.7 ± 559.8	2908.0 ± 36.7	-	-	-	-	-
Total phosphorus (mg/kg)	30210.0 ± 223.8	697.3 ± 6.1	-	-	-	-	-
Cu (mg/kg)	302.5 ± 2.0	77.6 ± 4.9	80	100	135	200	4300
Zn (mg/kg)	619.9 ± 6.6	113.5 ± 4.6	200	200	200	300	7500

* Data are reported as mean ± standard deviation (*n*=3). The full data are available in Table A.2.

4.3.3 Isotherm sorption of original digested sludge for Zn and Cu

4.3.3.1 Sorption capacity of original digested sludge

When Cu and Zn were added to the original digested sludge to a final concentration <2%, on average, more than 90% of the Cu and 85% of the Zn were absorbed to the original digested sludge (see Table 4.3). With further increases in the amount of Cu and Zn being added to the original digested sludge, an increasing proportion remained in the aqueous phase (particularly Zn). Interestingly, when 8% Cu and Zn were added to the original digested sludge, less Zn was sorbed than when 5% Cu and Zn were added to the original digested sludge.

Table 4.3 Sorption capacity of original digested sludge to Cu and Zn.

m_{metals} / $m_{\text{dry sludge solids}}$ ratio (%)	Liquid sludge volume (ml)	Dry sludge solids content (g/l)	Added metal amount to sludge (mg)		Absorbed metal amount to sludge (mg)		Sorption ratio (%)	
			Cu	Zn	Cu	Zn	Cu	Zn
0.4	200	45.6	36.48	36.48	34.54	32.11	95	88
0.8	200	45.6	72.96	72.96	68.18	67.42	93	92
1	200	45.6	91.20	91.20	92.68	76.32	101	84
2	200	45.6	182.40	182.40	166.14	155.72	91	85
5	200	45.6	456.00	456.00	368.70	258.09	81	57
8	200	45.6	729.60	729.60	571.42	195.54	78	27

4.3.3.2 Operationally defined metal speciation in original digested sludge and amended sludges

BCR sequential extractions indicated that the Cu was principally distributed in the H_2O_2 extract in the original digested sludge and its speciation pattern was H_2O_2 extract (78%) > aqua regia extract (16%) > $NH_2OH·HCl$ extract (4%) > CH_3COOH extract (2%) (see Figure 4.1A; specific concentration values of each Cu fraction were shown in Table 4.4). The similar speciation pattern of the Cu in the metal-amended sludges was observed when the added ratio of Cu was within 1%. The proportion of H_2O_2 extract-Cu in these amended sludges accounted for about 70%. A ~10% reduction in the proportion of H_2O_2 extract-Cu and a ~10% increase in the proportion of CH_3COOH extract-Cu were observed after adding the Cu to the original digested sludge at a ratio of 2%. The Cu speciation pattern in this amended sludge was H_2O_2 extract (60%) > aqua regia extract (14%) ≈ CH_3COOH extract (14%) ≈ $NH_2OH·HCl$ extract (12%). With the further increasing Cu content, a significant increase in the proportion of CH_3COOH extract-Cu occurred that reached to 42% and 61% at the added ratio of 5% and 8%, respectively. Concurrently, a dramatic reduction occurred in the proportion of H_2O_2 extract-Cu that reduced to 35% and 25%, respectively. Also, a slight reduction appeared in the proportion of aqua regia extract-Cu. As a result, the Cu speciation pattern in these two amended sludges was CH_3COOH extract > H_2O_2 extract > $NH_2OH·HCl$ extract > aqua regia extract.

With respect to the Zn, BCR sequential extractions (see Figure 4.1B; specific concentration values of each Zn fraction were shown in Table 4.5) showed that the Zn in the original digested sludge was distributed in between CH_3COOH extract (12%), $NH_2OH·HCl$ extract (31%), H_2O_2 extract (37%), and aqua regia extract (20%). There was no clearly dominant speciation for the Zn in the original digested sludge. With an initial addition of the Zn at a ratio of 0.4%, there was an immediately dramatic increase in the proportion of CH_3COOH extract-Zn (increased to 61%) and an apparent reduction in the proportion of H_2O_2 extract (reduced to 8%) and aqua regia extract (reduced to 5%). The CH_3COOH-extract became the dominant speciation for the Zn in the amended sludge. A continuous increase in the proportion of CH_3COOH

extract-Zn occurred with the increasing added ratios of the Zn. The proportion of CH_3COOH extract-Zn finally reached to over 90% when the added ratio was 5%. A similar high proportion of CH_3COOH extract-Zn was monitored when the added ratio of the Zn was 8%.

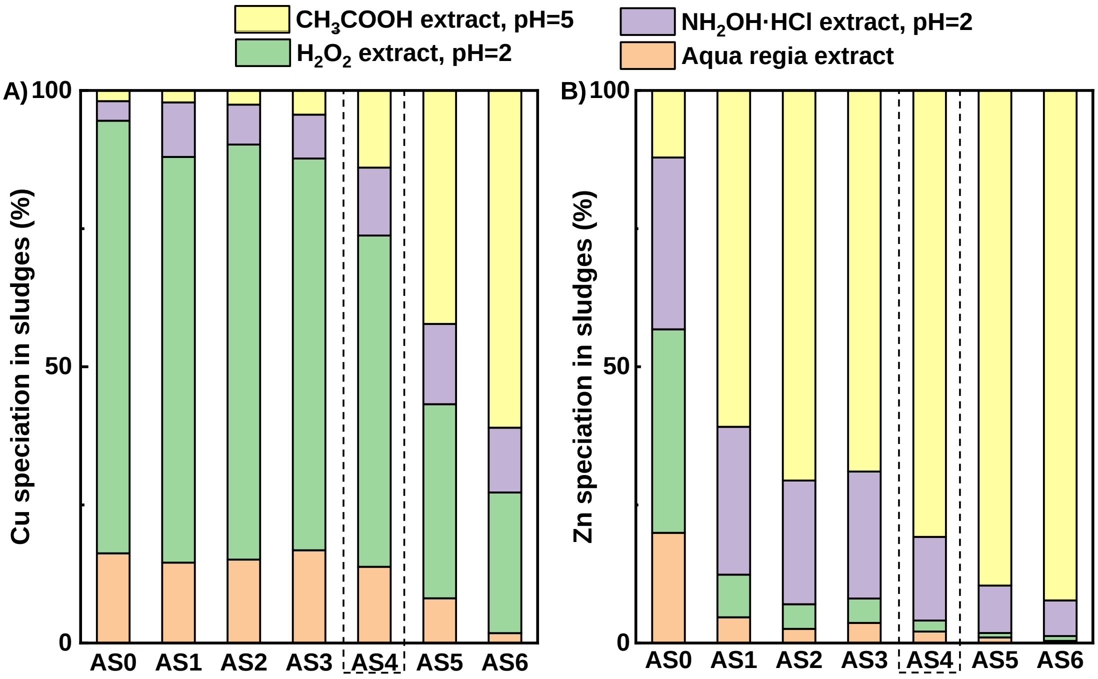

Figure 4.1 A) Cu and B) Zn speciation in original digested sludge and metal-amended sludges. AS0: original digested sludge; AS1-AS6: original digested sludge amended with metals at a ratio of 0.4%, 0.8%, 1%, 2%, 5% and 8%, respectively.

Table 4.4 Cu concentration of each fraction in original digested sludge and amended sludges (mg/kg of dry sludge).

Sludges	Cu concentration in each fraction (mg/kg)				Sum of four fractions (mg/kg)	Total Cu concentration by aqua regia (mg/kg)	Recovery rate (%)
	CH_3COOH extract	$NH_2OH{\cdot}HCl$ extract	H_2O_2 extract	Aqua regia extract			
AS0	6 ± 1	11 ± 3	242 ± 2	50 ± 6	309 ± 11	303 ± 2	102
AS1	86 ± 4	395 ± 12	2935 ± 28	581 ± 45	3997 ± 28	4090 ± 16	98
AS2	192 ± 4	541 ± 6	5629 ± 93	1131 ± 30	7493 ± 61	7778 ± 18	96
AS3	480 ± 25	871 ± 3	7788 ± 32	1844 ± 123	10983 ± 94	10465 ± 53	105
AS4	2724 ± 52	2398 ± 72	11699 ± 377	2690 ± 464	19511 ± 173	18520 ± 202	105
AS5	17702 ± 255	6082 ± 143	14723 ± 494	3390 ± 633	41896 ± 1039	40731 ± 176	103
AS6	37597 ± 371	7227 ± 150	15685 ± 38	1101 ± 15	61609 ± 503	62959 ± 490	98

Values are expressed as mean ± standard deviation (*n*=3). AS0: original digested sludge; AS1-AS6: original digested sludge amended with metals at a ratio of 0.4%, 0.8%, 1%, 2%, 5% and 8%, respectively. The full data are available in Table A.3 and Table A.5.

Table 4.5 Zn concentration of each fraction in original digested sludge and amended sludges (mg/kg of dry sludge).

Sludges	Zn concentration in each fraction (mg/kg)				Sum of four fractions (mg/kg)	Total Zn concentration by aqua regia (mg/kg)	Recovery rate (%)
	CH_3COOH extract	$NH_2OH{\cdot}HCl$ extract	H_2O_2 extract	Aqua regia extract			
AS0	76 ± 0	195 ± 3	231 ± 2	125 ± 1	628 ± 2	620 ± 7	101
AS1	2483 ± 35	1091 ± 31	315 ± 9	190 ± 2	4079 ± 21	4140 ± 30	99
AS2	5584 ± 23	1769 ± 17	353 ± 23	203 ± 6	7909 ± 40	8012 ± 56	99
AS3	6121 ± 291	2039 ± 115	392 ± 43	323 ± 72	8875 ± 63	8988 ± 89	99
AS4	14291 ± 272	2676 ± 159	354 ± 71	366 ± 71	17687 ± 476	17694 ± 129	100
AS5	25730 ± 291	2464 ± 193	225 ± 21	295 ± 70	28714 ± 285	28919 ± 280	99
AS6	19537 ± 185	1367 ± 90	181 ± 8	84 ± 4	21169 ± 267	22060 ± 270	96

Values are expressed as mean ± standard deviation (*n*=3). AS0: original digested sludge; AS1-AS6: original digested sludge amended with metals at a ratio of 0.4%, 0.8%, 1%, 2%, 5% and 8%, respectively. The full data are available in Table A.4 and Table A.6.

4.4 Discussion

4.4.1 Cu and Zn concentrations in the original digested sludge and amended sludges

In the original digested sludge, the concentration of Zn was about 2-fold greater than the Cu. It was consistent with the general concentration order of Cu and Zn present in the sludges summarised in Chapter 2. The Cu and Zn concentrations in this digested sludge were comparable to the typical Cu and Zn concentrations in the UK sludges (see Table 2.2). In addition, the original digested sludge showed a similar sorption capacity for both the Cu and Zn when the added ratio was within 2%. Approximately 90% of the added Cu and Zn can be absorbed by the original digested sludge within the added ratio of 2%. As a result, the concentrations of Cu and Zn in these amended sludges were comparable. As more metals were added to the original digested sludge, the difference in Zn and Cu concentrations in the amended sludges became pronounced increasingly. The Cu concentrations in the amended sludges were around 1.4-fold and 2.8-fold greater than the Zn concentrations when the added ratio was 5% and 8%, respectively. It probably suggests that Cu out competed Zn for some sorption sites with the increasing metal amount.

4.4.2 Changes in operationally defined speciation of Cu and Zn after amending procedure

In the original digested sludge, the Cu was primarily distributed in the H_2O_2-extract fraction (oxidizable). This was consistent with the general distribution pattern of Cu in the sludges reported in Chapter 2. The high proportion of oxidizable Cu fraction detected in the digested sludge was suggested to be highly related to the affinity of organic matter for Cu (Feng et al., 2023). The amending of original digested sludge with the soluble metals did not change the general speciation pattern of the Cu in the amended sludges when the added ratio was within 1%. Most of the Cu (approximately 70%) remained associated with the organic matter in the amended sludges. It is not surprising, as the organic matter content in the sludge exceeded 50%, offering abundant sorption sites for the added Cu. When the added ratio of the Cu increased to 2%, the proportion of oxidizable Cu reduced by about 10%. However, the oxidizable fraction was still the dominant chemical form for the Cu (~60%). This probably indicates that the sorption sites provided by the organic matter were insufficient for the amount of the added Cu when the ratio was 2%. A proportion of the Cu occurred to be adsorbed to the mineral surfaces or bound to the carbonates and Fe-Mn oxides. As a result, there was an increase in the proportion of exchangeable and reducible fractions when the added ratio was 2%. As would be anticipated from the increasing amount of the Cu being added to the digested sludge, chemical extractions showed that there was a dramatic decrease in the oxidizable Cu fraction when the added ratio increased to 5%, with a further reduction noted at an 8% ratio.

Unlike Cu speciation pattern, Zn was found to be fairly distributed in the CH_3COOH-extract, $NH_2OH{\cdot}HCl$-extract, H_2O_2-extract and aqua regia-extract fractions in the original digested sludge. This was in line with the typical speciation pattern of Zn in the sludges shown in Chapter 2. This result showed that the mobility of the Zn in the original digested sludge was higher than Cu, aligning with the typical mobility pattern of Cu and Zn in the sludges. Interestingly, the distribution of the Zn in the amended sludges differs significantly from that seen in the original digested sludge (even at a relatively low added ratio of 0.4%). The Zn was predominantly distributed in the CH_3COOH-extract fraction in the amended sludges. It indicated that most of the added Zn was preferentially adsorbed to the mineral surfaces or bound to the carbonates (indicated by BCR data).

4.4.3 Determination of appropriate amended sludge for use in soil amendment experiments

To experimentally investigate the transformation behaviour of contaminant metals in the sludge-soil/sludge-soil-plant system, it is essential to increase metal levels in the agricultural soil with the use of an appropriate sludge. Although the Cu and Zn concentrations in the original digested sludge were about 4-5 times greater than those in the agricultural soil, the use of original digested sludge can only slightly change the metal levels in the system when it is applied to the soil at a reasonable rate. As a result, it would be very difficult to experimentally determine the changes in the behaviour and fate of sludge-associated metals in the system. Therefore, the original digested sludge needs to be amended with metals first and then the appropriate metal-amended sludge is used in the later soil amendment experiments.

Results indicated that the sorption surfaces of original digested sludge were close to the limit of saturation when the added ratio of metals was within 2%. Noticeably, the metal-amended sludges only included the sorbed Cu and Zn (aqueous metals were largely removed by centrifugation). The absorbed metals were redistributed in the amended sludges. The Cu was still mainly distributed in the oxidizable fraction when the added ratio was within 2% (~60%), which was similar to the original digested sludge. With respect to the Zn, in the amended sludges it was predominantly distributed in the exchangeable fraction, which was different from that observed in the original digested sludge. Nevertheless, it showed that the Zn has a higher mobility in the amended sludges than the Cu, agreeing with the general mobility patterns of Cu and Zn in the sludges (Feng et al., 2023). Based on (1) the sorption capacity of original digested sludge to Cu and Zn, (2) Cu and Zn speciation pattern in the amended sludges, (3) typical application rate of sewage sludge and (4) the target metal concentrations in the sludge-amended soil for experimental monitoring of metal behaviour and fate, the amended sludge produced by adding metals to the original digested sludge at a ratio of 2% was finally used in the soil amendment experiments. The final metal concentrations in the amended sludge were about 50-100 times higher than the typical metal concentrations in the UK sludges, although they were within the range seen (0.5-4%) in heavily contaminated sludge (Bååth et al., 1998; Babel and del Mundo Dacera, 2006; Jain and Tyagi, 1992; Olthof and Lancy, 1979; Smith, 1996; Spence et al., 1995). Historically, the sludge applied to agricultural land has contained metal concentrations similar to those in this amended sludge (McLaren and Clucas, 2001). For example, Smith (1996) reported that the sludge with a Zn concentration of ~28,000 mg/kg had been applied to agricultural soil (heavily contaminated sludge may still be used in agriculture in areas where the monitoring is less effective).

The Cu and Zn distribution in this amended sludge was further investigated by SEM mapping. It showed that the Cu and Zn were uniformly distributed in this amended sludge (see Figure 4.2). Thus, by using this amended sludge, the target sludge-amended soil with elevated metal concentrations can be experimentally created (without applying the sludge at a very high rate) for later plant growth experiments. The elevated metal concentrations facilitate the experimental monitoring of transformation of metal behaviour and fate in the systems over time due to plant growth.

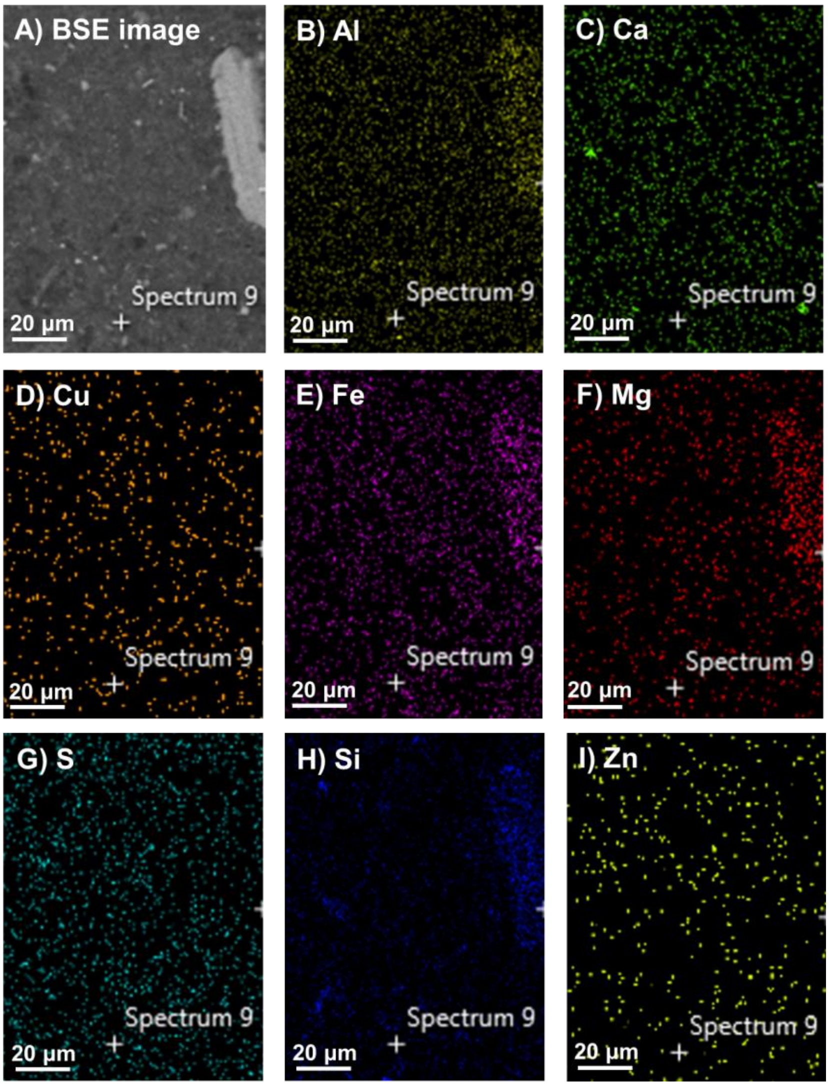

Figure 4.2 A) SEM backscatter image of the amended sludge adopted (at a ratio of 2%) and SEM-EDS elemental maps showing the distribution pattern of B) Al, C) Ca, D) Cu, E) Fe, F) Mg, G) S, H) Si and I) Zn in the amended sludge. BSE, Backscattered electron.

4.4.4. Comparison between Cu and Zn speciation in original digested sludge and amended sludge

The XANES spectra for Cu and Zn in Figure 4.3 provided a brief overview of the bonding environments of Cu and Zn in both the original digested sludge and amended sludge adopted in soil amendment experiments (sequential XANES scans of each sample see Figure 4.4). LCF analysis indicated that Cu(I)-S was the dominant phase for the Cu in both materials, although some Cu(I)-O and organo-Cu(II) phases were detected in the amended sludge that were not present in the original digested sludge (see Table 4.6). In addition, sequential extractions showed that most of the Cu was extractable by H_2O_2 in both the original digested sludge and amended sludge, although a slightly higher proportion of the Cu in the amended sludge was in the more easily leached fractions. Therefore, in this project, both BCR sequential extractions and XANES data showed that Cu speciation in the amended sludge was broadly similar to that seen in the original digested sludge. With respect to the Zn, about 80% of the Zn was in the Zn(II)-S phases in the original digested sludge, with the remainder in a Zn(II)-O_{IS} bonding environment (inner sphere bonding to metal oxide surfaces; see Table 4.7). The same two Zn(II) bonding environments were also present in the amended sludge, but the proportions were reversed. BCR sequential extractions showed that about 80% of the Zn in the amended sludge was in the exchangeable fraction, whereas it was distributed in the exchangeable, reducible, oxidizable and residual fractions in the original digested sludge. These results showed that there were bigger differences in the Zn speciation between the amended sludge and original digested sludge. However, the same two bonding environments for the Zn in both materials also indicate that the patterns of Zn behaviour are relevant. Overall, the experiments reported in this project are likely to reflect the behaviour and fate of Cu introduced to soil with digested sludge reasonably well, whereas the results for Zn probably require more careful interpretation.

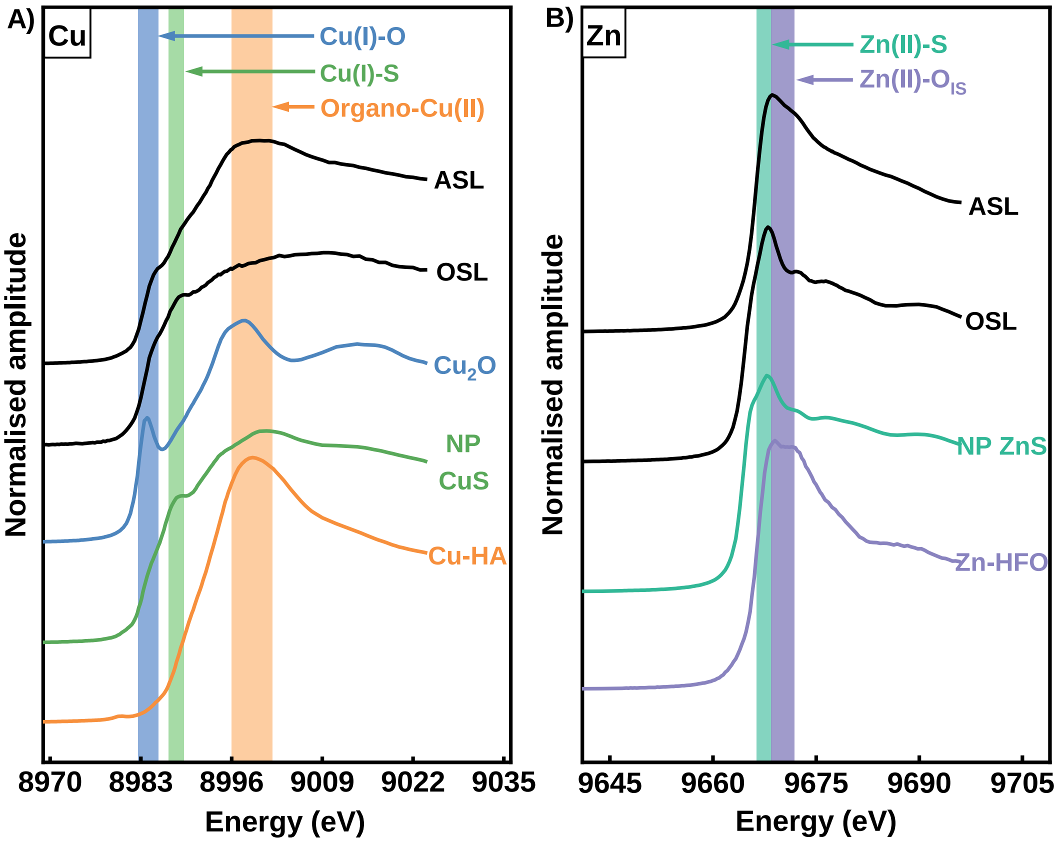

Figure 4.3 Bulk average K-edge XANES spectra for A) Cu and B) Zn collected from original digested sludge, amended sludge and selected standards. Colours match to the standards shown. ASL, amended sludge; OSL, original sludge; NP, nanoparticles; Cu-HA, Cu-humic acid complex; Zn^{2+} (aq), aqueous Zn^{2+}; Zn-HFO, Zn-hydrous ferric oxide; IS, inner sphere.

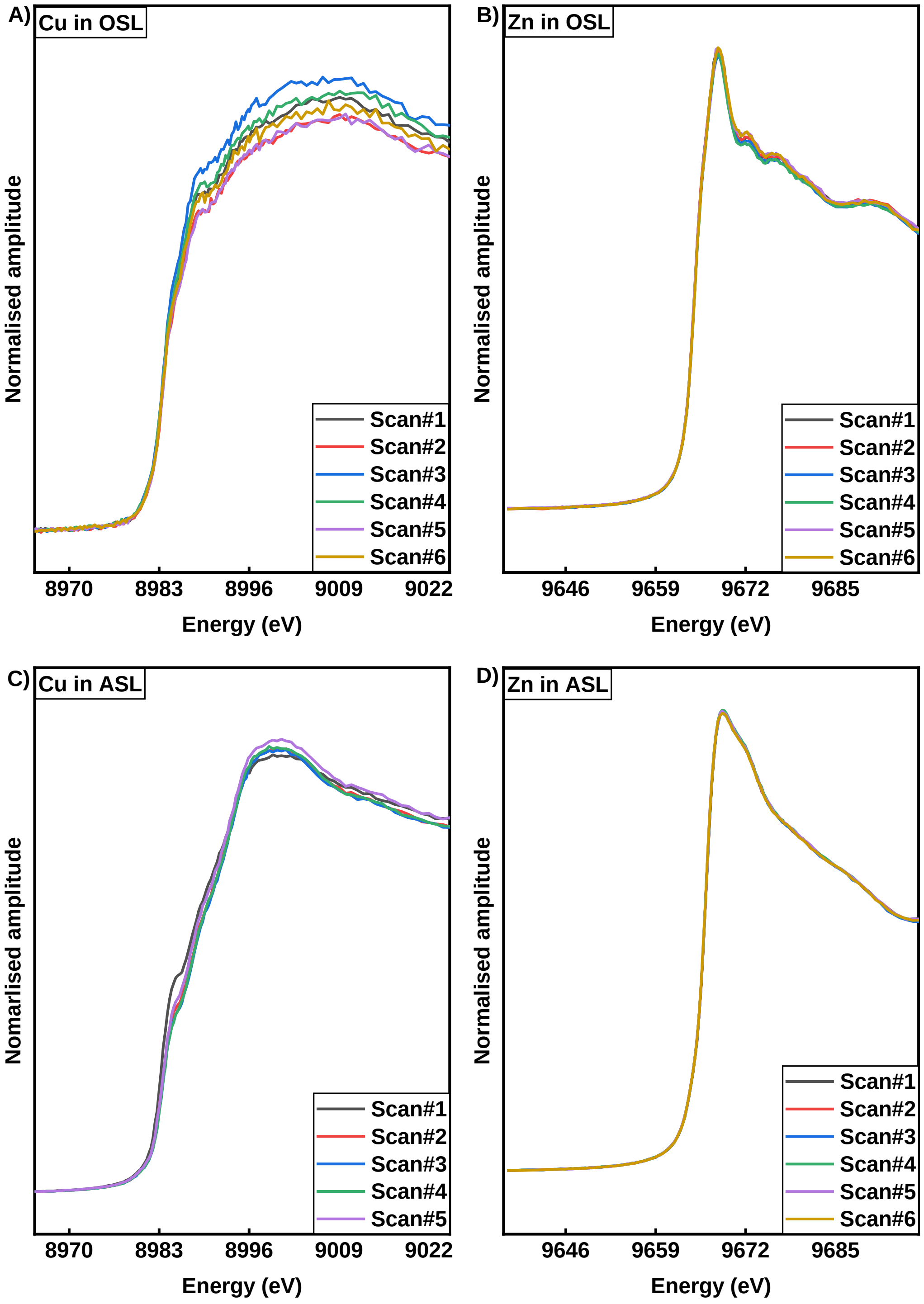

Figure 4.4 Sequential bulk XANES scans for A) Cu and B) Zn in the original digested sludge and C) Cu and D) Zn in the amended sludge (note: both the Cu and Zn spectra have been corrected for any drift in E_0 (+1.5 eV for Cu and +1.7 eV for Zn) using the data collected from Cu- and Zn- foil standards). OSL: original sludge; ASL: amended sludge.

Table 4.6 Relative abundance of Cu species determined from LCF analysis of Cu XANES spectra of the original digested sludge and the amended sludge. LCF derived errors are given in parentheses.

Samples	Cu species (%)			*R*-factor
	Cu_2O	NP Cu-S	Cu-HA	
Original digested sludge	2 (5)	98 (5)	-	0.0171
Amended sludge	16 (3)	69 (3)	15 (4)	0.0035

NP Cu-S, Cu(I)S nano particles; Cu-HA, Cu(II)-humic acid complex.

Table 4.7 Relative abundance of Zn species determined from LCF analysis of Zn XANES spectra of the original digested sludge and the amended sludge. LCF derived errors are given in parentheses.

Samples	Zn species (%)			*R*-factor
	NP Zn-S	Zn-HFO	Zn^{2+} (aq)	
Original digested sludge	79 (1)	21 (1)	-	0.0020
Amended sludge	22 (1)	78 (1)	-	0.0032

NP Zn-S, Zn(II)S nano particles; Zn-HFO, Zn(II)-hydrous ferric oxide; Zn^{2+} (aq), aqueous Zn^{2+}.

4.5 Conclusion

This chapter provides the investigation on isotherm sorption behaviour of original digested sludge for Cu and Zn and the characterization of original materials used in this project. The conclusions outlined below can be drawn from this chapter.

- Substantial proportions of Cu and Zn become incorporated into the digested sludge matrix when the sludge is amended with soluble Cu and Zn salts. The sorption surfaces of original digested sludge are close to saturation limits when the added ratio of Cu and Zn is within 2%. Within this ratio, on average, about 90% of the metals (Cu and Zn) can be absorbed to the digested sludge. A significant reduction in the sorption ratio of Zn and Cu occurs when the added ratio reaches 5% (particularly Zn).

- The fractional distribution of Cu between the original digested sludge and the amended sludges remains more or less the same when the added ratio is within 2%. Within this ratio, the oxidizable fraction is the predominant chemical form for Cu in the sludge. A dramatic increase in exchangeable Cu fraction in the amended sludge occurs when the added ratio increases to 5%.

- The fractional distribution of Zn differs considerably between the original digested sludge and amended sludges. The Zn is fairly distributed in between exchangeable, reducible, oxidizable and residual fractions in the original digested sludge (there is no dominant fraction). In contrast, the Zn is predominantly distributed in exchangeable fraction in the amended sludges (even at a ratio of 0.4%). The proportion of exchangeable Zn fraction is over 90% when the added ratio reaches 5%.

- Both sequential extractions and XAS data reveal that the Cu speciation in the amended sludge (at the ratio of 2%) was broadly similar to that seen in the original digested sludge. There are bigger differences in the Zn speciation between the amended sludge and original digested sludge. However, the same two bonding environments for the Zn in both materials suggest that the patterns of Zn behaviour are relevant. Therefore, in general, the experiments reported in this project will be likely to reflect the behaviour and fate of Cu introduced to the agricultural soil with digested sludge reasonably well and the results for Zn probably require more careful interpretation.

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Chapter 5: Evolution of Cu and Zn speciation in agricultural soil amended by digested sludge over time and repeated plant growth

5.1 Introduction

The presence of contaminant metals in sewage sludge (SS) is a major concern during its agricultural application as it may cause environmental problems and health risks (Urbaniak et al., 2024). It has been recognised that the risks posed by contaminant metals to plants and wider environment are both strongly dependent on their chemical forms (Goyal et al., 2023). Out of those metals present in SS, Zn and Cu are the two most abundant contaminant metals, so they will accumulate most quickly in the agricultural soil compared with other metals (You et al., 2020). Excessive Zn and Cu accumulation in the soil can adversely affect soil quality and plant growth although they are essential elements for plants (Liu et al., 2022; Meng et al., 2023; Yen et al., 2024). Based on the literature review in Chapter 2, in SS, generally, Cu is principally bound to organic matter, and Zn is adsorbed to mineral surfaces and/or bound to carbonates, Fe-Mn oxides, organic matter, and tightly bound to minerals (Illeraa et al., 2000; Morera et al., 2001), whereas Cu and Zn are both primarily tightly bound to the minerals in the agricultural soil (Doelsch et al., 2006; Jin et al., 2017). In the sludge-soil mixture, Cu and Zn bonding is typically intermediate between these two components (Feng et al., 2023).

Previously, most studies investigating the speciation of contaminant metals in the sludge-soil mixtures are usually conducted over relatively short periods after sludge application (Feng et al., 2023). Their aim is to understand the immediate changes in the metal speciation in the agricultural soil following sludge application. They do not recognise that when SS is applied to agricultural land, the metal speciation within the sludge-soil/sludge-soil-plant system can evolve over time due to organic matter degradation by soil microorganism, interaction with organic chemicals exuded by plants, and the effects of microbial metabolism on host phases such as sulfides and iron oxides (Caracciolo and Terenzi, 2021; De Conti et al., 2018; Gan et al., 2020). As a result, the contaminant metals in the system may be transformed into more bioavailable forms or sequestered in unreactive minerals. To date, little attention has been paid to the effects of contact time and/or repeated plant growth on metal speciation in the sludge-amended soil and this is becoming an obstacle to the agricultural use of SS.

Based on this general context, the primary work of this chapter is therefore to determine the evolution pattern of Cu and Zn speciation in an agricultural soil amended by digested sludge application, over time, and with repeated growth of spring barley (*Hordeum vulgare*), using a combination of sequential chemical extractions and X-ray absorption spectroscopy (XAS). The accumulation patterns of Zn and Cu in spring barley roots and shoots were also identified. This work can address the knowledge gap about the evolution of metal speciation during repeated plant growth and will inform better risk assessments for sludge use in agricultural land.

5.2 Materials and methods

The pot trial 1 was conducted in this chapter. The experimental details of pot trial 1 and the analytical techniques were introduced in the methodology of Chapter 3 (see Section 3.3). The characterization of original digested sludge and agricultural soil used in this work has been detailed in Chapter 4.

5.3 Results

5.3.1 Plant analysis

5.3.1.1 Plant height

Similar patterns of spring barley growth were observed in the three growth rounds (see Figure 5.1). Specifically, repeated measure ANOVA indicated that significant growth occurred in the first three weeks ($p<0.05$), followed by minor growth in the fourth and fifth week. The height of spring barley remained largely unchanged in the sixth week. After six weeks, the average height of plants for the three growth rounds was 31.8 cm, 32.7 cm, and 33.4 cm, respectively.

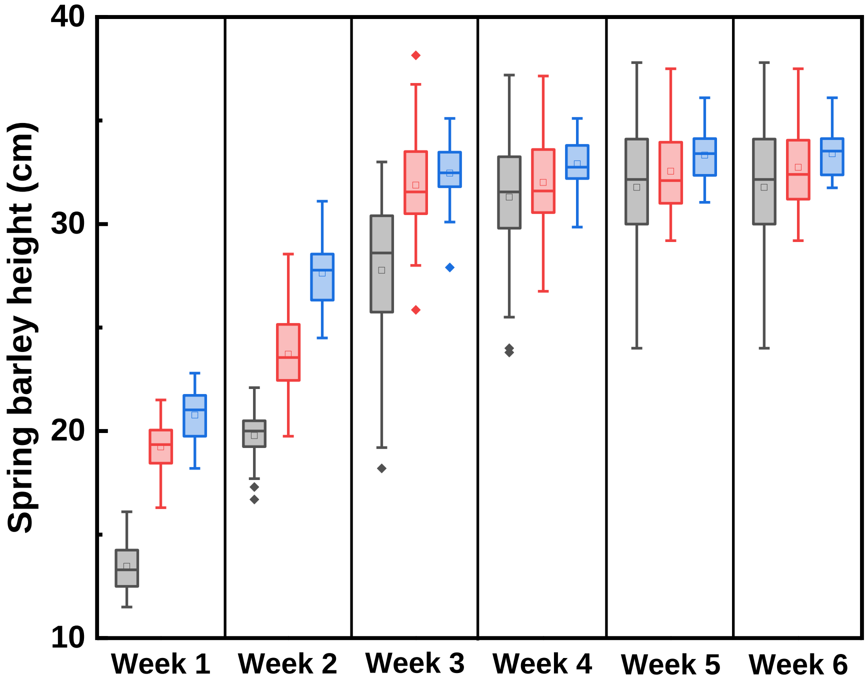

Figure 5.1 The recorded height of spring barley during growth period (grey: 1st round; red: 2nd round; blue: 3rd round; shaded boxes show the median values and interquartile range; tails indicate 1.5 × IQR; □: mean value; ♦: outliers).

5.3.1.2 Dry weight biomass

The dry weight biomass of spring barley shoots was more than 2-fold greater than that of the roots in each growth round (see Table 5.1). When there were two plants per pot, the ratio of shoots-to-roots biomass was greater than when a single plant in a pot. The dry weight biomass of per plant of three growth rounds was 0.13 g/plant, 0.16 g/plant and 0.17 g/plant, respectively.

Table 5.1 Dry weight biomass of spring barley roots and shoots.

Growth round	Dry weight biomass of shoots (g)	Dry weight biomass of roots (g)	Survived planted pots number	Harvested plants number	Dry weight biomass of each plant shoot (g/plant)	Dry weight biomass of each plant root (g/plant)
1st round	3.8	1.8	40	40	0.09	0.04
2nd round	8.7	2.6	37	74	0.12	0.04
3rd round	7.9	3.1	32	64	0.12	0.05

5.3.1.3 Zn and Cu uptake by spring barley

The average Cu and Zn concentrations in whole plants across the three growth rounds were about 760 and 970 mg/kg (see Figure 5.2; full data see Table B.1 and Table B.2). The Cu concentration in whole plants during the first growth round was slightly higher than the Zn. However, in the second and third growth rounds, the Zn concentration in whole plants was higher than the Cu. In total, <1% Cu and Zn in the sludge-amended soil was removed over three growth rounds. The ratio of Cu concentrations in the roots and shoots was 30±4 (mean ± standard deviation) which indicates that plant-associated Cu was strongly partitioned towards the roots. In contrast, the roots to shoots ratio for Zn concentrations was 1.5±0.3 (mean ± standard deviation). When corrected for the amount of dry weight biomass, the overall Zn uptake to shoots was approximately 60% of the total biomass associated Zn, whereas overall Cu uptake to shoots was only about 8% of the total biomass associated Cu.

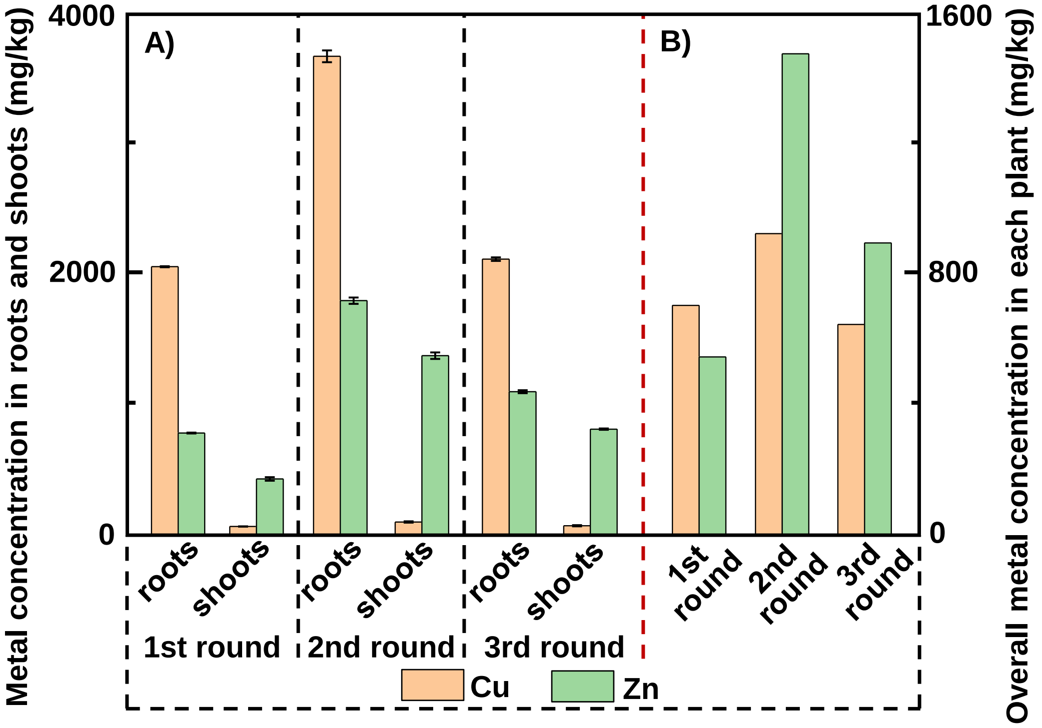

Figure 5.2 A) Zn and Cu concentrations in spring barley roots and shoots of three growth rounds (*n*=3) and B) overall Zn and Cu concentrations in each plant.

5.3.2 Cu and Zn speciation in sludges and soils

5.3.2.1 Cu and Zn concentrations in soils

The Cu and Zn concentrations in the control soils and bulk soils determined by aqua regia digestion were within ±3% of their concentrations in the amended soil in all three growth rounds, as shown in Table 5.2 and Table 5.3 (repeated measures ANOVA indicated no statistically significant difference at $p<0.05$). The total Cu concentration in the root-bound soils was ~3% lower than in the bulk soils in the second growth round (the Student's *t*-test indicated that this difference was significant at $p < 0.05$), and ~7% lower in the third growth round (likewise, this difference was significant). The total Zu concentration in the root-bound soils was ~6% higher than in the bulk soil in the second growth round (statistically significant at $p < 0.05$) but was ~2% lower in the third growth round (not statistically significant at $p < 0.05$).

Table 5.2 Cu concentration of each fraction in different samples (mg/kg of dry solids).

Samples	Cu concentration in each fraction (mg/kg)				Sum of four fractions (mg/kg)	Total metal content by aqua regia (mg/kg)	Recovery rate (%)
	CH_3COOH extract	$NH_2OH{\cdot}HCl$ extract	H_2O_2 extract	Aqua regia extract			
Original soil	11 ± 1	9 ± 1	13 ± 2	42 ± 2	74 ± 4	78 ± 5	96
Amended sludge	2724 ± 52	2398 ± 72	11699 ± 377	2690 ± 464	19511 ± 173	18520 ± 202	105
Amended soil	380 ± 4	356 ± 4	270 ± 16	61 ± 4	1067 ± 12	1058 ± 23	101
Control_1st round	340 ± 4	356 ± 11	291 ± 9	66 ± 3	1053 ± 16	1035 ± 7	102
Control_2nd round	318 ± 14	359 ± 14	301 ± 8	65 ± 1	1043 ± 24	1057 ± 5	99
Control_3rd round	298 ± 1	423 ± 6	275 ± 7	73 ± 2	1070 ± 14	1067 ± 23	100
Bulk_1st round	303 ± 15	423 ± 6	272 ± 9	68 ± 1	1067 ± 10	1066 ± 34	100
Bulk_2nd round	264 ± 7	442 ± 11	279 ± 8	70 ± 2	1055 ± 21	1068 ± 6	99
Bulk_3rd round	255 ± 5	413 ± 7	299 ± 10	80 ± 2	1047 ± 9	1064 ± 19	98
Root soil_2nd round	240 ± 3	386 ± 10	314 ± 11	76 ± 3	1016 ± 2	1032 ± 5	98
Root soil_3rd round	240 ± 5	375 ± 4	296 ± 2	88 ± 5	999 ± 3	993 ± 3	101

All values are expressed as mean ± standard deviation (n = 3). The full data are available in Table B.3 and Table B.5.

Table 5.3 Zn concentration of each fraction in different samples (mg/kg of dry solids).

Samples	The concentration of Zn in each fraction (mg/kg)				Sum of four fractions (mg/kg)	Total metal content by aqua regia (mg/kg)	Recovery rate (%)
	CH_3COOH extract	$NH_2OH·HCl$ extract	H_2O_2 extract	Aqua regia extract			
Original soil	1 ± 1	4 ± 2	2 ± 0	105 ± 3	112 ± 1	113 ± 5	99
Amended sludge	14291 ± 272	2676 ± 159	354 ± 71	366 ± 71	17687 ± 476	17694 ± 129	100
Amended soil	720 ± 9	193 ± 7	28 ± 5	90 ± 4	1031 ± 3	1065 ± 24	97
Control_1st round	683 ± 4	219 ± 2	47 ± 0	92 ± 2	1041 ± 8	1034 ± 9	101
Control_2nd round	655 ± 10	239 ± 5	51 ± 2	95 ± 0	1040 ± 15	1070 ± 9	97
Control_3rd round	602 ± 5	278 ± 7	46 ± 1	97 ± 3	1022 ± 17	1048 ± 12	98
Bulk_1st round	629 ± 21	281 ± 17	39 ± 1	106 ± 7	1056 ± 10	1050 ± 42	101
Bulk_2nd round	565 ± 12	322 ± 13	46 ± 2	107 ± 8	1039 ± 16	1080 ± 16	96
Bulk_3rd round	526 ± 6	292 ± 7	68 ± 2	106 ± 3	992 ± 9	1042 ± 15	95
Root soil_2nd round	498 ± 13	370 ± 2	102 ± 7	117 ± 10	1087 ± 9	1144 ± 13	95
Root soil_3rd round	481 ± 5	317 ± 3	89 ± 1	108 ± 0	995 ± 7	1026 ± 2	97

All values are expressed as mean ± standard deviation (*n* = 3). The full data are available in Table B.4 and Table B.6.

5.3.2.2 Cu and Zn distribution in amended sludge and amended soil

SEM-EDS elemental maps showed that both Cu and Zn were uniformly distributed in the metal-amended sludge (see Figure 5.3A and B). When the amended sludge was added to the agricultural soil, the remnant sludge particles were observed in the amended soil 21 days after mixing that had preferentially retained Cu relative to Zn (see Figure 5.3D and E; each individual elemental map in the amended soil was shown in Figure B.2). Similar features were also seen in the µXRF elemental maps of the sludge-amended soil (see Figure 5.3C). The µXRF maps further showed that Zn (and the remainder of the Cu) were evenly distributed within the soil matrix (which had an elemental composition consistent with a mix of Fe oxides and clay minerals, see Figure 5.3E).

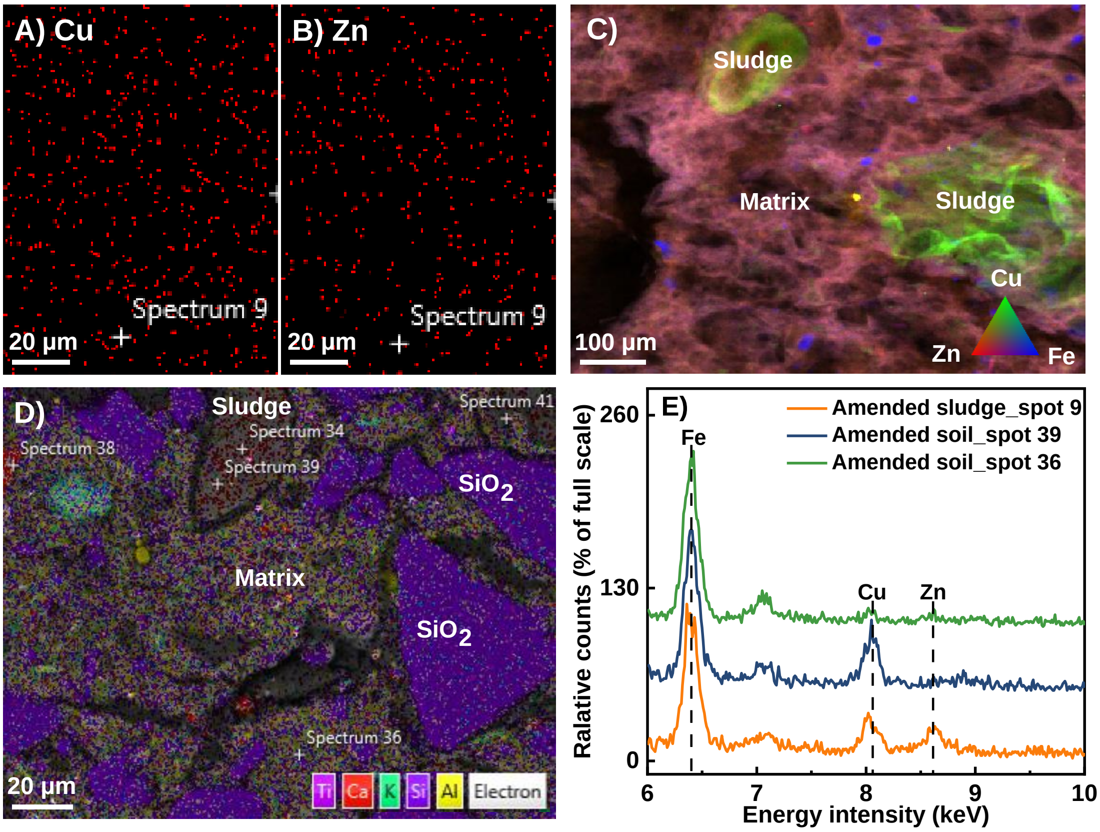

Figure 5.3 SEM-EDS elemental maps showing uniform distribution of both A) Cu, and B) Zn in the amended sludge (from same area of interest); C) false colour µXRF map showing the distribution of Cu-Zn-Fe in the amended soil; D) false colour SEM-EDS map of the amended soil (different areas of interest); E) truncated EDS spectra collected from the locations indicated in maps A), B) and D).

5.3.2.3 Cu speciation changes

The XANES spectra of Cu reference standards were shown in Table 5.4 and Figure 5.4. LCF of Cu XANES spectra indicated that Cu(I)-O (30%), Cu(I)-S (5%) and organo-Cu(II) (65%) atomic binding environments predominated in the original agricultural soil (see Figure 5.5 and Table 5.6; sequential scans of Cu XANES spectra from different samples see Figure B.3). Whereas, in the original digested sludge, Cu(I)-S containing phases were dominant (98%). Two thirds of the Cu in the amended sludge was also in a Cu(I)-S binding environment (69%), but one third was in Cu(I)-O (16%) and organo-Cu(II) (15%) binding environments. In the amended soil 21 days after mixing, there were roughly equal amounts of Cu(I)-O (43%) and Cu(I)-S (41%) containing phases, with the remaining 16% present as organo-Cu(II) phases.

In the control soils, the proportion of Cu in Cu(I)-O and Cu(I)-S phases decreased over 18 weeks to 39% and 32%, respectively, and the proportion of Cu in organo-Cu(II) phases increased to 29%. A similar change in Cu speciation was seen over the same period with repeated plant growth. The proportion of Cu in Cu(I)-O phases and Cu(I)-S phases decreased to 41% and 28%, respectively, and the proportion of Cu in organo-Cu(II) phases increased to 31%.

BCR analysis showed that 56% of the Cu in the original agricultural soil was in the aqua regia extraction, with smaller proportions distributed between the CH_3COOH (14%), $NH_2OH{\cdot}HCl$ (13%) and H_2O_2 (17%) extractions (see Figure 5.6 and Table 5.2). In contrast, 78% of the Cu in the original digested sludge was in the H_2O_2 extraction, with most of the remainder (16%) in the aqua regia extraction. The distribution of Cu in the amended sludge was similar to that in the original digested sludge, with 60% in the H_2O_2 extraction and 10-15% in each of the other extractions. In the amended soil, 36% of the Cu was in the CH_3COOH extraction, 33% was in the $NH_2OH{\cdot}HCl$ extraction, 25% was in the H_2O_2 extraction, with only 6% in the aqua regia extraction.

In the control soils, the proportion of Cu in the CH_3COOH extraction decreased over 18 weeks to 28%. The proportion of the Cu in the $NH_2OH{\cdot}HCl$ extraction increased to 40%, while the proportions in the H_2O_2 and aqua regia extractions were 26% and just over 7% after 18 weeks. A similar change in Cu distribution occurred with repeated plant growth in the bulk soil. However, the proportion of Cu in the CH_3COOH extraction decreased more than in the control soil, to 24%. The proportion of Cu in the $NH_2OH{\cdot}HCl$ extraction increased by a similar amount to the control soil to 39%. The proportion in the H_2O_2 extraction also increased more than in the control soil to 29%.

As there was a net loss of Cu from the root-bound soil, the proportions in each extraction from the root-bound soil have been calculated relative to the amount of extractable Cu in the amended soil. In both rounds, the proportion of Cu in the CH_3COOH extraction was just over 20%, while just over a third was in the $NH_2OH \cdot HCl$ extraction, nearly 30% was in the H_2O_2 extraction, and 5-10% was in the aqua regia digestion.

Table 5.4 Cu Reference standards label, chemical formula, and sources of reference compounds used for LCF analyses of Cu XANES data.

Reference standards sample (label/chemical formula)	Sources and/or synthesis method
Cu(II)CO_3	Purchased laboratory chemical
Cu(II)SO_4	Purchased laboratory chemical
Cu(II)O	Purchased laboratory chemical
$Cu(I)_2O$	Purchased laboratory chemical
Covellite (Cu(I)S)	Natural mineral sample
Cu(II)-humic complex (Cu-HA)	30 ml of 500 ppm Cu^{2+} (pH=3.28) and 3 g humic acid (Merck, UK) reacted for 24h, collected by centrifuging and dried at 40°C
$Cu^{2+}_{(aqueous)}$ (Cu^{2+} aq)	1000 mg/L $Cu(NO_3)_2$ solution
Cu(II)$(CH_3COO)_2$ ($Cu(OAc)_2$)	Purchased laboratory chemical
Cu(I)S nano particles (NP CuS)	Following the method of Adele et al. (2018), 50 ml of 100 mM Cu^{2+} and 50 ml of 500 mM S^{2-}·was reacted for 24h; separated solids recovered by centrifugation and dried at 20°C in a 95%N_2/5%H_2 atmosphere
$Cu(II)_3(PO_4)_2$	Purchased laboratory chemical
Cu(II)$(OH)_2$	10 ml of 6000 ppm $Cu_2(NO_3)_2$ solution and 10 ml of 1M KOH reacted and collected by filtration
Cu(II)-hydrous ferric oxide (Cu-HFO)	Hydrous ferric oxide synthesised according to the method of Cornell and Schwertmann (2003) (containing haematite, goethite and ferrihydrite by XRD) was reacted with Cu^{2+} for 24 hours at pH 7-8. Solids recovered by filtration and dried at 20°C

Table 5.5 Zn reference standards label, chemical formula, and sources of reference compounds used for LCF analyses of Zn XANES data.

Reference standards sample (label/chemical formula)	Sources and/or synthesis method
Zn(II)SO_4	Spectra shared by authors (Adele et al., 2018)
Zn(II)O	Spectra shared by authors (Adele et al., 2018)
Sphalerite (Zn(II)S)	Natural mineral sample
Zn(II)-FeOOH	Geothite synthesised according to the method of Cornell and Schwertmann (2003) was reacted with Zn^{2+} for 24 hours at pH 7-8. Solids recovered by filtration and dried at 40°C
Zn(II)S nano particles (NP ZnS)	Spectra shared by authors (Adele et al., 2018)
Zn(II)CO_3	Spectra shared by authors (Adele et al., 2018)
Zn(II)$(CH_3COO)_2$ (Zn$(OAc)_2$)	Spectra shared by authors (Adele et al., 2018)
Zn(II)$_3(PO_4)_2$	Spectra shared by authors (Adele et al., 2018)
$Zn^{2+}_{(aqueous)}$ (Zn^{2+} (aq))	1000 mg/L $ZnCl_2$ solution
Zn(II)-humic complex (Zn-HA)	30 ml 500 mg/kg Zn^{2+} (pH=3.28) and 3 g humic acid (Merck, UK) reacted for 24h, collected by centrifuging and dried at 40°C
Zn(II)-hydrous ferric oxide (Zn-HFO)	Hydrous ferric oxide synthesised according to the method of Cornell and Schwertmann (2003) (containing haematite, goethite and ferrihydrite by XRD) was reacted with Zn^{2+} for 24 hours at pH 7-8. Solids recovered by filtration and dried at 20°C
Zn(II)$(OH)_2$	Precipitated from 6000 ppm $ZnCl_2$ solution using 1M KOH and collected by filtration and dried at 40°C

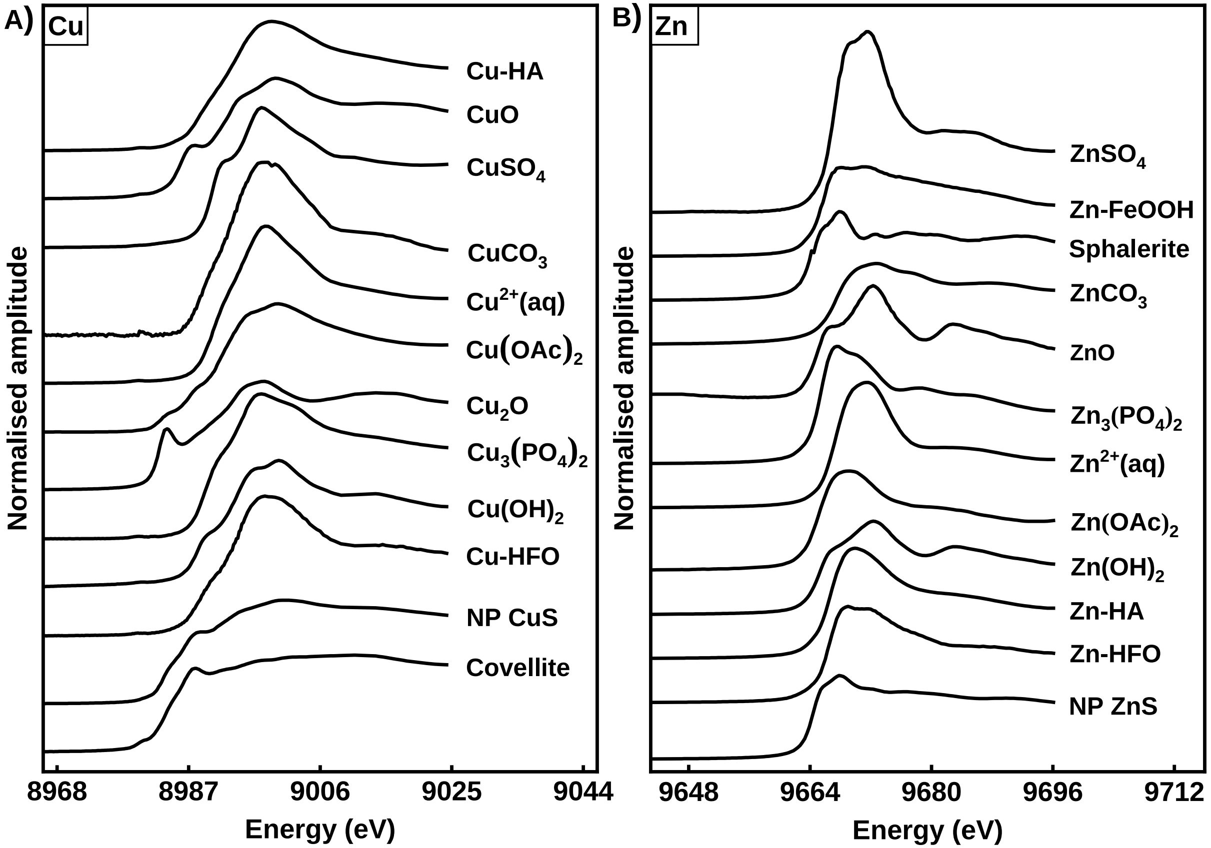

Figure 5.4 XANES spectra of collected reference standards of A) Cu and B) Zn.

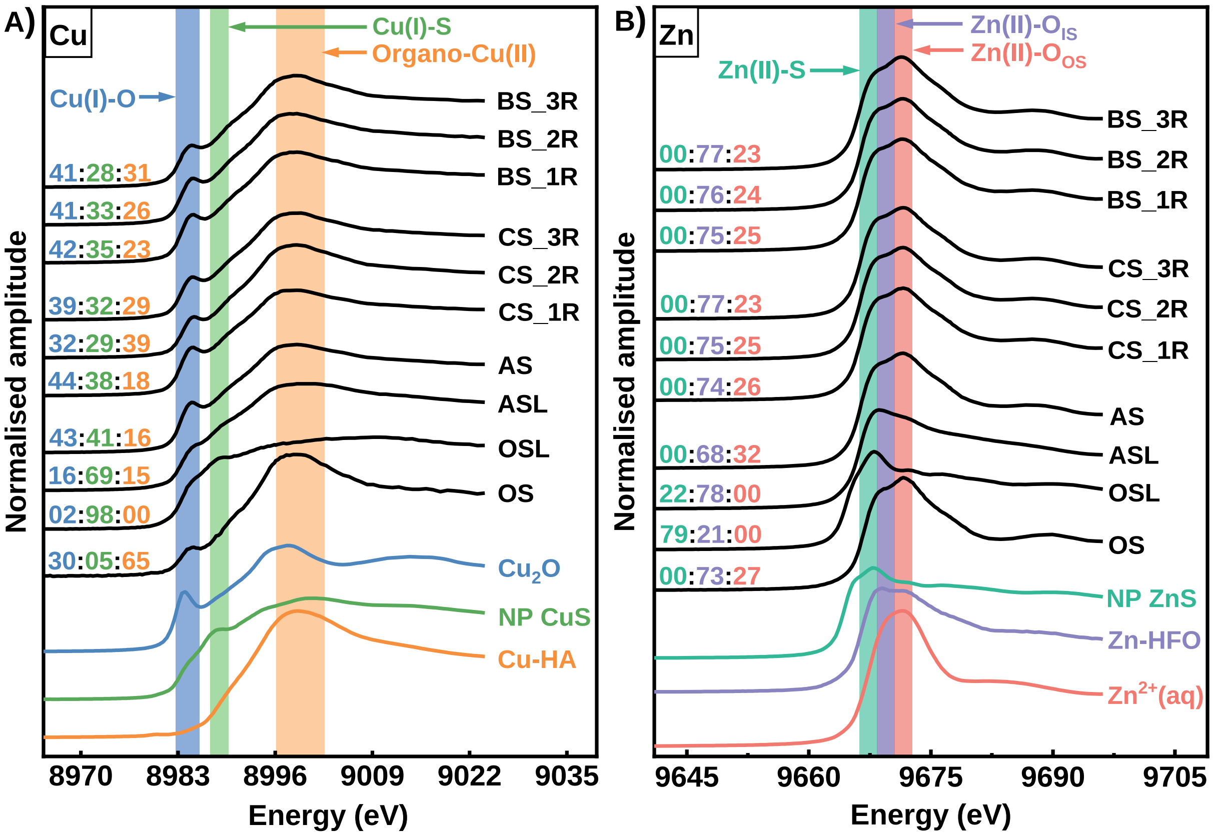

Figure 5.5 Bulk average K-edge XANES spectra for A) Cu and B) Zn collected from different samples and selected standards. Ratios presented above samples spectra are the results for LCF analysis of bulk samples spectra. Colours match to the standards shown. BS/CS_1R/2R/3R, bulk/control soil from the first/second/third round; AS, amended soil; ASL, amended sludge; OSL, original sludge; OS, original soil. NP, nanoparticles; Cu-HA, Cu-humic acid complex; Zn^{2+} (aq), aqueous Zn^{2+}; Zn-HFO, Zn-hydrous ferric oxide; IS and OS, inner sphere, and outer sphere.

Table 5.6 Relative abundance of Cu species determined from LCF analysis of Cu XANES spectra of samples. LCF derived errors are given in parentheses.

Samples	Cu species (%)			*R*-factor
	Cu_2O	NP CuS	Cu-HA	
Original soil	30 (2)	5 (2)	65 (3)	0.0027
Original sludge	2 (5)	98 (5)	-	0.0171
Amended sludge	16 (3)	69 (3)	15 (4)	0.0035
Amended soil	43 (3)	41 (3)	16 (4)	0.0036
Control_1st round	44 (2)	38 (2)	18 (3)	0.0028
Control_2nd round	32 (2)	29 (2)	39 (3)	0.0025
Control_3rd round	39 (2)	32 (3)	29 (3)	0.0032
Bulk_1st round	42 (3)	35 (3)	23 (4)	0.0041
Bulk_2nd round	41 (3)	33 (3)	26 (4)	0.0038
Bulk_3rd round	41 (2)	28 (2)	31 (3)	0.0020

NP CuS, Cu(I)S nano particles; Cu-HA, Cu(II)-humic acid complex.

Table 5.7 Relative abundance of Zn species determined from LCF analysis of Zn XANES spectra of samples. LCF derived errors are given in parentheses.

Samples	Zn species (%)			*R*-factor
	NP ZnS	Zn-HFO	Zn^{2+} (aq)	
Original soil	-	73 (3)	27 (3)	0.0104
Original sludge	79 (1)	21 (1)	-	0.0020
Amended sludge	22 (1)	78 (1)	-	0.0032
Amended soil	-	68 (2)	32 (2)	0.0045
Control_1st round	-	74 (2)	26 (1)	0.0022
Control_2nd round	-	75 (2)	25 (2)	0.0023
Control_3rd round	-	77 (2)	23 (1)	0.0020
Bulk_1st round	-	75 (2)	25 (1)	0.0021
Bulk_2nd round	-	76 (2)	24 (1)	0.0020
Bulk_3rd round	-	77 (2)	23 (2)	0.0040

NP ZnS, Zn(II)S nano particles; Zn-HFO, Zn(II)-hydrous ferric oxide; Zn^{2+} (aq), aqueous Zn^{2+}.

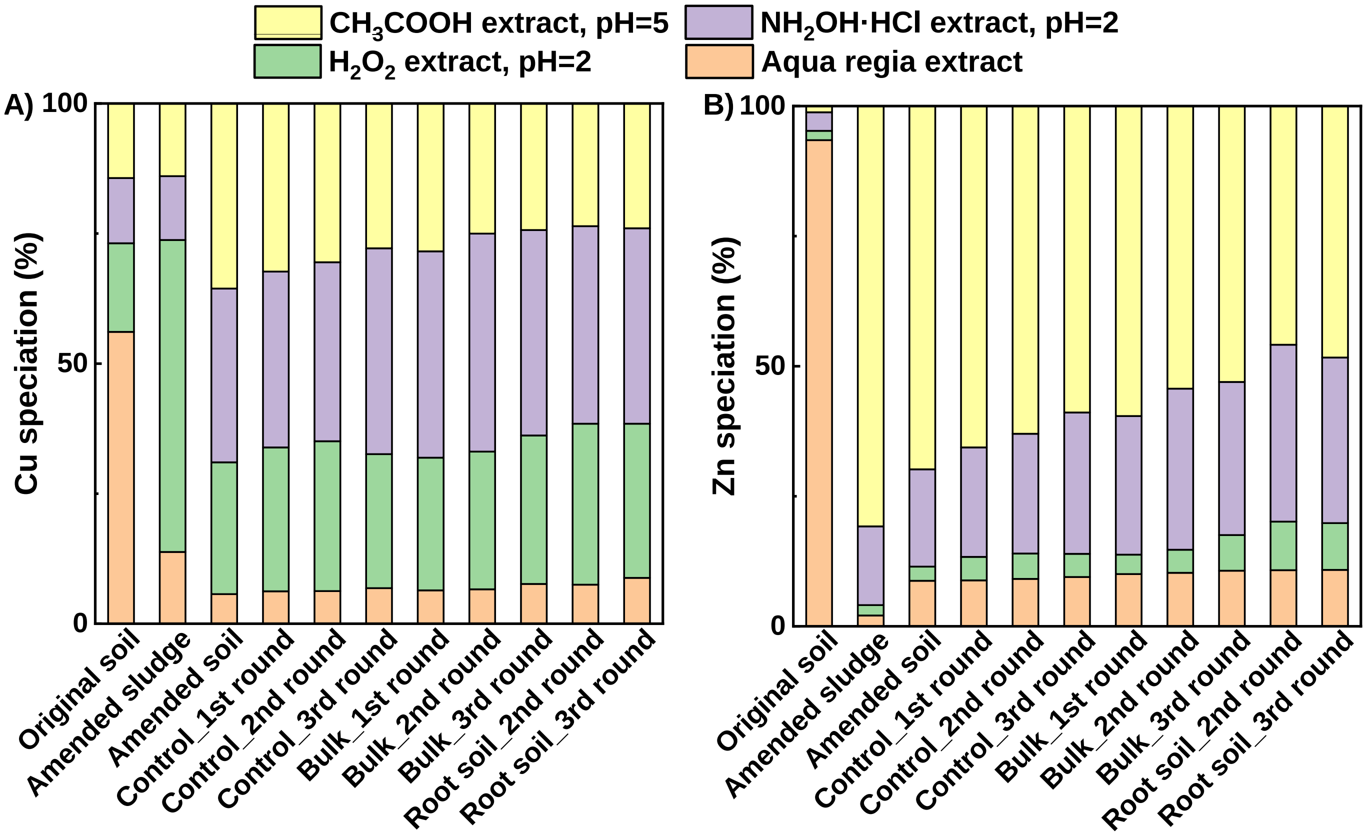

Figure 5.6 Evolution of operationally defined A) Cu and B) Zn speciation over time and with repeated plant growth.

5.3.2.4 Zn speciation changes

The XANES spectra of Zn reference standards were shown in Table 5.5 and Figure 5.4. LCF of Zn XANES spectra indicated that the Zn was in one of two distinct Zn(II)-O binding environments in the original agricultural soil. 27% of the Zn was in a binding environment similar to the hydrated aqueous Zn^{2+} solution species, which is also present in outer sphere binding complexes (Zn(II)-O_{OS}), while 73% was in a binding environment similar to inner sphere binding to metal oxide surfaces (Zn(II)-O_{IS}) (see Figure 5.5 and Table 5.7; sequential scans of Zn bulk XANES spectra from different samples see Figure B.4). In contrast, Zn(II)-S phases predominated (79%) in the original sludge, with the remaining Zn in a Zn(II)-O_{IS} binding environment (21%). The same two Zn(II) binding environments were present in the amended sludge as in the original sludge, but the proportions were reversed (78% Zn(II)-O_{IS} and 22% Zn(II)-S). The Zn binding environments in the amended soil were similar to the original soil (32% Zn(II)-O_{OS} and 68% Zn(II)-O_{IS}), and the Zn(II)-S binding was absent.

In the control soil, there was a significant reduction in the proportion of Zn(II)-O_{OS} phases to 26% after 6 weeks, with only a small further decrease in the remaining two experimental rounds. Concurrently, the proportion of Zn(II)-O_{IS} phases increased to 74% after one round, with small further increase in the subsequent rounds. The same changes in the Zn-binding environment were seen in the bulk soil.

BCR analysis showed that 93% of the Zn in the original agricultural soil was in the aqua regia extraction (see Figure 5.6 and Table 5.3). In contrast, 37% of the Zn in the original sludge was in the H_2O_2 extraction, with 31%, 20% and 12% in the $NH_2OH{\cdot}HCl$, aqua regia and CH_3COOH extractions, respectively. Whereas in the amended sludge Zn was primarily in the CH_3COOH extraction (81%), with most of the remainder in the $NH_2OH{\cdot}HCl$ extraction (15%). Generally, the distribution of Zn in the amended soil was similar to the distribution in the amended sludge, with 70% in the CH_3COOH extraction, 19% in the $NH_2OH{\cdot}HCl$ extraction, 3% in the H_2O_2 extraction and 10% in the aqua regia extraction.

In the control soil, the proportion of Zn in the CH_3COOH extraction decreased over 18 weeks to 59%, while the proportion of Zn in the $NH_2OH{\cdot}HCl$ extraction increased to 27%, with little change in the other extractions. A similar pattern of change in Zn distribution occurred with repeated plant growth in the bulk soil. However, the proportion of Zn in the CH_3COOH extraction decreased more than in the control soil to 53%, while the proportion in the $NH_2OH{\cdot}HCl$ extraction increased to 29%. There were also small increases in the proportion of Zn in the other two extractions.

As it is inappropriate to assume that there is no Zn flux to/from the root-bound soil, the proportions of Zn in each extraction from the root-bound soil have been calculated relative to the total amount of extractable Zn in the amended soil. In the root-bound soil, the proportion of Zn in the CH_3COOH extractions was between 45 and 50%, whilst the proportion in the $NH_2OH{\cdot}HCl$ extractions was between 35 and 30%, with about 10% of the Zn in each of the other two extractions.

5.4 Discussion

5.4.1 Speciation of Cu and Zn in anaerobic digestor sludge and agricultural soils

Sequential extractions on the original digested sludge showed that the Cu was predominately in the H_2O_2-extracted fraction (oxidizable). Similar operationally defined speciation has been reported for a wide range of sludges from several regions (Chen et al., 2008; Dabrowska, 2016; Tytla, 2020). It is generally suggested to be attributed to the high affinity of Cu for organic matter and high stability of Cu-organic matter complexes (Feng et al., 2023), although this has not been verified for the digested sludge. However, XANES analysis indicates that the Cu in the original digested sludge is primarily in Cu(I)-S phases, with no contribution from organo-Cu(II) complexes. This does not conflict with BCR extraction results as metal sulphides and metal-organic complexes are not easily separated in sequential extractions, with both commonly reporting to oxidizable fraction

(Rodgers et al., 2015). Furthermore, the formation of metal-sulphides during anaerobic digestion has been postulated by some researchers (Dabrowska and Rosinska, 2012; Feng et al., 2023; Thanh et al., 2016). Sequential extractions indicated that the Zn in the original digested sludge was fairly split between all four operationally defined fractions, but XANES analysis indicates most Zn was in the Zn(II)-S binding environment, and the remainder in an environment characteristic of Zn(II) in an inner sphere complex with metal oxides. This probably reflects the inherent weakness of sequential extractions, where a metal phase can be extracted in different fractions depending on the degree of crystallinity (Rennert, 2019).

The Cu binding in the amended sludge was similar to that in the original sludge with the majority in a Cu(I)-S environment, which is compatible with most of the Cu being extracted in the “oxidizable” fraction. However, Zn binding environments differed between in the amended sludge and original sludge, with the majority in inner sphere complexes with metal oxides, and a small proportion in a Zn(II)-S binding environment, the reverse of the pattern in the original sludge. This may have arisen because Cu out-competed Zn for reactive sulfides in the amended sludge. This difference explains why a lower proportion of Zn in the amended sludge is extracted in the “oxidizable” fraction, but the increase in the “exchangeable” fraction. This in turn suggest that at least part of the Zn present in recently formed inner sphere complexes on metal oxide surfaces can be extracted with the “exchangeable” fraction.

Cu and Zn were predominately organo-Cu(II) and Zn(II)-O_{IS} phases in the original soil. These major phases would be expected to be extracted by H_2O_2 and $NH_2OH·HCl$ extractants, respectively (Yang et al., 2018). However, both metals reported predominantly to the “residual” fraction in the sequential extractions, which was also reported in other sequential extraction studies (Kotoky et al., 2015; Topcuoğlu, 2014). The low extractability of these Cu and Zn adsorption complexes from the original agricultural soil may be associated with “aging” *in-situ*, as presumably they accumulated slowly over long timescales in the agricultural soil. Clearly, soil-associated Cu and Zn are not readily mobilised to solution, and most likely have low bioavailable, so pose a much lower environmental risk compared with these metals in the digested sludge.

5.4.2 Processes occurring during the mixing of metal-amended sludge and agricultural soil and over time

Remnant sludge particles were found in the amended soil 21 days after the mixing by SEM-EDS mapping (as shown in Figure 5.3). The EDS spectrum further showed Cu concentrations in these remnant sludge particles were higher than Zn concentrations, indicating Zn is preferentially lost from the amended sludge over a relatively short period. In addition, Zn binding environments in the amended soil reflected a transition to ‘soil-like’ speciation, with Zn still predominantly in the Zn(II)-O_{IS} phases, but with complete loss of Zn(II)-S phases. In contrast, sequential extractions indicated a very small change in Zn extractability beyond what would be anticipated simply from a mixture of the soil and the amended sludge. In comparison, Cu speciation in the amended soil reflected only a partial transition from ‘sludge-like’ Cu binding environment towards the ‘soil-like’ binding environment 21 days after mixing (possibly because Cu was retained more in the remnant sludge particles). There were increases in the proportions of organo-Cu(II) complexes and Cu(I)-O phases, and a decrease in the proportion of Cu(I)-S phases that are larger than what would be anticipated from mixing. As would be anticipated from the addition of sludge, sequential extractions showed an increase in Cu mobility where the exchangeable and reducible Cu fractions increased, and the oxidizable and residual Cu fractions decreased.

There were small further changes in the Zn binding environment with time shown by the control experiments, although there was a steady decrease in Zn extractability with time. The proportion of Zn in the exchangeable fraction decreased and there were very small increases in the other three fractions (most notably in the reducible fraction). The Cu binding environment also evolved slowly over time towards a “soil-like” state, with an increase in the proportion of organo-Cu(II) complexes and a decrease in the proportion of Cu(I)-S phases. Interestingly, after the initial increase in the proportion of Cu(I) associated with metal oxides upon mixing, that proportion decreased with more time towards that in the original soil, suggesting that this may be a transition speciation. Like Zn, sequential extractions also indicated a steady decrease in Cu extractability with the reduction in the proportion in the exchangeable fraction largely balanced by an increase in the reducible fraction. There was little change in the proportion in the H_2O_2 fraction probably because both organo-Cu(II) and Cu(I)-S are oxidizable. Therefore, over time Cu and Zn speciation in the control soil slowly evolved, tending towards the metal-phase fractional distribution pattern seen in the original soil. This evolution process is probably attributable to the reactions of the metals with the more stable components in the soil and their slow incorporation into these components (Han and Banin, 1999).

5.4.3 Evolution of metal speciation with plant growth and metal uptake to plants

The XANES data from the bulk soil showed the changes in both Cu and Zn binding environments were similar with and without plant growth. However, the decreases in the extractability of both these metals were more rapid when plants were present (the concentration of both metals in the exchangeable fraction decreased). It is notable that the decrease in the extractability of both these metals was greater in the root-bound soil, where there were increases in the concentrations of Cu in the residual fraction and Zn in the oxidizable fraction.

The decrease in Cu and Zn in the exchangeable fraction of the control soil was principally the result of transfer to the reducible fractions because there was no discernible reduction in the total Cu and Zn in the soil. The slightly larger decrease in Cu and Zn in the exchangeable fraction of the bulk soil and resulted in more transfer of Zn but less transfer of Cu to the reducible fraction. There were also small transfers of both metals to the oxidizable and residual fractions. The root-bound soil exhibited the largest decrease in Cu and Zn in the exchangeable fraction, which was associated with a net decrease in the soil’s metal concentrations. This resulted in most transfer of Zn but least transfer of Cu to the reducible fraction, and slightly more transfer to the oxidizable and residual fractions. The more pronounced decrease in

Cu and Zn in the exchangeable fraction of the root-bound soil is compatible with plant uptake being predominantly from the readily extractable phases (De Conti et al., 2018).

The finding that Cu and Zn concentrations were both higher in the roots than in the shoots has also been reported by Yu et al. (2011). With Cu, the ratio of roots to shoots concentration was very high (~30), whereas it was far lower for Zn (~1.5), indicating that Cu is strongly retained in the roots, but Zn is readily translocated into shoots. This tendency for Cu to be retained in plant roots has also been reported for other monocot and eudicot plant species by Hilber et al. (2007) and Juknys et al. (2009). In contrast, despite the total uptake of Cu and Zn being similar, less Zn is retained in the roots. It is probably because Zn is needed for the production of the growth hormone auxin, which is mainly produced in plants shoot meristems (without this hormone plant growth is compromised) (Deveshwar et al., 2020; Mapodzeke et al., 2021). In addition, the recommended dietary allowance for adults is ten-fold higher for Zn than Cu (NIH, 2022a), and the tolerable upper intake level is 3-5 folds higher for Zn than Cu (EFSA, 2006; Government of Canada, 2024; NIH, 2022b). This means that elevated concentrations of extractable Cu and Zn in the agricultural soil may pose a similar risk to food plants where the stem, leaves, flowers, and fruit are harvested, but elevated concentrations of extractable Cu are potentially very problematic for root vegetables.

5.4.4 Implications for anaerobic digestor sludge application to agricultural land

Anaerobic digestion is the most common method for treating SS before applying it to the agriculture (SEL, 2023). For example, about 70% of the UK sludge is treated by anaerobic digestion and over 80% of this treated sludge is disposed to agricultural land (BAS, 2024; Liu, 2019). During anaerobic digestion metal sulphides can become the primary host phase for chalcophile metals (see Cu and Zn XANES data presented above). Rapidly precipitated, poorly crystalline, metal sulphide phases are relatively reactive, easily remobilised and more bioavailable than the agricultural soil associated metals. The metals added to the agricultural soil with anaerobically digested sludge are likely to be rapidly transformed and redistributed to the soil matrix (particularly Zn), but remain more easily mobilised (at least for a time). Both Cu and Zn have the tendency to evolve towards the speciation seen in the original agricultural soil and the rate of transformation was slightly accelerated by repeated plant growth or close association with plant roots. However, it should be noted the transformation rate was element specific, which can be attributable to the amount of mobile metals in the sludge-soil mixture. With current data, it is difficult to establish the relationship between the transformation rate and the amounts of mobile metals that remains in the amended soils. In practice regular monitoring of mobile metal fraction would better define the overall risk from sludge application to soils.

Currently the agricultural utilization of SS is regulated only by the total metal concentrations (DEFRA, 2018; Hudcová et al., 2019; Ministry of Ecology and Environment of The People's Republic of China, 1985). These regulations are sufficient to prevent a large accumulation of sludge-borne metals in the agricultural soil. However, this will lead to more conservative restrictions on the sludge use in agriculture because the fraction of immobile metals in the sludge generally will not cause risks (Feng et al., 2023). Therefore, the total metal concentration data is insufficient to comprehensively understand the actual risk associated with the sludge use (Janaszek and Kowalik, 2023). There is a need to update the current regulations taking into account the bioavailability criteria of contaminant metals. This could be assessed by measuring the concentrations of weak acid accessible metals in the sludge-soil mixture, and setting limits on total leachable metals present during cropping rather than on bulk concentrations.

5.5 Conclusion

Both Cu and Zn are present in the digested sludge primarily as metal sulphide phases formed during anaerobic digestion. These phases are relatively reactive, easily remobilised and more bioavailable than the soil associated metals. Metals added to the agricultural soil with the amended sludge are rapidly transformed and redistributed to the soil matrix but remain more easily mobilised (at least for a time). Bulk XANES showed that about 40% of the Cu added as Cu(I)-S phases and all Zn added as Zn(II)-S phases in the amended sludge were converted to other phases after addition to the agricultural soil (mainly Cu(I)-O and outer sphere Zn(II)-O phases). Over time both Cu and Zn are transformed from easily mobilizable fractions to slowly mobilizable fractions; this transformation is enhanced in the presence of plant growth and close association with the plant roots. After 18 weeks of plant growth, about 60% of the Cu added as Cu(I)-S phases in the amended sludge was converted to other phases (this further decrease was associated with an increase in organo-Cu(II) phases). The proportions of Cu and Zn in the exchangeable fraction decreased over time from 36% and 70%, respectively, in the amended soil to 28% and 59% in the control experiments, and to 24% and 53% with plant growth. This trend is compatible with the observation that Cu and Zn in the soil tend to be in relatively unreactive phases. Current regulations governing the application of digested sewage sludge to agricultural land do not capture the changes in metal mobility during aging and plant growth.

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Chapter 6: Distribution and speciation of Cu and Zn near spring barley (*Hordeum vulgare*) roots in digested sludge-amended soil

6.1 Introduction

Sewage sludge (SS), as an alternative of organic fertilisers used for improving soil fertility, is strictly regulated to be used in agricultural land due to the presence of contaminant metals (Arteaga et al., 2024; Delibacak et al., 2020; Hechmi et al., 2021). To control the risk, many countries have produced regulations on guiding sludge use on farming (Dabrowska and Rosinska, 2012; DEFRA, 2018; EPA, 2022). They generally allow using SS to agricultural soils only if metal concentrations in SS do not exceed the permissible levels and require careful monitoring of bulk soil metal concentrations after sludge application to ensure them remain below the prescribed limits. However, these controls are being increasingly viewed as overly conservative as they do not recognise the risk posed by the metals which is largely dependent upon their chemical forms (Garcia-Delgado et al., 2002; Sekhar et al., 2002; Shivakumar et al., 2012). Sometimes, despite the total amount of a given metal exceeds the regulated limit, its environmental impacts may be limited if the metal is predominantly distributed in the residual fraction (tightly bound to minerals) (Fadiran et al., 2014). Furthermore, the metal speciation can evolve over time due to changes in soil properties and environmental conditions (Caracciolo and Terenzi, 2021; De Conti et al., 2018; Feng et al., 2023; Gan et al., 2020). As a result, this may lead to an overestimation of the associated risk, which in turn can limit the potential of sludge use in agriculture. To address this knowledge gap, in Chapter 5, the two most abundant metals in SS, Zn and Cu, were investigated. Their speciation changes in a digested sludge-amended soil that occurred over time and with repeated plant growth were determined. It indicated that over time both Cu and Zn in the sludge-amended soil become progressively less mobile, and the transformation is enhanced in the presence of plant growing.

In addition, it should be noted that most previous studies of the behaviour and fate of contaminant metals in the sludge-soil/sludge-soil-plant system have focussed on the bulk soil (at a distance from the plant root surfaces) in the plant root zone (Feng et al., 2024; Su et al., 2004; Youssef and Chino, 1988). However, plant roots can significantly influence the environment directly adjacent to them (rhizosphere) to obtain access to essential nutrients from the sludge-amended soil (Benedet et al., 2019; Castillo-Michel et al., 2012; Kissoon et al., 2010). As a result, the environmental conditions in the rhizosphere differ considerably from the bulk zone, which may further affect the transformation of metal behaviour and fate in the zone (Krishnamurti et al., 1996; Pirrone et al., 2013). To date, little attention has been paid to the transformation of contaminant metals in the rhizosphere and their dynamics adjacent to plant roots. Thus, there is a pressing need for more research on contaminant metal behaviour in close proximity to plant roots in the sludge-amended soil, to improve the management of risk when sludge is used in agriculture (Lin et al., 2003).

The primary objective of this chapter is therefore to understand the transformation of metal behaviour and fate in close proximity to spring barley (*Hordeum vulgare*) roots in the digested sludge-amended soil. Cu and Zn are the focus due to their 1) abundance in SS, 2) agronomic value as essential micro-elements and 3) environmental concerns. First the zone where Cu and Zn concentrations are significantly affected by spring barley roots is investigated at millimetre scale in a rhizo-pot system, where the plant roots and the sludge-amended soil are physically separated. Then the detailed *in-situ* spatial distribution and speciation of Cu and Zn in close proximity to spring barley roots are investigated at micrometre scale in a plug-tray system using electron probe microanalyzer (EPMA), microfocus X-ray fluorescence (µXRF) and microfocus X-ray absorption near edge spectroscopy (µXANES) techniques. This combined study elucidates how spring barley roots transform Cu and Zn within the rhizosphere and absorb them into their roots. The results will contribute to the knowledge needed for better risk assessments associated with sludge application in agricultural land.

6.2 Materials and methods

Two sludge-amended soils were used in this combined study. The methodology for the rhizo-pot experiment (pot trial 2) and the plug-tray experiment (pot trial 3) has been detailed in Chapter 3 (full details are available in Section 3.4).

6.3 Results

6.3.1 Rhizo-pot experiment results

6.3.1.1 Plants analysis

During the first growth round, the plant growth rate was slower than in the subsequent growth rounds, with little change in the plant height after 3 weeks (see Figure 6.1). The growth rate was slightly higher in the second round, but again there was little change in the plant height after 3 weeks. Growth in the third, fourth and fifth rounds was similar, with a higher initial growth rate than in the earlier rounds, and with growth continuing over the six-week growth period. As a result, the average plant height after six weeks was 15.4 cm, 19.6 cm, 29.7 cm, 29.2 cm, and 33.3 cm in five growth rounds. At the end of each growth period, the dry weight biomass of per plant was 0.04 g/plant, 0.04 g/plant, 0.09 g/plant, 0.10 g/plant and 0.14 g/plant, respectively (see Table 6.1). The dry weight biomass ratio of shoots to roots was approximately 2 in all growth rounds.

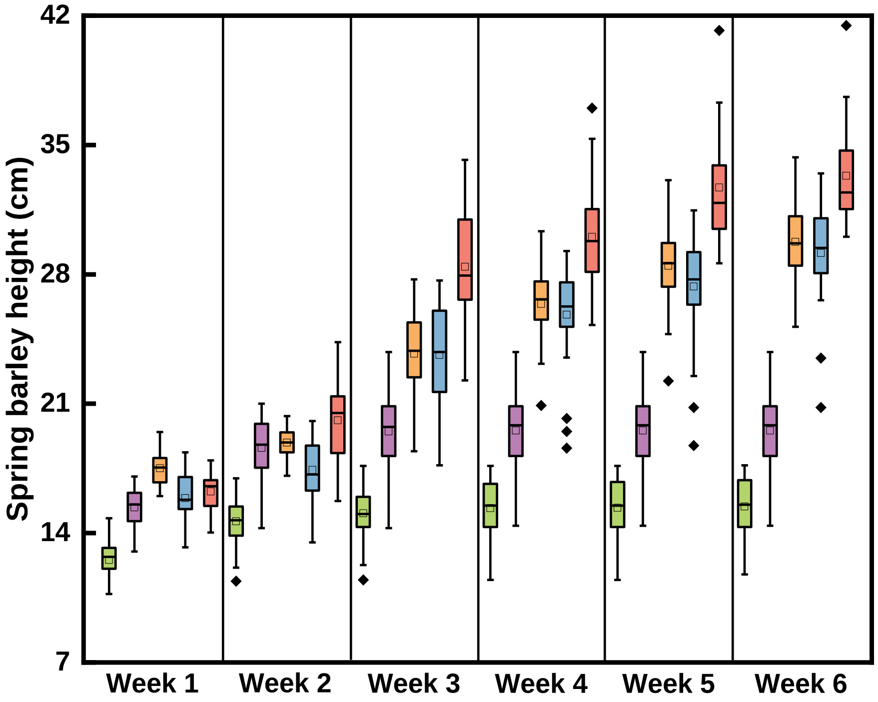

Figure 6.1 The recorded height of spring barley during each growth period (green: 1st round; purple: 2nd round; yellow: 3rd round; bule: 4th round; pink: 5th round. shaded boxes show the median values and interquartile range; tails indicate 1.5 × IQR; □: mean value; ♦: outliers).

Table 6.1 Dry weight biomass of spring barley roots and shoots from the rhizo-pot experiment.

Growth round	Dry weight biomass of shoots (g)	Dry weight biomass of roots (g)	Survived planted pots number	Harvested plants number	Dry weight biomass of each plant shoot (g/plant)	Dry weight biomass of each plant root (g/plant)
1st round	4.4	1.2	42	126	0.03	0.01
2nd round	2.9	1.3	32	96	0.03	0.01
3rd round	5.9	3.2	32	96	0.06	0.03
4th round	5.2	3.1	27	81	0.06	0.04
5th round	7.2	4.2	27	81	0.09	0.05

The average Cu concentration in the spring barley was about 210 mg/kg in the first three growth rounds, decreasing to 150 and 120 mg/kg in the fourth and fifth growth rounds (see Figure 6.2; full data are available in Table C.2 and Table C.3). Interestingly, the Cu was partitioned strongly to plant roots, where the average Cu concentration was about 570 mg/kg in the first three growth rounds, decreasing to about 320 and 240 mg/kg in the fourth and fifth growth rounds. The average ratio of roots to shoots for Cu concentrations was about 8. The average Zn concentration in the spring barley increased in the first four growth rounds, before decreasing in the fifth growth round (about 260 mg/kg, 510 mg/kg, 960 mg/kg, 1110 mg/kg and 670 mg/kg, respectively). The Zn concentration in the roots followed same pattern over the five rounds of growth (about 370 mg/kg, 530 mg/kg, 1360 mg/kg, 1,400 mg/kg and 770 mg/kg respectively), but Zn partitioned less strongly to the plant roots than Cu. The average ratio of roots to shoots for Zn concentrations was about 1.5.

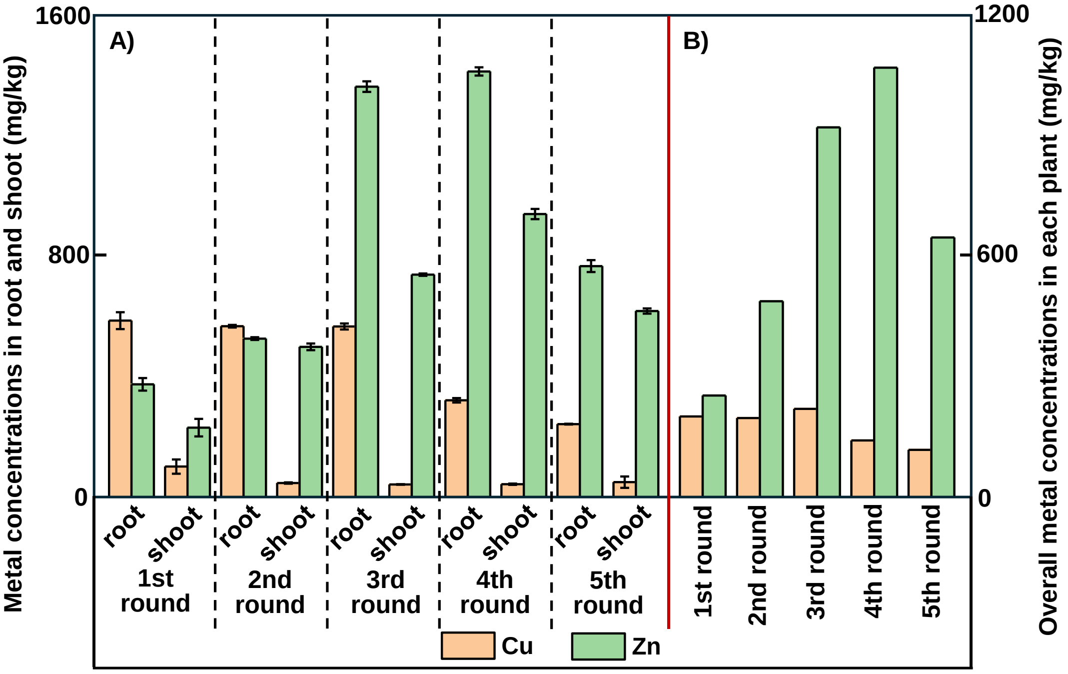

Figure 6.2 A) Zn and Cu concentrations in spring barley roots and shoots of five growth rounds (*n*=3) and B) overall Zn and Cu concentrations in each plant for five growth rounds.

6.3.1.2 Cu and Zn concentrations in the rhizo-pot soil sublayers

One-way ANOVA indicated that there were no significant differences in Cu concentrations between 0-20 mm control soil sublayers after the first round ($p<0.05$; see Table 6.2). Whereas for both the third and fifth rounds, Cu concentrations in the 0-2 mm control soil sublayers were significantly lower than the 4-20 mm sublayers. After these rounds the Cu concentration in the 2-4 mm sublayer was intermediate between the 0-2 mm and 4-20 mm layers, but not statistically different from either. In the planted soils, after the first and third rounds, one-way ANOVA indicated that Cu concentrations in the 0-2 mm sublayer were significantly lower than 4-20 mm sublayers. As seen in the control, the Cu concentration in the 2-4 mm sublayer of the planted soil was intermediate between the 0-2 mm and 4-20 mm sublayers, but not statistically different from either. After five growth rounds, Cu concentrations in the 0-4 mm sublayers of the planted soil were significantly lower than the 4-20 mm sublayers. Also, the Cu concentration in the 0-2 mm sublayer was significantly lower than in the 2-4 mm sublayer. The Cu concentrations in the 0-2 mm and the 2-4 mm sublayers of the planted soils were in each case lower than in the equivalent control sublayers, but only one of the six comparisons passed the Student's *t*-test for significance ($p<0.05$). This suggests that the observed reduction in Cu concentration in the near surface soils was not directly associated with plant growth, and that five rounds of plant growth had little influence on Cu concentration in adjacent soil at the mm scale of this experiment.

For Zn, one-way ANOVA showed that there were no significant differences between control soil sublayers after any of the growth rounds ($p<0.05$; see Table 6.3). In the planted soils, Zn concentrations after the first round were significantly lower in the 0-2 mm sublayer than in the 2-20 mm sublayers, with little difference between the other sublayers. After three rounds, the Zn concentration in the 0-2 mm sublayer was significantly lower than in the 2-4 mm sublayer, which in turn was significantly lower than the Zn concentrations in the 4-20 mm sublayers. After five rounds, the Zn concentrations in the 0-2 mm and 2-4 mm sublayers were comparable, but both were significantly lower than the Zn concentration in the 4-7 mm sublayer, which in turn was significantly lower than the Zn concentration in the underlying sublayers. The Zn concentrations in the 0-2 mm and 2-4 mm sublayers of the planted soils were in each case lower than in the equivalent control sublayers, and for five comparisons this difference was significant (the Student's *t*-test; $p<0.05$). This suggests that the observed reduction in Zn concentration in the near surface soils was enhanced by the plant growth.

Table 6.2 Mean Cu concentrations at different sublayers of control and planted rhizo-pot soils after first, third and fifth growth rounds (mg/kg of dry soils).

Sublayers (mm)	1st round			3rd round			5th round		
	Control	Planted	*t*-test *p*-value	Control	Planted	*t*-test *p*-value	Control	Planted	*t*-test *p*-value
0-2	4839 ± 150 ^{ab}	4705 ± 58 ^{c}	0.259	4892 ± 76 ^{c}	4708 ± 80 ^{c}	**0.019**	4731 ± 75 ^{c}	4654 ± 89 ^{c}	0.259
2-4	4797 ± 92 ^{ab}	4751 ± 52 ^{bc}	0.390	4984 ± 109 ^{bc}	4861 ± 73 ^{bc}	0.100	4835 ± 130 ^{bc}	4815 ± 112 ^{b}	0.824
4-7	4900 ± 67 ^{ab}	4913 ± 104 ^{a}	0.857	5039 ± 68 ^{ab}	5000 ± 188 ^{ab}	0.745	4988 ± 83 ^{ab}	5012 ± 57 ^{a}	0.642
7-10	4944 ± 103 ^{a}	4849 ± 58 ^{ab}	0.138	5097 ± 51 ^{ab}	5080 ± 241 ^{a}	0.907	5106 ± 49 ^{a}	5122 ± 82 ^{a}	0.769
10-15	4900 ± 12 ^{ab}	4930 ± 104 ^{a}	0.560	5150 ± 66 ^{a}	5051 ± 85 ^{ab}	0.138	5095 ± 46 ^{a}	5100 ±184 ^{a}	0.967
15-20	4725 ± 86 ^{b}	4902 ± 87 ^{a}	**0.031**	5085 ± 83 ^{ab}	5024 ± 77 ^{ab}	0.332	5146 ± 195 ^{a}	5082 ± 49 ^{a}	0.489

Values are expressed as mean ± standard deviation (n=3 for control soils and n=5 for planted soils). One-way ANOVA has been applied to each column of data (p<0.05). Different superscript letters indicate a significant difference in mean Cu concentration between each sublayer for that test condition. For example, a population labelled ^{a} is significantly different from ^{b} or ^{c}, while ^{ab} would not be significantly different from a population annotated as ^{a} or ^{b}, but would be significantly different from those labelled ^{c}. The Students' *t*-test was used to determine the significance of the difference in mean Cu concentration between the sub-layers of planted soil and control soil in each growth round. The full data are available in Table C.4 and Table C.5.

Table 6.3 Mean Zn concentrations at different sublayers of control and planted rhizo-pot soils after first, third and fifth growth rounds (mg/kg of dry soils).

Sublayers (mm)	1st round			3rd round			5th round		
	Control	Planted	*t*-test *p*-value	Control	Planted	*t*-test *p*-value	Control	Planted	*t*-test *p*-value
0-2	4824 ± 19 ^{bc}	4579 ± 110 ^{c}	**0.010**	4754 ± 257 ^{b}	4085 ± 82 ^{d}	**0.001**	4667 ± 151 ^{a}	3635 ± 347 ^{c}	**0.003**
2-4	4976 ± 54 ^{abc}	4834 ± 32 ^{b}	**0.003**	4943 ± 46 ^{ab}	4563 ± 119 ^{c}	**0.001**	4693 ± 378 ^{a}	3970 ± 473 ^{c}	0.067
4-7	5041 ± 71 ^{abc}	4987 ± 49 ^{ab}	0.242	4903 ± 163 ^{b}	4926 ± 394 ^{b}	0.928	4695 ± 389 ^{a}	4605 ± 165 ^{b}	0.655
7-10	5088 ± 156 ^{ab}	5043 ± 167 ^{a}	0.718	4867 ± 209 ^{b}	5184 ± 264 ^{ab}	0.129	4842 ± 499 ^{a}	5015 ± 161 ^{a}	0.483
10-15	5104 ± 90 ^{a}	5066 ± 82 ^{a}	0.559	5045 ± 186 ^{ab}	5245 ± 138 ^{a}	0.129	4821 ± 127 ^{a}	5056 ± 273 ^{a}	0.219
15-20	4798 ± 286 ^{c}	4929 ± 203 ^{ab}	0.471	5259 ± 70 ^{a}	5046 ± 74 ^{ab}	**0.007**	5049 ± 215 ^{a}	5079 ± 64 ^{a}	0.773

Values are expressed as mean ± standard deviation (n=3 for control soils and n=5 for planted soils). One-way ANOVA has been applied to each column of data ($p < 0.05$). Different superscript letters indicate a significant difference in mean Zn concentration between each sublayer for that test condition. For example, a population labelled ^{a} is significantly different from ^{b} or ^{c}, while ^{ab} would not be significantly different from a population annotated as ^{a} or ^{b}, but would be significantly different from those labelled ^{c}. The Students' *t*-test was used to determine the significance of the difference in mean Zn concentration between the sub-layers of planted soil and control soil in each growth round. Full data see Table C.4 and Table C.5.

6.3.2 Plug-tray experiment results

6.3.2.1 Plant height

A rapid growth occurred in the first three weeks, followed by minor growth in the fourth and fifth week (see Figure 6.3). The height of spring barley remained largely unchanged in the sixth week. After six weeks growth, the average height of plants was about 27 cm.

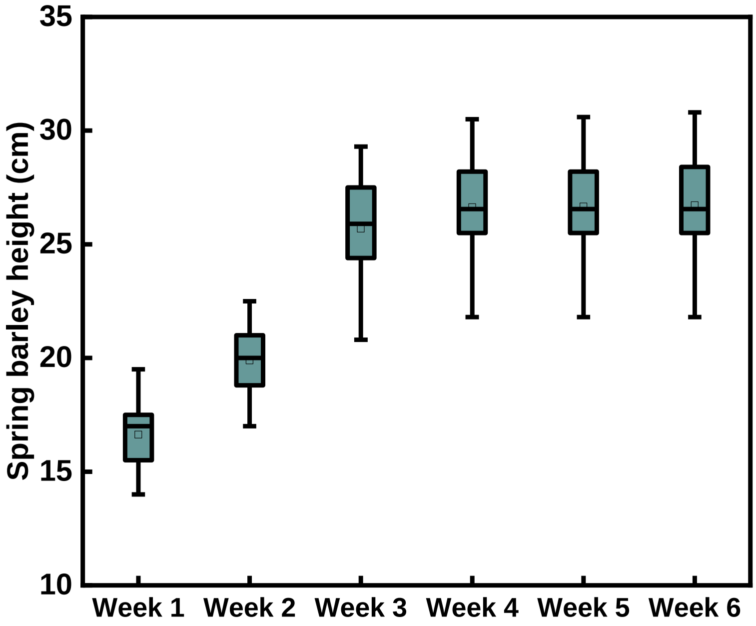

Figure 6.3 Changes in spring barley height during 6-week growth phase in the plug-tray experiment (shaded boxes show the median values and interquartile range; tails indicate 1.5 × IQR; □: mean value).

6.3.2.2. Initial assessment of plant root structures

After six weeks growth period, both the cross section and longitudinal section of spring barley roots were monitored by SEM (see Figure 6.4). It indicated that the size of spring barley root reached over hundreds micro-meters after six weeks growth. The root cortex and vascular tissue were clearly observed. The cortex was featured with honeycomb-like structure. Interestingly, a few small Zn-rich spots were found in the vicinity of root surfaces.

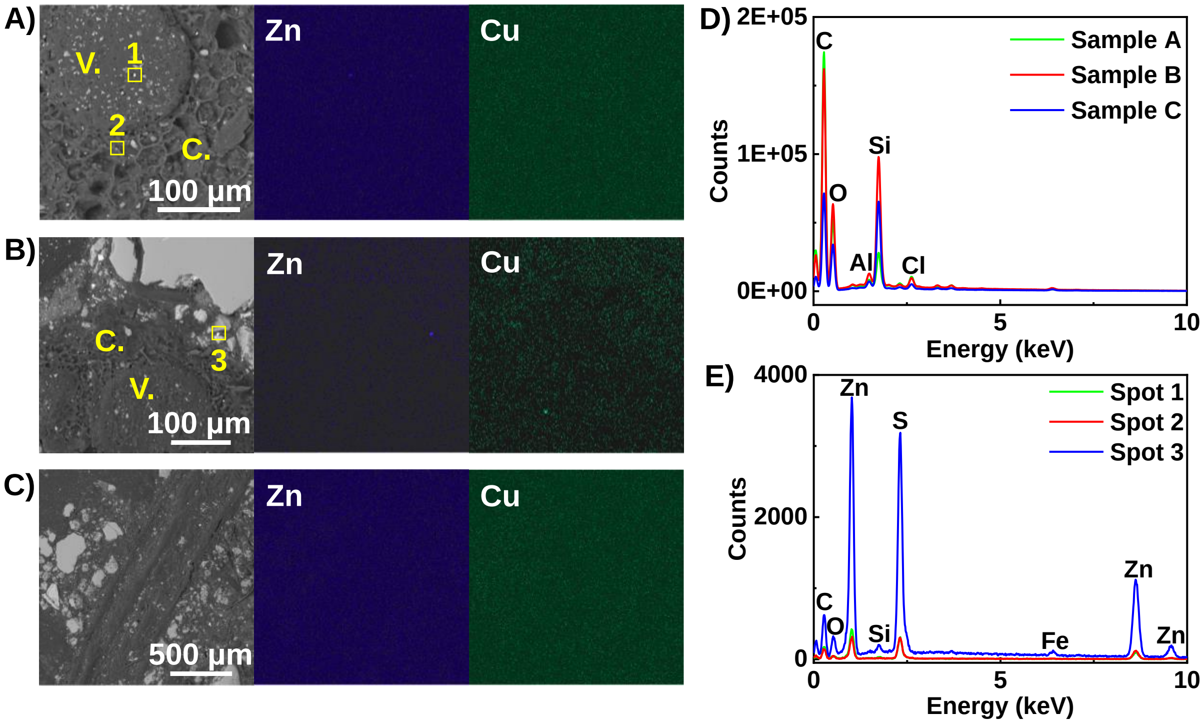

Figure 6.4 SEM backscattered images of A) spring barley roots, B) cross section and C) longitudinal section of close proximity to the spring barley roots; D) the sum spectrum of the sample A), B and C). E) the spectrum of the three Zn-rich spots. Labelling: V. - vascular structure; C. - cortex. Spots 1-3 are the Zn-rich spots.

6.3.2.3 Cu and Zn elemental distribution

The resin-impregnated soil samples from the plug-tray experiment were viewed under an optical microscope, and locations where spring barley roots were orientated roughly perpendicular and roughly parallel to the polished plane were identified for subsequent analysis. When a root is perpendicular to the polished plane (see Figure 6.5), it is visible on the µXRF Si-Al-Ca map as a “Ca” hot spot. The Si-Cu-Zn µXRF map of the same location showed that Cu was present throughout the root structures, but with higher relative concentrations in the outer cortex. Small Cu-rich spots were seen distributed in the vicinity of the root surfaces. A similar Cu distribution pattern was observed in the longitudinal section of a different root imaged by EPMA (see Figure 6.6). The element map suggests that the Cu concentration is higher near the root surface and within the root outer cortex, than within the vascular region of the root (see Figure 6.6C). This pattern is clearer when pixels within the same horizontal row of the strip map are averaged (see Figure 6.6E), and suggests that the Cu concentration within the root outer cortex is higher than it is outside the root (immediately adjacent to the root).

The Si-Cu-Zn µXRF elemental maps showed that the Zn concentrations were similar in the root outer cortex and inner vascular region. Like Cu, small Zn-rich spots were found in the rhizosphere (also seen in the SEM elemental maps shown in Figure 6.4). The average intensity within the vertical strip map across a root orientated parallel to plane being imaged showed that Zn concentrations were similar in the root (cortex and vascular tissues) and rhizosphere soil immediately adjacent to the root.

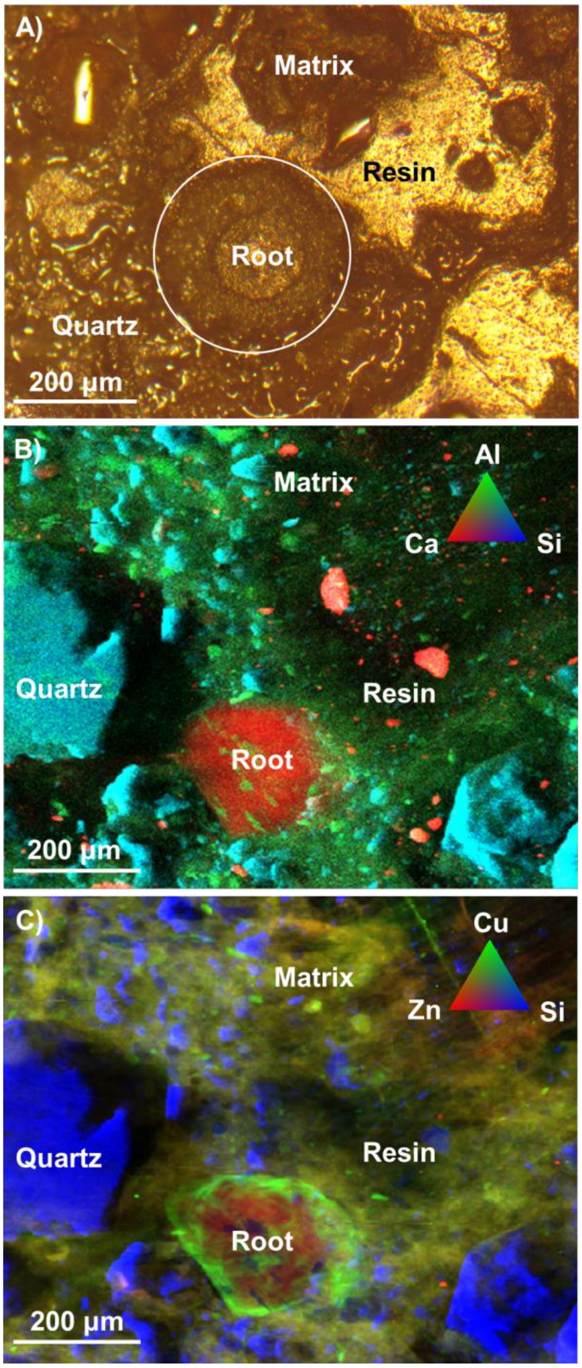

Figure 6.5 A) Optical image cross section of resin embedded spring barley root grown in sludge-amended soil showing location of a plant root. µXRF false colour maps showing distribution of B) Si-Al-Ca, and C) Si-Cu-Zn in close proximity to the root of A). Note: there is a small difference in the frame of reference for optical microscopy and µXRF mapping.

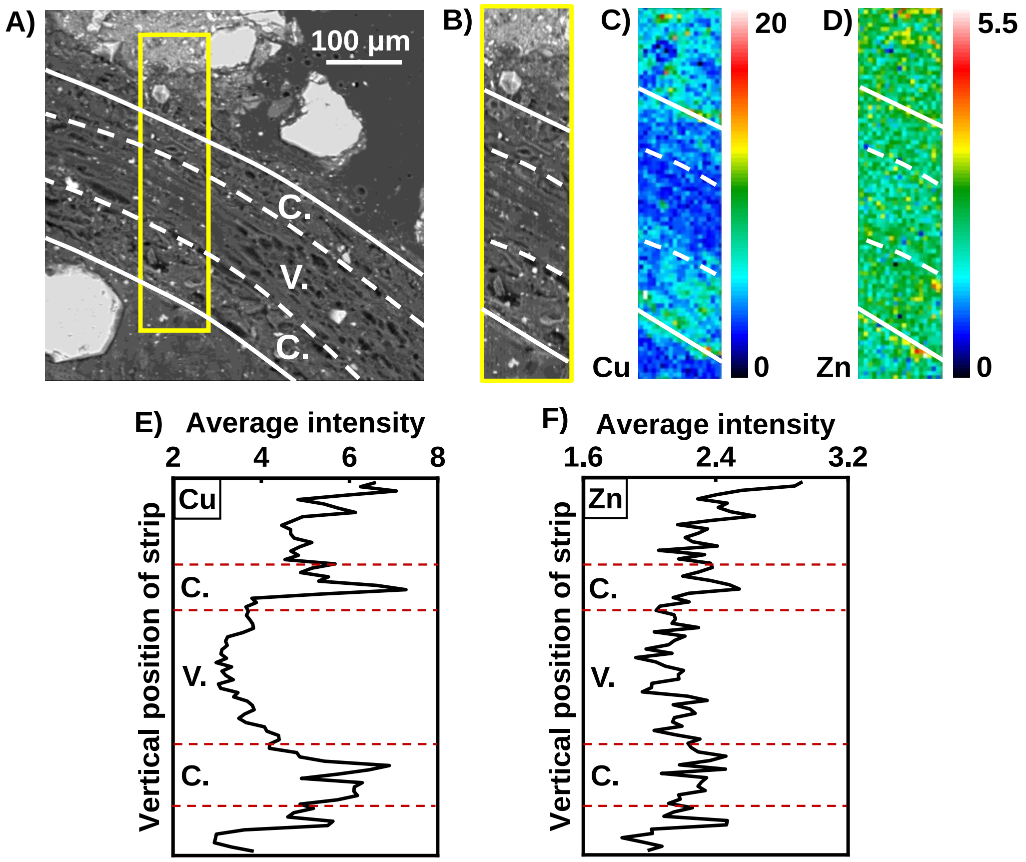

Figure 6.6 Distribution of Cu and Zn in the vicinity of a spring barley root (longitudinal section). A) EPMA backscattered electron image; B) the subregion of the image selected for EPMA analysis; C) Cu and D) Zn maps of the selected region; E) and F) average intensity within the selected region as a function of vertical position. Labelling: C. - cortex; V. - vascular structure.

6.3.2.4 Cu and Zn speciation

The Cu µXANES spectra collected from different locations within Figure 6.5 showed that the Cu binding environments varied between the plant root structures, the rhizosphere and the sludge-amended soil (see Figure 6.7: locations from which the spectra were collected are shown in Figure C.1). LCF results indicate that the three most abundant Cu bonding environments in the sludge-amened soil were Cu(I)-O (~45%), Cu(I)-S (~30%) and organo-Cu(II) (~25%) (see Table 6.4). However, neither Cu(I)-S nor organo-Cu(II) were among the three most abundant bonding environments in either the rhizosphere or the root structures. The dominant Cu bonding environments in the rhizosphere were Cu(I)-O (~60%) and Cu(II)-O_{PO4} (~40%, absorbed/bonded to phosphate), with similar proportions of Cu in these two binding environments in the root cortex. The same two binding environments were also found in the vascular tissue, but with a larger proportion of Cu(I)-O (~70%) and a lower proportion of Cu(II)-O_{PO4} (~30%).

LCF of Zn µXANES spectra indicated that the dominant Zn bonding environments in the sludge-amended soil, rhizosphere and plant root structures were also different (see Figure 6.7 and Table 6.5). In the sludge-amended soil, Zn was predominantly found in the bonding environments similar to inner sphere complexes with metal oxides (Zn(II)-O_{IS}, ~70%), aqueous Zn^{2+} (which is indistinguishable from outer sphere bonding complexes; Zn(II)-O_{OS}, ~20%) and Zn absorbed/bonded to phosphate (Zn(II)-O_{PO4}, ~10%). The dominant Zn bonding environments in the rhizosphere were absorbed to/incorporated into carbonates (Zn(II)-O_{CO3}, ~40%), Zn(II)-S (~35%) and Zn(II)-O_{IS} (~25%). In the root structures, the dominant bonding environments were Zn(II)-O_{CO3}, Zn(II)-S and Zn acetate complexes (organo-Zn(II)). In the root cortex the relative proportions were ~55%, ~30%, and ~15%, respectively, while in the vascular tissue they were ~50%, ~20% and ~30%.

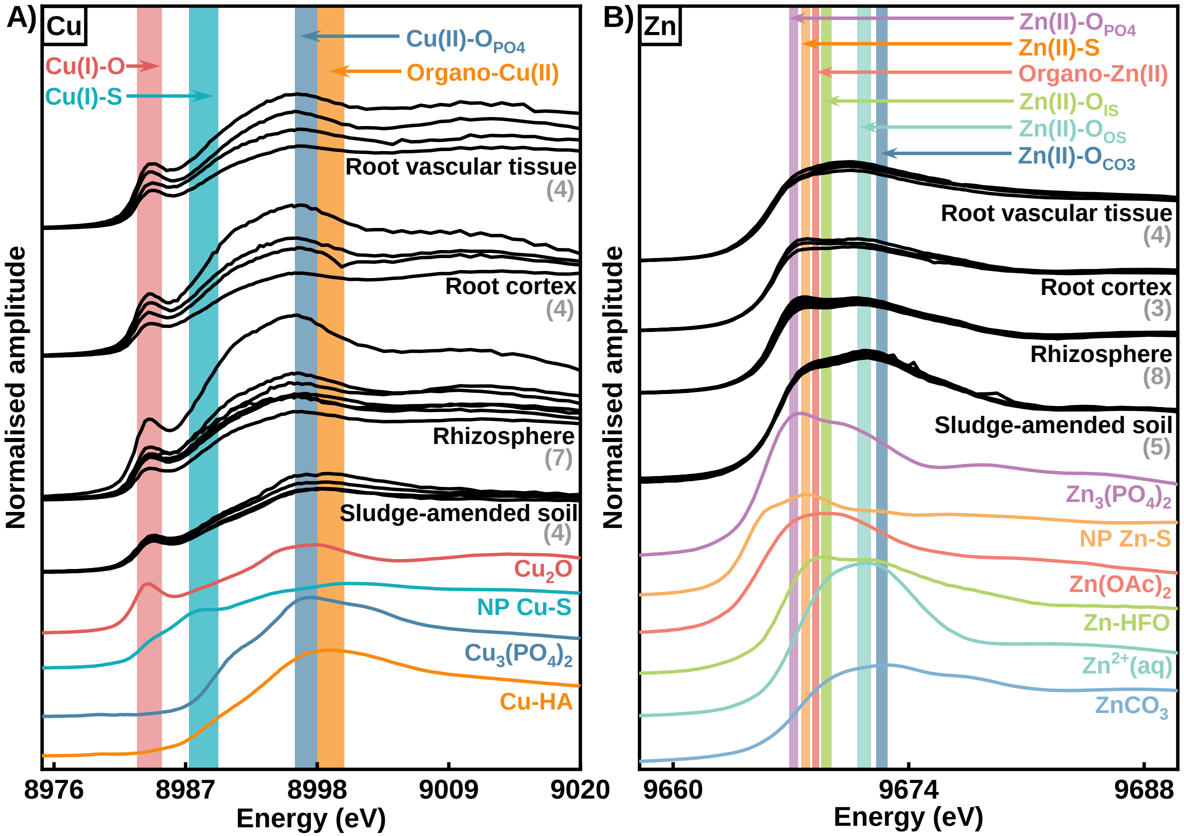

Figure 6.7 Average K-edge µXANES spectra for A) Cu and B) Zn collected from different spots of interests based on µXRF elemental map of Figure 6.5 and bulk XANES spectra of selected reference standards. The number in grey brackets is the number of spot analyses shown. The coloured bands are provided to guide the eye to significant spectra feature present in reference standards spectra.

Table 6.4 Relative abundance of Cu species determined from LCF analysis of Cu µXANES spectra of samples. LCF derived errors are given in parentheses.

Samples	Spots	Cu species (%)				*R*-factor
		Cu_2O	Cu-HA	NP Cu-S	$Cu_3(PO_4)_2$	
Sludge-amended soil	1	44 (5)	25 (2)	31 (6)	/	0.0137
	2	42 (4)	26 (5)	33 (4)	/	0.0086
	3	49 (6)	26 (13)	25 (6)	/	0.0162
	4	44 (2)	23 (5)	33 (2)	/	0.0028
	Average	**45**	**25**	**30**	**/**	**/**
Rhizosphere	5	52 (10)	/	/	48 (12)	0.2313
	6	49 (9)	/	/	51 (14)	0.1783
	7	7 (25)	/	/	93 (25)	0.6840
	8	64 (5)	/	/	36 (5)	0.0802
	9	75 (9)	/	/	25 (5)	0.0742
	10	79 (2)	/	/	21 (5)	0.0148
	11	79 (8)	/	/	21 (6)	0.1195
	Average	**58**	**/**	**/**	**42**	**/**
Root cortex	12	84 (3)	/	/	16 (3)	0.0237
	13	64 (9)	/	/	36 (9)	0.1830
	14	31 (17)	/	/	69 (16)	0.4020
	15	66 (6)	/	/	34 (6)	0.1079
	Average	**61**	**/**	**/**	**39**	**/**
Root vascular tissue	16	65 (9)	/	/	35 (9)	0.1797
	17	49 (14)	/	/	51 (14)	0.3508
	18	80 (5)	/	/	21 (5)	0.0650
	19	91 (2)	/	/	9 (5)	0.0139
	Average	**71**	**/**	**/**	**29**	**/**

The selected spots of interests for Cu analysis are shown in Figure C.1. Cu-HA, Cu-humic complex; NP Cu-S, Cu(I)S nano particles.

Table 6.5 Relative abundance of Zn species determined from LCF analysis of Zn µXANES spectra of samples. LCF derived errors are given in parentheses.

Samples	Spots	Zn species (%)						*R*-factor
		$Zn_3(PO_4)_2$	Zn^{2+} aq	Zn-HFO	NP Zn-S	$ZnCO_3$	$Zn(OAc)_2$	
Sludge-amended soil	1	16 (4)	16 (2)	68 (2)	/	/	/	0.0028
	2	9 (2)	13 (2)	77 (3)	/	/	/	0.0041
	3	9 (7)	24 (3)	67 (3)	/	/	/	0.0054
	4	13 (1)	29 (2)	58 (7)	/	/	/	0.0038
	5	11 (5)	15 (2)	74 (2)	/	/	/	0.0029
	Average	**12**	**19**	**69**	**/**	**/**	**/**	**/**
Rhizosphere	6	/	/	19 (8)	35 (12)	46 (7)	/	0.0968
	7	/	/	18 (8)	31 (16)	51 (12)	/	0.0997
	8	/	/	40 (14)	36 (5)	25 (5)	/	0.0567
	9	/	/	29 (7.0)	37 (13)	35 (6)	/	0.0714
	10	/	/	32 (7)	30 (11)	38 (6)	/	0.0691
	11	/	/	34 (7)	33 (7)	33 (6)	/	0.0649
	12	/	/	20 (8)	36 (16)	44 (17)	/	0.0938
	13	/	/	22 (8)	39 (6)	39 (12)	/	0.0876
	Average	**/**	**/**	**27**	**35**	**39**	**/**	**/**
Root cortex	14	/	/	/	43 (5)	57 (9)	0 (0)	0.1164
	15	/	/	/	34 (9)	52 (13)	14 (8)	0.0834
	16	/	/	/	21 (8)	52 (12)	27 (7)	0.5955
	Average	**/**	**/**	**/**	**33**	**54**	**14**	**/**
Root vascular tissue	17	/	/	/	24 (5)	53 (10)	23 (5)	0.0264
	18	/	/	/	18 (5)	50 (10)	32 (4)	0.0221
	19	/	/	/	12 (4)	49 (9)	39 (4)	0.0152
	20	/	/	/	31 (7)	55 (4)	14 (8)	0.0531
	Average	**/**	**/**	**/**	**21**	**52**	**27**	**/**

The selected spots of interests for Zn analysis are shown in Figure C.1. Zn^{2+} aq, aqueous Zn^{2+}; Zn-HFO, Zn(II)-hydrous ferric oxide; NP Zn-S, Zn(II)S nano particles.

6.4 Discussion

6.4.1 Macro-scale mobilization of contaminant metals from sludge-amended soil

The changes in surface soil metal concentrations are balanced by the total metal amount taken up by the plants and the metal transfer to lower surface levels. In the rhizo-pot experiment, Zn was removed from near surface soil layers in both the planted pots and the control pots. However, at all three sampling times, Zn removal from the sludge-amended soil to a depth of 4 mm was significantly greater in the presence of plants than in the controls, with the amount of Zn removed increasing with number of growth rounds. After 5 rounds, 28% of the Zn in the surface layer was removed in the planted pots whereas 7% was removed in the controls.

Cu was also removed from the near surface soil layers in both the planted pots and the control pots. However, whilst the average Cu concentration in the planted pots was lower than that in the associated controls at all three sampling points in both the 0-2 mm and 2-4 mm soils, this difference was not large enough to be statistically significant. After 5 rounds, 10% of the Cu in the surface layer was removed in the planted pots and 9% was removed in the controls. The Cu uptake per plant was only about 25% of the Zn uptake, which may partially explain why Cu removal by the plants was not statistically significant from the control. The distance from the roots over which metal removal occurs may also be a factor. Previous work on the sludge-amended soil used in the plug-tray experiment found that the Cu concentration in soil particles on the surface of plant roots was significantly lower than the average value in the root zone, whereas the Zn concentration in the soil at the root surface was similar to the average value in the root zone (see Chapter 5). This suggests that the Cu uptake comes from sludge-amended soil in relatively close proximity to the roots (<< 1mm), whereas Zn can be taken up from a larger region of the root soil (> 1mm). The differences in metal removal behaviour probably reflect the differences in Zn and Cu speciation in the sludge-amended soil.

The average Zn µXANES LCF results for the sludge-amended soil used in the plug-tray experiment showed that Zn was present as Zn(II) adsorbed to metal oxide surfaces in inner-sphere and outer-sphere Zn(II) complexes, as well as a small amount as Zn(II) absorbed/bonded to phosphate. This is broadly consistent with previous bulk XANES data on this sludge-amended soil (which was prepared for the work reported in Chapter 5). The bulk XANES identified the Zn coordination environments as Zn(II) adsorbed to metal oxide surfaces in both inner-sphere (70-80%) and outer-sphere (20-30%) complexes (full details see Chapter 5). It is likely that the Zn bonding environments in the sludge-amended soil of the rhizo-pot experiment will have been similar to that of the plug-tray experiment, although the higher amended-sludge loading in the rhizo-pot experiment may have affected the exact proportions of the two dominant bonding environments. As long-range (mm scale) interaction between plant roots and contaminant metals will only occur by diffusion (outward diffusion in plant exudates, inward diffusion of contaminant metals), it is suggested that the Zn removal from the rhizosphere soil created a concentration gradient that resulted in the Zn diffusion from the bulk soil to the rhizosphere. Also, it is likely that the Zn removal is from the pool of more bioavailable outer-sphere adsorbed Zn(II) ions.

The average Cu µXANES LCF results for the sludge-amended soil used in the plug-tray experiment showed Cu in a coordination environment characteristic of Cu(I) oxides, Cu(I) sulphides, and Cu(II)-organic complexes. This is also consistent with the previous bulk XANES data, which identified Cu bonding environments in the same sample as Cu(I) oxides (40-50%), Cu(I) sulphides (20-30%), and Cu(II)-organic complexes (30-40%) phases (full details see Chapter 5). It is likely that the Cu bonding environments in the sludge-amended soil of the rhizo-pot experiment will have been similar to that seen in the plug-tray experiment. Generally, Cu in the above three coordination environments has limited mobility in the sludge-amended soil, which is probably why very little Cu removal was observed at mm scale. The modest removal in both the planted and control soils was probably the result of irrigation with distilled water (a small proportion of Cu(II)-organic complexes may have been associated with soluble soil organics such as fulvic acids; Wu et al., 2002).

6.4.2 Cu and Zn speciation in rhizosphere soils

Cu in the rhizosphere soil was predominately in bonding environments characteristic of Cu(I) oxides (seen in the sludge-amended soil) and Cu(II) bonded to/incorporated into phosphates (not detected in the sludge-amended soil). The increase in the proportion of Cu in Cu(I) oxides relative to the bulk sludge amended soil may reflect the removal of other Cu phases close to plant roots, whilst the absence of Cu(I) sulphides and Cu(II)-organic complexes suggests that Cu initially in these bonding environments is either taken-up by the plant roots, or converted to Cu(II) phosphates. The presence of Cu(II) bonded to phosphate may suggest that the complexation of Cu(II) by P-containing ligands is an important process in the rhizosphere. P is usually sparingly available in soils, so plants have evolved chemical and biological changes strategies for mobilising P in the rhizosphere (Ding et al., 2021; Shen et al., 2011). The formation Cu(II)-phosphate phases in the rhizosphere may result from a reaction with plant mobilised P.

Zn in the rhizosphere soil was in bonding environments characteristic of Zn(II) inner-sphere complexes with metal oxide surfaces, Zn(II) sulphides and Zn(II) bonded to/incorporated into carbonates. As neither Zn(II) sulphides and Zn(II) carbonates were observed in this sludge-amended soil, they must be due to the close proximity of the roots of growing plants. The presence of Zn(II) bonded to/incorporated into carbonates is likely due to the reaction of Zn(II) with bicarbonate produced by plant and/or microbial metabolism. However, the reason for the Zn(II) sulphide-like bonding environment is less clear, but may be associated with changes in Cu chemistry in rhizosphere (conversion of Cu(I) sulphides to Cu(II)-phosphates).

6.4.3 Cu and Zn speciation in plant roots

Zn and Cu are both essential micronutrients for healthy plant growth, but both can disrupt plant metabolic processes at high concentrations (Behtash et al., 2022). Typically, plants require less Cu than Zn for healthy growth, and Cu becomes toxic to plants at lower concentrations (Behtash et al., 2022; Premier Tech Growers and Consumers, 2014). The rhizo-pot experiments showed that both Zn and Cu were taken up into the plants, with on-average ~4x more Zn up-take per plant than Cu. Although Zn was far more readily translocated to the plant shoots (the ratio of roots to shoots concentrations was ~8 for Cu and ~1.5 for Zn), the average Zn concentrations in the roots were still about twice the average Cu concentrations. Further, whilst Zn concentrations were similar across the roots, Cu concentrations were higher in the root cortex than in the vascular tissue. One mechanism by which plants can respond to metal stress is to sequester them in the inactive tissues such as root epidermal cells (Cao et al., 2019; Wang et al., 2024). The resolutions of µXRF and EMPA cannot differentiate between the epidermis and the cortex, but it appears that spring barley has sequestered excess Cu, but not excess Zn, in the outer tissues of the root.

The Zn bonding environments in the root cortex differed from that seen in the rhizosphere. The root cortex did not contain Zn(II) in inner sphere complexes. Instead, Zn was found as Zn(II) absorbed to/incorporated into the carbonates, Zn(II) sulphides and Zn(II)-organic complexes. Plants acquire Zn from the sludge-amended soil solution, and transport it towards the root vasculature, primarily as aqueous Zn(II), but also as Zn-chelates (Zn(II)-organic complexes; Balafrej et al., 2020; Broadley et al., 2007; Stanton et al., 2022). It is not possible to comment on the bonding environment of mobile Zn, as mobile species can be lost during resin impregnation. However, the presence of Zn(II) absorbed to/incorporated to carbonates is likely to have resulted from a reaction with bicarbonate in the roots. Thiol moieties (R-SH) in amino acids such as cysteine may account for Zn(II) sulphide in the cortex, as cysteine residues have a high affinity for aqueous Zn(II) ions (Zn(II)-cysteine complexes are critical mediators/precursors in protein synthesis present ~9% of the eukaryotic proteome; Pace and Weerapana, 2014; Stanton et al., 2022; Zeng et al., 2011). The same Zn bonding environments were present in the vascular tissue as that seen in the cortex, but there is a slightly higher proportion of Zn(II)-organic complexes (at the expense of Zn(II) sulphides). This may be because Zn transport from roots to shoots in the xylem involves predominantly as Zn(II)-organic complexes, probably as chelate complexes with nicotianamine, amino acids, or organic acids (Balafrej et al., 2020; Clemens et al., 2013).

The Cu bonding environments in the root cortex were similar to that seen in the rhizosphere (60% Cu(I) oxides and 40% Cu(II) absorbed/bonded to phosphate). In vascular tissue it was 70% Cu(I) oxides and 30% Cu(II) absorbed/bonded to phosphate. Cu is transferred within plants roots mainly as Cu(I)- or Cu(II)-complexes with chaperone proteins (Kumar et al., 2021; Wang et al., 2024). The µXANES LCF suggests that the majority of the Cu in the plant roots is immobile, which is supported by low Cu translocation to above ground structures, and localisation to the root cortex. Cell vacuoles are important plant organelles used to store mineral nutrients such as iron and phosphate (Peng and Gong, 2014; Wieczorek et al., 2022). Cu is also stored in vacuoles when absorbed in excess of nutritional needs (Peng and Gong, 2014). The µXANES data is compatible with Cu storage in cell vacuoles, particularly within the root cortex.

6.5 Conclusions an implications

- Zn introduced with digested sewage sludge is mobile on a mm scale, whereas Cu introduced with digested sewage sludge is only mobile on a sub-mm scale. As a result, young spring barley plants took-up 4x more Zn than Cu even though these metals were present at similar concentrations in the sludge-amended soil.

- The speciation of Cu and Zn close to plant roots (*i.e.* in the rhizosphere soil) is different from that found more widely in sludge-amended soil. Local to the plant roots, Zn and Cu are in chemical environments that suggest their mobility has been reduced by presence of plant roots.

- Zn that is taken-up by spring barley is relatively evenly distributed through the roots, with similar concentrations in the plant shoots, whereas most Cu that is taken-up is sequestered in the roots (particularly the root cortex), with only a small proportion translocated to above ground plant structures.

- Understanding changes in metal speciation during plant uptake is important in determining the relative risks of different metals (*i.e*. Cu is hazardous at lower concentrations than Zn, but Cu is trapped in the rhizosphere whereas Zn is translocated to plant shoots).

- Better understanding of the mobility and uptake of metals that have elevated concentration in sewage sludge could be used to undertake element specific risk assessments for sludge use in agriculture.

6.6 References

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Chapter 7: General discussion

This chapter aims to provide more discussions on the important findings determined in this project, serving as a reference for informing better risk assessments for sewage sludge (SS) use in agricultural land. The limitation of this project is also discussed, offering the guidance for future studies related to the behaviour and fate of contaminant metals in sludge-soil/sludge-soil-plant systems.

7.1 Comparison between sequential extractions and X-ray absorption spectroscopy

The chemical forms of contaminant metals significantly control the risk (Lasheen and Ammar, 2009). The analysis of metal speciation can provide an understanding of the extent of metal bioavailability and help in more reasonable decision disposing SS to agricultural land (Fadiran et al., 2014). X-ray absorption spectroscopy (XAS) and sequential extractions are the two most common methods for determining metal speciation in samples. XAS is a well-established method for determining metal bonding environments and oxidation state (Nevidomskaya et al., 2021; Tiberg et al., 2021). However, it should be stressed that the metal bonding environments determined by XAS may be not the same as the metal phases because multiple phases can have the same bonding structure (Ekanayake et al., 2024). Besides, XAS cannot fully explain the metal leachability. The metal leachability can be revealed by sequential extractions, but this approach also has some intrinsic limitations: (1) the chemical oxidation state of contaminant metals cannot be reflected accurately (Wang et al., 2021). For example, in this project, sequential extractions failed to observe the coordination environment of Cu(I) oxides in the sludge-amended soil; (2) it is difficult to accurately determine the metal speciation in waste materials because the phase composition of this material is significantly different from the material for which the sequential extractions are originally designed (Tessier et al., 1979). Given the limitations of these two methods, their combination was used in this project to provide the complementary speciation and leachability information on Zn and Cu in the complex sludge-soil/sludge-soil-plant systems. Specifically, XAS provided the information on the molecular-scale chemical oxidation state of Cu and Zn, which was then used to trace the changes of their bonding environments over geochemical and microbiological processes. Sequential extractions provided more insights into the changes of potential leachability of solid phases upon contact with environmental media. Their combination enhanced the understanding of transformation behaviours of Cu and Zn in the systems. For future studies, it is recommended to use a combination of spectroscopic methods and sequential extractions as their combination enables a more accurate determination of metal speciation in the samples, providing invaluable information about the potential bioavailability of metals.

7.2 Comparison of metal behaviour between original digested sludge and amended sludge

Instead of using original digested sludge, the metal-amended sludge (approximately 20,000 mg/kg Cu and Zn) was applied to the soil to elevate metal levels in the system. This decision was made based on the aim of this project: to investigate the transformation of behaviour and fate of Cu and Zn introduced to the agricultural soil by digested sludge over time due to repeated plant growth. To ensure the observed changes in metal behaviour within the system were principally due to the changes in the behaviour of sludge-associated metals, the original digested sludge was amended with soluble Cu and Zn salts to final concentrations about 50-100 times higher than the typical metal concentrations in the UK sludges. Historically, the metal concentrations in sludges applied to agricultural land have been comparable to, or in some cases greater than, those in the amended sludge used in this project; in some areas with less effective regulations, such kind of moderately/heavily contaminated sludge is still possibly applied to agricultural soils (Babel and del Mundo Dacera, 2006; Jain and Tyagi, 1992; Olthof and Lancy, 1979; Smith, 1996). The amending of original digested sludge with metals is a widely used technique for experimental purposes to escalate metal levels in the soil due to one sludge application (Kandpal et al., 2004; McLaren and Clucas, 2001; Street et al., 1977). The use of amended sludge can prevent the need for excessive use of original digested sludge to elevate metal levels. Also, compared with the metals directly added to the soil, this amending procedure can allow the added metals to react with and become incorporated into the sludge matrix (Kandpal et al., 2004). For example, the work in Chater 4 indicated that ~90% of the Cu and Zn can rapidly become incorporated into the sludge matrix when the added ratio of metals is within 2%. However, it should be noted that the behaviour of metals in the amended sludge may differ slightly from the metal behaviour naturally present in the original digested sludge. As discussed in Section 4.4.4, BCR extractions and XAS data both showed that Cu speciation in the amended sludge was broadly similar to that seen in the original digested sludge. Thus, the experiments reported in this project are likely to reflect the behaviour and fate of Cu introduced to soils with digested sludge reasonably well. With respect to the Zn, it probably requires more careful interpretation because there were bigger differences in Zn speciation between the amended sludge and original digested sludge (indicated by both BCR and XANES data). Nevertheless, the conversion of Zn(II)-S phase in the amended sludge predominantly to Zn(II)-O_{OS} phase upon mixing with the agricultural soil, and the subsequent slow conversion of Zn(II)-O_{OS} phase to Zn(II)-O_{IS} phase over time/repeated plant growth that reflects a transition in Zn speciation towards that seen in the original soil, are likely to occur in a real system. The rapid large-scale redistribution of sludge-introduced Zn to the soil matrix and the presence of Zn(II)-S, Zn(II)-O_{CO3} and organo-Zn(II) phases in the plant roots are also likely to occur in a real system.

7.3 Transformation of Cu and Zn behaviour and fate over time and with repeated plant growth

7.3.1 Sludge-soil system

In a single sludge system (the original digested sludge or the amended sludge), Cu was mainly distributed in the oxidizable fraction. It aligned with the bulk XANES results showing Cu(I)-S was the dominant bonding environment for Cu in both sludges. For Zn, sequential extractions indicated that it was distributed in exchangeable, reducible, oxidizable and residual fractions in the original digested sludge. Differently, in the amended sludge, Zn was predominantly distributed in the exchangeable fraction. It indicated that the added Zn was preferentially adsorbed to mineral surfaces or bound to carbonates. In a single agricultural soil system, Cu and Zn were both dominantly distributed in the residual fraction. This distribution pattern was consistent with the general conclusion drawn from literature review that aqua regia extract is the primary speciation for metals in the soil (Dudka and Chlopecka, 1990; Kotoky et al., 2015; Sposito et al., 1982; Topcuoğlu, 2014). The presence of a significant component of contaminant metals in the residual fraction indicates that the soil-associated metals are highly stable and immobile, posing minimal environmental risks under the existing physico-chemical environments.

Due to significant differences in metal speciation patterns between the amended sludge and the agricultural soil, a redistribution of sludge-borne and soil-borne metals into different fractions is expected following their mixing. This redistribution process is highly related to the amount of metals introduced to the soil via sludge. Typically, the higher the amount of metals introduced via sludge, the more the pattern of metal speciation in the sludge-soil system tends to be the pattern of metal speciation in the adopted sludge (see Chapter 2). This conclusion is supported by the results of this project. In Chapter 5, compared with the original agricultural soil, metal speciation in the sludge-amended soil (21 days after mixing) was more like the amended sludge because over 90% of the metals in the sludge-soil system were introduced by the amended sludge. Interestingly, after the amended sludge application, the sludge-introduced Zn was rapidly redistributed to the soil matrix on a large scale, and the affinity of Zn for these sludge particles was relatively weak (not at all like the very strong bonding seen in the original agricultural soil). By comparison, the sludge-borne Cu was partly redistributed to the soil matrix with a lot of Cu still residing within the amended sludge particles and predominantly retained as Cu(I)-S phase. Partial sulphide and Cu oxidation lead to the increases in organo-Cu(II) phases and the proportion of Cu that is more easily extractable was greater in the amended soil. Overall, the application of amended sludge to the agricultural soil resulted in a rapid redistribution of much of the sludge-associated metals (particularly Zn), but this was not accompanied by a reduction in metal mobility (indicated by BCR data).

Noticeably, the determined metal speciation pattern in the sludge-amended soil only reflected the redistribution state on the 21st day after the mixing. The redistribution process of metals in the system is still ongoing and will continue until the final equilibrium is reached. Indeed, it was observed that over time, in the control series, there were further modest changes in the metal speciation and leaching behaviour. The Cu and Zn phase composition (indicated by XANES) evolved over time towards that observed in the original agricultural soil. Their leaching behaviour (indicated by BCR) also evolved with slight reduction in the proportions of exchangeable Cu and Zn fractions. It appears that with time the sludge-introduced Cu and Zn became immobilised within the sludge-soil system and were transferred from easily extractable forms to slowly extractable forms. This is likely due to the microbial degradation of sludge organic carbon (a reduction in the carbon content was detected from average 3.36 wt% in the freshly amended soil to average 3.18 wt% in the first-round control soil; Terry et al., 1979). Therefore, although the amended sludge application introduces a significant amount of easily mobile metals to the agricultural soil, these metals tend to become slowly mobile over contact time under natural processes.

7.3.2 Sludge-soil-plant system

In comparison to the transformation of contaminant metals in sludge-soil systems, the presence of plant growth can further affect the transformation process (sludge-soil-plant system). In Chapter 5, LCF results of Cu and Zn XANES data of the bulk soils showed a similar distribution pattern of metal phases compared to the control soils. It indicates the same evolution path of metal speciation with repeated plant growth as that observed in the control soils. Sequential extractions showed a decrease in the concentration of CH_3COOH-extracted metals over three growth rounds of spring barley, and it was more pronounced than the control soils. This transformation of operationally defined Cu and Zn speciation in the bulk soils was also observed in the root-bound soils and the transformation was even more pronounced than the bulk soils. Therefore, it appears that repeated spring barley growth did not change the transformation direction of metal speciation in the sludge-soil-plant system as that seen in the sludge-soil system, but it may slightly accelerate the transformation process. This acceleration can be ascribed to serval processes taking place concurrently, such as the precipitation of easily extractable metals with exudations from plants and the enhanced microbial degradation of sludge organic carbon (Yamaguchi et al., 2014; Yan et al., 2020). Noticeably, sequential extractions illustrated the major difference in the operationally defined metal speciation between control soils and bulk soils was reflected in the exchangeable and reducible fractions. The metals of these two fractions are both bioavailable for plants uptake. It probably suggests that the acceleration was also related to the metal acquisition by spring barley growth (De Conti et al., 2018).

In the vicinity of spring barley roots in the sludge-amended soil, there were variations in metal behaviour and fate compared to the bulk sludge-amended soil (see Chapter 6). The µXANES results showed that in the amended soil, Cu was predominantly in the Cu(I)-O, Cu(I)-S and organo-Cu(II) bonding environments (which agree with the bulk XANES data reported in Chapter 5). Cu(I)-O was also the primary bonding environment for Cu in the rhizosphere, but organo-Cu(II) and Cu(I)-S were absent. Interestingly, Cu(II)-O_{PO4} containing phase was detected, suggesting that the complexation by phosphorus-containing ligands may be important for Cu species in the rhizosphere. The average proportion of Cu(I)-O containing phase in the rhizosphere was greater than in the amended soil. Cu(I)-O and Cu(II)-O_{PO4} phases were also the two bonding environments for Cu in the plant root structures as that seen in the rhizosphere. For Zn, the average LCF results of Zn µXANES spectra revealed that in the amended soil, Zn was predominantly in Zn(II)-O_{IS} phase, with smaller proportions of Zn(II)-O_{OS} and Zn(II)-O_{PO4} phases. In contrast, in the rhizosphere both Zn(II)-O_{PO4} and Zn(II)-O_{IS} bonding environments were not detected, but Zn(II)-O_{IS} bonding environment was still detected. Also, Zn(II)-S and Zn(II)-O_{CO3} containing phases were found in the rhizosphere and the proportions of these two Zn bonding environments were almost equal. The formation of Zn(II)-S containing phase can promote plant growth as it can easily penetrate the plant and then provide more surface area for various metabolic reactions within the plant system (Kanwal et al., 2022). The occurrence of Zn(II)-O_{CO3} was probably due to the reaction of available Zn with bicarbonates. Within the plant structures, Zn(II)-S and Zn(II)-O_{CO3} containing phases were also the two important bonding environments for Zn as that seen in the rhizosphere. However, instead of Zn(II)-O_{IS} phase, organo-Zn(II) containing phase became important for the Zn within the plant structures. This result was not surprising as the Zn can be complexed with organic acid chelates within the plant structures, and then translocated into the shoots via the xylem (Al Jabri et al., 2022; Rizwan et al., 2019; Saifullah et al., 2016). The observation of organo-Zn(II) phase in the inner vascular tissue (including the xylem part) supported this conclusion.

In the sludge-soil-plant system, spring barley can only absorb the Cu from the immediate vicinity of the roots (sub-mm scale), but Zn can be taken up from further afield (at similar concentrations; mm-scale indicated by rhizo-pot experiment). Cu and Zn taken up by spring barley were primarily concentrated in the roots (particularly Cu), showing the order of accumulation ability for Zn and Cu in spring barley tissues was roots > shoots. Interestingly, a very high Cu concentration was detected in the plant roots, with only a limited allocated to the shoots. Although Cu is an essential element for plant metabolism and can be effectively translocated from roots to shoots at a low external concentration (Žaltauskaitė and Šliumpaitė, 2013), its translocation to aboveground parts was significantly limited when the plant is exposed to a higher metal concentration (such as 1000 mg/kg and 5000 mg/kg). It suggests that spring barley roots can act as an effective barrier against Cu uptake when the bulk soil metal concentrations reach a critical limit, protecting the food chain by restricting Cu movement to the shoots (Kovacevic et al., 1999). Therefore, for Cu, although a large proportion of bioavailable Cu was introduced to the soil via sludge, it may cause limited risk due to the plant-soil barrier. In contrast, in the sludge-soil-plant system, a large proportion of Zn accumulated in the roots can be translocated to the shoots. It was observed that in both contact patterns between sludge-amended soil and plants (direct contact in chapter 5 and separated by nylon mesh in chapter 6). It is ascribed to the little Zn sequestration in the root surfaces. This also explains why significantly more Zn than Cu was removed in the vicinity of spring barley roots in the digested sludge-amended soil observed in the rhizo-pot experiment.

7.4 Implications

7.4.1 *In-situ* spatial distribution and speciation of contaminant metals near plant roots in digested sludge-amended soil

The rhizosphere is a micro-environment with characters that differ markedly from those of non-rhizosphere (Su et al., 2004). The plant-induced changes to the rhizosphere (such as pH alteration, exudation of organic molecules) are known to have impacts on the bioavailability of metals (Naftel et al., 2007). Currently, little attention has been paid to the extent to which plant roots affect the bioavailability and distribution of metals in the vicinity of plant roots in digested sludge-amended soil. One reason why few studies explore the rhizosphere can be ascribed to the difficulty in physically separating the rhizosphere soil from the roots and the very narrow region of greatest interest (Naftel et al., 2007). A few studies used rhizo-pot systems to investigate the metal distribution across the proximity to plant roots in the sludge-amended soil (as that seen in Chapter 6). However, their soil sampling protocols were typically conducted in mm-scale (Su et al., 2004; Youssef and Chino, 1989). As a result, valuable insights into the behaviour of metals in the immediate vicinity of plant roots may be masked (Jacobson et al., 2007). Indeed, experimental results from Chapter 6 approved that the microscale effects closer to plant roots in the digested sludge-amended soil will have been masked by sampling to an initial depth of 2 mm.

This project for the first time employed the developed fluid displacive drying and resin-embedding techniques to prepare *in-situ* sludge-amended soil samples containing plant roots from the plug-tray experiment. This combined technique avoided the samples cracking due to physical drying and the fabric was always supported by the fluid throughout (Pike and Kemp, 1996). The samples prepared by this technique can preserve the most detail for later microscopic observation. By using this technique, the sludge-amended soil containing spring barley roots was successfully prepared and for the first time to elucidate the *in-situ* spatial distribution of Cu and Zn in close proximity to spring barley roots in the digested sludge-amended soil. It enhances the understanding of Cu and Zn transformations in sludge-soil-plant systems.

7.4.2 Sludge application in agriculture and risk assessments

Current regulations focusing solely on total metal concentrations are overly conservative for managing sludge use in agriculture. A consensus has been recognised that the risk posed by contaminant metals is more closely related to their speciation rather than total metal levels. Meanwhile, it should be stressed that while the metal speciation analysis is useful insight in assessing the bioavailability of metals, it still needs to be supported by the ongoing monitoring of transformations of metal speciation over time. A good example is the results reported in Chapter 5. It showed that the application of amended sludge increased overall metal concentrations in the system. However, positively, the metal mobility tended to decrease over time. Zn and Cu were both transformed from easily mobile fractions to slowly mobile fractions over contact time. The presence of repeated plant growth did not change the transformation direction, and it potentially increased the initial transformation rate. The immobilisation of metals in the system indicated that the risk associated with sludge-borne metals can be decreased over time/repeated spring barley growth. Therefore, for better defining the overall risk from sludge application to agricultural soils, regular monitoring of metals in the mobile fractions should be taken account into future regulations.

In addition, the interaction between metals and plants in the system is also crucial for assessing the risk as it can control metals availability to plants. The rhizo-pot experiment indicated spring barley can only absorb Cu from the immediate vicinity of the roots in the digested sludge-amended soil (sub-mm scale), but Zn can be taken up from further afield (mm scale). The soil heterogeneity in terms of distribution and chemical forms of soil metals inevitably increases with plants growth process at the level of the rhizosphere or the rooting zone. In future regulations the spatial limitation of metals uptake should be considered because soil metals are positionally unavailable to plants. Such soil heterogeneity should also be considered when assessing the risk posed by the metals in sludge-soil-plant systems (Yanai, et al., 2004). Also, in this study spring barley exhibited different responses to excessive Cu and Zn in the system. At both moderate- and heavy- metal levels (~1000 mg/kg and ~5000 mg/kg respectively), spring barley showed a stronger absorption capacity for Zn than Cu, which can be related to the significantly higher translocation from roots to shoots for Zn. Unlike Zn (which was almost equally distributed in plant roots and shoots), Cu was highly concentrated in the roots (mainly in the epidermis and/or cortex structure), with very small amount transferred to aboveground parts. Also, a large proportion of Cu(I)-O containing phase was found to be distributed in the root structures. Based on these findings, it suggests that for the agricultural soil with single Cu contamination from sludge use, spring barley may be a suitable plant for use because 1) excessive Cu levels (at least ~1000 mg/kg) do not threaten spring barley growth, 2) Cu is mostly sequestered in spring barley roots, 3) Cu mobility has the tendency to decrease over spring barley growth and 4) soil fertility can be beneficially improved with sludge application. Logically, single metal contamination may occur in a real system as each metal concentration in sewage sludge differs, potentially causing one metal to reach regulated limits first while others remain below. In such a scenario, the use of suitable plant species can maximize the benefits of sludge application to soils while minimizing the risk posed by sludge-introduced metals. Future studies can focus more on the impacts of different plant species on the transformation of metal behaviour and fate in sludge-soil-plant systems to identify suitable plant species for metal-contaminated agricultural soils due to sludge application. In turn, the understanding of interactions between contaminant metals and different plant species in sludge-amended soils can also improve the risk assessments associated with sludge use in agriculture.

7.5 Limitations

Cu and Zn are the metals of interests in this project due to 1) their abundance in SS, 2) their role as essential micronutrients for plants growth and 3) concerns about their environmental risk (Juknys et al., 2012; Luo and Christie, 1998; Medas et al., 2019; Shahid et al., 2014). In addition to Cu and Zn, SS also contains other common metals such as Cr, Cd, Pb and Ni. Their transformation behaviours are not investigated in this project. Out of them, Cd is generally the least abundant metal in SS, with concentrations typically below 5 mg/kg. As a result, its accumulation in the soil is relatively slower than other metals. The concentration of Pb is slightly higher than Cd and sometimes it is a concern when it accumulates in the soil (Liang et al., 2011; Sivapatham et al., 2014; Zhao et al., 2016). However, Pb in SS is predominantly distributed in between oxidizable and residual fractions, showing a relatively low bioavailability. Also, Pb is not the essential micronutrient for plants (He et al., 2020). Like Pb, Cr also shows a low mobility in SS and its concentration is relatively low. Ni in SS is typically distributed in between exchangeable, reducible, oxidizable, and residual fractions, showing a high mobility like Zn. However, its concentration is significantly lower than Zn. Based on this general context, this project primarily investigated the transformation of representative metals, Cu and Zn, in the sludge-soil /sludge-soil-plant system. In future studies, there is a need for investigating the transformation behaviour of other contaminant metals in the system to better assess the risk posed by sludge-borne metals.

7.6 References

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Chapter 8: Conclusions and recommendations for future work

8.1 Summary

Annually, a huge amount of sewage sludge can be produced globally, so its safe disposal is an enormous problem for many countries (Guo et al., 2024; Saha et al., 2017; Wang, 2021; Yang et al., 2020). The problem can be reduced if it is applied to agricultural land (Feng et al., 2023; Iticescu et al., 2021). Meanwhile, the agricultural use of sewage sludge has numerous benefits to humankind as (1) it can return the organic matter and plant nutrients enriched in sewage sludge to agriculture (Basopo et al., 2022; Saffari et al., 2020); (2) the agricultural soil fertility can be significantly improved, particularly for barren soils with significantly depleted organic matter (Alvarez et al., 2000; Namdari et al., 2024) and (3) the dependce on mineral fertilisers can be decreased (Walter et al., 2006). Therefore, sewage sludge use in agriculture is considered a way of sludge disposal that aligns with the principles of circular economy and eco-friendliness (Ye et al., 2022). However, due to the presence of contaminant metals in sewage sludge, it must be carefully managed during its agricultural application.

To control the risk posed by sludge-introduced metals and safely manage sludge use, there is a need for understanding the transformation behaviour of metals in the sludge-soil/sludge-soil-plant system (including rhizosphere and non-rhizosphere). The knowledge is crucial in establishing appropriate safe thresholds for metal concentrations in the sludge that will be applied to agricultural soils, refining the safe limits for metal accumulation in the agricultural soil and developing a more reasonable scheme for sludge application in agricultural land. Based on this general context, four important research questions were proposed in this project. To address these questions, a comprehensive literature review alongside a series of pot trials were conducted. The key findings from this project are as follows.

- Based on the most recently published data, it is definitively estimated that the annual global production of sewage sludge may rise from ~53 million tons dry solids currently to ~160 million tons if global wastewater were to be treated to a similar level as in the EU-27/UK.

- Industrial wastewater, domestic wastewater and urban runoff are three main sources of contaminant metals present in sewage sludge. The conventional treatment processes generally result in the partitioning of over 70% of contaminant metals from wastewater into primary and secondary sludges. Typically, the order of metal concentrations in sewage sludge is Zn > Cu > Cr ≈ Pb ≈ Ni > Cd. In sewage sludge, the proportion of these contaminant metals that are easily mobilised is highest for Zn and Ni, followed by Cd and Cu, then Pb and Cr.

- Substantial proportions of Cu and Zn can become incorporated into the digested sludge matrix when the sludge is amended with the soluble Cu and Zn salts. The sorption surfaces of original digested sludge are close to saturation limits when the added ratio of Cu and Zn to original digested sludge is within 2% ($m_{\text{metals}}/m_{\text{dry sludge solids}}$). Within this ratio, on average, more than 90% of the Cu and 85% of the Zn can be absorbed to the sludge matrix. A significant reduction in the sorption ratio of Zn and Cu occurs when the added ratio is 5% (particularly Zn).

- Both sequential extractions and X-ray absorption spectroscopy data reveal that the Cu speciation in the amended sludge (at the ratio of 2%) is broadly similar to that seen in the original digested sludge. There are bigger differences in the Zn speciation between the amended sludge and original digested sludge. However, the same two bonding environments (Zn(II)-S and inner sphere Zn(II)-O phases) for Zn are detected in both materials.

- Cu and Zn are present in the anaerobic digested sludge primarily as metal sulphide phases formed during anaerobic digestion. These phases are relatively reactive, easily remobilised and more bioavailable than the agricultural soil associated metals. The contaminant metals added to the agricultural soil with the amended sludge are rapidly transformed and redistributed to the soil matrix but remain more easily mobilised (at least in the short-term).

- Over time, Zn and Cu become progressively less mobile, showing a tendency to return to a fractional distribution pattern of metals similar to that seen in the original agricultural soil. Repeated spring barley growth potentially increases the initial transformation rates of metals but does not affect the transformation direction of metals in the sludge-amended soil. Overall, while sludge application to land will probably increase the overall metal concentrations, metal bioavailability tends to reduce over time. Therefore, safety assessments for sludge application in agriculture should be based on both metal concentrations present and their specific binding strength within the system.

- Zn introduced with digested sludge is mobile on a mm scale, whereas Cu introduced with digested sludge is only mobile on a sub-mm scale. As a result, young spring barley plants took-up 4x more Zn than Cu even though these metals were present at similar concentrations in the sludge-amended soil. Zn that is taken-up by spring barley is relatively evenly distributed through the roots, with similar concentrations in the plant shoots, whereas most Cu that is taken-up is sequestered in the roots (particularly the root cortex), with only a small proportion translocated to above ground plant structures.

- The speciation of Cu and Zn close to plant roots (*i.e.* in the rhizosphere soil) is different from that found more widely in sludge-amended soil. Local to the plant roots, Zn and Cu are in chemical environments that suggest their mobility has been reduced by presence of plant roots.

- Understanding changes in metal speciation during plant uptake is important in determining the relative risks of different metals (*i.e.* Cu is hazardous at lower concentrations than Zn, but Cu is trapped in the rhizosphere whereas Zn is translocated to plant shoots). Better understanding of the mobility and uptake of metals that have elevated concentration in sewage sludge could be used to undertake element specific risk assessments for sludge use in agriculture.

8.2 Recommendations

To make sewage sludge more sustainable and safer for agricultural application, more studies are required to understand the transformation behaviour of contaminant metals in the sludge-soil/sludge-soil-plant system. This project primarily investigated the isotherm sorption behaviour of original digested sludge for Cu and Zn, the evolution pattern of Cu and Zn speciation in the digested sludge-amended soil over time and with repeated plant growth, the effect of root zone on Cu and Zn uptake, and the *in-situ* spatial distribution and speciation patterns of Cu and Zn in close proximity to spring barley roots in the digested sludge-amended soil. The accumulation characteristics of Cu and Zn in the spring barley roots and shoots and the potential mechanisms underlying these characteristics were also elucidated. These important findings can inform better risk assessments associated with the sludge-introduced metals and provide guidance for updating current regulations on the use of sewage sludge in agriculture. Due to time constraints, some other interesting aspects are not explored in this project, but they are also crucial for improving the sustainable application of sewage sludge in agricultural land. Herein, the following research needs are recommended for future studies.

8.2.1 Impacts of long-term repeated sludge application

This project used the metal-amended sludge in the soil amendment experiments. In Chapter 5, this amended sludge was used to produce the sludge-amended soil containing about 1000 mg/kg Cu and Zn (this sludge-amended soil was also used in the plug-tray experiment in Chapter 6). As a result, the transformations of Cu and Zn speciation in the sludge-soil/sludge-soil-plant system can be experimentally monitored by complementary chemical extractions and XAS techniques. The use of this amended sludge ensured that the changes in metal behaviour and fate in the system were principally due to the changes in the behaviour and fate of sludge-associated metals. Also, it should be noted that in the agricultural use of sewage sludge, the increase in bulk soil metal concentrations can be related to the gradual accumulation of metals from long-term repeated sludge application. In that scenario, each single sludge application can alter soil properties and environmental conditions, affecting the redistribution of metals within the system. As a result, in that scenario metals may behave differently compared to the scenario established in this project. Currently, less attention has been paid to the transformation behaviour of contaminant metals when they accumulate in the soil via long-term repeated application. This can be attributable to the need of a very long time for establishing such a scenario. However, considering its practical significance, more studies are required to understand the metal behaviour and fate in that scenario. It can be expected that based on the results from the impacts of long-term repeated sludge application on metal behaviour (such as metal accumulation rates in plants, and transformation rates of bioavailable metals into more stable phases during repeated sludge application), a more reasonable scheme can be developed for applying sewage sludge to the agricultural soil. The knowledge established from this project (such as watering and lighting schemes, root system washing procedures) can contribute to the study of long-term sludge application. It will then enable a comparison between the two scenarios, further enhancing the understanding of metal behaviour in the system.

8.2.2 Plant uptake studies

In this project, spring barley (*Hordeum vulgare*) was selected for investigating the impacts of its repeated growth on the transformation of metals behaviour and fate in the sludge-soil-plant system. The results revealed that spring barley can be tolerant for excessive metals in the system (at least 1000-5000 mg/kg Cu and Zn). It keeps a very low translocation from roots to shoots for Cu to prevent its entry into the edible parts (“excluder” mechanism). Also, in the sludge-amended soil the metal bioavailability tends to decrease over repeated spring barley growth. Considering the high retention capacity of Cu in the spring barley roots and its ability to decrease Cu mobility, it probably suggests that spring barley is an appropriate plant for use in the Cu-contaminated soil due to sludge use.

It should be stressed that the results of spring barley may be not universally applicable for other plant species as the response of different plant species to metals varies. Specifically, (1) there are variabilities in metals absorption capabilities among different plant species (Rashid et al., 2023); (2) the relationship between the metal bioavailability and the amounts of metals taken-up by different plants can also be different (Feng et al., 2023); (3) the survival ability of different plant species varies within different soil metal levels (the sensitivity of plants to higher metal levels can lead to a slow plant growth and this can further affect the transformation behaviour of metals) (Kafle et al., 2022). Therefore, more plant uptake studies are needed including the investigation on the impacts of different plant species on the changes of metal behaviour and fate in the sludge-soil-plant system and the determination of relationship between metals bioavailability and the amounts of metal uptake by different plants. The obtained information will be valuable for screening out the appropriate plant for use in the agricultural soil affected by specific metals from sludge use to maximize the contribution of plants.

8.2.3 Large-scale field experiments

Most studies on metal behaviour and fate in the sludge-soil/sludge-soil-plant system are based on the laboratory-scale experiments within a small-time frame (Kafle et al., 2022). In laboratory-scale experiments, the impacts of important individual factor on the transformation of metals can be explored benefiting from controlling other variables (Humphries, 2020). However, the results yield from laboratory-scale experiments may be not fully representative of the metal behaviour under field conditions. In the large-scale field experiments, a wide range of variables can be present, which may have a combined effect on the metal behaviour in the systems. So far, in the sludge-soil-plant system, the impacts of repeated plant growth on the transformation of metals have not yet been systematically investigated by the large-scale field research. To figure out the true potential of a specific plant growth on the influence of metal behaviours in the system, there is a need for conducting more long-term field experiments across a wide range of climatic and agricultural contexts (Allen et al., 2023). The results from the large-scale filed experiments will be more representative of the real-world conditions and further increase the environmental sustainability and agricultural applicability of sewage sludge. It will also allow a comparison with the results from laboratory-scale experiments, enhancing a better understanding of the transformation behaviour of metals in the complex system.

8.2.4 More targeted regulation of sludge application to agricultural land

In current regulations, it is required to monitor the total metal concentrations to control the risk posed by sludge-introduced metals (DEFRA, 2018; Duan et al., 2017; EPA, 2022). This control is sufficient to prevent secondary metal contamination resulted from sludge use. However, it is poorly indicative of the risk assessments as the changes in the risk associated with the metals does not necessarily result from the changes in total soil metal concentrations (Burges et al., 2018; Sánchez-Martín et al., 2007; Wang et al., 2006). In this project, the experimental data showed that the fraction of easily bioavailable Cu and Zn decreased over contact time, showing a decrease in the risk associated with the sludge-introduced metals. However, this decrease in the risk cannot be reflected based on total metal concentrations as there were no changes in total metal concentrations in the control series. For future safety assessments of sludge application in agriculture, metal bioavailability should be considered, as it significantly represents the labile fraction that is readily available for plant uptake. Also, this project demonstrated that the plant may have high retention capacity and detoxification ability for contaminant metals in the system. Thus, in future regulations, the specific behaviour of contaminant metals and their translocation differences within the system should also be incorporated for a better sludge reuse, and less conservative restrictions on sludge application, as well as cost-effective recovery of valuable components from sewage sludge (summary of these research recommendations outlined above is shown in Table 8.1).

Table 8.1 Summary of future research priorities for improved agricultural application of sewage sludge.

Research recommendations	Research outcome / Impact
Impacts of long-term repeated sludge application - Understand the transformation of metal behaviour and fate due to repeatedly long-term sludge application.	- Develop schemes for applying sewage sludge to agricultural soil determined according to metal accumulation rates, and rate of transformation of bioavailable metals into more stable phases.
Plant uptake studies - Understand the influence of different types of plants on metal behaviour and fate in the sludge-amended soil. - Determine the relationship between metals bioavailability and the amount of metals of uptake by different plants.	- Screen out groups of plants to reduce metal uptake to plants during growth.
Large-scale field experiments - Understand the impacts of repeated plant growth on the transformation of metal behaviour and fate in a real system.	- Allow a comparison with laboratory-scale experiment results. - Enhance a better understanding of transformations of metal behaviour and fate in sludge-soil-plant system.
More targeted regulation of sludge application to agricultural land - Critical multi-scale analyses of factors (such as soil properties, environmental conditions) determining how land management decisions determine the long-term fate of contaminant metals in sewage sludge. - Incorporation of chemical forms, specific behaviour and translocation difference of contaminant metals into regulations on sewage sludge use in agriculture.	- Better reuse of sewage sludge in agriculture. - Less conservative restrictions on sludge application. - Cost effective recovery of valuable components of sewage sludge.

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Appendix A: Appendices for Chapter 4

Table A.1 Total Zn and Cu concentrations in a certified reference material (by aqua regia digestion) in full.

Sample		Metal concentration in samples (mg/kg)		Average metal concentration in samples (mg/kg)		Standard deviation	
		Cu	Zn	Cu	Zn	Cu	Zn
Certified material (lake sediment)	1	36	207	36	208	0	5
	2	36	213				
	3	35	205				

Table A.2 Characteristics of original digested sludge and agricultural soil in full.

Parameters	Number	Original digested sludge	Agricultural soil
pH	1	8.0	7.5
	2	7.9	7.5
	3	7.9	7.5
	Average value	7.9	7.5
	Standard deviation	0.0	0.0
Organic matter (%)	1	51.4	6.6
	2	51.3	6.5
	3	50.6	6.7
	Average value	51.1	6.6
	Standard deviation	0.4	0.1
Total Kjeldahl nitrogen (mg/kg)	1	75018	2940
	2	75433	2868
	3	76126	2916
	Average value	75525.7	2908.0
	Standard deviation	559.8	36.7

Continued from Table A.2.

Total phosphorus (mg/kg)	1	30007	696.0
	2	30173	704.0
	3	30450	692.0
	Average value	30210.0	697.3
	Standard deviation	223.8	6.1
Total Cu concentrations (mg/kg)	1	302.1	81.9
	2	300.8	72.2
	3	304.7	78.7
	Average value	302.5	77.6
	Standard deviation	2.0	4.9
Total Zn concentrations (mg/kg)	1	622.4	118.8
	2	612.4	110.9
	3	624.9	110.8
	Average value	619.9	113.5
	Standard deviation	6.6	4.6

Table A.3 Total Cu concentrations in original digested sludge and amended sludges (by aqua regia digestion) in full.

Samples		Cu concentrations (mg/kg)	Average Cu concentration (mg/kg)	Standard deviation
AS0	1	302	303	2
	2	301		
	3	305		
AS1	1	4072	4090	16
	2	4101		
	3	4097		
AS2	1	7763	7778	18
	2	7773		
	3	7798		
AS3	1	10524	10465	53
	2	10423		
	3	10447		
AS4	1	18440	18520	202
	2	18371		
	3	18751		
AS5	1	40577	40731	176
	2	40693		
	3	40922		
AS6	1	63326	62959	490
	2	62403		
	3	63148		

AS0: original digested sludge; AS1-AS6: original digested sludge amended with metals at a ratio of 0.4%, 0.8%, 1%, 2%, 5% and 8% respectively.

Table A.4 Total Zn concentrations in original digested sludge and amended sludges (by aqua regia digestion) in full.

Samples		Zn concentrations (mg/kg)	Average Zn concentration (mg/kg)	Standard deviation
AS0	1	622	620	7
	2	612		
	3	625		
AS1	1	4165	4140	30
	2	4149		
	3	4106		
AS2	1	7954	8012	56
	2	8016		
	3	8066		
AS3	1	8894	8988	89
	2	9072		
	3	8997		
AS4	1	17704	17694	129
	2	17816		
	3	17559		
AS5	1	28659	28919	280
	2	29215		
	3	28883		
AS6	1	21789	22060	270
	2	22330		
	3	22063		

AS0: original digested sludge; AS1-AS6: original digested sludge spiked with metals at a ratio of 0.4%, 0.8%, 1%, 2%, 5% and 8% respectively.

Table A.5 Cu concentrations of each fraction in original digested sludge and amended sludges (by BCR extraction) in full.

Samples		Cu concentration in each fraction (mg/kg)				Sum of four fractions (mg/kg)
		CH_3COOH extract	$NH_2OH·HCl$ extract	H_2O_2 extract	Aqua regia extract	
AS0	1	7	15	244	56	322
	2	5	9	242	44	300
	3	5	9	240	51	305
	Average value	6	11	242	50	309
	Standard deviation	1	3	2	6	11
AS1	1	86	408	2966	534	3994
	2	82	390	2912	586	3970
	3	90	386	2926	624	4026
	Average value	86	395	2935	581	3997
	Standard deviation	4	12	28	45	28
AS2	1	192	546	5662	1104	7504
	2	196	544	5524	1164	7428
	3	188	534	5700	1126	7548
	Average value	192	541	5629	1131	7493
	Standard deviation	4	6	93	30	61

Continued from Table A.5.

AS3	1	475	875	7751	1986	11087
	2	459	868	7801	1772	10900
	3	508	871	7811	1775	10965
	Average value	480	871	7788	1844	10983
	Standard deviation	25	3	32	123	94
AS4	1	2709	2479	11319	3202	19709
	2	2781	2375	11707	2571	19434
	3	2680	2340	12072	2298	19390
	Average value	2724	2398	11699	2690	19511
	Standard deviation	52	72	377	464	173
AS5	1	17687	6245	15199	3844	42975
	2	17964	5977	14212	3659	41812
	3	17455	6023	14757	2667	40902
	Average value	17702	6082	14723	3390	41896
	Standard deviation	255	143	494	633	1039
AS6	1	37950	7400	15702	1104	62156
	2	37630	7150	15642	1084	61506
	3	37210	7130	15712	1114	61166
	Average value	37597	7227	15685	1101	61609
	Standard deviation	371	150	38	15	503

AS0: original digested sludge; AS1-AS6: original digested sludge amended with metals at a ratio of 0.4%, 0.8%, 1%, 2%, 5% and 8% respectively.

Table A.6 Zn concentrations of each fraction in original digested sludge and amended sludges (by BCR extraction) in full.

Samples		Zn concentration in each fraction (mg/kg)				Sum of four fractions (mg/kg)
		CH_3COOH extract	$NH_2OH{\cdot}HCl$ extract	H_2O_2 extract	Aqua regia extract	
AS0	1	77	193	233	124	627
	2	76	195	232	126	629
	3	76	198	229	126	629
	Average value	76	195	231	125	628
	Standard deviation	0	3	2	1	2
AS1	1	2444	1126	322	188	4080
	2	2510	1080	318	192	4100
	3	2496	1068	304	190	4058
	Average value	2483	1091	315	190	4079
	Standard deviation	35	31	9	2	21
AS2	1	5610	1754	354	200	7918
	2	5572	1766	330	198	7866
	3	5570	1788	376	210	7944
	Average value	5584	1769	353	203	7909
	Standard deviation	23	17	23	6	40

Continued from Table A.6.

AS3	1	5879	2121	427	397	8824
	2	6040	2088	407	318	8853
	3	6444	1908	344	253	8949
	Average value	6121	2039	392	323	8875
	Standard deviation	291	115	43	72	63
AS4	1	14063	2505	307	433	17308
	2	14219	2704	319	291	17533
	3	14592	2820	436	374	18222
	Average value	14291	2676	354	366	17687
	Standard deviation	272	159	71	71	476
AS5	1	25883	2637	220	298	29038
	2	25912	2256	207	224	28599
	3	25395	2499	248	364	28506
	Average value	25730	2464	225	295	28714
	Standard deviation	291	193	21	70	285
AS6	1	19720	1470	188	80	21458
	2	19540	1320	172	84	21116
	3	19350	1310	184	88	20932
	Average value	19537	1367	181	84	21169
	Standard deviation	185	90	8	4	267

AS0: original digested sludge; AS1-AS6: original digested sludge amended with metals at a ratio of 0.4%, 0.8%, 1%, 2%, 5% and 8% respectively.

Appendix B: Appendices for Chapter 5

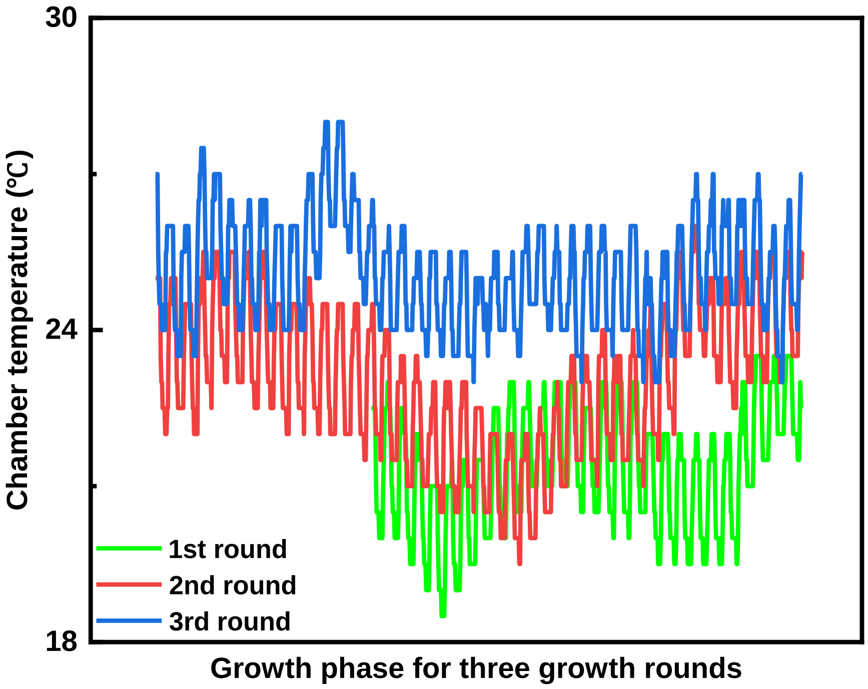

Figure B.1 Chamber temperature data for the three growth rounds in the pot trial 1.

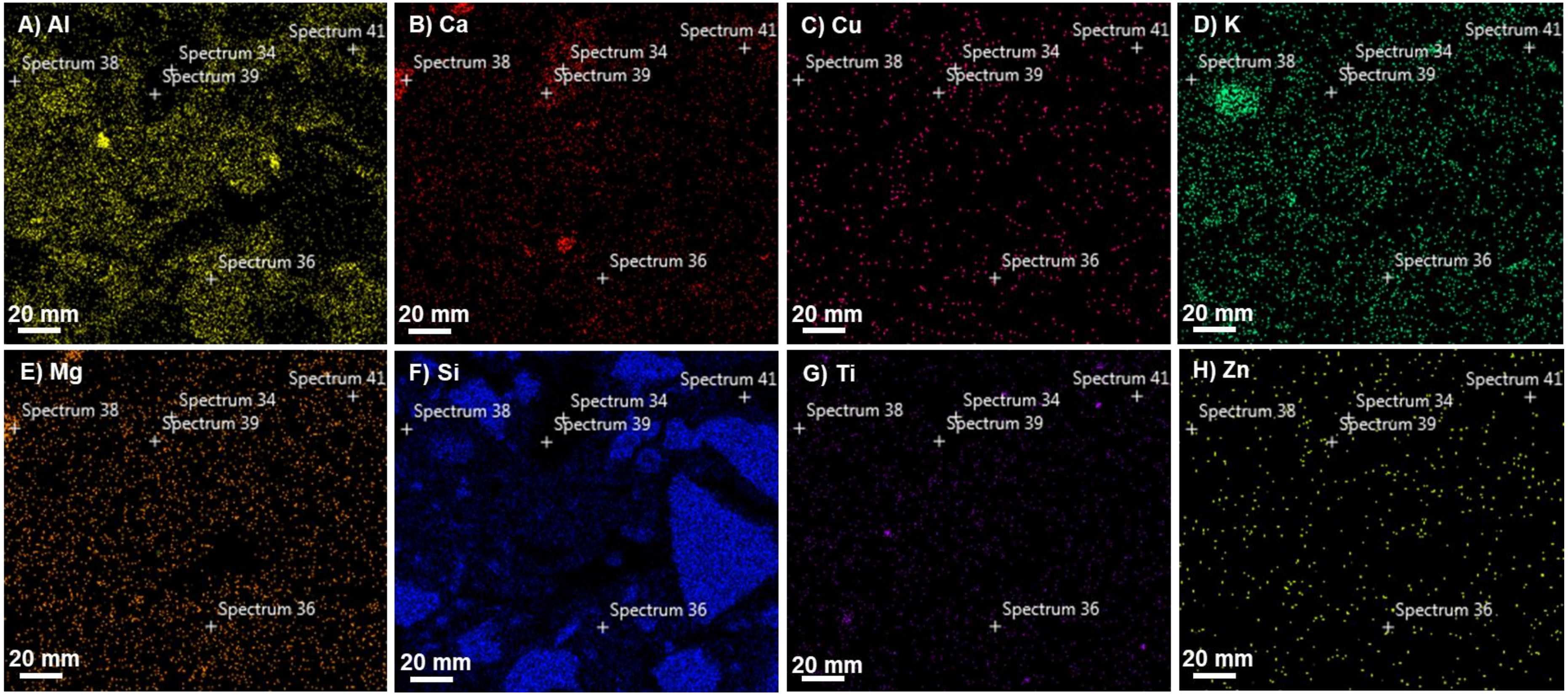

Figure B.2 Elemental distribution in the amended soil 21 days after mixing by SEM-EDS.

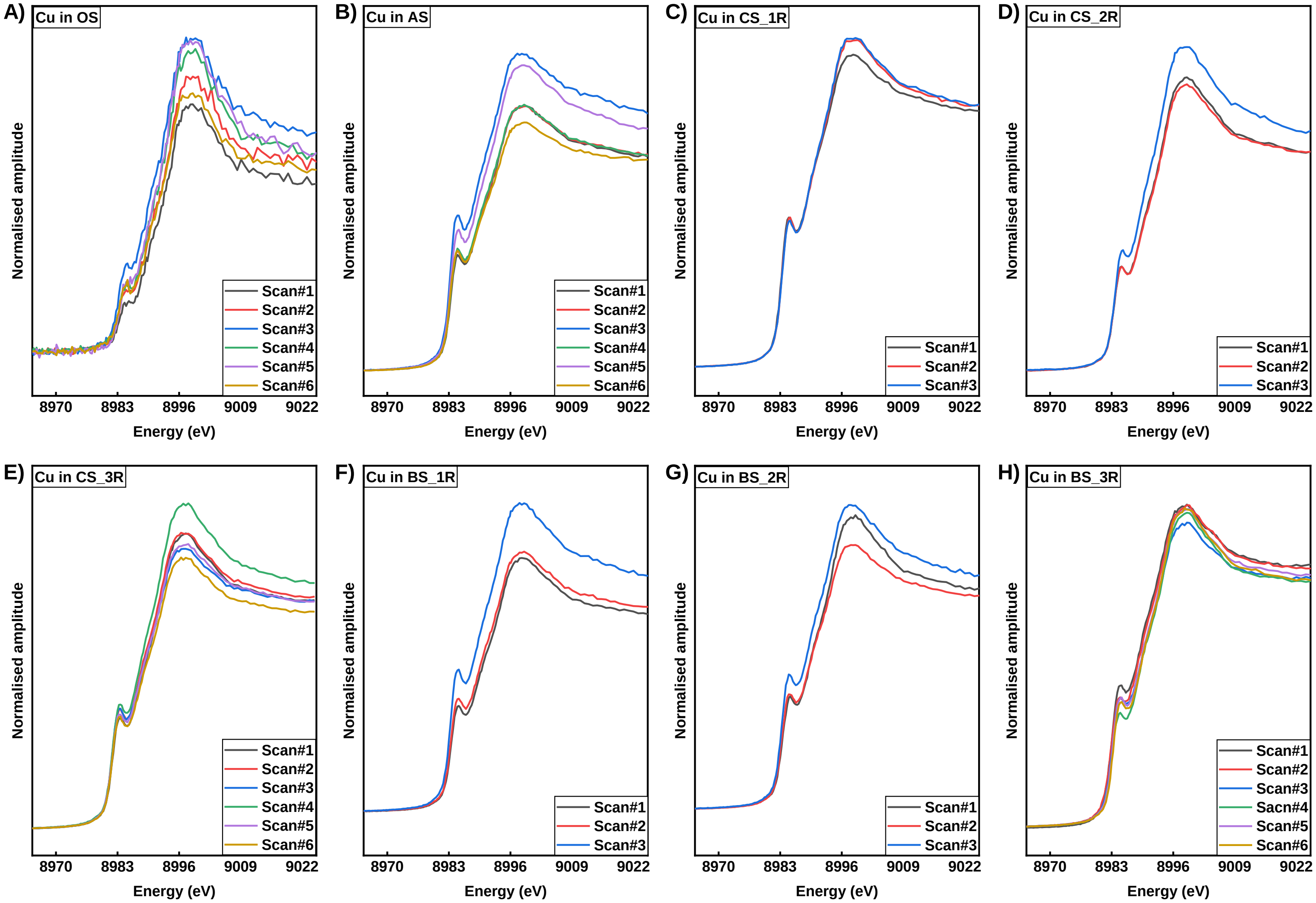

Figure B.3 Sequential scans of Cu bulk XANES spectra from different samples. OS, original soil; AS, amended soil; BS/CS_1R/2R/3R, bulk/control soil from the first/second/third round. Note: sequential scans of Cu bulk XANES spectra in the original digested sludge and the amended sludge have been shown in Figure 4.4.

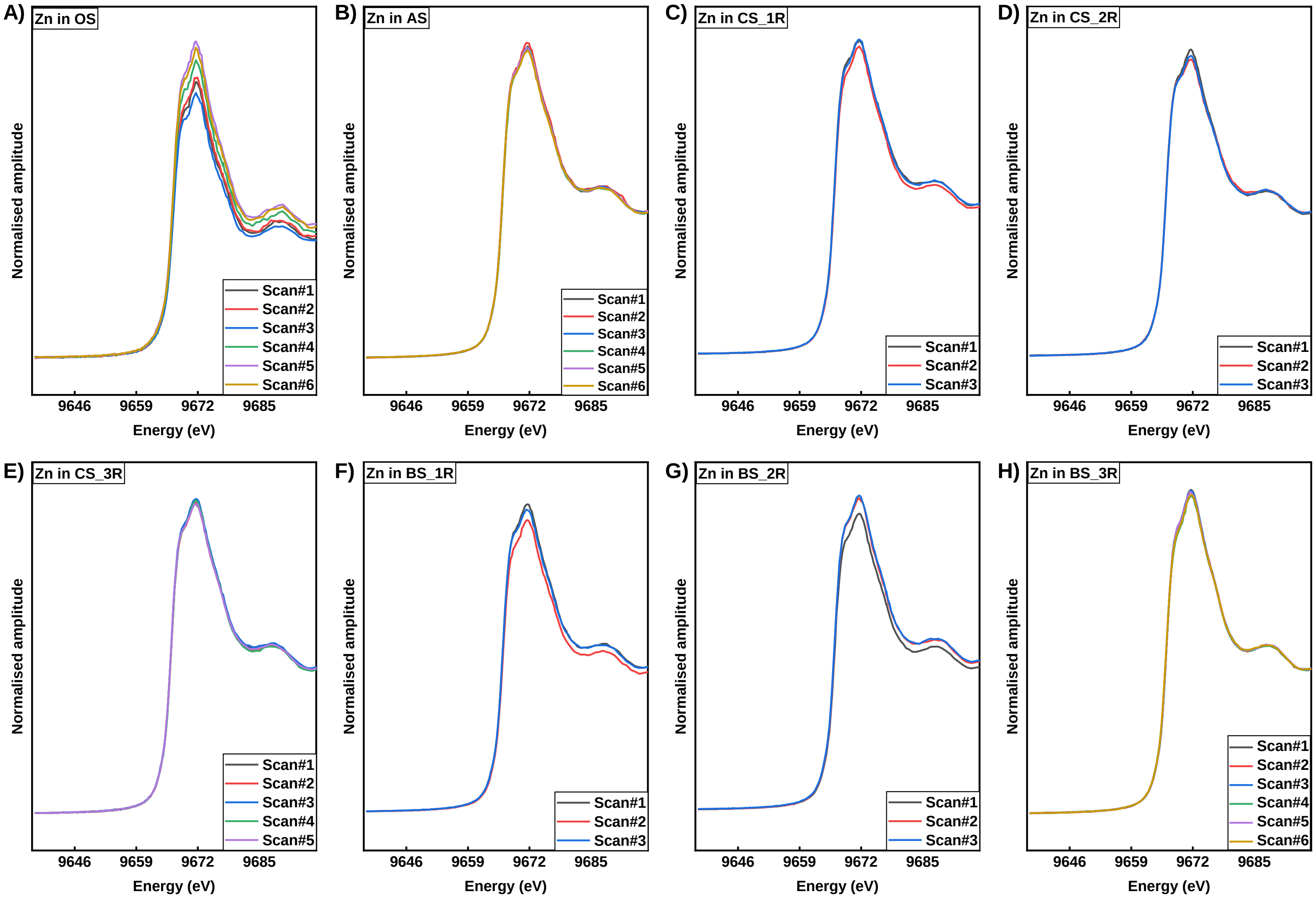

Figure B.4 Sequential scans of Zn bulk XANES spectra from different samples. OS, original soil; AS, amended soil; BS/CS_1R/2R/3R, bulk/control soil from the first/second/third round. Note: sequential scans of Zn bulk XANES spectra in the original digested sludge and the amended sludge have been shown in Figure 4.4.

Table B.1 Cu concentrations in spring barley roots and shoots in the pot trial 1 in full.

Growth round	Spring barley	Sample	Cu concentrations (mg/kg)	Average Cu concentration (mg/kg)	Standard deviation
1st round	Roots	1	2066	2063	4
		2	2063		
		3	2060		
	Shoots	1	73	72	1
		2	71		
		3	73		
2nd round	Roots	1	3727	3675	45
		2	3649		
		3	3650		
	Shoots	1	113	107	5
		2	104		
		3	104		
3rd round	Roots	1	2136	2121	13
		2	2113		
		3	2114		
	Shoots	1	83	78	4
		2	76		
		3	75		

Table B.2 Zn concentrations in spring barley roots and shoots in the pot trial 1 in full.

Growth round	Spring barley	Sample	Zn concentrations (mg/kg)	Average Zn concentration (mg/kg)	Standard deviation
1st round	Roots	1	786	788	4
		2	788		
		3	791		
	Shoots	1	452	437	13
		2	433		
		3	426		
2nd round	Roots	1	1830	1803	25
		2	1782		
		3	1797		
	Shoots	1	1362	1381	25
		2	1410		
		3	1372		
3rd round	Roots	1	1118	1106	11
		2	1103		
		3	1096		
	Shoots	1	813	818	5
		2	818		
		3	823		

Table B.3 Total Cu concentrations in different samples (by aqua regia digestion) in full.

Samples		Cu concentrations (mg/kg)	Average Cu concentration (mg/kg)	Standard deviation
Original soil	1	82	78	5
	2	72		
	3	79		
Amended sludge	1	18440	18520	202
	2	18371		
	3	18751		
Amended soil	1	1079	1058	23
	2	1041		
	3	1055		
Control_1st round	1	1034	1035	7
	2	1028		
	3	1042		
Control_2nd round	1	1059	1057	5
	2	1052		
	3	1061		
Control_3rd round	1	1046	1067	23
	2	1092		
	3	1064		

Continued from Table B.3.

Bulk soil_1st round	1	1091	1066	34
	2	1030		
	3	1078		
Bulk soil_2nd round	1	1063	1068	6
	2	1075		
	3	1067		
Bulk soil_3rd round	1	1050	1064	19
	2	1085		
	3	1056		
Root soil_2nd round	1	1029	1032	5
	2	1038		
	3	1030		
Root soil_3rd round	1	996	993	3
	2	990		
	3	994		

Table B.4 Total Zn concentrations in different samples (by aqua regia digestion) in full.

Samples		Zn concentrations (mg/kg)	Average Zn concentration (mg/kg)	Standard deviation
Original soil	1	119	113	5
	2	111		
	3	111		
Amended sludge	1	17705	17694	129
	2	17816		
	3	17559		
Amended soil	1	1089	1065	24
	2	1046		
	3	1060		
Control_1st round	1	1025	1034	9
	2	1038		
	3	1040		
Control_2nd round	1	1070	1070	9
	2	1061		
	3	1078		
Control_3rd round	1	1037	1048	12
	2	1060		
	3	1048		

Continued from Table B.4.

Bulk soil_1st round	1	1068	1050	42
	2	1012		
	3	1072		
Bulk soil_2nd round	1	1064	1080	16
	2	1078		
	3	1096		
Bulk soil_3rd round	1	1032	1042	15
	2	1060		
	3	1036		
Root soil_2nd round	1	1130	1144	13
	2	1156		
	3	1144		
Root soil_3rd round	1	1029	1026	2
	2	1025		
	3	1026		

Table B.5 Cu concentration of each fraction in different samples (by BCR extraction) in full.

Samples		Cu concentration in each fraction (mg/kg)				Sum of four fractions (mg/kg)
		CH_3COOH extract	$NH_2OH·HCl$ extract	H_2O_2 extract	Aqua regia extract	
Original soil	1	12	8	14	44	78
	2	10	10	14	41	75
	3	10	10	10	40	70
	Average value	11	9	13	42	74
	Standard deviation	1	1	2	2	4
Amended sludge	1	2709	2479	11319	3202	19709
	2	2781	2375	11707	2571	19434
	3	2680	2340	12072	2298	19390
	Average value	2724	2398	11699	2690	19511
	Standard deviation	52	72	377	464	173
Amended soil	1	383	352	257	62	1054
	2	376	357	288	56	1077
	3	379	359	266	64	1068
	Average value	380	356	270	61	1067
	Standard deviation	4	4	16	4	12

Continued from Table B.5.

	1	343	366	291	69	1069
	2	335	357	283	62	1037
Control_1st round	3	343	345	300	66	1054
	Average value	340	356	291	66	1053
	Standard deviation	4	11	9	3	16
	1	307	355	310	66	1038
	2	313	347	295	66	1021
Control_ 2nd round	3	335	374	297	64	1070
	Average value	318	359	301	65	1043
	Standard deviation	14	14	8	1	24
	1	299	423	281	73	1076
	2	296	429	278	75	1078
Control_3rd round	3	298	417	267	71	1053
	Average value	298	423	275	73	1070
	Standard deviation	1	6	7	2	14
	1	320	418	268	69	1075
	2	292	430	266	67	1055
Bulk_1st round	3	298	422	282	69	1071
	Average value	303	423	272	68	1067
	Standard deviation	15	6	9	1	10

Continued from Table B.5.

Bulk_2nd round	1	268	453	287	70	1078
	2	255	442	279	72	1048
	3	268	431	271	67	1037
	Average value	264	442	279	70	1055
	Standard deviation	7	11	8	2	21
Bulk_3rd round	1	254	414	308	78	1054
	2	260	420	288	82	1050
	3	250	406	300	80	1036
	Average value	255	413	299	80	1047
	Standard deviation	5	7	10	2	9
Root soil_2nd round	1	237	381	321	79	1018
	2	243	379	320	75	1017
	3	239	398	302	75	1014
	Average value	240	386	314	76	1016
	Standard deviation	3	10	11	3	2
Root soil_3rd round	1	245	380	294	82	1001
	2	238	374	296	90	998
	3	236	372	298	92	998
	Average value	240	375	296	88	999
	Standard deviation	5	4	2	5	3

Table B.6 Zn concentration of each fraction in different samples (by BCR extraction) in full.

Samples		Cu concentration in each fraction (mg/kg)				Sum of four fractions (mg/kg)
		CH_3COOH extract	$NH_2OH{\cdot}HCl$ extract	H_2O_2 extract	Aqua regia extract	
Original soil	1	2	2	2	107	113
	2	2	6	2	101	111
	3	0	4	2	105	111
	Average value	1	4	2	105	112
	Standard deviation	1	2	0	3	1
Amended sludge	1	14063	2505	307	433	17308
	2	14219	2704	319	291	17533
	3	14592	2820	436	374	18222
	Average value	14291	2676	354	366	17687
	Standard deviation	272	159	71	71	476
Amended soil	1	728	185	25	90	1028
	2	711	200	33	86	1030
	3	721	193	25	94	1033
	Average value	720	193	28	90	1031
	Standard deviation	9	7	5	4	3

Continued from Table B.6.

Control_1st round	1	687	220	47	93	1047
	2	678	217	47	90	1032
	3	684	221	47	93	1045
	Average value	683	219	47	92	1041
	Standard deviation	4	2	0	2	8
Control_ 2nd round	1	651	244	53	95	1043
	2	647	233	49	95	1024
	3	667	241	51	95	1054
	Average value	655	239	51	95	1040
	Standard deviation	10	5	2	0	15
Control_3rd round	1	603	281	47	97	1028
	2	607	283	46	100	1036
	3	596	269	44	93	1002
	Average value	602	278	46	97	1022
	Standard deviation	5	7	1	3	17
Bulk_1st round	1	653	262	39	105	1059
	2	613	293	39	100	1045
	3	622	289	40	113	1064
	Average value	629	281	39	106	1056
	Standard deviation	21	17	1	7	10

Continued from Table B.6.

Bulk_2nd round	1	571	330	47	109	1057
	2	551	329	47	98	1025
	3	572	307	44	114	1037
	Average value	565	322	46	107	1039
	Standard deviation	12	13	2	8	16
Bulk_3rd round	1	525	294	70	104	993
	2	532	298	66	104	1000
	3	521	284	68	110	983
	Average value	526	292	68	106	992
	Standard deviation	6	7	2	3	9
Root soil_2nd round	1	483	372	107	125	1087
	2	505	367	103	119	1094
	3	507	369	94	107	1077
	Average value	498	370	102	117	1087
	Standard deviation	13	2	7	10	9
Root soil_3rd round	1	485	320	90	108	1003
	2	482	314	88	108	992
	3	476	316	90	108	990
	Average value	481	317	89	108	995
	Standard deviation	5	3	1	0	7

Appendix C: Appendices for Chapter 6

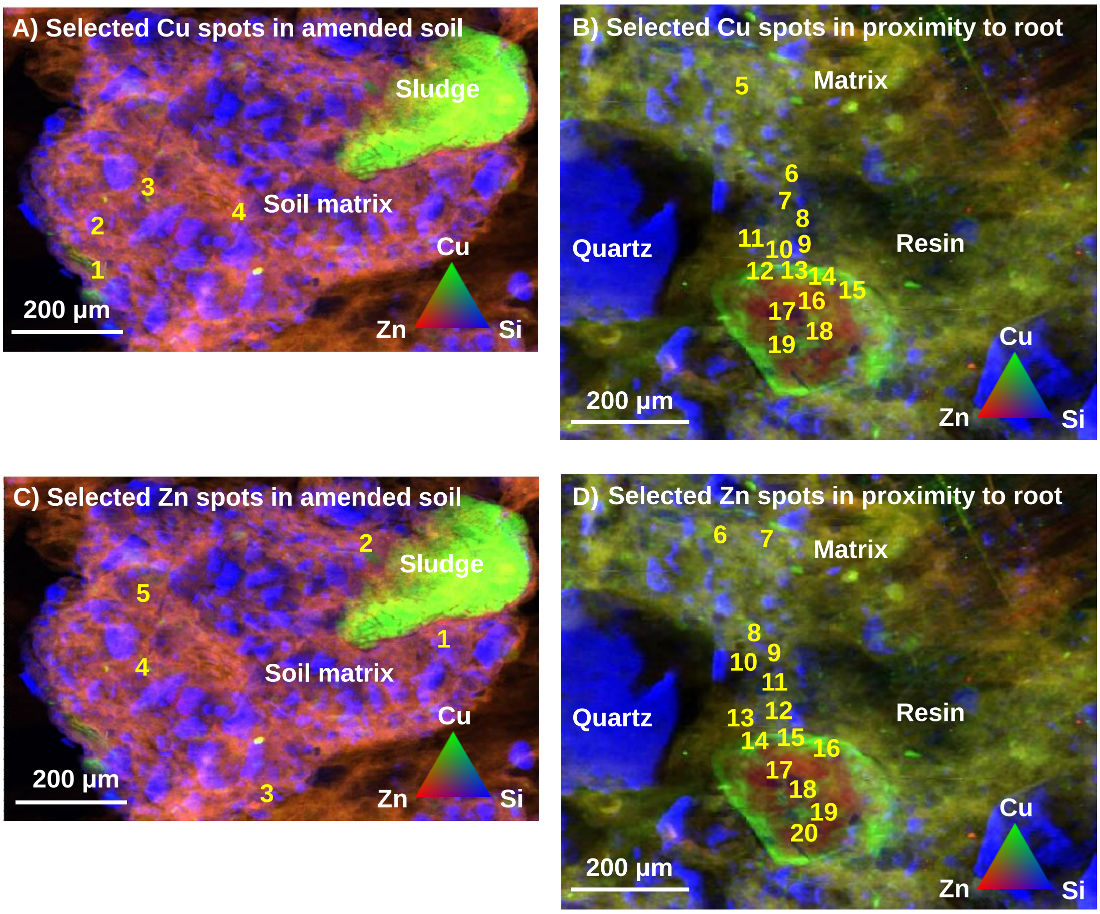

Figure C.1 Selected Cu spots in A) the sludge-amended soil (before the plug-tray growth experiment) and B) close proximity to the plant root shown in Figure 6.5. Selected Zn spots in C) the sludge-amended soil (before the plug-tray growth experiment) and D) close proximity to the plant root shown in Figure 6.5.

Table C.1 Metal concentrations in original digested sludge, original agricultural soil, metal-amended sludge and sludge-amended soil used in the rhizo-pot experiment (mg/kg of dry solids).

Samples	Metal concentrations in samples (mg/kg)	
	Cu	Zn
Original digested sludge	183 ± 2	600 ± 3
Original agricultural soil	78 ± 5	113 ± 5
Metal-amended sludge	20236 ± 18	18351 ± 151
Sludge-amended soil	5184 ± 76	5031 ± 60

Values are expressed as mean ± standard deviation (*n*=3).

Table C.2 Cu concentration in plant roots and shoots (rhizo-pot experiment; by aqua regia digestion) in full.

Growth round	Plant	Sample	Cu concentrations (mg/kg)	Average Cu concentration (mg/kg)	Standard deviation
1st round	Roots	1	617	585	28
		2	573		
		3	565		
	Shoots	1	80	102	24
		2	127		
		3	98		
2nd round	Roots	1	569	566	4
		2	569		
		3	561		
	Shoots	1	46	47	2
		2	46		
		3	49		
3rd round	Roots	1	568	566	10
		2	575		
		3	555		
	Shoots	1	41	42	1
		2	43		
		3	43		

Continued from Table C.2.

4th round	Roots	1	313	321	7
		2	322		
		3	328		
	Shoots	1	46	43	2
		2	43		
		3	42		
5th round	Roots	1	241	242	1
		2	244		
		3	243		
	Shoots	1	72	50	19
		2	38		
		3	41		

Table C.3 Zn concentration in plant roots and shoots (rhizo-pot experiment; by aqua regia digestion) in full.

Growth round	Plant	Sample	Zn concentrations (mg/kg)	Average Zn concentration (mg/kg)	Standard deviation
1st round	Roots	1	393	374	21
		2	377		
		3	352		
	Shoots	1	201	231	29
		2	259		
		3	231		
2nd round	Roots	1	528	525	5
		2	528		
		3	520		
	Shoots	1	511	498	11
		2	493		
		3	491		
3rd round	Roots	1	1364	1360	17
		2	1374		
		3	1340		
	Shoots	1	740	737	4
		2	738		
		3	733		

Continued from Table C.3.

4th round	Roots	1	1395	1410	14
		2	1422		
		3	1413		
	Shoots	1	993	974	17
		2	964		
		3	964		
5th round	Roots	1	744	766	20
		2	769		
		3	783		
	Shoots	1	626	617	9
		2	610		
		3	613		

Table C.4 Cu and Zn concentrations in control soil sublayers (rhizo-pot experiment; by aqua regia digestion) in full.

Growth round	Sublayers (mm)	Samples	Metal concentrations (mg/kg)		Average Cu concentrations (mg/kg)		Standard deviation	
			Cu	Zn	Cu	Zn	Cu	Zn
1st round	0-2	1	4729	4803	4839	4824	150	19
		2	5010	4841				
		3	4777	4827				
	2-4	1	4752	4934	4797	4976	92	54
		2	4736	4958				
		3	4903	5037				
	4-7	1	4976	5005	4900	5041	67	71
		2	4873	5123				
		3	4851	4995				
	7-10	1	4827	4913	4944	5088	103	156
		2	5022	5141				
		3	4983	5212				
	10-15	1	4914	5153	4900	5104	12	90
		2	4892	5000				
		3	4895	5159				
	15-20	1	4795	5010	4725	4798	86	286
		2	4628	4472				
		3	4751	4911				

Continued from Table C.4.

Growth round	Sublayers (mm)	Samples	Metal concentrations (mg/kg)		Average Cu concentration (mg/kg)		Standard deviation	
			Cu	Zn	Cu	Zn	Cu	Zn
3rd round	0-2	1	4975	4789	4892	4754	76	257
		2	4877	4992				
		3	4826	4481				
	2-4	1	5026	4908	4984	4943	109	46
		2	4861	4927				
		3	5066	4996				
	4-7	1	5117	4887	5039	4903	68	163
		2	4994	5073				
		3	5006	4749				
	7-10	1	5041	4740	5097	4867	51	209
		2	5112	5107				
		3	5140	4753				
	10-15	1	5225	5240	5150	5045	66	186
		2	5102	5027				
		3	5122	4870				
	15-20	1	5142	5250	5085	5259	83	70
		2	4990	5193				
		3	5123	5333				

Continued from Table C.4.

Growth rounds	Sublayers (mm)	Samples	Metal concentrations (mg/kg)		Average Cu concentration (mg/kg)		Standard deviation	
			Cu	Zn	Cu	Zn	Cu	Zn
5th round	0-2	1	4653	4829	4731	4667	75	151
		2	4804	4530				
		3	4735	4642				
	2-4	1	4803	4789	4835	4693	130	378
		2	4725	4277				
		3	4978	5014				
	4-7	1	4974	4803	4988	4695	83	389
		2	4913	4264				
		3	5078	5018				
	7-10	1	5061	4679	5106	4842	49	499
		2	5100	4445				
		3	5158	5402				
	10-15	1	5148	4803	5095	4821	46	127
		2	5064	4705				
		3	5073	4957				
	15-20	1	5025	4955	5146	5049	195	215
		2	5042	5295				
		3	5371	4897				

Table C.5 Cu and Zn concentrations in planted soil sublayers (rhizo-pot experiment; by aqua regia digestion) in full.

Growth round	Sublayers (mm)	Samples	Metal concentrations (mg/kg)		Average Cu concentration (mg/kg)		Standard deviation	
			Cu	Zn	Cu	Zn	Cu	Zn
1st round	0-2	1	4675	4575	4705	4579	58	110
		2	4662	4480				
		3	4776	4762				
		4	4655	4572				
		5	4760	4507				
	2-4	1	4783	4817	4751	4834	52	32
		2	4751	4823				
		3	4713	4821				
		4	4818	4891				
		5	4690	4815				
	4-7	1	5068	5034	4913	4987	104	49
		2	4956	4951				
		3	4855	4919				
		4	4798	5019				
		5	4887	5009				

Continued from Table C.5.

Growth round	Sublayers (mm)	Samples	Metal concentrations (mg/kg)		Average Cu concentration (mg/kg)		Standard deviation	
			Cu	Zn	Cu	Zn	Cu	Zn
1st round	7-10	1	4882	5167	4849	5043	58	167
		2	4768	4813				
		3	4891	4951				
		4	4809	5230				
		5	4897	5054				
	10-15	1	4995	5126	4930	5066	104	82
		2	4891	5032				
		3	4893	5082				
		4	5069	5146				
		5	4802	4942				
	15-20	1	4762	4913	4902	4929	87	203
		2	4966	5188				
		3	4880	4933				
		4	4973	4623				
		5	4930	4988				

Continued from Table C.5.

Growth round	Sublayers (mm)	Samples	Metal concentrations (mg/kg)		Average Cu concentration (mg/kg)		Standard deviation	
			Cu	Zn	Cu	Zn	Cu	Zn
3rd round	0-2	1	4692	4106	4708	4085	80	82
		2	4750	4095				
		3	4726	4064				
		4	4580	4193				
		5	4792	3966				
	2-4	1	4822	4495	4861	4563	73	119
		2	4907	4479				
		3	4871	4464				
		4	4759	4644				
		5	4946	4732				
	4-7	1	4955	4803	5000	4926	188	394
		2	4846	4611				
		3	4836	4589				
		4	5072	5101				
		5	5289	5528				

Continued from Table C.5.

Growth round	Sublayers (mm)	Samples	Metal concentrations (mg/kg)		Average Cu concentration (mg/kg)		Standard deviation	
			Cu	Zn	Cu	Zn	Cu	Zn
3rd round	7-10	1	5031	5164	5080	5184	241	264
		2	4768	4777				
		3	5440	5187				
		4	5112	5285				
		5	5048	5506				
	10-15	1	5007	5142	5051	5245	85	138
		2	4967	5164				
		3	5184	5134				
		4	5080	5429				
		5	5015	5358				
	15-20	1	5013	5098	5024	5046	77	74
		2	4968	5058				
		3	5144	5019				
		4	5048	4934				
		5	4949	5120				

Continued from Table C.5.

Growth round	Sublayers (mm)	Samples	Metal concentrations (mg/kg)		Average Cu concentration (mg/kg)		Standard deviation	
			Cu	Zn	Cu	Zn	Cu	Zn
5th round	0-2	1	4526	3836	4654	3635	89	347
		2	4660	3729				
		3	4662	3044				
		4	4643	3642				
		5	4777	3923				
	2-4	1	4870	4309	4815	3970	112	473
		2	4766	3957				
		3	4648	3160				
		4	4855	4164				
		5	4938	4262				
	4-7	1	4980	4447	5012	4605	57	165
		2	4992	4711				
		3	5084	4467				
		4	4946	4567				
		5	5059	4834				

Continued from Table C.5.

Growth round	Sublayers (mm)	Samples	Metal concentrations (mg/kg)		Average Cu concentration (mg/kg)		Standard deviation	
			Cu	Zn	Cu	Zn	Cu	Zn
5th round	7-10	1	5100	4762	5122	5015	82	161
		2	5243	5158				
		3	5014	5022				
		4	5133	4981				
		5	5121	5151				
	10-15	1	5009	4657	5100	5056	184	273
		2	4850	4952				
		3	5265	5082				
		4	5293	5376				
		5	5084	5215				
	15-20	1	5139	5159	5082	5079	49	64
		2	5115	5082				
		3	5019	5104				
		4	5048	5065				
		5	5086	4983				