CRANFIELD UNIVERSITY

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DIAGNOSTIC STRATEGY AND RISK ASSESSMENT FRAMEWORK FOR COMPLEX CHEMICAL MIXTURES

SCHOOL OF WATER, ENERGY AND ENVIRONMENT
ENVIRONMENTAL TECHNOLOGY

PhD
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Supervisor: Prof. Frédéric Coulon
Associate Supervisor: Dr. Pablo Campo Moreno
October 2018

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OCTOBER 2018

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ABSTRACT

Environmental contamination comprises a complex mixture of both organic and inorganic contaminants. Understanding their distribution, behaviour and chemical interactions provides the evidence necessary to make informed decision and implement robust remediation strategies. However most of the current risk assessment frameworks, used to manage land contamination, are based on the total contaminant concentration rather than the concentration likely to pose significant risk, the bioavailable concentration. Further to this, the exposure assessments embedded within the frameworks do not explicitly address the partitioning and bioavailability of chemical mixtures. This inability may contribute to an overestimation of both the eco-toxicological effects of the fractions and their mobility in air and water; leading to an overestimation of health and environmental effects. In turn, this may limit the efficacy of the risk assessment frameworks to inform targeted and proportionate remediation strategies. The aim of this PhD study was to address this gap by delivering an integrated risk assessment framework for sites contaminated with complex chemical mixtures. Specifically, this PhD study investigated the fate and behaviour of complex mixtures of petroleum hydrocarbons, metals and metalloids in soils and its implication for partitioning, bioavailability and risk assessment through a 12 month mesocosms study. Further to this, an integrated approach, where contaminants bioavailability and distribution changes along with a range of microbiological indicators and ecotoxicological bioassays, was used to provide multiple lines of evidence to support the risk characterisation and assess the remediation end-point over a 6 month study. From the empirical data obtained from the two mesocosm studies, two Machine Leaning (ML) approaches have been developed to provide a quick and reliable tool to assess multi-contaminated sites with Visible and Near-Infrared Spectroscopy (Vis-NIRS), and to predict bioavailability and toxicity changes occurring during bioremediation. Overall this PhD study shed light on the behaviour of bioavailability, and toxicity of complex chemical mixtures in soils genuinely contaminated. This was supported through a comprehensive and integrated analytical framework providing the necessary lines of evidence to evaluate the implications for risk assessment and identify the end point remediation. The developed framework can significantly help to identify optimal remediation strategies and contribute to change the over-conservative nature of the current risk assessments.

Keywords: contaminated land, bioavailability, toxicity, bioremediation, machine learning.

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LIST OF ABBREVIATIONS

ALK	Alkanes
ANOVA	Analysis of variance
ASD	Analytical spectral device
BS	British standard
BSPT	Basic solid phase test procedure (Microtox assay®)
CFU	Colony-forming units
CI	Condition index
CISED	Chemometric identification of substrates and elements distribution
CL:AIRE	Contaminated land: applications in real environments
C/N	Carbon nitrogen ratio
CSM	Conceptual site model
CRA	Cumulative risk assessment
DCM	Dichloromethane
EC_{50}	Half maximal effective concentration
EA	Environment agency
EU	European union
GC-MS	Gas chromatography-mass spectrometry
GC-TCD	Gas chromatography with thermal conductivity detector
GC-FID	Gas chromatography with flame-ionisation detector
HCl	Hydrochloric acid
HEX	Hexane
HM	Heavy metals (and metalloids)
HMW	High molecular weight
HPCD	Hydroxypropyl-cyclodextrin and hydroxypropyl-β -cyclodextrin
ICP-MS	Inductively coupled plasma mass spectrometry
ISTD	Internal standard
LOOCV	Leave-one-out-cross-validation
LOI	Loss of ignition
LMW	Low molecular weight
MeOH	Methanol
MDI	Mean decrease in impurity

MIR	Mid-infrared
ML	Machine learning
MLP	Multi-layer perceptron
MMW	Medium molecular weight
NN	Neural network
PAH	Polycyclic aromatic hydrocarbons
PCA	Principal components analysis
PERMANOVA	Permutational multivariate analysis of variance
PHC	Petroleum hydrocarbons compounds comprising aliphatic and aromatic hydrocarbons
pH	“Power of hydrogen” negative log of hydrogen ion concentration
PLFA	Phospholipid fatty acids
RA	Risk assessment
RBLM	Risk based land management
r^2	Coefficient of determination
RF	Random forest
RMSEP	Root mean square error of prediction
RPD	Residual prediction deviation
RPIQ	Ratio of the performance to interquartile distance
RSD	Relative standard deviation
SPE	Solid phase extraction
SGV	UK soil guideline values
SMMR	Self-modelling mixture resolution algorithm
SuRF-UK	UK sustainable remediation forum
TC	Total carbon
TOC	Total organic carbon
TN	Total nitrogen
TSA	Tryptone soya agar
TP	Total phosphorous
UK	United kingdom
USA	United States of America
Vis-NIRS	Visible and near infrared spectroscopy

1. Introduction

The last 40 years of ‘environmental revolution’ in Europe and beyond has helped to establish comprehensive frameworks built around preventing pollution and risk-based management. After various lessons learnt, several countries, namely the United Kingdom (UK), Netherlands, Belgium, the United States of America (USA), and Australia have now a set of mature policy frameworks and successful track records of sustainable integrated remediation strategies (Bardos et al., 2016). The risk-based approach of their contaminated land legislative regimes has further allowed more innovative and cost effective approaches to be applied elsewhere in the world. Nevertheless, tackling the protection and recovery of soils impacted by complex chemical mixtures such as among others, polycyclic aromatic hydrocarbons (PAH), heavy metals (HM) and metalloids remain a key challenge because of its consequences for water resources and land use (Van Liedekerke et al., 2014; Kienzler et al., 2016).

Petroleum hydrocarbons originate from incomplete combustion of organic materials, petroleum-based products, coke or aluminium production, and accidental spills (Abdel-Shafy and Mansour, 2016) while heavy metals usually come from vehicle emission, industrial wastes, and mining activities (Yuan et al., 2014). These contaminants are ubiquitous and persistent in soil (Ivshina et al., 2016). They can negatively impact both human and ecological receptors (Duan et al., 2015). Thus the importance of studying these groups of contaminants is related to their co-occurrence in polluted soils, which challenges the risk evaluation and complicates the achievement of site-specific remediation objectives (Renoux et al., 2013).

Risk assessment (RA) is recognised as a robust process to support decision-making practice for contaminated land and to prevent further damage to the environment and human health (Cipullo et al., 2016). It has been further shown that measuring only the total concentration of contaminants in soil does not give a useful basis for the evaluation of the potential risks to human and the environment. Thus, in the last decade in the UK, and increasingly across the world, the end-point of remedial activity is defined not by the total concentration of the chemicals of concern but by the concentration likely to pose significant risk, the bioavailable concentration (Ortega et al., 2015; Kuppusamy et al., 2017; Cipullo et al., 2018).

Several risk-based frameworks for contaminated soils have been published, under the auspices of national and international regulatory organizations, each reflecting national legislation and a range of expert judgments and socioeconomic issues (Rodrigues et al., 2009). Typically, these frameworks use a tiered assessment approaches. However the limitation of such frameworks, similar to that of exposure assessment methods, is the inability to assess the risk posed by complex chemical mixtures. Unlike single contaminant, the physico-chemical interactions of chemical mixtures are still not fully understood as the additive, synergistic or antagonistic effects of mixtures will often yield bioavailability values that differ from those of individual contaminants (Ramakrishnan et al., 2011; Kienzler et al., 2016). It is also recognised that bioavailability of complex chemical mixtures is strongly influenced by sorption/desorption processes occurring in the soil matrix (Caporale and Violante, 2016; Yu et al., 2018). These processes are controlled by a number of biotic and abiotic factors including; soil characteristics, contaminants physico-chemical properties, co-contaminants interactions, and biological/environmental factors (Wuana et al., 2014).

These oversights may contribute to an overestimation of both the eco-toxicological effects of the fractions and the mobility of contaminants. In turn, this may limit the efficacy of the risk frameworks to inform targeted and proportionate remediation strategies. Thus, understanding the distribution, behaviour, and interactions of complex chemical mixtures is key for providing the evidence necessary to make informed decisions and implement robust remediation strategies.

While bioavailability of chemical mixtures is still poorly understood and rarely incorporated into risk decision making, it represents a significant area of research to be tackled in order to move forward the over-conservative nature of the current risk assessments.

1.1. Aim and objectives

The aim of the PhD research is to deliver a comprehensive and integrated analytical framework for historically contaminated sites where complex chemical mixtures are present. It will yield underpinning science from the areas of environmental fate and behaviour modelling, chemical and risk analysis that will guide new remediation strategies. It will also help to determine if remediation is required and inform planning by defining safe post-remediation contamination levels.

To achieve the research aim, the following specific objectives have been addressed:

- **Objective 1**: To critically review relevant literature to highlight how different mechanisms, partitioning, and bioavailability of chemical mixtures, can affect the risk estimation.
- **Objective 2**: To understand the influence of physico-chemical factors affecting chemical mixtures behaviour including inorganic and organic contaminants
- **Objective 3**: To link bioavailability of complex chemical mixtures to toxicity data informing risk assessment and end-point remediation.
- **Objective 4**: To evaluate the feasibility of Visible and Near-Infrared Spectroscopy (Vis-NIRS) as rapid-measurement tool for chemical mixtures.
- **Objective 5**: To develop machine learning (ML) predictive tools for complex chemical mixtures behaviour and fate.

1.2. Thesis structure and format

The PhD thesis is comprised of seven chapters, of which five have been written as paper format (Figure 1.1). A brief description of each chapter is provided hereinafter:

Chapter 1: This chapter presents a general introduction and provide research context, background, aim and objectives of the research.

Chapter 2: This chapter provides a critical review of the state of the art regarding bioavailability of chemical mixtures; it helped to identify gaps within the literature, to set the research focus, and to structure the research plan. This chapter has been published in Science of the Total Environment.

Chapter 3: A 12-month mesocosms experiment was setup to investigate the effect of physico-chemical factors (pH, moisture, and temperature) and weathering (time) on (i) heavy metals/metalloids fractionation, and (ii) petroleum hydrocarbons degradation in five different soils genuinely contaminated (3 industrial contaminated soils, and 2 rural contaminated soils) ranging from low/medium (HM ≤ 800 mg/kg, PHC ≤ 500 mg/kg) to high (HM ≥ 6200 mg/kg, PHC ≥ 1000 mg/kg) contamination. Total exhaustive extraction of organic compound was performed with dichloromethane: hexane, and pseudo-total element digestion was performed according to the ISO 11047 method with aqua regia. Moreover, non-exhaustive extractions with methanol or hydroxypropyl-β-cyclodextrin (HP-β-CD) solutions (organics), and sequential extraction with weak-acid solutions (inorganics) were applied. The complex environmental datasets, obtained in this study, were used to evaluate metal and organic contaminants persistence, fate, and distribution pattern in soils, through chemometric analysis.

Chapter 4: The use of Visible and near-Infrared Spectroscopy Analysis (Vis-NIRS) coupled with the empirical data obtained in the 12 month experimental setup (Chapter 3) have been evaluated as a potentially better technique for delivering cost-effective and fast analyses to support site investigation and reduce the analytical cost associated with complex-contaminant assessments. Infrared spectroscopy scanning in parallel with chemical extraction of petroleum hydrocarbons (PHC) and heavy metals (HM) were used to assess the performance of random forest (RF) to predict total and bioavailable concentrations changes in soils contaminated with complex chemical mixtures.

Chapter 5: A 6-month laboratory scale study was carried out to assess the effect of biochar and compost amendment on the fate and behaviour of complex chemical mixtures in two genuine contaminated soils collected from former gaswork sites (Soil 1, 450 mg/kg HM/metalloids and 9000 mg/kg PHC, and Soil 2, 500 mg/g HM/metalloids and 2000 mg/kg PHC). The total and bioavailable PHC and HM were monitored throughout 180 days incubation. Additionally, to define the end point of remediation and link the bioavailability to the toxicity changes, a range of biological and ecological indicators including: microbial biomass, total bacteria count, soil respiration, phospholipid fatty acids analysis, seeds germination (mustard, rye grass, and pea), earthworm's lethality, and Microtox® basic solid phase test were assessed to provide complementary evidence of the risk posed by multiple contaminants present in soil.

Chapter 6: Empirical data obtained from the 6-month mesocosm experiment (Chapter 5) were used to assess the ability and performance of two machine learning (ML) models to predict the temporal bioavailability and toxicity changes of PHC, HM and metalloids in contaminated soils amended with compost or biochar. The models included artificial neural network (NN) and random forest (RF). ML models can be a powerful tool to support site-investigation, inform decision making, action plans for remediation, and risk-reduction approaches.

Chapter 7: This chapter provides an overall discussion and summary of the key outputs from each chapter. Further it describes how each chapter contributed to the achievement of the aim of the research and the overall implications of the study. This chapter summarized the novelty of this research, and provides recommendations for further studies.

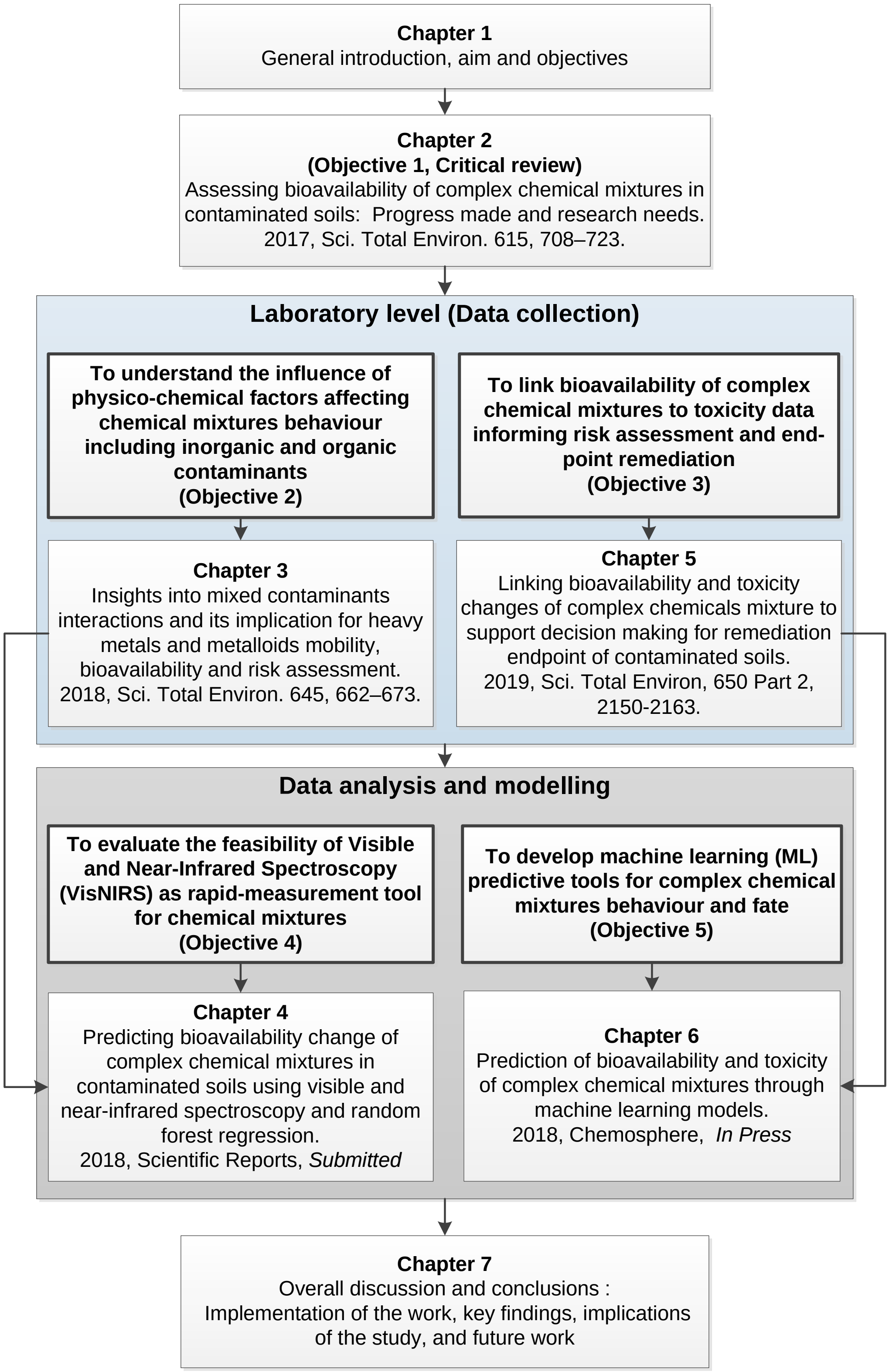

Figure 1.1: Thesis structure and chapters mapped against each objective

1.3. Publications

At the time of writing this thesis, four papers have been accepted for publication in international peer-reviewed journals, and one is currently under revision as listed below. Further to this, one book chapter as lead author and five papers as co-author have been published.

Publications included in the thesis

- **Cipullo, S.**, Prpich, G., Campo, P., Coulon, F., 2018. “Assessing bioavailability of complex chemical mixtures in contaminated soils: Progress made and research needs”. Sci. Total Environ. 615, 708–723. **(Chapter 2)**
- **Cipullo, S.**, Snapir, B., Tardif, S., Campo, P., Prpich, G., Coulon, F., 2018. “Insights into mixed contaminants interactions and its implication for heavy metals and metalloids mobility, bioavailability and risk assessment”. Sci. Total Environ. 645, 662–673. **(Chapter 3)**
- **Cipullo, S.**, Nawar, S., K., Mouazen, A.M., Campo, P., Coulon, F., 2018. “Feasibility of Visible and Near-Infrared Spectroscopy and random forest for predicting complex chemical mixtures bioavailability in multi-contaminated soils”. Scientific Reports, *Submitted, under revision* **(Chapter 4)**
- **Cipullo, S.**, Negrin, I., Claveau, L., Snapir, B., Tardif, S., Pulleyblank, C., Campo, P., Prpich, G., Coulon, F., 2018. “Linking bioavailability and toxicity changes of complex chemicals mixture to support decision making for remediation endpoint of contaminated soils”. Sci. Total Environ, 650 Part 2, 2150-2163 **(Chapter 5)**
- **Cipullo, S.**, Snapir, B., Prpich, G., Campo, P., Coulon, F., 2018. “Prediction of bioavailability and toxicity of complex chemical mixtures through machine learning models”. Chemosphere, *in press* **(Chapter 6)**.

Other publications

- **Cipullo S.,** Brassington K.J., Pollard S.J.T., Coulon F. 2016. Weathered hydrocarbons biotransformation: implications for bioremediation, analysis and risk assessment. Chapter 5. In: Steffan R. (eds) Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation. Handbook of Hydrocarbon and Lipid Microbiology. Springer, Cham, 18 pp, https://doi.org/10.1007/978-3-319-44535-9_4-1
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2. Assessing bioavailability of complex chemical mixtures in contaminated soils: Progress made and research needs

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Abstract: Understanding the distribution, behaviour and interactions of complex chemical mixtures is key for providing the evidence necessary to make informed decisions and implement robust remediation strategies. Much of the current risk assessment frameworks, applied to manage land contamination, are based on total contaminant concentrations, and the exposure assessments embedded within them do not explicitly address the partitioning and bioavailability of chemical mixtures. These oversights may contribute to an overestimation of both the eco-toxicological effects of the fractions and the mobility of contaminants. In turn, this may limit the efficacy of risk frameworks to inform targeted and proportionate remediation strategies. In this review we analyse the science surrounding bioavailability, its regulatory inclusion and the challenges of incorporating bioavailability in the decision making process. While a number of physical and chemical techniques have proven to be valuable tools for estimating bioavailability of organic and inorganic contaminants in soils, doubts have been cast on its implementation into risk management soil frameworks mainly due to a general disagreement on the interchangeable use of bioavailability and bioaccessibility, and the associated methods which are still not standardised. This review focuses on the role of biotic and abiotic factors affecting bioavailability along with soil physico-chemical properties and contaminant composition. We also included advantages and disadvantages of different extraction techniques and their implications for bioavailability quantitative estimation. In order to move forward the integration of bioavailability into site-specific risk assessments we should (1) account for soil and contaminant physico-chemical characteristics and their effect on bioavailability; (2) evaluate receptor's potential exposure and uptake based on mild-extraction;

(3) adopt a combined approach where chemical-techniques are used along with biological methods; (4) consider a simplified and cost-effective methodology to apply at regulatory and industry setting; (5) use single-contaminant exposure assessments to inform and predict complex chemical mixture behaviour and bioavailability.

Keywords: bioavailability, partitioning, contaminated land, risk assessment, sorption, ageing.

2.1. Introduction

Contaminated sites are often impacted by a wide range of organic and inorganic chemical mixtures, and among them heavy metals/metalloids and petroleum hydrocarbons are the most commonly found (European Environment Agency, 2012; British Geological Survey, 2014; Coulon et al., 2014). These contaminants will often form complex mixtures in soil that complicate the assessment of risk, and the achievement of site-specific remediation objectives (Renoux et al., 2013). Unlike single contaminants, the physico-chemical interactions of mixed contaminants are not well understood as the additive, synergistic or antagonistic effects of mixtures will often yield bioavailability values that differ from those of individual contaminants (Ramakrishnan et al., 2011). Poor understanding about the fate and behaviour of contaminant mixtures in soil limits the effectiveness of risk-based contaminated land management decisions.

Risk assessment is an established methodology that is employed to assess the potential impacts of contaminants on human and ecological health (Vegter et al., 2002). Reflecting regional legislation, expertise, and socio-economic issues, several risk-based contaminated land management frameworks have been published to support environmental management decisions (Brassington et al., 2016; Kabari et al., 2016). Typically, these frameworks use a tiered assessment approach. A limitation of such frameworks, similar to that of exposure assessment methods, is the inability to assess the risk posed by complex chemical mixtures. In fact, these frameworks are based on conservative risk screening levels, and therefore tend to overestimate the risk, as they do not take into account the amounts of chemicals potentially bioavailable in soil and bioaccessible to organisms. Determining appropriate site specific measures and remedial objectives depends on our understanding of contaminant partitioning and interaction with the soil matrix over time. Measuring the total concentration of contaminants in soil does not provide a useful basis for the evaluation of the potential risks to humans and the environment. The variety of physical–chemical properties, and thus differences in the migration and fate of individual compounds, as well as the toxicity of different fractions and compounds, must be taken into account in risk assessments.

Over the last 30 years, accounting for the bioavailable nature of soil contaminants has received increasing attention. As a result, great amounts of scientific literature have reported on the development of methods to estimate the bioavailable fraction of these contaminants. Despite this progress, implementation of these methods into contaminated land decision-making processes has not yet been statutorily defined, and uncertainties remain on how bioavailability should be assessed and integrated into existing risk based management frameworks (Ortega-Calvo et al., 2015; Wu et al., 2013; Harmsen and Naidu, 2013; Naidu et al., 2015).

In this review we will highlight the factors that influence the bioavailability of chemical in soil, and will discuss the challenges that complex chemical mixtures pose. We will critically review the existent literature to assess the use of bioavailability in contaminated land risk assessments. Finally, we will offer suggestions for how bioavailability could be integrated into existing contaminated land risk assessment frameworks.

2.2. Bioavailability concept

Defined from a chemical perspective, bioavailability is the fraction of freely available (not sorbed or sequestrated) contaminant in the environment that is mobile, and thus most likely to lead to human exposure (Dean and Scott, 2004; Ruby et al., 1996). Similarly, Semple et al. (2004) defined bioavailability as the contaminant fraction “freely available” in a medium and able to reach the cellular membrane of an organism over a given time. Thus, for a contaminant to be bioavailable it must be mobile and there must be likelihood for exposure with a biological membrane.

2.2.1. Factors affecting bioavailability

Managing risk associated with chemical mixture in the environment requires an understanding of how contaminants are released, transported, and taken up by a target receptor. The different transportation and uptake pathways, that affect the quantitative estimation of bioavailable fractions of metals and oil-derived products in soil, depends on both the physico-chemical characteristics (Table 2.1), the receptors (Table 2.2), and other additional factors (Table 2.3). Among the physico-chemical factors, soil characteristics (pH, soil composition, organic carbon percentage, and salinity), compound properties (hydrophobicity, aqueous solubility, and acid dissociation constant), and transformation/degradation processes are generally responsible for interactions occurring between the soil matrix and the chemical compounds (Table 2.1). Biological processes (e.g. bioaccumulation, biotransformation) whereby contaminants are transported into an organism, are highly dependent on the type of organism and its biology (Table 2.2). It is important to recognize that any combination of individual physico-chemical and biological processes will affect contaminant bioavailability and exposure of receptors.

Soil matrix heterogeneity will also affect bioavailability (Farmer, 1997). Among physico-chemical factors, sorption is the main factor influencing the biotic and abiotic transformations happening over time (i.e. ageing) in solid environmental matrices, which normally yield to a more stable solid-associated compound and therefore a decrease in bioavailability (Zhang et al., 2014; Moyo et al., 2014; Dube et al., 2001; Kleber et al., 2007).

Sorption, which includes absorption and adsorption, is the process whereby a chemical compound adheres (reversibly or otherwise) to the surface of a soil particle (Olu-Owolabi et al., 2014). The sorbed substance is referred to as the sorbate (compound), and the material that it is sorbed to is referred to as the sorbent (solid phase). When contaminants are released in the soil, the chemistry of the particles and the equilibrium between phases will influence the pathways and interactions between sorbate and sorbent. Contaminants will interact with both the mineral and organic content of soil, either sorbing to surfaces, or migrating within the porous structure of soil compartments (Reid et al., 2000). Contaminants can also dissolve into the pore water of a soil matrix, making it available for biodegradation (Figure 2.1). The interaction between contaminant and soil particle will lead to different degrees of desorption (1) rapid — compounds can easily desorb and return to the pore water; (2) slow — reversible but over a longer timeframe (Ren et al., 2018); (3) non reversible —

rate of contaminant removal is low and compounds are bound (sequestered) to the soil (Kuppusamy et al., 2017). The non-reversible fraction is generally believed not to be relevant for bioavailability assessment.

Partitioning of a contaminant at the solid-water interface will depend on the chemical structure of the contaminant. For example, small organic contaminants and low molecular weight PAH could dissolve into the soil pore water, or could be rapidly sorbed onto the particle surface (Vicent et al., 2013; Abdel-Shafy and Mansour, 2016). PAH with high molecular and larger organic molecules with non-polar structures, on the other hand, tend to sorb onto the non-polar, condensed organic domains of soils (Loibner et al., 2000). These fractions resist degradation and will persist in soil. However, even small molecules can become persistent environmental pollutants; for example, chloro-organic compounds show a great stability and recalcitrance due to their C–Cl bond (Nikel et al., 2013). Metals also sorb to soil particles, particularly iron hydroxides, clays, and carbonate minerals, and can form solid stable compounds with oxygen and sulphur, becoming irreversibly enclosed and thus, not bioavailable.

Table 2.1: Physico-chemical factors influencing bioavailability of metals and oil-derived products in soil (similarities and differences).

Factors	**Metals**	**Implication for element behaviour and bioavailability**	**Oil-derived products**	**Implication for oil-derived compounds behaviour and bioavailability**	**Reference of special interest**
Contaminant characteristics	Present in different elemental forms (metal speciation).	Metals' bioavailability can increase or decrease depending on their chemical form. Formation of sulphide cause low solubility (low bioavailability).	Molecular weight, polarity, hydrophobicity, solubility, octanol partitioning coefficient (K_{OW}), sorption coefficients (K_{OC}, K_d), acid dissociation constant (pK_a).	Highly complex chemical mixture and concentration constantly changing due to transformations and interactions with environmental media over time.	Violante et al., 2010; Rinklebe et al., 2016; Liu et al., 2016; Shahid et al., 2017; Duan & Naidu, 2013; National Research Council, 2014; Trellu et al., 2017.
Soil characteristic and sorption, desorption	Influenced by both geochemical processes (e.g., redox/pH) and soil characteristics (e.g. particle size, organic content).	High pH form insoluble metal (decrease in bioavailability), low pH form free ionic species or organo-metals (increase in bioavailability). The presence of mineral phosphates and carbonates decreases bioavailability.	Quantity and type/quality of organic carbon, clay content, organic content (condensed humic material, soot particles), and soil organic matter can influence bioavailability.	Binding of PAH to condensed organic domains rend these compounds less bioavailable. Adsorption can be also influenced by pH, depending on the presence or absence of intrinsic positive or negative charges on the compounds functional groups.	Lomaglio et al., 2017; Wang et al., 2016; Pauget et al., 2011; Pan et al., 2016; Tahervand & Jalali, 2016; Cecchin et al., 2016; Lukić et al., 2016; M. Zhang et al., 2014; Sabljic & Nakagawa, 2014; Wu et al., 2013; Chen et al., 2017; Yu et al., 2018.

Factors	Metals	Implication for element behaviour and bioavailability	Oil-derived products	Implication for oil-derived compounds behaviour and bioavailability	Reference of special interest
Transformation, degradation (biological/chemical)	No degradation.	HM can only be bio-accumulated or sequestrated (Olaniran et al., 2013). Their bioavailability depends on partitioning and distribution across soil substrates. presence of metals (if bioavailable) can inhibit organic compounds degradation interfering with microbial processes.	Both biotic (microbial degradation), and abiotic degradation (volatilisation, leaching, and photodegradation) can lead to transformation and degradation of organic compounds in soil.	Bioavailability of organic compounds over time tends to decrease due to diffusion into soil particles, formation of stabile complexes, and to microbial degradation.	Yu et al., 2016; Palleiro et al., 2016; Young, 2013; Sihag et al., 2014; Vila et al., 2015; Ghosal et al., 2016; Marquès et al., 2016; Alegbeleye et al., 2017.
Oxidation/reduction cation exchange capacity, and soil pH	Influenced by the presence of organo-mineral colloids (adsorption). Complexation with humus, precipitation in presence of clay mineral and Fe, Mn, Al oxides and carbonates.	Reducing conditions, due to a high content of organic carbon and/or sulphide, can cause formation of less soluble species e.g. Cr (III).	Changes in pH can influence mostly ionizable organic compounds, impacting sorption and removal of organic solutes from solution (Naidu, 2011). Changes in redox potential and pH can accelerate oxidation of organic contaminants (Eggleton and Thomas, 2004).	Both mineral and humic substances can impact bioavailability of organic pollutants via oxidative and reductive transformation processes.	Ashraf et al., 2012; Yu et al., 2016; An et al. ,2015; Schneider et al., 2016; Kunhikrishnan et al., 2016; Ling et al., 2015; Xiao et al., 2014; Zhang & Fan, 2016; Zhang et al., 2015.

Table 2.2: Biological factors influencing bioavailability of metals and oil-derived products in soil (similarities and differences).

Factors	Metals	Implication for element behaviour and bioavailability	Oil-derived products	Implication for oil-derived compounds behaviour and bioavailability	Reference of special interest
Uptake	Metals uptake is typically based upon bioassay exposures to a dissolved chemical, therefore highly dependent on the metals solubility in solution, and oxidation states.	Highly dependent on the system considered for example in plants the bioavailability of a certain metal in the water phase, depends on root structure, but also presence/absence of organic acids exudates (such as citrate and oxalate). For aquatic species bioavailability depends on both ingestion of metal-enriched sediment during feeding, and uptake of metal suspended particles from solution (Du Bray, 1995).	Depend on multiple factors such as concentration in soil, its chemical form, soil pH, biological species, and uptake pathways of specific species.	Depend on where and how an organism lives and feeds in the soil or sediment.	Wyszkowska et al., 2012; Seshadri et al., 2015; Tangahu et al., 2011; Rüdel et al., 2015; Peters et al., 2016; Juhasz et al., 2014; Beriro et al., 2016; Lal et al., 2015; Rostami & Juhasz, 2011.
Bio- concentration, bio-accumulation, and bio-transformation	Metal bioaccumulation (in bacteria, fungi, and plants) can happend throught biosorprion or absorption and uptake. Metal can potentially interact and affect funtion of enzymes involved in biodegradation of chlorinated organic compounds.	Depend on uptake, levels of fats (lipids) within the organism, metabolism, age, growth life stage, and gender.	Strong correlations between the bio-concentration factor, bioaccumulation factor and the octanol: water partition coefficient (K_{OW}).	Depend on uptake, levels of fats (lipids) within the organism, metabolism, age, growth life stage, and gender.	Berthelot et al., 2008; Jaishankar et al., 2014; Tchounwou et al., 2012; Khan et al., 2015; Fantke et al., 2016; McLachlan et al., 2011; Pampanin 2017; Vasseur & Bonnard, 2014.

Table 2.3: Additional factors influencing bioavailability of metals and oil-derived products in soil (similarities and differences).

Factors	Metals	Implication for element behaviour and bioavailability	Oil-derived products	Implication for oil-derived compounds behaviour and bioavailability	Reference of special interest
Ageing	A rapid uptake via electrostatic adsorption is usually followed by a secondary transformation that form a more stable complex.	Ageing can have an effect on inorganic contaminants, where metal precipitation can occur rapidly causing a decrease in bioavailability and toxicity. However is less clear how stable/reversible is the process (Hamon et al., 2006).	Different processes might occur: incorporation into natural organic matter (absorption), slow diffusion into small pores (soil intraparticle).	Overall a decrease in bioavailability has been observed during time due to different factors (dilution, dispersion, biodegradation, volatilisation, and irreversible sorption).	Wijayawardena et al., 2015; Liang et al., 2014; Wang et al., 2017; Romero-Freire et al., 2017; Jiang et al., 2017; Duan et al., 2014, 2015; An et al., 2017; Smith et al., 2011; Liu & Haderlein, 2013.
Co-contaminant interaction	(1) Metal-metal interaction is mostly competitive affecting affinity for soil-surface and sorption sites (e.g. Zn is a competitor for Cd and Pb). (2)Metal-organic interaction can henance HM transport (Chigbo et al., 2013) due to: association with mobile colloids, and formation of metal-organic complexes that are not sorbed onto the surfaces.	Metals that compete for the same sorption sites can trigger the release of the competitor metals, enhancing their bioavailability. Necessity of addressing contaminant as a mixture.	(1) Organic-metal interactions: high concentration of inorganics might influence mobility of PAH. (2) Organic-organic interaction: competitive displacement, and co-solvency. Molecules with similar structure are highly competitive (interchangeability).	The non-linear behaviour affecting sorption/desorption rate can challenge bioavailability predictions. Some natural compounds might also share a similar structure and therefore displace contaminants increasing their bioavailability. Necessity of addressing contaminant as a mixture.	Sun & Zhou, 2010; Van Genderen et al., 2015; Meyer et al., 2015; Zhao et al., 2016; Chigbo et al., 2013; Olaniran et al., 2013; Gauthier et al., 2014; Biswas et al., 2015; Wuana et al., 2014.

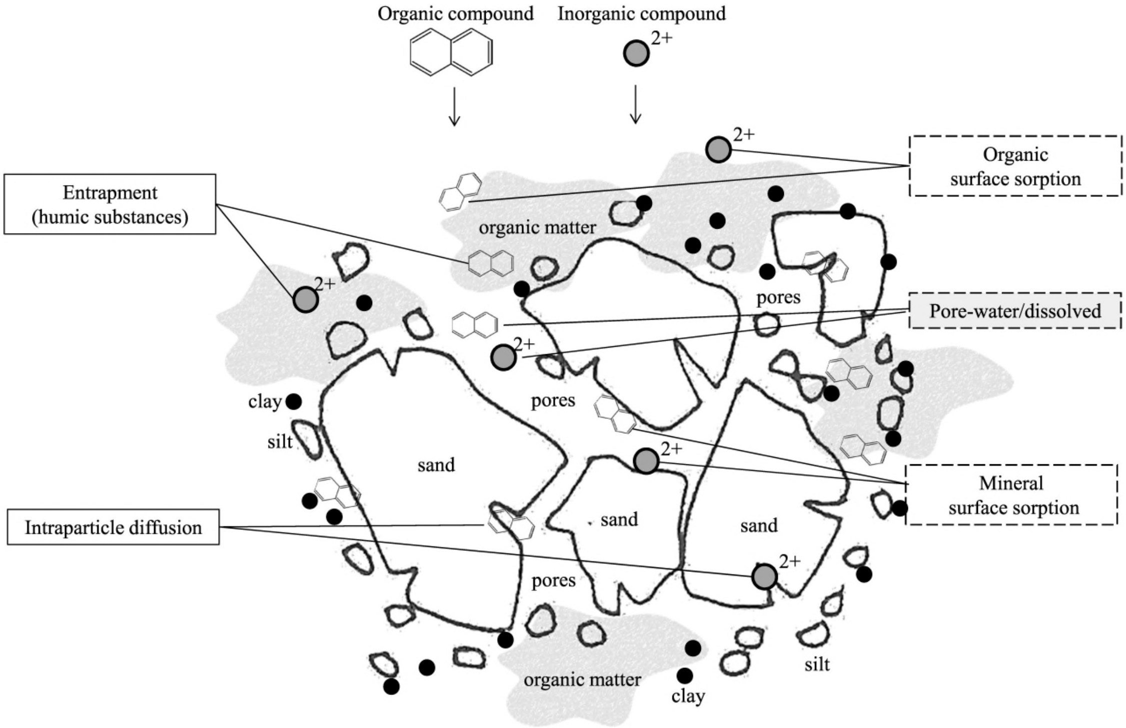

Figure 2.1: Process of sequestration of the compounds in soil, adapted from (Reid et al., 2000); bold textbox indicates the non-desorbing fraction (irreversible processes), dashed textbox indicates the rapidly-desorbing fraction (reversible processes), and the highlighted textbox represent the dissolved fraction (bioavailable).

Contaminant retention is largely regulated by soil particle size distribution (Table 2.1). Smaller particle sizes provide a greater surface for interactions with hydrophobic organic chemicals (Capri et al., 2004). Clays and fine-grained sediments have the greatest surface area and therefore a high capacity to retain organic and inorganic compounds. Further to this, the presence of oxides (Fe and Al oxides, hydroxides, and oxyhydroxides) along with reactive calcium carbonate ($CaCO_3$) can enhance organic and inorganic contaminants retention (Loibner et al., 2006) and therefore favour the biological stabilization of organic carbon (encapsulation) (Heng et al., 2010). Such mechanisms will however hinder microbial degradation of the compounds of concern due to reduced accessibility (Krull et al., 2001) (Figure 2.1). Hard- and soft-organic matters are also associated with retention and ageing processes (Table 2.3). Soil organic matter (SOM) is formed from natural organic matter (e.g. vegetal decomposed material), animal residues at various stages of decomposition, fulvic acids, and humic acids (Sharma et al., 2010). Generally, SOM is thought to be composed of “soft carbon” (amorphous or hydrolysable carbon), and “hard carbon” (condensed or non-hydrolysable carbon) constitute of kerogen, black carbon, and lignin (Weber et al., 1992). Black carbon and kerogen, in particular, can bind tightly the organic contaminants reducing their solubility and/or dissolution rate, and thus bioavailability (Stroud et al., 2007; Van Elsas et al., 2006; Berkowitz et al., 2008). Large amounts of organic matter in the soil have also been shown to effect the residence time of organic matter-associated metals. For example, when organic matter is oxidized, the associated metals are likely to be released, becoming more bioavailable. Conversely, soil with low organic matter content will often accumulate oxide minerals (e.g. clay) that favour the complexation of both metals and metalloids, thus reducing the bioavailable fraction (John and Leventhal, 1995). Soil properties are site specific and will vary from one site to another, therefore, if two sites contain equivalent amounts of a certain contaminant, their bioavailability may still vary significantly, depending on how tightly the chemical is bound to the soil.

The ratio between bioavailable and non-bioavailable fractions is shown in Figure 2.2. Over time, the proportion of bioavailable contaminant will decrease, relative to the non-bioavailable fraction. Pollutants in soil and sediment do not disperse quickly and the desorption and remobilization of metals and oil-derived products in soil are considered long-term processes. For example, heavy metals associated with fluvial sediments can display a residence time from days to years, on the order of 100 – 1000 years (depending on stream-flow dynamics) (Ciszewski and Grygar, 2016; Coulthard and Macklin, 2003). Heavy metals also have a long residence time in soil (Sayadi et al., 2017), ranging from 500 to 3000 years (Lepp, 2012; Jørgensen, 2000; Ayres, 1992; Alloway, 1995). The process of aging can enhance the amount of absorbed heavy metals in soil, where a redistribution from weakly bound fractions to more strongly bound fractions can be observed (Settimio et al., 2014; Wang et al., 2015). Recent metal contamination tends to be more reactive and prone to dissolution compared to older contamination where the elements might be in crystalline forms (Lynch et al., 2014), which presents a lower environmental risk

(Baran et al., 2015; Environmental Agency, 2008). Sediment and soil-bound organic contaminants can persist over decades without significant concentration reductions. Chlorinated or hydrophobic contaminants, in particular, tend to desorb very slowly over time with a desorption rate on the order of years, due to their hydrophobicity (Eggleton and Thomas, 2004). Though oil-derived products tend to persist in soil, the PAH bioavailable fraction will decline exponentially over time (Yang et al., 2016). Weathered hydrocarbon residues pose negligible risks to human health, and this is reflected in post-treatment remedial objectives (Coulon et al., 2010; Jiang et al., 2016). When bioavailable fractions are high (despite being below risk-based clean-up levels) further treatment and more stringent clean-up levels should be mandated to reduce the elevated risk of exposure that is present (Cipullo et al., 2016).

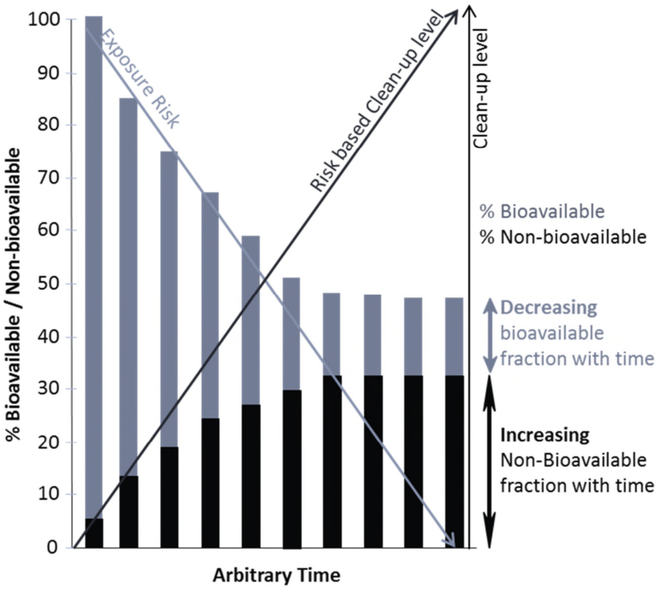

Figure 2.2: Relationship between the percentage of bioavailable and non-bioavailable contaminants in soil, exposure risks and risk based clean-up level (adapted from Reid et al., 2000; and Tri-Service Ecological Risk Assessment Workgroup, 2003).

The necessity of collecting case-specific parameters can challenge the development of a unified methodology to assess the bioavailable fraction, and to determine the risks to human and environment in a straightforward way. A number of analytical methods to assess readily (bio) available compounds in soil and sediments are available, and we discuss these in the following sections.

2.2.2. Methods for estimating bioavailability of heavy metals

Metals can be present in soil as either free metal ions, forming various soluble complexes with inorganic or organic ligands; or associated with colloidal and mineral materials (McLean and Bledsoe, 1992) becoming strongly incorporated with the soil matrix (inert). Most of the divalent heavy metal cations (e.g., Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) share a relatively similar structure and therefore display similar behaviour in soil (Olaniran et al., 2013).

Total metal concentration estimated with acid digestion (e.g. aqua regia) mobilizes all forms of metal in soils and sediment. This measure refers to both metal content in particulate (sorbed + precipitated), and dissolved (inorganic complexes + organic complexes + free ionic forms) fractions. However, particulate metals do not contribute to the solid-solution distribution (potentially bioavailable), and may only become available through very slow desorption processes. This fraction does not provide appropriate basis for expressing metal bioavailable (labile) concentrations in soil, thus presumably not readily available for receptors' uptake.

Several approaches are used to determine the pool of labile metals (concentration and distribution) in soils and sediments; including exchange resins, diffusive gradient in thin films (DGT), conventional single-step extractions, and sequential extractions (Table 2.4).

Passive samplers (e.g. exchange resins) act as ion sinks and are used to quantify free ion activities, soluble fractions, and labile pool concentrations of metals in soils (Qian and Schoenau, 2002; Ge et al., 2005). Free ion activity in solution represents the most relevant parameter for assessing bioavailability and toxicity of metals in contaminated soils. The use of exchange resins has been successfully applied to predict uptake of metal in the environment (e.g. in plants (Peijnenburg et al., 2007)), (Table 2.4), however, to date no standard technique has been validated.

Diffusive gradients in thin films (DGT), is a relatively cost-effective technique, based on a layer of hydrogel and resin gel. The DGT devices allow for the passive accumulation of labile species from a solution (in real time), and have been used to assess in-situ the fraction of metals dissolved in water (Zhang, 1998; Hooda et al., 1999; Parker et al., 2016). As an in situ sampling technique, DGT can be also used in soil to determine the labile (bioavailable) fraction of elements, and to understand how this fraction changes in the environment. However, continuous depletion of metals from pore water can limit the diffusion of analytes to the DGT sampler, affecting the estimation of the available pool of metals (Peijnenburg et al., 2014). In addition, development and validation of a DGT method to establish accumulation in biological endpoints remains a challenge, due to the complexity of the uptake by model organisms and inconsistent results often obtained (Menegário et al., 2017).

Single-step extraction (Table 2.4) uses a wide range of extractants including: salt solutions ($CaCl_2$, $NaNO_3$, NH_4NO_3, $Ca(NO_3)_2$, $AlCl_3$, $MgCl_2$), acid solutions (HNO_3, CH_3COOH, HCl) and chelating agents (EDTA, DTPA). These tests were initially designed to predict nutrient deficiency in soil, and generally contain organic chelates and acids in order to mimic plant metals uptake (National Research Council, 2003). Single-step extractions were lately adapted to measure the labile concentration of metals in soil and sediments, potentially available for ecological receptors uptake (Alvarez et al., 2011). Complex organic reagents (EDTA, DPTA) are also used to mimic the organic exudates produced by plants, and have been positively correlated with metals concentration found in plants (Domínguez, 2008), and further exploited to mimic the bioavailable fraction. Chelating agents, due to their high affinity for metal ions, are used to enhance the solubilisation of metal(loids) through the formation of soluble chelates (Bolan et al., 2014). Caution is needed, however, because studies have shown that results are not consistent and robust across different types of soil (National Research Council, 2003), and are highly dependent on extraction conditions and trace elements speciation (Cappuyns, 2012). Single-step extraction techniques widely vary in type of extractant used, its concentration, soil: solution ratio, and extraction time. They can partially dissolve trace elements associated with different fractions (e.g. pore water and exchangeable), which provides useful information about metals behaviour (Alvarez et al., 2011). Though relatively simple to use (minor sample handling), in single-step extraction mode the non-labile metal fraction might also become solubilized, which might cause an overestimation of the labile pool of several metals (Moreno et al., 2005; Qasim et al., 2015).

While passive samplers and single-step sequential extractions have been used to measure labile metals, these methods do not provide information on the fate and behaviour of contaminants over time.

Sequential extraction, however, can be used to quantify the distribution of metals and assess the mobility of potentially harmful elements over time (Sungur et al., 2015). The procedure involves leaching successive fractions of metals by increasing the strength of an acid solution (HNO_3, HF-$HClO_4$, $HClO_4$, HCl, and CH_3COOH) or other reagents (such as $Na_4P_2O_7$ and NH_2OH) used for each phase association (Zimmerman and Weindorf, 2010). The number of step may vary from 3 to 7 (Table 2.4). Sequential extraction has been used for over 30 years (Tessier et al., 1979) and has been modified to create the community Bureau of Reference Method (BCR) (Rauret et al., 2000) and the Chemometric Identification of Substrates and Element Distribution (CISED) method (Cave et al., 2004), which overcome non-selectivity and redistribution of trace elements. The BCR method has been validated against a sediment reference material (BCR-701) and provides extractable concentrations for several metals (Rodgers et al., 2015). The non-specific sequential extraction CISED has been validated against a sediment reference material (BGS-102) and uses chemometric data processing to provide mechanistic information about metal-soil phase associations (Gál et al., 2006). Results from CISED could be a powerful tool to understand how mineralogical forms might affect bioavailability, yet considerably more chemometric and geochemistry information need to be applied (Giacomino et al., 2011). Despite the large amount of information that sequential approaches can provide the standardisation and application of these technique in commercial laboratories is limited because of the laborious and time-consuming nature of these techniques and the difficulty of interpreting the results (Alvarez et al., 2011).

Table 2.4: Methods to characterize labile fraction and bioavailability of metals in contaminated soils.

Method	Advantages	Disadvantages	Specifics	Reference
Passive sampler	• Passive samplers are relatively low-cost, can be used for in-situ measurements (Menegário et al., 2017), can achieve low detection limits (Peijnenburg et al., 2014), and allow long-term trends assessments. . • DGT was found to be a good tool for measuring in situ metal bioavailability in sediments (Ren et al., 2015). • DMG is a relevant tool for in-situ assessment of environmental risks posed by metals (Perez et al., 2016). • Passive sampler measurement can be translated into fugacity models and equilibrium studies to understand chemical potential activity and estimate potential risk (Amiard-Triquet et al., 2015).	• Little attention is given to the effects that water chemistry and method of field deployment may have on uptake kinetics (Mills et al., 2014). • In some cases require a time-consuming elution step with acids, in order to retrieve the analyte from the solid sorbent phase (Almeida et al., 2014). • DGT and DMT not yet considered suitable for routine analysis due to poor detection limits, time-consuming procedures, and a lack of validation (Brand et al., 2009). • Deployment of DGT samplers, where nanoparticles are high, may result in an overestimation of dissolved metals concentrations (Pham et al., 2015).	Exchange resins Diffusive gradient thin film (DGT) Donnan membrane technique (DMT) Diffusive milli-gels (DMG)	Cantwell et al., 1982; Holm et al., 1995; Lorenz et al., 1997; Christensen & Christensen, 1999; Davlson & Zhang, 1994; Agbenin & Welp, 2012; Koopmans et al., 2008; Pampura et al., 2006; Weng et al., 2005; Perez et al., 2015.
Stripping voltammetry	• High sensitivity, high reproducibility, and mainly used for the detection of trace levels of heavy metal ions (Almeida et al., 2014). • Found to be suitable for assessment of heavy metals bioavailability to plants (Dytrtova et al., 2008).	• Limitations for on field measurements, sample perturbations due to sample handling and storage (Rurikova & Kudrava, 2006). • Adsorption effects of humic and fulvic acids in soil accompanied by the metal complexation can limit the success of this method (Rurikova & Kudrava, 2006).	Anodic (or cathodic) stripping voltammetry	Sauvé et al., 1997; Zima & Van Den Berg, 1994; Davidson & Smyth, 1979.
Competitive chelation	• Method has good sensitivity and can provide reliable estimates of ion activities (Amacher, 1984).	• Equilibrium between chelate and soil can take a long time to be attained (Norvell & Lindsay, 1972; Norvell & Lindsay, 1969). • The success of the method depends on abundancy of metal of interest and the selected competing metal (Workman & Lindsay, 1990).		Xue et al., 1995.

Method	Advantages	Disadvantages	Specifics	Reference
Ion exchange	• Results were comparable to ion selective electrode, and anodic stripping voltammetry (Ge et al., 2005). • Simple, cost-effective, relatively easy to use, and applicable to different soil types (Qian & Schoenau, 2002). • Possibility of simultaneous multi-metal measurement (Weng et al., 2005).	• Requires a characterization of the resin adsorption properties (Weng et al., 2005). • Soil solution composition need to be considered during speciation analysis (Fotovat & Naidu, 1997).	Cation exchange resin	Sunda, 1984; Apte & Batley, 1995; Qian & Schoenau, 2002; Ge et al., 2005.
Single extraction with (1) salt solutions,(2) acid solutions, (3) chelating agents	• Can be used to perform fast screening analysis of the labile pool of elements in soils and sediments (Sakan et al., 2016). • Significant positive correlations between the single extractions methods results and lettuce shoot content were obtained for several metals (Pinto et al., 2015). • $CaCl_2$ extraction has been reported as being a good proxy for bioavailability of metals in soils to plants (Houben et al., 2013) and was correlated with concentrations of potential harmful elements in plant (Qasim et al., 2015). • Leaching test employing a neutral salt solution ($CaCl_2$ or NH_4NO_3) is considered to be sufficient to measure the bioavailable fraction of mobile metals (in particular Cd, Ni, and Zn) (Kim et al., 2015). • The single extraction method involving EDTA presented good precision (Sahito et al., 2015). • Using the single-step extraction (EDTA or acetic acid) allows predicting metal extractable content (bioavailable) in vineyard soil-grapevine system (Vázquez et al., 2016).	• Chemical extractions can hardly account for the complex processes involved in metals uptake by plants therefore not sufficient for estimating soil metal bioavailability to plants (Krishnamurti et al., 2015). • No consensus on best single step extraction conditions to extract and maintain integrity of arsenic species (Sun et al., 2015). • At low reactive element to organic matter ratios, diluted nitric acid extraction (0.43 M) can underestimate concentrations of geochemically reactive elements with a particularly high affinity for organic matter or oxides (Groenenberg et al., 2017). • Complexing and chelating extractants (EDTA and DTPA) showed poor correlation with potential harmful elements concentrations in plant (Qasim et al., 2015).	0.01-1 M $CaCl_2$ 0.1 M $NaNO_3$ NH_4NO_3 0.1 M $Ca(NO_3)_2$ 0.3 M $AlCl_3$ 0.02-0.1 M $MgCl_2$ different concentrations of HNO_3 CH_3COOH HCl EDTA DTPA	Houba et al., 2000; Young et al., 2000; Novozamsky et al., 1993; Ure, 1996; Gupta & Aten, 1993; Novozamsky et al., 1993; Meers et al., 2007; Hughes & Noble, 1991; Makino et al., 2006; Tipping et al., 2003; Almås et al., 2007; Novozamsky et al., 1993; Cappuyns, 2012; Leggett & Argyle, 1983; Lindsay & Norvell, 1978.

Method	Advantages	Disadvantages	Specifics	Reference
Sequential extractions	• These methods are simple, low cost, applicable to different soil type, and results are often comparable (Rosado et al., 2016). • BCR method provide relevant information on the relationships between soil characteristics and metal potential fractions for uptake by plants (Sungur et al., 2014). • BCR method showed correlations between available metal and the plant uptake (Fernández-Ondoño et al., 2017). • The modified version of the three-step procedure proposed and validated by the BCR (Community Bureau of Reference) could potentially be accepted as the standard method (Ahmadipour et al., 2014). • CISED method can provide a powerful tool for understanding metal fractionation in soils (Cave et al., 2015). • CISED is a valuable methodology for studying the solid-phase fractionation of potential harmful element in soil and potential bioavailability (Reis et al., 2014; Palumbo-Roe et al., 2013; Cox et al., 2013). • Sequential leaching studies can help understanding leachability, solubility, and mobility of heavy metal, therefore allowing to make assumptions on metal bioavailability for risk assessment (Kaakinen et al., 2015).	• Sequential extraction are inadequate for determining the extraction kinetics, and subjected to high risk of bias due to re-adsorption processes (Rosas-Castor et al., 2015). • BCR method drawbacks include lacks of specificity and difficulty in interpreting results (Huang et al., 2014). • BCR method results tedious and time-consuming due to long shaking time and filtration requirements (Matong et al., 2016). • Interpretation of data from the CISED extraction may be more challenging than selective chemical extractions (Reis et al., 2014). • Main limitation associated with sequential extraction procedures is the long time associated with extraction (Khan et al., 2013). • Limitations associated with sequential extraction methods includes; redistribution of analytes among phases, incomplete extraction, non-selectivity of reagents, and precipitation of other minerals (Selim, 2015). • Laborious and time consuming techniques, results often of difficult interpretation (Domini et al., 2011).	modified BCR extraction four-step chemical fractionation procedure five-step chemical fractionation procedure six-step chemical fractionation procedure seven-step chemical fractionation procedure non-specific sequential extraction (CISED)	Ure et al., 1993; Rauret et al., 2000; Tessier et al., 1979; Elliott et al., 1990; Mclaren & Crawford, 1973; Miller et al., 1986; Krishnamurti et al., 1995; Cave et al., 2004.

2.2.3. Methods for estimating bioavailability of oil-derived products

Estimating the bioavailability of organic compounds and integrating it into the decision-making processes remains a scientific and regulatory challenge. Research into the bioavailability of oil-derived contaminants has received considerable attention in the last 20 years (Thompson, 2016). In the context of implementing bioavailability into regulatory frameworks it is important to both quantify the (potentially) bioavailable fraction, but to also understand the mobility and behaviour of contaminants in soil in order to assess potential effects of complex contaminations on receptors. Empirical approaches are often used to predict contaminant toxicity or assess the effectiveness of remediation treatments (Environment Agency 2006, 2010). Computational methods which integrate the multitude of compounds and molecular structures have proven to be the most challenging. In particular, the prediction of complex contaminants' toxicity, such as crude oil, requires understanding and forecasting the potential effects of several hundreds of different organic compounds, which possesses different chemical characteristics and different behaviours that might influence the rate and efficacy of degradation (Weng et al., 2015). Molecular weights have been shown to most strongly affect compounds' persistence in soil (Atlas, 1995). Moreover, complex physico-chemical interactions between different compounds can lead to unexpected or poorly understood reactions (e.g. co-solvency), which might alter the bioavailable nature of a compound in mixture. A wide range of analytical procedures have been used to estimate the bioavailable fractions of organic contaminants in mixtures (e.g. oil constituents) (see for review Brand et al., 2012; Ortega-Calvo et al., 2015). These procedures can measure different fractions: freely dissolved fraction (passive samplers), which measure the dissolved (actual) concentration in a matrix, and rapidly desorbed fraction (non-exhaustive techniques), which uses extractants to recover compounds from soil (Table 2.5).

Table 2.5: Extraction methods in relation to bioavailability of organic compounds.

Type	Method	Advantages	Disadvantages
Passive sampler (solid phase)	Polydimethylsiloxane (PDMS) Solid-phase microextraction (SPME) Polyoxymethylene solid phase extraction (POM-SPE) Triolein embedded cellulose acetate membrane (TECAM) C18 membrane disks Diffusive gradients in thin films (DGT) Semipermeable membrane devices	• Consistent relationship between chlorobenzenes levels in biota and in the PDMS-SPME fibres (Van Der Wal et al., 2004). • SPME can accurately measure freely dissolved pore water concentrations to estimates earthworms uptake (Van Der Wal et al., 2004). • PDMS is very sensitive and able to detect PAH freely dissolved pore water concentrations (ng/L) (Laak et al., 2006). • SPME shows good correlation between bioaccumulation of organic contaminants on a wide range of organisms(You & Landrum, 2006). • SPME and POM-SPE generally are able to predict PAH concentrations in earthworms (Gomez-Eyles et al., 2011).	• SPME and POM-SPE tend to under-predict PAH root concentrations (Gomez-Eyles et al., 2011). • SPME measures truly dissolved concentrations but it is not able to measure compounds associated with dissolved organic matter (ECETOC, 2014). • Poor correlation between availability of PAH in soil and bio-concentration factors in earthworms (Bergknut et al., 2007).
Fluid-phase extractions	Subcritical water extraction (SWE), superheated water technique (SWAT) Supercritical fluid extraction (SFE) Sequential supercritical fluid extraction (SSFE)	• Good correlation between SWE extractions of 14C-activity fraction mineralized by catabolically active *Pseudomonas* (Latawiec et al., 2008). • Successfully used to predict rates of long-term release of organic compounds (Weber, 2001; Hawthorne et al., 2000; Miller & Hawthorne, 1998). • SWE selectively extracts the PAH relative to the readily extracted fraction (Smith, 2002). • SFE recoveries of the ‘‘mobile’’ fraction of PAH were greater than 90% (Librando & Aresta, 2004). • The amount of PCBs extracted by SFE was very close to the estimated bioavailable fraction in earthworms (Hallgren et al., 2006).	• Potential degradation of analytes subjected to high temperatures. • Lack of significant correlation between the amounts desorbed/amount assimilated by earthworms (Weber et al., 2002). • Contaminants with high molecular weight (decreasing polarity and increasing K_{aw} coefficients) showed reduced recoveries when applying SSFE (Loibner et al., 2000). • Mild SFE was not able to differentiate pyrene availability in unaged soils (Sun & Li, 2005). • Using SFE for predicting bioavailability can be limited due to great variability of soil matrix (Cajthaml & Väclav, 2005)

Type	Method	Advantages	Disadvantages
Non-exhaustive extraction techniques (i)	Mild-solvents (butanol, methanol, n-propanol, or ethyl acetate) Combination of solvent and $CaCl_2$ solution Surfactants (Triton X-100)	• Methanol-water and n-butanol extraction of chemical mixtures were correlated with uptake by earthworms (Elsey & Lexander, 1997). • Good correlation between extractable fraction/ uptake earthworms(Kelsey et al., 1997; Tang et al., 1999). • Good correlations between extractable fractions/ bacterial genotoxicity assay (Alexander & Alexander, 2000; Tang et al., 2002).	• Little consistency among different soils (Chung & Alexander, 1998). • High variability in technical operation (type of mixture, shaking time) limits the comparability of data (Cachada et al., 2014). • PAH extracted by mild solvent extraction show a similar composition to the total soil PAH (Bergknut et al., 2007). • Mild solvent extractions consistently over predicted PAH biotic concentrations (Gomez-Eyles et al., 2011). • PAH bioavailability (estimated with butanol) and earthworm bioavailability were found to be non-related (Johnson et al., 2002). • Triton X-100 failed to predict PAH bioavailability in contaminated sediments because extracted both readily and poorly bioavailable PAH (Cuypers et al., 2002).
Non-exhaustive extraction techniques (ii)	Tenax®	• Tenax® was found to be a matrix-independent, cheap and less time-consuming chemical method of estimating bioavailable fraction in PCB-contaminated field and sediments (Trimble et al., 2008). • Successfully used to assess the bioavailability of aromatic compounds in sediment (Morrison et al., 2000; Cal et al., 2008; Harwood et al., 2012). • Good correlation between bioavailable fraction in the sediment and quantity measured by Tenax® extraction (You & Pehkonen, 2007). • Rapidly desorbing fractions of PAH measured by Tenax® have been linked to biodegradation rate (Braida et al., 2004; Shor et al., 2003; Cornelissen et al., 1998). • Tenax® extraction of PAH, PCB, and organochlorine pesticides were correlated to bioavailability to worms (Hulscher et al., 2003). • Tenax® extraction is a good technique to predict bioavailability to earthworms of aged PAH in soil (Lu et al., 2011).	• The process involves a lot of steps in order to estimate the rapidly desorbing fraction (Xing et al., 2011). • Contaminant desorbing fractions in river sediments extracted by Tenax® over-estimated the bioavailable fraction of benthic invertebrates (Leppanen et al., 2003). • Few studies where correlations between Tenax® extractable amount and biota-sediment accumulation were not related (Sormunen et al., 2008, 2009; Leppa & Kukkonen, 2006). • The Tenax® measure of rapidly desorbing fraction is not the only factor contributing to the bioavailability. A number of ecological factors (species and feeding habits) can affect the bioavailable fraction in model organisms, and remain undetected by Tenax® extractions (Akkanen et al., 2007).

Type	Method	Advantages	Disadvantages
Non-exhaustive extraction techniques (iii)	Aqueous hydroxypropyl-cyclodextrin (HPCD)	• A 1:1 correlation between phenantrene extracted and phenantrene degraded by microorganisms was obtained (Reid et al., 2000). • HPCD was successfully used to predict PAH bioavailability in contaminated sediments (Cuypers et al., 2002). • HPCD was successfully used to predict the microbial bioaccessibility and mineralisation rate of aliphatic hydrocarbons (Stroud et al., 2008). • A significant relationship ($p < 0.01$) between HPCD extractability and mineralization was observed (Rhodes et al., 2008). • HPCD β was found to be a good estimation of bioavailable fraction in both single, and multiple contaminants conditions (Stroud et al., 2009). • HPCD ease in sample handling and that no additional device is needed (Cui et al., 2013).	• Predictability of HPCD extraction decreased for higher organisms such as earthworms (Barthe & Pelletier, 2007; Hartnik et al., 2008). • Poor indicator of PAH accumulation in benthic invertebrates (Barthe & Pelletier, 2007). • Cyclodextrin size and structure can limit the complexation of some PAH (size dependent) (Villaverde & Pe, 2012; Stokes et al., 2005). • Cyclodextrin extractions consistently over predicted PAH biotic concentrations (Gomez-Eyles et al., 2011).

2.2.3.1. Passive sampler methods (PSMs)

Passive sampler methods (PSM) (Table 2.5), commonly defined as biomimetic extractions (ECETOC, 2014), are used to measure the freely dissolved concentration (Cfree) of contaminants (Parkerton et al., 2012) in equilibrium with the rapidly desorbing fraction. These techniques are based on the molecular diffusion principle, and often use polymer materials such as polydimethylsiloxane, polyethylene, polyoxymethylene, and ethylvinylacetate (Parkerton et al., 2012). Passive sampler methods have been used to predict PAH bioavailability, and have been shown to correlate well with model organism PAH uptake and bioaccumulation (Jonker et al., 2007; Gomez-Eyles et al., 2011; Muijs and Jonker, 2011), (Table 2.5). Although PSM are valuable tools for providing weight of evidence and informing regulatory decision-making, there exists a lack of consensus about its implementation and standardization. The following issues were identified and need to be overcome in order to further develop and implement these techniques: (1) PSM have been applied to only a limited number of target compounds; (2) the complexity of the PSM equilibrium requires better characterization of potential errors when applied in-situ; (3) adoption of robust quality assurance and control strategies are needed (Mayer et al., 2014).

Correlation with in-vivo measurements and bioaccessibility data are not fully validated, and the complexity of (bio)accumulation mechanisms (Cachada et al., 2014) that govern toxicity responses are not yet fully understood. More information and guidance on the application of passive sampling for the management contaminated sediment sites can be found in the SERDP and ESTCP national guidance documents (Burgess and Driscoll, 2016; Driscoll and Thompson, 2016).

2.2.3.2. Fluid-phase extractions

Supercritical Fluid Extraction (SFE) (Table 2.5) uses supercritical fluids (e.g. CO_2 in combination with solvents) to extract a compound from a soil matrix. This technique can be used to study sorption/desorption processes, and to estimate bioavailability of organic pollutants in soil and sediment. Data obtained from SFE can be fit to a prediction model to obtain information about the PAH bioavailable fraction. Different experiments found that the amount of organic compounds (PCB) extracted by SFE was able to represent the readily extracted fraction (Smith, 2002) and the bioavailable fraction uptaken by earthworms (Hallgren et al., 2006), (Table 2.5). SFE was also found to recover over 90% of the mobile fraction of PAH from soil and sediments samples (Librando and Aresta, 2004). Though SFE can measure the freely dissolved fraction, it is likely to underestimate the concentration/uptake in benthic organisms (with other uptake routes) and its use can be limited due to great variability of soil matrix (Cajthaml and Väclav, 2005). In addition, SFE was found to be able to predict degradation for low molecular weight PAH (three and four ring), with a good correlation between biodegradability and bioavailability (Naidu, 2011), but often overestimated bioavailability of high molecular weight PAH due to their different extractability.

2.2.3.3. Non-exhaustive techniques

Non-exhaustive techniques to assess the bioavailability of organic compounds in soil (Table 2.5) include mild solvent extraction (Kelsey et al., 1997; Liste and Alexander, 2002), solid sorbents (e.g. Tenax®) (Cornelissen et al., 2001; Hulscher et al., 2003; Lydy et al., 2015), and hydroxypropyl-β-cyclodextrin (HPCD) (Reid et al., 2000; Cuypers et al., 2002; Stokes et al., 2005).

Mild-solvent extraction consists of adding a polar solvent or mixture of solvents and water (e.g. butanol, ethanol, methanol, methanol-water) to a sediment or soil sample, agitating the mixture, and then analysing the extract for contaminants' presence (Cui et al., 2013). This technique has shown good correlation between extractable fraction and uptake in earthworms (Kelsey et al., 1997) and bacterial genotoxicity assay (Alexander and Alexander, 2000; Liste and Alexander, 2002), (Table 2.5). Mild-solvent extraction could also be used as a proxy to estimate the contaminant bioaccessible fraction, however it shows little consistency between different soil types (Chung and Alexander, 1998).

Tenax® is a porous polymeric resin that when mixed with contaminated sediment, will recover target compounds. Sorbed compounds are eluted from the resin with a solvent, and fresh polymeric beads can be added several times (multiple steps) and harvested to measure the recoverable hydrocarbons fraction (Cui et al., 2013). Tenax® has been largely used to assess the bioavailability of aromatic compounds in soil and sediment (Morrison et al., 2000; Cal et al., 2008; Harwood et al,. 2012).

Hydroxypropyl-cyclodextrin (HPCD) is a cyclic oligosaccharide formed by α-D-glucopyranoside units linked 1-4 and bound together in a ring (Riding et al., 2013). This structure is highly soluble (hydrophilic outside), and creates a hydrophobic cavity, capable of forming inclusion complexes with hydrophobic compounds such as organic contaminants (Bardi et al., 2000). In these inclusion complex formations, water molecules are released from the HPCD cavity through displacement by a more hydrophobic molecule in solution (Del Valle, 2004). HPCD vary in size (α, β, γ) depending on the number of glucose monomers (from six to eight units) present in the ring. Generally, an aqueous solution of HPCD is mixed with soil or sediment (1:20 ratio), and then the aqueous phase recovered via centrifugation and the supernatant is analysed for the presence of target contaminants (Cui et al., 2013). Alternatively, the supernatant is discarded and the soil pellet resuspended and extracted using exhaustive-solvents (total extraction), and cyclodextrin uptake measured by subtraction (comparing the sum totals of PAH extracted by HPCD against the total amount extracted by exhaustive solvent) (Papadopoulos et al., 2007). Reid et al. (2000) showed a reliable prediction of the microbial available concentration of PAH in soil compared to classical methods based on Soxhlet extraction. Positive correlation has been also observed between the amount of HPCD extracted and microbial mineralization in activated carbon-amended soils (Rhodes et al., 2008) (Table 2.5). Potential of HPCD for indicating bioavailable organic contaminants has been recognized, but no clean-up thresholds for HPCD-extractable PAH have been implemented yet (Canadian Council of Ministers of the Environment, 2006). Main limitations associated with HPCD extraction are the reduction of potential for indicating bioavailable fraction in higher organisms (earthworms, benthic invertebrates) (Barthe and Pelletier, 2007; Hartnik et al., 2008) and the size of the HPCD cavity that might prevent PAH complexation, owing to steric constraints, which would result in poor extraction efficiencies (Stokes et al., 2005).

In summary, while passive samplers can be used to estimate the readily-available (pore water concentration) for most soil organisms (Brand et al., 2013), bioavailable concentrations measured by Tenax® and HPCD correspond to the sum of the fraction dissolved in pore water and the concentration that could become available on a longer term (i.e. rapid and slow desorbing fraction). Methods to estimate potential bioavailable concentrations can be considered more conservative; therefore we believe that both Tenax® and HPCD could be more suitable compared to PSM for the evaluation of receptor's potential exposure and implementation into the risk assessment. Overall the number of laboratory studies and publications on less exhaustive techniques is promising, but they require further efforts to obtain an optimised and enhanced procedure that can be applied across different soil samples and a wider range of contaminants. Such methods could assist in evaluating exposure of ecological receptors and facilitate a more proportionate definition of risk. In addition, these measurements may have implication when establishing remediation end-points.

2.3. Challenges in assessing complex chemical mixtures bioavailability

Methods to assess bioavailability predominantly focus on assessments carried out on individual substances, or a limited number of substances. Humans and ecological receptors, however, are exposed to a wide variety of chemicals and therefore understanding the potential adverse effects of interactions between these chemicals in a mixture is fundamental to assess risk. We can summarise the challenges of assessing the bioavailability of complex chemical mixtures in three key points (1) standards for mixed-pollutants are absent; (2) combination effects studies on complex chemical mixtures are limited; (3) bioavailability is often neglected.

In co-contaminated sites, the presence of both inorganic and oil-derived products, in mixture compositions of near infinite character can produce unpredictable effects (Borgert, 2004). Uncertainty in the behaviour of these mixtures reflects not only the complexity of the soil matrix, but also the heterogeneous nature of contaminants in soil, as well as temporal variations in chemical structure and concentrations. Conventional risk assessments apply risk-based criteria (guideline values) to deterministic models to make decisions about soil remediation, and establish clean-up standards. However, exposure-risk relationships are established on specific quantitative values (maximum acceptable risk), which can largely vary among different countries (Aqeel et al., 2014), depending on the assumption made when modelling exposure. Whereas conventional exposure assessment relied on the measurable effects of individual chemical species (De Zwart and Posthuma, 2005), predictive models for exposure assessment, are unlikely to account for (and interprete) the combative effects of chemical mixtures (Cornelis et al., 2010). Regulatory frameworks address chemical mixtures based on the contribution of each individual compound present in the mixture, if individual compound toxicity does not exceed the threshold, the overall mixture is often considered non-toxic (Heys et al., 2016). Over the last decade, the increasing interest in complex chemical mixtures has been reflected by legislative developments and scientific improvement in understanding of the role of bioavailability of single (Elgh-Dalgren, 2009; Bradham et al., 2015; Chen et al., 2015; Fadaei et al., 2015; Henry et al., 2015; Juhasz et al., 2015; Ortega-Calvo et al., 2015; Stegemeier et al., 2015; Tao et al., 2015) and multiple contaminants (Allan et al., 2012; Gouliarmou and Mayer, 2012; Cain et al., 2013; Liu et al., 2013; Kuhn and Maurice, 2014; Amato et al., 2014; Jia et al., 2014; Arp et al., 2014); however to date potential risks of combined chemicals are rarely examined in risk assessment (Kienzler et al., 2016).

Only a few laboratory based studies have attempted at studying complex chemical mixtures toxic effect on in vitro or biological systems (European Environment Agency, 2012), more data are required on synergistic and antagonistic interactions of these compounds. While the concepts of mixture toxicity have been discussed for decades, their use has been limited by the absence of toxicological data associated with specific substances, the lack of bioavailability data, and generally the uncertainty associated with knowledge on mixtures of compound. The greatest knowledge gap at the present time is the lack of understanding regarding the mode of action of mixture of compounds which limits the definition of a set of criteria, and therefore requires a careful case-by case approach (EU Scientific Committee on Health and Environmental Risks, 2011).

Cumulative risk assessment (CRA) is a relatively new approach that aims to quantify the health, or environmental risk, by estimating the level of exposure to multiple contaminants (U.S. Environmental Protection Agency, 2003). CRA represents a conceptual innovation in the decision making process by moving from a single effect approach to a multiple ecological and human approach to the effects caused by multiple exposure of contaminants (Fox, 2002). Although cumulative risk assessment appears to be pragmatic, few ecotoxicological specific guidelines (e.g. pesticides regulations) account for it (European Environment Agency, 2012). At present, CRA may be the best way to add a health dimension to basic contaminant concentration evaluation. It also might support the decision making process creating a more comprehensive understanding of chemicals behaviour in the environment. Further development and additional studies to verify if CRA is a fair representation of the combine risk for compounds, that might not be equal in toxicity, ecotoxicity and chemical behaviour are needed. Ultimately, CRA should not be the only measure of risk, but a valuable support to other analytical tools for investigating environmental risk. Moreover, as highlighted in this review, bioavailability is influenced by a wide range of physico-chemical (including both soil and nature of contaminant) (Table 2.1) and biological factors (Table 2.2) and it can greatly differ among different organisms; therefore designing suitable one-fit-all extraction approach can be challenging. In order to estimate the bioavailable fraction we should question which of these methods provide a good representation for the specific species we intend to protect. Several chemical analytical methods have been developed to assess the bioavailability of inorganic and organic compounds, yet few of them were found to correlate with uptake in model organisms. As such, none of these techniques have been applied to complex chemical mixtures (Muijs and Jonker, 2011).

2.4. Conclusions

Given the multiple variables affecting the availability of chemicals in soil, we should look at bioavailability not as a fixed value (concentration), but as a dynamic process between an organism and the chemical-uptake over time (ageing). Methods to estimate bioavailability are still not sufficiently cost-effective and standardised. While a great deal of studies and results have been achieved in regards to estimating bioavailability of inorganic contaminants, there is still more work to be done for organic contaminants. Bioavailability and bioaccessibility estimations are seen as useful means to inform human health risk assessment and improving cost-effective management of contaminated land. For instance, when the exceedance of the guideline values is minor, bioaccessibility become the main driver on large site investigations, where costs associated with soil removal are considerably high. Nevertheless, lack of information regarding other potential routes of exposure (dermal contact and inhalation) contributes in limiting our confidence in integrating these findings into risk assessment. Similarly, bioavaliability is still not fully understood and implemented in existing frameworks, because of both multiple definition across different disciplines and lack of standardised tests to measure it. Also, a large number of studies and chemical methods have shown that bioavailable fractions can be positively correlated with uptakes in model organisms and microbial mineralization; obtained data are however inconsistent among different type of contaminants and across different receptors tested. An approach based on weigh-of-evidence should apply chemical techniques to measure the bioavailable and bioaccessible fractions, along with biological methods (bioassays) to better understand effects of contaminants uptake and relate it to bioavailability in potential receptors. Bioassays provide a direct measure of contaminant’s (bioavailable) concentration for a specific organism over time and are able to quantify and detect a wide range of toxins at relatively low cost.

Understanding and implementing site-specific bioaccessibility and bioavailability data means being able to represent more realistically the on-site conditions. Implementation of bioavailability can help the revision of exposure estimate, reducing the cost of remediation, and bringing a greater degree of judgment when assessing risk, and allowing greater levels of contamination left safely in soil. However, in order to provide increased confidence in using bioavailability, further investigation is needed on how to incorporate it into risk assessment. Moreover new approaches are required to tackle the complexity of chemical mixtures and the likely effect of exposure. The challenges are understanding the potential risk connected to a complex chemical mixture, and assessing how the physico-chemical interactions, such as co-solvency, sorption, desorption, and saturation, can affect the potential toxicological response. Understanding which chemicals are effectively bioavailable may be the key for future risk assessment.

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3. Insights into mixed contaminants interactions and its implication for heavy metals and metalloids mobility, bioavailability and risk assessment

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Abstract: Mobility of heavy metals at contaminated sites is mainly influenced by the soil physico-chemical properties and environmental conditions, therefore assessing heavy metals (HM) and metalloids fractionation can provide insights into their potential risk and the mechanisms that regulate bioavailability. A 12-month mesocosms experiment was setup to investigate the effect of physico-chemical factors (pH, moisture, and temperature) and weathering (time) on HM and metalloids fractionation in three different multi-contaminated soil matrices (low, medium, and high contamination) collected from a soil treatment facility located in the United Kingdom, and two rural contaminated soil samples. The study demonstrates that even though Pb and Zn were found associated with the exchangeable fraction in the soil with the highest contamination (total average Pb 3400 mg/kg, and total average Zn 2100 mg/kg in Soil 3), neither the conditions applied nor the weathering caused an increase in their mobility. Although it was expected that lower pH (4.5) would favours the dissociation of HM and metalloids, no significant differences were observed, potentially due to the initial alkaline pH of the genuine-contaminated soil samples. The results show that even though total concentration of Pb, Cu, and Zn exceed the soil standards and guideline values, HM were predominantly associated with the non-exchangeable fraction, while only 5% were dissolved in the pore water fraction (potentially bioavailable). In addition, the mobility and bioavailability of HM remained constant over the 12 month monitoring, suggesting that these soils pose negligible risk to the environment.

Keywords: chemical mixtures, fractionation, mobility, ageing, risk assessment.

3.1. Introduction

Anthropogenic activities such as mining, waste disposal, combustion of leaded fuels, the use of fertilizers and pesticides, and petrochemical spills all contribute to the presence, accumulation, and persistence of heavy metals (HM) in soil (Tóth et al., 2016; Suresh et al., 2012; Wuana and Okieimen, 2011). While organic contaminants might be degraded as they persist in the environment, inorganic contaminants, such as heavy metals and metalloids, are non-degradable and display long-term persistence in soils (Lu et al., 2017), which can potentially cause risk for plants, animals, and humans (Bolan et al., 2014).

European environmental regulatory frameworks, to manage HM pollution, define risk based on the total extractable concentration of metals in soil. This approach does not consider how likely an HM is to be bioavailable, which can lead to an over/under estimation of risk (Cipullo et al., 2018). In relation to contaminated land risk assessment and remediation, bioavailability can be interpreted as the fraction of contaminant that is freely available in the environment (not sorbed or sequestrated), and mobile (extractable by mild extraction), thus the most likely to lead to receptor exposure (Adedigba and Semple, 2015; Dean and Scott, 2004).

Sorption and desorption are the main processes controlling bioavailability of HM (Caporale and Violante, 2016); in particular soil components responsible for the sorption includes; amorphous materials, silicates, clay minerals, carbonates, and organic matter (Leleyter et al., 2012). How a HM interacts with the different soil compartments will influence its bioavailability, and it is bioavailability that can inform the likelihood that a HM might leach into the broader environment (Ashraf et al., 2012). For example, HM that are dissolved in pore water can be easily mobilized, and are considered readily available for uptake by plants (Chang et al., 2014) or available for interaction with biological systems (Hodson et al., 2011); while those dissolved in labile fractions are potentially bioavailable, if physico-chemical conditions were to change (e.g. pH decrease) (Di Bonito et al., 2018). Many physico-chemical factors such as soil pH, composition, organic carbon content, and redox potential, can impact partitioning between soil-solid phase and pore water, which will consequently have an impact on HM bioavailability (Islam et al., 2015; Venegas et al., 2016). In contrast, HM associated with non-exchangeable or non-mobile soil fractions are characterized by a stronger binding (weaker reversibility), therefore unlikely to leach into the surrounding environment.

Despite the recent shift toward risk-based approaches for assessing contaminated sites, risk characterization remains a conservative approach (Harmsen and Naidu, 2013; Naidu et al., 2015; Ortega-Calvo et al., 2015), because it relies on total contaminant concentration, rather than assessing the fraction of the total (bioavailable) that can potentially interact with biological and environmental targets. For bioavailability to be implemented and support regulatory decisions, the bioavailable estimation should rely on standardized methods, however to date there exists no systematic method of assessment (Alvarez et al., 2011; Kim et al., 2015).

A number of techniques have been developed over the past two decades, and are still used, to estimate HM bioavailability in soil; including diffusive gradient in thin films (Agbenin and Welp, 2012; Menegário et al., 2017; Parker et al., 2016; Ren et al., 2015), ion exchange (Ge et al., 2005; Qian and Schoenau, 2002), single-step extractions (Kim et al., 2015; Pinto et al., 2015; Sakan et al., 2016), and sequential extractions (Cox et al., 2013; Fernández-Ondoño et al., 2017; Palumbo-Roe et al., 2013; Reis et al., 2014; Sungur et al., 2014).

Sequential extractions, in particular, are simple low cost methods, that can be applied to different soil types (Rosado et al., 2016), and can help understanding HM and metalloids leachability, solubility, and mobility (Kaakinen et al., 2015), providing the most information about the fate, transport, and behaviour of HM in soil. However, most studies focus on (1) assessing effects and toxicity of one contaminant in isolation (Cui et al., 2005), (2) using sequential chemical extraction on synthetic models or spiked samples (Kim et al., 2015; Ma et al., 2015) rather than genuine contaminated soil samples (Ma et al., 2015). Limitations associated with these approaches include; metal transfer among phases (Bermond, 1992) when performing sequential extractions resulting in an overestimation of metals concentration and risk, and the inability of an artificial contamination to reproduce the actual geochemistry encountered in real site conditions (Ribeiro and Mexia, 1997). While it is challenging to establish a one-size fits all approach for assessing HM behaviour in contaminated soil, the choice of procedure should be based on a more realistic prediction of elemental mobility and characterisation of their association with the soil matrix.

In this study a modified version of a non-specific sequential extraction method coupled to chemometric analysis known as the Chemometric Identification of Substrates and Element Distributions (CISED), was used and applied to five different genuine contaminated soils. Our objectives were (1) to apply a sequential extraction technique in genuine-contaminated soil samples and identify the common soil phases, (2) to evaluate the spatial distribution of HM/metalloids and potential changes over time in order to ascertain the bioavailability of HM/metalloids and potential risk, (3) to determine the influence of different physico-chemical factors on HM/metalloids solid phase distribution and bioavailability, and (4) to evaluate the effect of co-occurrence of hydrocarbons on HM/metalloids partitioning in soil samples.

The novelty of this study lies in the fact that it highlights the importance of taking into account the effects of a range of environmental stressor conditions (pH, moisture, and temperature) and weathering (time), on HM/metalloids potentially labile fractions; including both dissolved elements (pore water), and exchangeable fraction in genuine co-contaminated soil samples. A special emphasis on the effectiveness of this protocol with multi-contaminated samples of different nature (with and without stabilisation treatment), origins (industrial and rural), and with a wide range of HM contents has also been verified. This information can be used as additional lines of evidence to support risk-based decisions about endpoint remediation and to evaluate potential reuse of remediated soil.

3.2. Materials and methods

3.2.1. Sample collection and preparation

Since soil contaminants are often present in the environment in a complex mixture, including both inorganic and organic compounds as by-products of industrial activities (Wawra et al., 2018), this study investigates five multi-contaminated soil samples.

Three genuine contaminated soils, denoted as Soil 1, Soil 2 (treated), and Soil 3, were collected from a soil treatment facility located in the United Kingdom (UK). Information regarding original location of the soil samples collected, and specific details regarding the treatment applied (Soil 2), were not disclosed to maintain anonymity and confidentiality. Two additional samples were collected from a rural site contaminated by HM/metalloids and diesel range organic (DRO) compounds (EC_{10} - EC_{24}) (Soil 4), and HM/metalloids mineral oil range organic (MRO) compounds (EC_{22} - EC_{34}) (Soil 5). The mutual presence of organic and inorganic contaminants in these soil samples could potentially enhance (or inhibit) HM transport by competitive sorption, where metal-organic complexes are formed, limiting their capacity to interact with soil-surfaces (Wuana et al., 2014). Information about the soil matrix and type of contamination is provided in Table 3.1.

All samples were collected randomly by disturbing sampling soil, up to a depth of 30 cm and immediately stored at 4°C to minimise biological transformation and other chemical reactions. Soil 1 was a sandy loam soil heavily contaminated with tar and HM (petroleum hydrocarbons compounds (PHC) > 1000 mg/kg of soil, HM > 800 mg/kg of soil); Soil 2 was similar to Soil 1 except that the former was stabilised with a cement-binder mixture.

Soil 3 was a sandy loam soil presenting low petroleum hydrocarbon content (PHC < 1000 mg/kg) but high concentration of HM (HM > 6200 mg/kg of soil). In addition, two different rural soils contaminated with HM and diesel (Soil 4), and HM and mineral oil (Soil 5) (PHC < 500 mg/kg of soil, HM < 800 mg/kg of soil) were used.

Table 3.1: Soils samples and soil characteristics used in the mesocosms experimental setup.

Soil	Treatment	Soil type	Contamination type	Soil matrix
Soil 1	Pre- treatment*	Industrial	PHC > 1000 mg/kg (high), HM > 800 mg/kg	Sandy loam
Soil 2	Post- treatment**			
Soil 3	No treatment	Industrial	PHC <1000 mg/kg (medium), HM > 6200 mg/kg	Sandy loam
Soil 4	No treatment	Rural	PHC < 500 mg/kg (low), HM < 800 mg/kg	Clay loam
Soil 5	No treatment			

PHC: petroleum hydrocarbons, HM: heavy metals
* No stabiliser, ** application of cement stabiliser

3.2.2. Mesocosms experimental design

Duplicate soil mesocosms were set up for each condition studied using a 10 L polypropylene bucket. Each bucket was filled with approximately 5 kg of loosely packed soil, and amended with buffer or moisture as according to the experimental conditions described in Table 3.2. All experiments were tested over a 12 month period. Different temperature conditions were simulated by storing samples in controlled temperature rooms at 20°C and 4°C. Those experiments treated under outdoor conditions were placed outside and subject to seasonal UK temperature variations. Soil samples were amended with a mixture of sulphuric acid and water to achieve different pH conditions. Redox reactions are a relevant aspect of soil chemistry as they can affect speciation and solubility of heavy metals and metalloids in soil, altering the biochemistry of soils (Kuhlbusch and Crutzen, 2018; Tuor, 1990). Therefore this experiment was conducted in presence of atmospheric O_2 for all the soil samples and all the conditions tested. Moisture content was maintained by adding deionized water up to 20 and 70% of the soils' maximum water holding capacity (WHC) and moisture content was re-assessed bi-monthly. The moisture content for Condition 6, which was kept outdoors, was not altered. Soil samples were taken from each mesocosm at 0, 6, and 12 months' time. All samples were analysed for pseudo-total and bioavailable HM content.

Table 3.2: Experimental design, conditions applied to all soils.

Condition	pH	Temperature	Moisture content
Cond 1	Buffered to 6-7	20°C	As received
Cond 2	Constant acid rain simulation (pH 4.5)	20°C	As received
Cond 3	As received	20°C	20% WHC
Cond 4	As received	20°C	70% WHC
Cond 5	As received	20°C	As received
Cond 6	As received	Outdoor	As received
Cond 7	As received	4°C	As received

Cond: condition, WHC: water holding capacity

3.2.3. Physico-chemical characterisation

Soil samples were sieved using a 2 mm mesh to separate large particles (e.g. roots, stems, and pebbles). Each soil sample was divided and processed for analysis in the following way: (1) 5 g of sample was used for dry matter and water content analysis, (2) a volume of 225 cm^3 of sample was used for water holding capacity measurement. Additionally, a large aliquot of each soil sample, approximately 500 g, was air dried for 7 days to perform multiple analysis where individual air-dried samples were used as follow: (1) 10 mL of sample (measured with 10 mL brass scoop) was used for pH analysis, (2) 10 mL of sample was used for particle size distribution, (3) 5 g of sample was used for loss of ignition (LOI), (3) 0.001 mg was used for total Nitrogen (TN) and total carbon (TC), (4) 5 g of sample was used for total phosphorous (TP) and available phosphorous (AP).

For dry matter and water content analysis, 5 g of fresh soil samples were weighted in a crucible and dried at 105°C ± 5°C for 24 hrs; the difference in mass of an amount of soil before and after drying was used to calculate the dry matter and water contents on a mass basis. Maximum water holding capacity was determined according to ISO 11274 (1998). Soil samples were air-dried and then flooded on a wetting-up bath for 7 days; the mass recorded was used to determine the moisture content at saturation.

Soil pH was measured using a pH meter. Samples were prepared by adding distilled water to create a slurry (1 part soil: 5 parts water). Samples were shaken for 60 min and allowed to equilibrate for an additional 30 min before pH was measured (ISO 10390:2005).

Particle size distribution was determined by the sieving and sedimentation method. In short, soil organic matter was discomposed with hydrogen peroxide, and the resulting slurry dispersed with a buffered sodium hexametaphosphate solution, then the different particle size fractions was determined by a combination of sieving and sedimentation (ISO 11277:2009).

The soil organic content was determined by loss of ignition (LOI); air-dried soil was dehydrated at 105°C then ashed at 450°C, loss on ignition is expressed as a percentage of the dehydrated sample (British Standard BS EN 13039:2000).

Total carbon and total nitrogen in soil material were determined by heating to a temperature of at least 900°C in the presence of oxygen gas, the amount of nitrogen and carbon is then measured by a thermal conductivity detector (TCD) (British Standard BS EN 13654-2:2001).

Total phosphorous was measured with a hydrochloric/nitric acid mixture extraction; the phosphorus content was then determined by a spectrometric measurement in solution (ISO 11047:1998). Available phosphorous was measured by treating the soil with a 0.5 mol/L sodium hydrogen carbonate solution at pH 8.5, the extract is then analysed by a spectrometric method (ISO 11263:1994).

3.2.4. Extraction and quantification of total PHC

The method used to determine total petroleum hydrocarbons compounds (PHC), including both aliphatic (ALK) and aromatic (PAH) fractions in soil, was based on the Risdon et al. (2008) procedure. Briefly 2.5 g of soil were weighted and chemically dried with 2 g anhydrous sodium sulphate. At the same time as weighing samples for extraction, moisture content was measured to provide the appropriate correction factors. The chemically dried samples were extracted for PHC content with a mixture of 15 mL of dichloromethane:hexane sonicated for 20 min at room temperature, and shaken at 150 rpm for 16 h. On the following day, samples were again sonicated for 20 min at room temperature and centrifuged at 2000 g for 10 min to separate the solid and liquid fractions. The liquid fraction was then cleaned onto a 6 mL SPE DSC-Si silica tubes, concentrated to dryness (on ice) under a gentle stream of nitrogen, and re-suspended in 0.5 mL dichloromethane:hexane (1:1) with addition 0.5 mL of internal standards comprised of a deuterated alkanes mix ($C10^{d22}$, $C19^{d40}$ and $C30^{d62}$) and deuterated PAH mix (1,4-dichlorobenzene^{d4}, naphthalene^{d8}, anthracene^{d10}, chrysene^{d12}, and perylene^{d12}) at 10 µg/mL each, respectively. Concentration of petroleum hydrocarbons were identified and quantified by gas chromatography-mass spectrometry (GC-MS) using an Agilent gas chromatograph coupled to a Turbomass Gold mass spectrometer operated at 70 eV in positive ion mode. The column used was a Restek fused silica capillary column (30 x 0.25 mm internal diameter) coated with RTX®-5MS (0.25 µm film thickness). Splitless injection with a sample volume of 1 µL was applied. The oven temperature was increased from 60°C to 220°C at 20°C/min then to 310°C at 6°C/min and held at this temperature for 15 min; for a total run time of 38 min. The mass spectrometer was operated using the full scan mode (range m/z 50-500) for quantitative analysis of target aliphatic and aromatic hydrocarbons. For each compound, quantification was performed by integrating the peak at specific m/z. External multilevel calibrations were carried out using alkane (standard solution (EC_8-EC_{40}) Sigma Aldrich, Dorset, UK) and PAH (EPA 525 PAH Mix A; Sigma Aldrich, Dorset, UK) standards, the concentration of which ranged from 2.5 to 50 µg/mL respectively. For quality control, blank controls and a 500 µg/mL diesel standard solution (ASTM EC_{12}-EC_{60} quantitative, Supelco) were analyzed every 20 samples. The variation of the reproducibility of extraction and quantification of soil samples were determined by successive injections ($n = 7$) of the same sample and estimated to ± 8%. In addition, duplicate reagent control and reference material were systematically used. The reagent control was treated following the same procedure as the samples without adding soil sample. The reference material was an uncontaminated soil of known characteristics, and was spiked with a diesel and mineral oil standard at a concentration equivalent to 16,000 mg/kg. Relative standard deviation (RSD) values for all the soils were < 10%.

3.2.5. Modified sequential extraction and pseudo-total element digestion

Assessing metal partitioning through the non-specific sequential extraction with Chemometric Identification of Substrates and Element Distributions (CISED) can (1) limit the re-adsorption and re-distribution of elements among phases during extraction, often happening in genuine contaminated samples, (2) overcome problems linked with “operational speciation”, where soil phases (operationally defined metal forms) are identified strictly based on their response to the extraction reagents, which not necessarily reflects the behaviour of natural samples (Adamo and Zampella, 2008).

In this work, a modified procedure for sequential extraction was conducted similar to that described in Cave et.al. (2004). Soil samples of approximately 2 g were consecutively extracted by addition of 10 mL of an extraction solution (Table 3.3) which contained an increasing concentration of nitric acid (i.e. from 0 to 5 M). After adding 10 mL of extraction solution, samples were mixed on an end-over-end shaker for 10 minutes, and the liquid phase was recovered via centrifugation (4350 g for 5 min) and used for analysis; the soil pellet was resuspended again with the following extraction solution. Each extraction solution (7 solutions) was used twice to obtain a total of 14 extracts (10 mL). As highlighted in Table 3.3, in the last 8 extractions (E_7 to E_{14}) increasing amount of H_2O_2 were added to the extraction solutions to enhance degradation of organic matter and favour the dissociation of Fe–Mn oxides (Filgueiras et al., 2002). However addition of H_2O_2 caused a high release of gas in the genuine contaminated soil samples, rendering the centrifugation and separation phase not possible without losing significant amount of soil material. We hypothesised that this was due to both (1) high calcium and phosphorus content typical of the content of cement-based stabilisers (Saeed, 2012), (2) the high reactive organic content soils caused by the presence of co-contamination (petroleum hydrocarbons), often observed in multi-contaminated soil matrix, such as the industrially-polluted soils used in this study. Therefore in our approach we implemented a modified version of the Cave et.al. (2004) extraction procedure, which required the inclusion of an additional step. Hence, when 10 mL of solution 4 (9.75 mL of 0.10 M HNO_3, and 0.25 mL of H_2O_2 100 volumes > 30% w/v) were added to the samples, tubes were placed in a water bath for 30 min at 70ºC, to favour the reaction and limit the gas production. This additional step was sufficient to reduce the re-mixing of the solution allowing a proper separation when centrifuging.

Table 3.3: Sequential extraction steps.

Extraction number	Solution number	Concentration (M)	Deionised water	Volume HNO_3 (mL)	Volume H_2O_2 (mL)	Total volume (mL)
E_{1-2}	Sol 1	0.00	10.0	0.00	0.00	10.00
E_{3-4}	Sol 2	0.01	0.00	10.00	0.00	10.00
E_{5-6}	Sol 3	0.05	0.00	10.00	0.00	10.00
E_{7-8}	Sol 4	0.10	0.00	9.75	0.25	10.00
E_{9-10}	Sol 5	0.50	0.00	9.50	0.50	10.00
E_{11-12}	Sol 6	1.00	0.00	9.25	0.75	10.00
E_{13-14}	Sol 7	5.00	0.00	9.00	1.00	10.00

E: extraction, Sol: solution, M: molar, HNO_3: nitric acid, H_2O_2: hydrogen peroxide

The pseudo-total element digestion was performed according to the ISO 11047 method with aqua regia (ISO 11047:1998). Briefly, 0.5 g of soil was extracted with 8 mL hydrochloric/nitric acid mixture using a microwave digestion system. The extract was then filtered with 0.45 µm 25 mm nylon syringe filters and made up to 50 mL volume with water.

All pseudo-total and sequential solutions extracted were filtered with 0.45 µm 25 mm nylon syringe filters and diluted 4 times with 1% HNO_3 before analysis by inductively coupled plasma mass spectrometry (NexION® 350D ICP-MS, Perkin Elmer). The ICP-MS was calibrated using a mixture of both major (Ca, Fe, K, Mg, Mn, Na, S, Si, P) and trace (Al, As, Ba, Cd, Co, Cr, Cu, Hg, Li, Mo, Ni, Pb, Sb, Se, Sr, V, Zn) elements. The concertation ranges were 1, 5, 15, 20, 40 µg/mL for major elements and 0.01, 0.1, 0.5, 1, 2 µg/mL for trace elements. In both cases, working standards were prepared in matching sample matrix solutions (nitric acid 1%). Calibration standards and samples extracts were spiked with the following mix of four internal standards: Sc, Ge, Rh, and Bi. ICP–MS was calibrated after each sample (14 sequential extracts). Limits of detection (LOD) were estimated as the concentrations corresponding to three times the standard deviation of measurements of analytes in a series of blank solutions (MilliQ water with 1% HNO_3 and buffer solution) (n = 40) treated the same way as the samples. The results are given in Appendix in Table 3.6. Additionally, acid blanks (1% nitric acid), digestion blank, and guidance materials (BGS_{102}) were analysed every batch of 7 samples along with an adequate rinse time programmed in between samples; to monitor blank contamination, sensitivity, operating conditions, and extraction's accuracy. For the quantitative analyses, no blank correction was necessary as the calibration standards and samples were treated exactly in the same way adding the same amount and concentration of HNO_3. The blank value was therefore taken into account in the calibration curve.

Mean repeatability of guidance materials (BGS_{102}) (expressed as relative standard deviation %) was lower than 6 and 8% for sequential and aqua regia digestion respectively. All elements' concentrations have been converted into mg/kg extracted from the soil-solid matrix. Descriptive statistics for the metals and metalloids concentrations (expressed in mg/kg) is presented in Appendix in Table 3.5.

Soil samples extraction recoveries obtained with the CISED method compared with pseudo-total metal concentration averages were lower. The reason these extraction recoveries are not 100% is that the CISED extraction protocol mainly targets the easily soluble surface coatings, without attacking the silicate matrix of soil. However, assessing the pore-water, carbonates, and oxides fractions, it is sufficient to make assumption on HM and metalloids fractionation, as in contaminated soil the input of HM (anthropogenic contamination) is mostly provided by non-silicate bound forms (Wuana et al., 2014).

3.2.6. Modelling

Data obtained from the HM/metalloids sequential extraction were analysed using MatLab$^{®}$ (Version R2015a) following the protocol developed by Cave et al. (2004). The non-specific sequential extraction method named Chemometric Identification of Substrates and Element Distributions (CISED) assumes that the chemical composition data for each extract is made up from different proportions of the physico-chemical components in the soil. Since the algorithm is designed to identify the number of components based on principal component analysis and by Varimax rotation (Giacomino et al., 2011), for the purpose of the modelling, the soil samples extracted were grouped according to soil matrix type (Soil 1, Soil 2, Soil 3, Soil 4, and Soil 5) and metals concentration (low, medium, and high contamination), in order to derive a more homogeneous data matrix for processing. Data processing of the sequential extraction was carried out on 5 multiple data matrices, each comprising the elemental extraction data (25 elements) for the 14 extracts for each test soil, over 7 conditions at 3 sampling times (294 rows of data per matrix). The data were processed using a self-modelling mixture resolution (SMMR) algorithm in MatLab® (Cave et al., 2004). The algorithm output is based on three main data matrices: profile (PRF), distribution (DST), and composition (CMP). The PRF of each modelled soil component is calculated as the overall amount extracted (mg/kg) in each of the 14 extractions. The DST expressed in mg/kg represents the concentration of each element across the different soil components identified by the model. The CMP data is expressed as a percentage of each element present in the identified component. Both PRF and CMP are then used to calculate the single element concentrations (mg/kg). More details are provided in paragraph **3.7 Annex**.

3.2.7. Cluster analysis and complex associations between variables

Modelled soil components and element distribution data, obtained from the MatLab® algorithm, have been post-processed in RStudio to create a matrix, which has been further categorised using a clustering methodology, and visualised in a heatmap as previously described by Wragg et al. (2014) and Cox et al. (2013). More details are provided in paragraph 3.7 Annex.

The SMMR algorithm produced distinct sets of physico-chemical phases for each of the 5 multiple data matrices analysed. Briefly, representative samples for each soil were selected and arranged in a data matrix containing on the left side the elements composition (CMP) (Na, Mg, Al, Si, P, S, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Li, Mo, Cd, Sb, Sr, Ba, Pb, Se) expressed as percentage, and on the right side the extraction profile (PRF) of each soil under investigation (expresses in mg/kg). The matrix was then imported in RStudio and subjected to hierarchical clustering where the data were mean centred and scaled with Euclidean distance and linkage using Ward's method (Ward, 1963) and the '*Agnes*' function in the cluster package (Maechler et al., 2012) in RStudio (Version 1.1.423 – $^{©}$ 2009-2018 RStudio, Inc.). Clustering results were visualized using a heatmap (presented in Figure 3.1 for overall soils and treatments, and from Figure 3.4 to Figure 3.8 in Appendix for individual soils and treatments) created using *ggplot2*, *reshape2*, *grid*, and *ggdendro* packages (Wickham, 2007; Kahle and Wickham, 2013), where each row represents a physico-chemical soil components found for a given soil. Soil name is indicated by the 'soil name' previously used in Table 3.1 (Soil 1, Soil 2, Soil 3, Soil 4, and Soil 5), followed by the elements name (e.g. Ca, Ca-K-Si, Fe-Mg) that make up >10% of the physico-chemical component composition. The hierarchal clustering obtained was used in parallel with chemical profile to provide interpretations and classify the components into common distinct soil phases; pore water (readily available or bioavailable), carbonates (potentially available with time), and oxides (bounded, non-available) and to assess the partitioning and bioavailable concentrations of HM/metalloids in soil.

3.2.8. Data analysis and descriptive statistics

In the context of this research, PERMANOVA was used to investigate the significance and relationship between conditions tested (Cond 1, Cond 2, Cond 3, Cond 4, cond 5, Cond 6, and Cond 7), and PHC concentration (high, medium, low) on (1) pore water, (2) exchangeable, and (3) non-exchangeable fractions of inorganic contaminants in the soil samples. Permutational Multivariate Analysis of Variance (PERMANOVA) is a Multivariate ANOVA with permutations; it was applied by using "*adonis*" function of the vegan library in R Studio (Oksanen et al., 2011).

Additionally using the "*describe"* function of *Hmisc* package (Harrell, 2018), descriptive statistics for the metals and metalloids concentrations in the different fractions and the 40 blank measurements and limit of detection (LOD) were calculated, and are provided in Appendix in Table 3.5, Table 3.6, and Table 3.7.

To establish a direct or indirect correlation between HM, which might be indicative of similar elements behaviour in multi-contaminated soil, univariate linear regression analysis was used by applying Pearson correlation coefficient with the “*corrplot*” package in R Studio (Oksanen et al., 2011). The output returned a correlation matrix for each soil (available in Appendix Table 3.8, Table 3.9, Table 3.10, Table 3.11, and Table 3.12) which allows assessment of relationships between all the HM.

3.3. Results and discussion

3.3.1. Soils characteristics and pseudo-total HM and PHC content

All soil samples physico-chemical properties, HM/metalloids pseudo-total concentrations, and hydrocarbons total content are summarised in Table 3.4.

Soils 1, 2, and 3 are representative of industrial sites with low total nitrogen (700, 800 and 1200 mg/kg) and phosphorus (450, 430 and 500 mg/kg) contents as well as alkaline pH; conditions often found in urban settings (Vodyanitskii and Savichev, 2017). Soil samples collected at the treatment facility (Soil 1, 2, and 3) belonged to a manufacturing gas plant, where often in addition to co-presence of PAH and heavy metals, the coal ash and wood are generally characterised by alkaline pH (Hatheway and Speight, 2017). Soil 2 had high calcium and phosphorus content typical of the content of cement-based stabilisers (Saeed, 2012). For the majority of metals, alkaline conditions can potentially increase their adsorption (Horváth et al., 2015), and reduce their mobility and thus limiting risk of exposure. However, some metals (Cr (VI), Mo (V)) and metalloids (e.g. As and Se) are mainly present in stable oxyanions forms (e.g. arsenate, selenite, vanadate, Cr (VI) chromate, and molybdate) under alkaline pH. Oxyanionic species are negatively charged and can be more mobile compared to the cationic species due to their high solubility and lack of adsorption on the surface of soil minerals (Cornelis et al., 2008).

Soil 4 and Soil 5 texture was clay loam (sand content < 35%), and pH 7.0 - 8.0, and presented a higher nutrient content overall. In these soils the presence of soil particles smaller than 0.002 mm, such as clay, could contribute to increase the HM retention capacity due to the larger specific surface area (Ander et al., 2011).

The C/N ratio of Soil 1 and 2 was more than 5 times higher than for the rural contaminated soils (Soil 4 and 5), because of the larger amount of hydrocarbons present in the industrial contaminated soil. The high organic content might also be responsible for higher HM retention (Almeida et al., 2008; Millward et al., 2004). For all the soils investigated, the pseudo-total metal concentrations of Pb and Zn exceeded 8 times and 4 times the UK Soil Guideline Values (SGV) and the European Directive 86/278/EEC; with an average pseudo-total concentration of respectively 3400 mg/kg (Pb) and 2130 mg/kg (Zn) for the most contaminated sample (Soil 3).

Descriptive statistics for the total concentration of ALK, PAH, and PHC compounds are provided in Table 3.4, where total maximum concentrations values in rural contaminated soil samples were half compared to the most contaminated industrial sample (Soil 1) ; 1295, 552, 286, 592, 427 mg/kg PHC for Soil 1, Soil 2, Soil 3 , Soil 4 and Soil 5, respectively.

Table 3.4: Physico-chemical properties of the five soil samples including pseudo-total heavy metals/metalloids and total petroleum hydrocarbon concentrations.

		Industrial			Rural	
Characteristics	**Analysis**	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5
Nutrients	Total N (%)	0.07	0.08	0.12	0.23	0.25
	Total C (%)	4.00	4.14	3.87	2.39	2.78
	C:N	57.28	52.86	31.71	10.44	10.93
	Total P (mg/kg)	453.37	433.73	499.60	798.59	801.12
	Available P (mg/kg)	31.55	30.55	42.18	35.22	36.72
Physical properties	70% of WHC_{max} (% m/m)	21.92	20.37	19.64	39.21	40.02
	20% of WHC_{max} (% m/m)	6.26	5.99	5.61	11.20	12.00
	Dry matter content W_{dm} (%)	78.40	76.37	79.88	68.24	68.28
	Water content (%)	27.55	30.94	25.19	46.53	46.46
Chemical properties	pH	9.71	9.56	9.22	7.99	7.54
	LOI (%)	4.28	3.97	5.44	5.99	6.49
Stone/gravel content	% > 5.5 mm	25.97	23.47	20.13	0.00	0.00
	% 5.5 mm< > 2 mm	24.54	27.00	38.73	24.69	23.57
	% < 2 mm	49.48	49.54	41.15	75.31	76.43
Particle size	% 0.6 - 2 mm (Coarse sand)	11.88	13.65	16.86	3.55	4.36
	% 0.2 – 0.6 mm (Medium sand)	29.86	33.41	34.58	14.90	14.46
	% 0.06 - 0.2 mm (Fine sand)	30.37	27.04	20.24	11.70	11.29
	Overall sand content	72.11	74.10	71.68	30.16	30.10
	% 0.002 mm - 0.06 mm (Silt)	19.67	16.70	16.14	40.57	36.07
	% < 0.002 mm (Clay)	8.22	9.20	12.17	29.28	33.83

		Industrial			Rural	
Characteristics	**Analysis**	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5
HM and metalloids pseudo-total concentrations (mg/kg) *	As	1.38 - 22.05	4.13 - 22.4	3.31 - 46.99	3.95 - 25.89	5.88 - 33.29
	Cd	0.08 - 3.6	0.26 - 2.29	0.27 - 1.9	0.05 - 0.4	0.08 - 0.33
	Cr	3.08 - 44.02	8.7 - 99.99	5.2 - 51.23	7.7 - 85.17	19.93 - 61.81
	Cu	5.19 - 169.82	10.42 - 99.08	9.25 - 128.08	4.01 - 34.28	8.47 - 30.49
	Hg	0 - 1.63	0 - 5.68	0 - 0.24	0 - 0.06	0 - 0.04
	Ni	2.16 - 29.76	6.39 - 34.88	4.1 - 36.54	7.04 - 49.14	10.64 - 34.44
	Pb	18.49 - 794.1	9.21 - 672.67	337.38 - 6603.57	11.51 - 66.85	20.44 - 59.73
	Se	0.42 - 45.24	0.61 - 44.95	0.55 - 4.11	0.81 - 4.08	0.97 - 3.97
	Zn	15.42 - 272.17	66.2 - 281.63	277.81 - 3527.2	30.03 - 156.62	44.57 - 130.83
PHC Total concentrations (mg/kg)*	TOT ALK	83.2 - 496.1	49.5 - 147.9	81.3 - 323.0	78.5 - 323.0	78.8 - 184.2
	TOT PAH	59.1 - 796.1	135.0 - 405.4	4.9 -140.8	0.34 - 267.0	0.5 - 293.9

*across all condition tested and time points analysed

N: nitrogen; C: carbon; P: phosphorous, WHC: water holding capacity; LOI: loss of ignition; As: arsenic; Cd: cadmium; Cr: chromium; Cu: copper; Hg: mercury; Ni: nickel; Pb: lead; Se: selenium; Zn: zinc; HM: heavy metals; PHC: petroleum hydrocarbons compounds; ALK: alkanes; PAH: polycyclic aromatic hydrocarbons.

3.3.2. HM solid phase distribution

Soil samples were subjected to the CISED sequential extraction procedure to determine the physico-chemical soil components (substrates) being extracted from the soil (e.g. carbonates, clays, exchangeable phases); and the solid phase distribution of HM/metalloids between each identified soil component. Figure 3.1 indicates the presence of 10 distinct physico-chemical clusters (blocks) which have been further grouped as: (1) Pore water, (2) Carbonates (low and high carbonates) and (3) oxides (Al-oxides, Mn-Oxides, and Fe-oxides) (Figure 3.2). HM chemical partitioning results, obtained by sequential extraction, are essential to understand their mobility; the results obtained show that the extractable amounts obtained from each fraction can vary widely (Figure 3.1 and Figure 3.2). The first physico-chemical cluster (Figure 3.1, clusters 3, 5, and 8) is dominated by the presence of Na, Ca, S, and K, and was extracted by the initial step of CISED, when deionized water (E_1-E_2) or low acid concentration (HNO_3 0.01 M, E_3-E_4) was used. This step was used to extract elements that are soluble, highly mobile, and most likely associated with the pore water fraction. The second physico-chemical cluster, Ca dominated, is well identified in these samples (in particular Soil 2) and mainly composed of Ca and, to lesser extent, of K, Si, and S (Figure 3.1, clusters 1, 2, 7, and 9). The elevated presence of Ca in this fraction is linked to the fact that common binders are calcium-based. This fraction can be divided into low carbonate (extracted with low acid strength HNO_3 0.05 – 0.1 M, E_5-E_6, E_7-E_8) and high carbonate (extracted with HNO_3 0.5 M, E_9-E_{10}). The third physico-chemical cluster identified through the modelling corresponds to oxides including Mn-oxides, Al-oxides, and Fe-oxides (Figure 3.1, clusters 4, 6, and 10). This cluster was associated with elements (e.g. Mn, Al, and Fe) released after H_2O_2 addition and dissolved by the concentrated HNO_3 (E_7 to E_{14}). These elements were extracted with very strong acid concentrations (E_9-E_{14}) and likely associated with the clay components of the soil, therefore being overall immobile under natural environmental conditions.

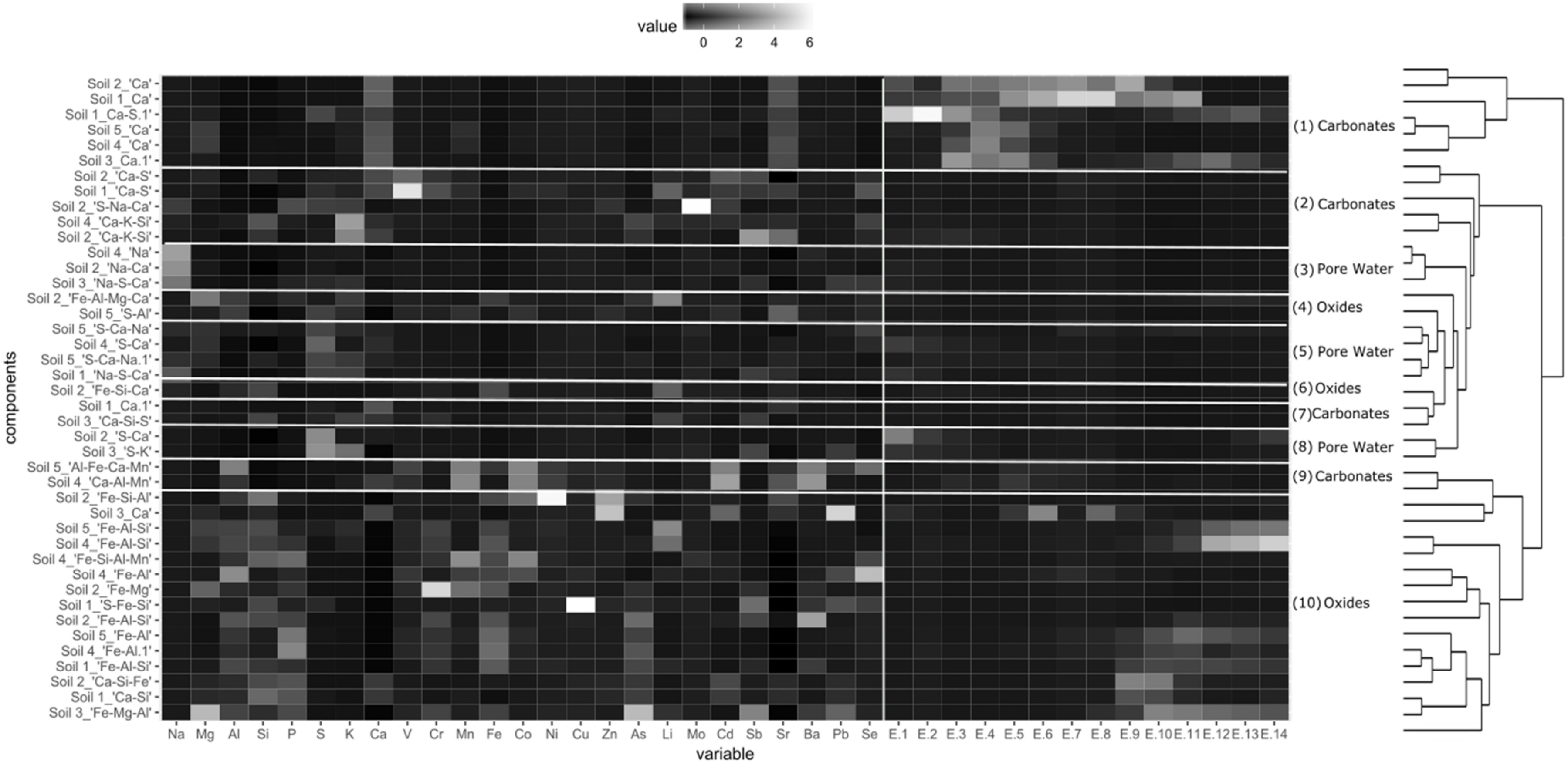

Figure 3.1: Heatmap and associated clustergram for CISED extraction data for a selection of test soils. The horizontal white lines divide the heatmap into the 10 different clusters, The vertical white line divides the elements composition data on the left side (e.g. Na, Mg, Al) from the extraction number data (E_1 to E_{14}) on the right side. A high proportion of each component and an indication of its composition are shown by a white or pale grey colouration with a low proportion as dark grey or black.

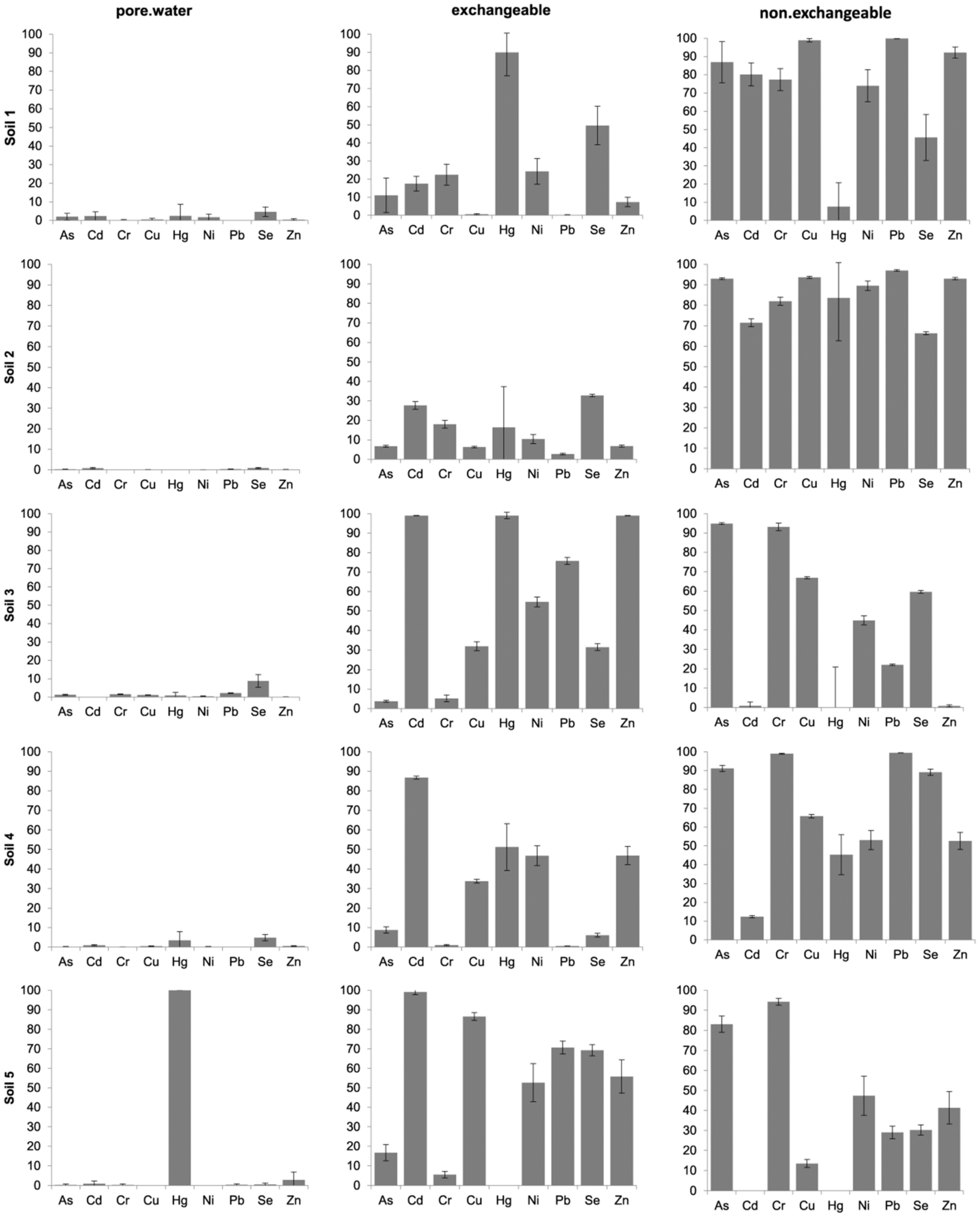

Figure 3.2: Overall HM/metalloids distribution for Soil 1, Soil 2, Soil 3 , Soil 4, and Soil 5 across the 7 conditions for all sampling times (T0, 6, and 12 months) expressed as percentage. Concentrations have been averaged across time and conditions in order to provide an overview of the overall metal behaviour.

3.3.3. Relationship between HM and metalloids distribution and bioavailability

The compositional data and distribution of HM and metalloids, for all soil samples in these fractions, were obtained by transforming each original raw concentration (i.e. mg/kg) into proportions of the total (100%) and are shown in Figure 3.2**.** Concentrations have been averaged across time and conditions in order to provide an overview of the overall metals behaviour in the five soils types (Soil 1, Soil 2, Soil 3, Soil 4, and Soil 5) (see for details Appendix, Table 3.7). The most mobilised elements in the exchangeable fraction were the following: Hg and Se for Soil 1; Cd and Se for Soil 2; Cd and Hg for Soil 3; Cd for Soil 4, and Cd, Cu for Soil 5. Conversely, As and Cr showed the least mobility. The order of mobility of the metals in the exchangeable fraction was as follows: Hg > Se > Ni > Cr > Cd > As > Zn > Cu > Pb (Soil 1); Se > Cd > Cr Hg > Ni > As > Cu > Zn > Pb (Soil 2); Cd – Hg **-** Zn > Pb > Ni > Cu > Se > Cr >As (Soil 3); Cd > Hg > Ni – Zn > Cu > As > Se > Pb > Cr (Soil 4); and Cd > Cu > Pb **-** Se > Zn > Ni > As > Cr > Hg (Soil 5). Interestingly As was the least mobile, while Cd was very mobile at pH > 9 in the industrial soil samples (Soil 1, Soil 2, and Soil 3). Previous literature showed that As adsorption tend to decrease under alkaline conditions (pH > 9) due to the presence of negatively charged H_2AsO_3, $HAsO_3$, and AsO_3 (forming soluble species of As(III)) (Dias et al., 2009). However in these samples, As was found mostly associated with the non-exchangeable fraction (Mn-Al and Fe oxides). Therefore we can assume that the majority of As was present as inorganic oxyanion As(V) forming H_2AsO_4 and $HAsO_4^2$, which is known to strongly interact with oxides (positively charged) in both un-contaminated and contaminated soils (Lin and Puls, 2000; Sarkar, 2002).

In Soil 3, 4, and 5, Cd sorption was limited as the element was predominantly found in the exchangeable fraction which is likely due to the influence of the soil-solid particle distribution on Cd behaviour. Previous studies highlighted that Cd binding on clay minerals is weaker compared to binding to organic matter (Janssen, 1997; Prokop et al., 2003) which was the case for these soils.

In Soil 1, both Mn and Fe were below detection limit in the pore water fraction. Changes in Mn and Fe concentrations, in the pore water, were negligible for Soil 2 and Soil 3. In contrast for Soil 5, both Fe and Mn concentrations decreased overtime in the pore water, suggesting that pore water Fe (II) was oxidised to insoluble Fe (III).

The partitioning, mobility, and distribution of HM and metalloids assessed in these soil samples can provide different level of information, such as (i) information on the origin of the contamination, (ii) the effectiveness of cement-stabiliser and potential reuse of soil material, (iii) the limitation often associated with guideline values thresholds. HM/metalloids partitioning can provide information on the origin of the contamination, where often HM from anthropogenic sources usually bind to the exchangeable fractions (Frentiu et al., 2008; Hu et al., 2006; Iwegbue, 2015); as observed for Soil 1 where 90, 50, and 25% of Hg, Se and Ni were distributed in the exchangeable fraction. Over 33, 28, and 20% of Se, Cd, and Cr (Soil 2), and over 80% of Cd, Hg, Pb, and Zn (Soil 3) were found in the exchangeable fraction; while Soil 4 and 5 showed lower values below 50% for Zn and Ni. Nevertheless, HM and metalloids present in the exchangeable fraction can also become mobilised over time (Baran and Tarnawski, 2015) and should therefore be considered for a more complete assessment of the entire pool of mobilisable elements. However data regarding the soil origin and underline geology must also be considered in order to estimate the weight of the effect of geogenic or (anthropogenic) contribution on HM/metalloids bioavailability (Borgese et al., 2013). Being the soil samples provided anonymously from a treatment facility, no further information on the geology, location, or origins of the contamination were provided.

The potential of re-using soil that has been treated or remediated is a viable and sustainable strategy (Mehta et al., 2018); however concerns regarding safety of the re-used material and the possible further spreading of contaminants still exists. Results obtained from sequential extraction highlighted that even though Soil 1 and 2 showed a similar HM mobility pattern, some of the less mobile elements including Cr, As, and Zn were significantly more associated with the non-exchangeable fraction of Soil 2 (treated with stabiliser). The presence of the cement stabiliser was able to reduce HM solubility, increasing adsorption and incorporation to the porous surfaces, as previously observed in the literature (Jiang et al., 2006; Johnson, 2004). Since no information was available on the type of cement stabiliser used in Soil 2, it was not possible to draw further conclusion on the mechanism dominating the fixation of HM. Ultimately, whilst providing information on the target HM metalloids for risk assessment, total concentration cannot provide sufficient information about elements mobility and bioavailability in soil; highlighting that soil guideline values (SGV) are useful, but their application in the detailed quantitative risk assessment is limited. Sequential extraction instead provided specific information on the solid-phase fractionation of HM/metalloids in soil (Cox et al., 2013; Palumbo-Roe et al., 2013; Reis et al., 2014), therefore allowing relevant stakeholders and regulators to make informed assumptions on bioavailability for risk assessment (Kaakinen et al., 2015).

3.3.4. Behaviour of exchangeable metal fraction over time

Average HM/metalloids content and distribution across the three fractions (pore water, exchangeable, and non-exchangeable) in the five soil samples for the 7 conditions tested are presented in Figure 3.3. In Soil 1, no changes were found for Cd, Cr, Cu, Ni, Pb, and Zn over time. On the other hand, As and Se concentrations changes can be explained by the good As-Se-metals correlation (Appendix, Table 3.8) suggesting that As and Se metals could come from sulphides. The mobility and toxicity of As and Se depends on their redox state. In neutral to alkaline soils, As and Se mobility increases because of the formation of arsenate ($H_2AsO_4^-$) and selenate (SeO_4^{-2}) ions (Soukup, 2013), which weakly bond to oxides and other minerals. For Soil 2, all the HM/metalloids showed little or no difference in distribution across the three sampling events (T0, T6, and T12). Most of HM/metalloids were almost entirely found in the non-exchangeable fraction. Such behaviour can be explained because either (1) the addition of the stabilisers was effective in retaining the contamination over time, as highlighted in previous paragraph, or (2) these metals were mainly associated with clay related elements (e.g. Mn, Al, and Fe) released after H_2O_2 addition, and dissolved by the concentrated HNO_3. This suggests that Pb, Cu, and Zn are unlikely to become available with time. For example, Pb quantities present in the exchangeable fraction (Soil 2) fell within the range of the median concentration for UK urban topsoil G-BASE data (48-128 mg/kg), with a 75th percentile of 253 mg/kg (Ander et al., 2011) (Appendix, Table 3.7).

Similarly, As, Cd, Cr, and Zn in Soil 3 were not affected by ageing. In particular As (93%) and Cr (81%) were almost exclusively present in the non-exchangeable fraction. Pb, Cu, Ni, and Se distributions barely changed overtime being exclusively in the exchangeable fraction. Even though Pb is one of the main contaminant of concern with high concentration in exchangeable fraction (average conc. 1500 mg/kg), its concentration persisted over the 12-month incubation. This is probably due to the formation of insoluble Pb compounds such as phosphates, carbonates, and oxides typically formed when the pH is above 6 (Wuana and Okieimen, 2011). Zn was almost entirely associated with the exchangeable fraction: it is well known to generally display strong affinity to the non-residual fraction of the soil (Naji et al., 2010). Soil 4 and 5 presented a very similar distribution with the exception of Cu, Hg, Pb, and Se, which were more exchangeable in Soil 5. In both soils and similarly to Soil 2, As, Cd, Cr, Cu, Pb, and Se were not affected by ageing. The increase of Zn concentration in the exchangeable fraction observed for Soils 5 and 6 during mesocosms incubation was attributed to Zn affinity for hydroxides and carbonates, which can promote remobilization of this element in soil (Kumar, 2016). Ni also showed trends similar to Zn in metal release and its concertation increased in the mobile fraction after incubation. Ageing, has been previously identified as a main driver for leachability of metals in soil. However, an inverse relationship between time of residence in soil and amount of metal leachable exists: the shorter the time, the larger is the amount that can be released (Kumar, 2016). Regarding the time when the contamination occurred, there was no information associated to the soil samples collected. Nevertheless, it was assumed that contamination in Soils 4 and 5 was more recent when compared to Soils 1, 2, and 3.

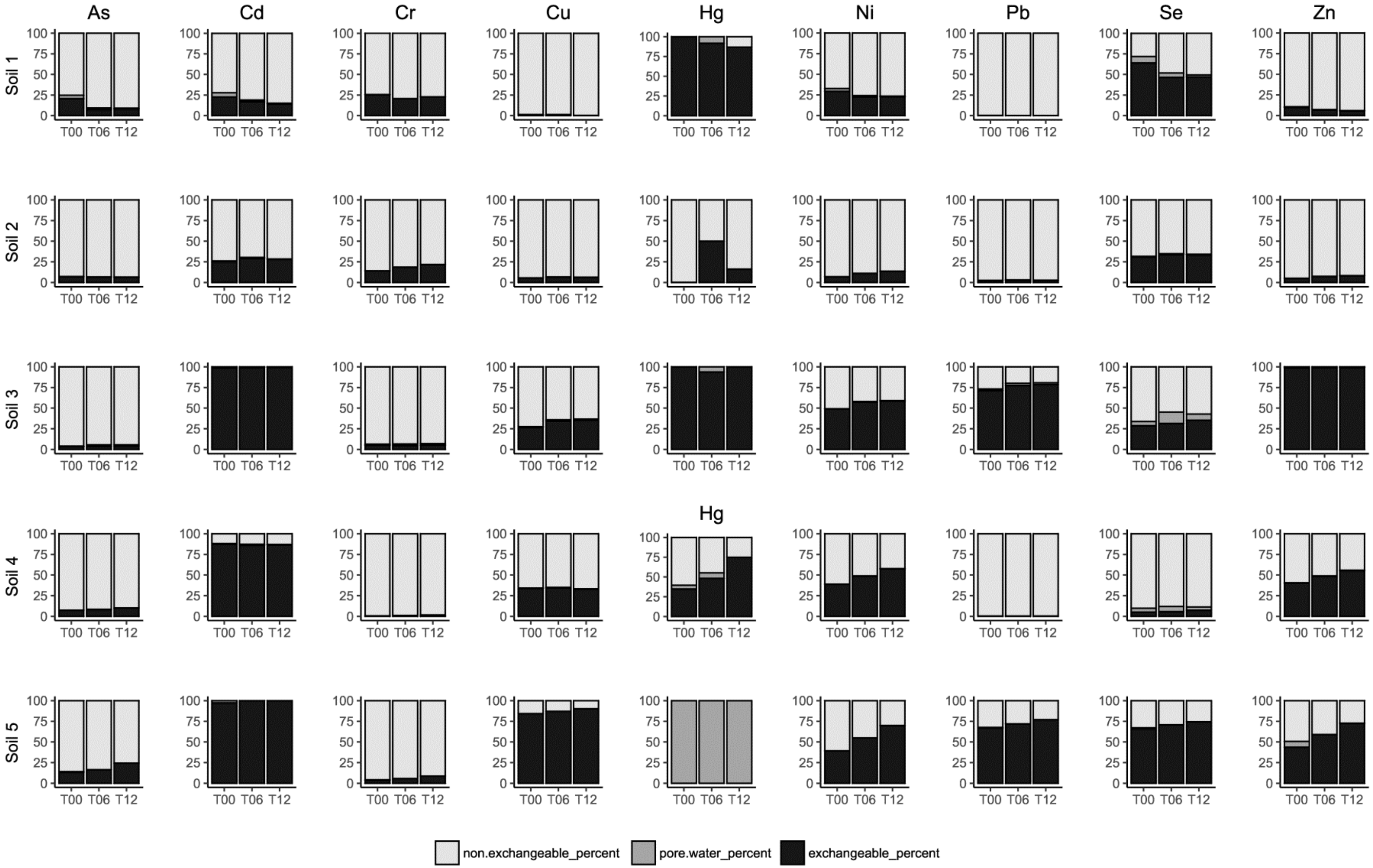

Figure 3.3: Heavy metals and metalloids distribution expressed as percentage (pore water, exchangeable, and non-exchangeable), within the 5 soil samples under the 7 mesocosm conditions tested at T0, 6 and 12 months.

3.3.5. Influence of the environmental parameters on HM and metalloids behaviour and fate

Sequentially extracted fractions were compared to gain a mechanistic understanding on how measurements varied when different conditions were applied at the different times. After evaluating the concentration and distribution of the HM/metalloids in the 5 soils, a detailed investigation of the 3 metal pool fractions behaviour under the 7 mesocosms conditions was carried out. No significant differences were found for the same soil samples exposed to different pH (Conds 1 and 2), different moisture content (Conds 3 and 4) and different temperature (Conds 6 and 7) (data not shown).

PERMANOVA was used to investigate the significance and relationship between conditions tested (Cond 1 - 7), and PHC concentration (high, medium, low), on (1) pore water, (2) exchangeable, and (3) non-exchangeable fractions of inorganic contaminants in the soil samples. For all industrial contaminated samples (Soil 1, Soil 2, and Soil 3) no significant effect ($p > 0.5$) of conditions, nor PHC concentrations on pore water, exchangeable, and non-exchangeable fractions were recorded. This confirms that difference observed in HM partitioning among different fractions, in different soil samples was minimal, and that these soil materials potentially pose low risk to the environment. For both Soil 4 and 5 no significant effect ($p > 0.5$) of conditions, PHC concentrations on pore water concentrations was observed. However, in Soil 4 only, a significant effect of PHC concentrations on exchangeable ($p = 0.001$) and non exchangeable ($p = 0.003$), fractions were found; suggesting that additional factors may play a role in contaminant concentration changes (e.g. degradation of organic contaminants, volatilisation, and interaction with soil organic matter), rather than the condition applied, which did not cause a significant difference between groups. The co-presence of PHC increased the HM/metalloids redistribution into the exchangeable fraction for Zn, Hg, Ni, while no changes were observed for As, Cd, Cr, Cu, Pb, and Se.

While individual compounds in a complex chemical mixture are assumed to have independent sorption behaviour, at high concentrations co-presence of contaminants can influence sorption as a results of changes in the soil-solution equilibrium (Gao et al., 2006). Thus, co-presence of mixed contaminants such as petroleum hydrocarbons and heavy metals may influence/change mobility, behaviour and bioavailability of HM. The degree and type of combined effects obtained from mixtures is highly dependent on both concentration and time of persistence in soil (Wuana and Okieimen, 2011). Most studies report a negative effect of PAH-HM co-occurrence, due to the negative influence of HM on soil microbial community which can hamper the biodegradation. However some other studies highlighted the positive interaction of heavy metals and PAH; Saison et al. (2004), Gao et al. (2006), and Zhang et al. (2011) observed an increase in adsorption of phenanthrene in presence of HM; additionally a positive interaction between Zn, Cd, and phenanthrene towards microbial enzyme activity was observed in Shen et al. (2005) study. Some examples of studies assessing effects of co-contamination are present in literature (Ding et al., 2017; Iwegbue, 2015; Lin et al., 2008), however mechanisms that regulates sequestration, displacement, and partitioning of HM in complex contaminated sites is still poorly understood.

Since pH governs trace metal solubility (low pH decreases sorption and increases bioavailability and mobility), a greater variation in HM/metalloids distribution associated to Conds 1 and 2 were expected: this was not observed in any of the soils tested. Different authors have previously reported that pH has less or no effect on Cu, Pb and Zn sorption (De Matos et al., 2001; Gomes et al., 2001; Katyal and Sharma, 1991). Such behaviour suggests that soil chemical properties (e.g. pH) are not the only parameters affecting HM remobilisation. This finding can be attributed to the addition of mild acidic sulfuric acid solutions (Conds 1 and 2) which were not sufficient to permanently modify the soils’ pH. This acid addition could just cause a transient decrease in pH, and thus revealed the strong pH buffer capacity of these soils. Our finding were consistent to previous results in the literature which highlighted the minor effect on HM leaching behaviour affected by mildly acidic and neutral pH soil values (Du et al., 2014). The lack of changes in the HM stability was more remarkable in samples with higher sorption capacity owing to the presence of porous material such as high clay content (Soil 4) or presence of cement stabiliser (Soil 2), which could have played a role in increasing the retention of soluble HM.

Higher interstitial water (Cond 4) can cause a decrease in soil redox potential and change HM oxidation states (Mukwaturi and Lin, 2015). This influences the fate and transport of metals and, combined with pH, could also control solubility or the formation of coordination complexes. Changes in pH and moisture content (Conds 3 and 4) did not affect however, the behaviour and transformation pathways of the metals in the soils tested. Among the factors that could influence metal distribution, temperature (Conds 6 and 7) has previously been found not to exert any measurable effects on Pb solubility (Kalbasi et al., 1978) or Cd leaching (Basta and Tabatabai, 1992). A similar trend was observed for moisture on paddy soil (Liu et al., 2016).

3.3.6. Descriptive statistics

Descriptive statistics were conducted on the data to identify relationships between HM and metalloids, details are provided in Appendix. A positive correlation coefficient among HM suggests that those elements may share common sources, mutual dependence, and have identical behaviour during the transport (Kennou et al., 2015; Suresh et al., 2012, 2011). Information on potential sources and pathways of HM can be obtained based on inter-element relationships (Altan et al., 2016).

Elements, namely Zn, As, Cd and Pb, were positively correlated for Soil 1, whereas Cu did not relate with any of the studied metals and Se was only correlated with Hg (Table 3.8). Similarly, a significant positive correlation was also detected between Zn, As, Cd, Pb and Se for Soil 2 (Table 3.9). In Soil 3, a significant positive correlation was also observed for Zn, Cd and Pb (Table 3.10). Table 3.11 and Table 3.12 further showed high Pearson’s correlation coefficients between Cu, Zn, As, Pb, and Se in Soil 4 and between Ni, Cu, Zn, Cd, Pb, and Se in Soil 5.

Cu, Zn, and Cd in particular belong to the groups 12 and 13 of the periodic table and share similar physico-chemical characteristics therefore behaving similarly.

Absence of correlation between Hg-Cu (- 0.052) or Pb-Hg (- 0.080) for Soil 1, Cr-Hg (0.304) Soil 2, Ni-Cr (0.436), Pb-Hg (0.273) for Soil 3, Cd-Cr (- 0.296), Cd-As (- 0.270) for Soil 4, and Cr-Hg (- 0.067), Cu-Cr (0.125) for Soil 5 suggests that these metals behave very differently and their fate and distribution are not controlled by a single common factor (Kennou et al., 2015).

3.4. Conclusions

Assessing the partitioning of HM and metalloids in soil is a more suitable tool to understand distribution and fate, rather than total concentration and the generic guideline values which commonly assumes that 100% of the contaminant of concern is bioavailable. The solid phase distribution highlighted the following: (1) while pseudo-total concentration shows that Pb, Cu, and Zn exceed the guideline values, only a negligible fraction of these HM were dissolved in pore water, which confirm that these metals were not readily-available; (2) the concentration of Zn and Pb in the mobile fractions (exchangeable) was higher than those in the non-mobile fraction (non-exchangeable), both fractions remained stable during weathering and under the different treatment applied; (3) a clear difference was observed between Soil 1 and Soil 2, where HM were significantly more bounded in Soil 2, a fact confirming that the stabilisation was a successful technique to minimize element’s mobility. We assessed the behaviour of exchangeable metal fraction over time, results showed that HM were stable and, similar behaviours were observed for both industrial contaminated soils (Soil 1, 2 and 3), and rural contaminated soil (Soil 4 and 5) at 0, 6 and 12 months. In addition, the conditions applied such as different pH (Cond 1 and Cond 2), different moisture (Cond 3 and Cond 4) and temperature (Cond 6 and Cond 7) did not have a clear pattern/effect on metals concentration over time. This finding can be attributed to both the soils having a strong soil pH buffer capacity, and the initial alkaline pH of the soil samples. Lastly, the Pearson’s correlation coefficients showed similarities between investigated HM/metalloids and their observed distribution pattern among the three phases (pore water, exchangeable, and non-exchangeable), and helped to classify these HM in groups. Overall, the limited changes in metal fractionation in these soil samples, including limited removal from more recalcitrant fractions, suggest that stable soil-complexes and interaction with the soil matrix were formed and may render the elements less mobile over time, therefore reducing environmental risk. In conclusion, standard guidelines values can provide initial information on the target HM for risk assessment, but they are not sufficient to understand the role of metal partitioning and soil properties on metal bioavailability and their potential effects (risk). Using sequential extraction to measure the HM concentration allows site specific assessment criteria to be determined and refined, providing a better estimate of the HM/metalloids potential bioavailable concentration.

3.5. References

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3.6. Appendix

Table 3.5: Descriptive statistics for metals and concentrations (expressed in mg/kg), obtained from replicates CISED sequential extraction of guidance materials.

Elements	min	max	range	sum	median	mean	SE.mean	CI.mean.0.95	var	std.dev	coef.var
Al	244.59	379.45	134.87	1682.34	342.91	336.47	24.65	68.45	3039.10	55.13	0.16
As	0.39	0.81	0.43	2.49	0.43	0.50	0.08	0.22	0.03	0.18	0.36
Ba	8.45	10.38	1.93	47.71	10.18	9.54	0.44	1.23	0.98	0.99	0.10
Ca	1108.26	1535.07	426.81	6375.74	1221.39	1275.15	72.74	201.95	26454.26	162.65	0.13
Cd	0.02	0.05	0.03	0.16	0.03	0.03	0.00	0.01	0.00	0.01	0.33
Co	1.37	1.83	0.46	8.13	1.72	1.63	0.09	0.24	0.04	0.20	0.12
Cr	0.37	3.45	3.08	11.46	2.85	2.29	0.54	1.51	1.48	1.22	0.53
Cu	0.73	1.24	0.51	5.12	1.12	1.02	0.09	0.26	0.04	0.21	0.20
Fe	319.69	857.41	537.72	3392.79	762.15	678.56	94.22	261.59	44383.96	210.68	0.31
K	63.99	107.52	43.54	435.88	81.22	87.18	8.39	23.30	352.23	18.77	0.22
Li	0.24	0.60	0.36	2.04	0.46	0.41	0.07	0.19	0.02	0.15	0.37
Mg	42.30	77.94	35.64	319.76	63.17	63.95	6.47	17.95	209.06	14.46	0.23
Mn	364.95	442.46	77.51	1996.96	404.15	399.39	13.99	38.85	979.00	31.29	0.08
Mo	0.04	0.38	0.34	0.95	0.20	0.19	0.05	0.15	0.02	0.12	0.64
Ni	1.17	2.04	0.87	8.60	1.84	1.72	0.16	0.45	0.13	0.36	0.21
P	43.34	84.58	41.24	317.31	63.71	63.46	6.56	18.22	215.22	14.67	0.23
Pb	2.42	4.07	1.65	16.28	3.29	3.26	0.36	1.00	0.65	0.81	0.25
S	19.19	53.78	34.59	162.83	33.81	32.57	6.14	17.06	188.78	13.74	0.42
Se	0.24	0.58	0.33	1.62	0.27	0.32	0.06	0.18	0.02	0.14	0.44
Si	178.17	294.16	115.99	1204.00	266.29	240.80	23.00	63.87	2645.73	51.44	0.21
Sr	2.49	3.03	0.54	14.19	2.97	2.84	0.11	0.31	0.06	0.25	0.09
V	1.43	2.62	1.19	10.73	2.25	2.15	0.23	0.62	0.25	0.50	0.23
Zn	2.79	5.04	2.26	20.93	4.36	4.19	0.37	1.04	0.70	0.84	0.20

SE.mean: Standard error on the mean; CI.mean: confidence interval of the mean at the p level; var: variance; std.dev: standard deviation; coef.var: variation coefficient, defined as the standard deviation divided by the mean.

Table 3.6: Descriptive statistics of 40 blank measurements (expressed in counts per second), and limit of detection (LOD, expressed in µg/L) of the analytical method applied.

Elements	min	max	range	sum	median	mean	SE mean	CI.mean. 0.95	std.dev	coef. var	stdev	stdev*3	slope	LOD
Al	116.0	322.0	206.0	1029.1	148.6	171.5	30.9	79.3	75.6	0.4	51.6	154.8	136.8	1.1
As	1.0	6.0	5.0	19.3	3.1	3.2	0.7	1.8	1.7	0.5	1.2	3.5	318.1	0.0
Ba	1961.0	3850.0	1889.0	14543.5	2193.0	2423.9	290.3	746.1	711.0	0.3	411.5	1234.5	66104.0	0.0
Ca	2.0	12.0	10.0	38.0	6.0	6.3	1.4	3.6	3.4	0.5	2.1	6.4	4.7	1.4
Cd	5.0	26.0	21.0	59.8	7.4	10.0	3.2	8.3	7.9	0.8	3.5	10.5	6069.7	0.0
Co	4.0	448.0	444.0	497.7	7.5	82.9	73.1	187.9	179.0	2.2	81.3	244.0	7670.5	0.0
Cr	30.0	1450.0	1420.0	1681.4	43.5	280.2	234.1	601.7	573.3	2.1	214.7	644.2	3768.5	0.2
Cu	145.0	835.0	690.0	1783.9	201.0	297.3	108.4	278.7	265.6	0.9	151.1	453.2	5688.6	0.1
Fe	2606.0	30202.0	27596.0	44906.0	2922.0	7484.0	4545.0	11684.0	11134.0	1.5	4171.0	12515.0	2943.0	4.3
Hg	79.0	159.0	80.0	644.5	101.5	107.4	11.3	29.0	27.6	0.3	16.9	50.6	6151.6	0.0
K	7777.0	10162.0	2385.0	54500.9	9138.7	9083.5	310.9	799.2	761.6	0.1	298.6	895.9	299.7	3.0
Li	391.0	713.0	322.0	3080.9	488.0	513.5	47.5	122.0	116.3	0.2	96.1	288.4	81705.3	0.0
Mg	168.0	832.0	664.0	1826.8	205.8	304.5	105.9	272.2	259.4	0.9	110.1	330.2	486.4	0.7
Mn	8.0	363.0	355.0	433.1	14.5	72.2	58.2	149.6	142.6	2.0	53.4	160.3	1685.4	0.1
Mo	14.0	48.0	34.0	162.4	24.9	27.1	4.7	11.9	11.4	0.4	7.0	20.9	13962.6	0.0
Ni	19.0	663.0	644.0	831.0	31.3	138.5	105.1	270.1	257.4	1.9	128.7	386.2	2242.0	0.2
Na	4654.0	11725.0	7071.0	37876.2	5386.8	6312.7	1101.6	2831.8	2698.4	0.4	1631.2	4893.5	1139.9	4.3
P	161.0	197.0	36.0	1086.1	182.1	181.0	5.0	12.8	12.2	0.1	8.6	25.9	8.7	3.0
Pb	776.0	3336.0	2560.0	7708.6	904.5	1284.8	411.2	1056.9	1007.1	0.8	384.7	1154.1	88169.8	0.0
S	2597.0	3306.0	709.0	18408.3	3124.9	3068.1	99.7	256.4	244.3	0.1	114.1	342.3	3.1	111.9
Sb	23.0	86.0	63.0	271.5	41.8	45.3	8.9	22.9	21.8	0.5	12.5	37.6	19894.6	0.0
Se	2.0	11.0	9.0	39.5	6.8	6.6	1.2	3.2	3.0	0.5	2.1	6.3	24.3	0.3
Si	34440.0	41612.0	7172.0	236813.4	40173.7	39468.9	1037.5	2667.0	2541.3	0.1	1046.5	3139.6	116.6	26.9
Sr	732.0	1548.0	816.0	5612.9	840.5	935.5	124.5	320.1	305.0	0.3	153.7	461.1	64139.7	0.0
V	1.0	10.0	9.0	23.3	3.1	3.9	1.3	3.3	3.2	0.8	1.7	5.1	2962.4	0.0
Zn	39.0	491.0	452.0	792.0	65.8	132.0	72.0	185.2	176.5	1.3	74.7	224.1	494.8	0.5

SE.mean: Standard error on the mean; CI.mean: confidence interval of the mean at the p level; var: variance; std.dev: standard deviation; coef.var: variation coefficient, defined as the standard deviation divided by the mean.

Table 3.7: Descriptive statistics of heavy metals (HM) and metalloids concentrations in pore water, exchangeable and non-exchangeable fraction (expressed in mg/kg) in the soil samples analysed (Soil 1, Soil 2, Soil 3, Soil 4, and Soil 5).

Sample	Element	Pore water		Exchangeable		Non-exchangeable	
		Range	Median	Range	Median	Range	Median
Soil 1	As	0.050 - 0.113	0.080	0.494 - 0.615	0.599	6.031 - 7.345	7.050
	Cd	0.002 - 0.005	0.003	0.042 - 0.051	0.047	0.216 - 0.259	0.234
	Cr	0.003 - 0.006	0.005	0.882 - 1.092	1.029	3.070 - 4.716	3.648
	Cu	0.056 - 0.126	0.089	0.168 - 0.213	0.193	25.918 - 41.040	33.170
	Hg	0.000 - 0.000	0.000	0.001 - 0.002	0.002	0.000 - 0.000	0.000
	Ni	0.035 - 0.079	0.056	1.256 - 1.506	1.388	4.197 - 5.605	4.984
	Pb	0.000 - 0.000	0.000	0.044 - 0.056	0.048	96.378 - 122.121	106.981
	Se	0.033 - 0.075	0.053	0.691 - 0.845	0.791	0.732 - 0.918	0.864
	Zn	0.106 - 0.238	0.168	4.769 - 5.756	5.320	70.648 - 81.294	73.908
Soil 2	As	0.016 - 0.028	0.021	0.542 - 0.607	0.582	7.678 - 8.684	7.929
	Cd	0.004 - 0.008	0.006	0.214 - 0.256	0.250	0.582 - 0.705	0.656
	Cr	0.000 - 0.000	0.000	1.091 - 1.354	1.263	4.910 - 6.364	5.375
	Cu	0.024 - 0.036	0.029	2.528 - 3.079	2.867	41.476 - 46.504	43.960
	Hg	0.000 - 0.000	0.000	0.001 - 0.001	0.001	0.001 - 0.006	0.002
	Ni	0.004 - 0.005	0.004	0.874 - 0.990	0.894	5.945 - 9.796	6.501
	Pb	0.357 - 0.648	0.468	3.848 - 4.699	4.486	149.771 - 186.686	171.491
	Se	0.010 - 0.017	0.013	0.544 - 0.590	0.569	1.102 - 1.229	1.139
	Zn	0.161 - 0.315	0.214	8.978 - 11.568	10.750	131.240 - 150.798	140.395
Soil 3	As	0.168 - 0.246	0.192	0.590 - 0.741	0.671	13.142 - 20.037	16.334
	Cd	0.000 - 0.000	0.000	1.129 - 1.446	1.340	0.010 - 0.015	0.012
	Cr	0.126 - 0.201	0.154	0.390 - 0.536	0.472	7.415 - 11.306	9.216
	Cu	0.389 - 0.581	0.461	12.106 - 15.287	14.005	22.493 - 34.295	27.957
	Hg	0.000 - 0.000	0.000	0.007 - 0.016	0.009	0.000 - 0.000	0.000
	Ni	0.029 - 0.043	0.036	5.545 - 7.054	6.614	4.092 - 6.239	5.086
	Pb	42.420 - 62.902	52.165	1624.888 - 2064.239	1891.046	420.330 - 640.887	522.437
	Se	0.087 - 0.126	0.104	0.437 - 0.561	0.510	0.747 - 1.139	0.929
	Zn	2.052 - 3.118	2.540	1313.302 - 1657.856	1535.797	9.358 - 14.268	11.631

Sample	Element	Pore water		Exchangeable		Non-exchangeable	
		Range	Median	Range	Median	Range	Median
Soil 4	As	0.002 - 0.005	0.004	0.325 - 0.385	0.343	3.094 - 4.406	3.622
	Cd	0.001 - 0.002	0.002	0.170 - 0.204	0.187	0.024 - 0.032	0.028
	Cr	0.001 - 0.002	0.001	0.060 - 0.073	0.063	4.267 - 9.207	5.086
	Cu	0.048 - 0.090	0.069	5.286 - 6.041	5.708	10.286 - 12.040	10.661
	Hg	0.000 - 0.000	0.000	0.002 - 0.003	0.002	0.001 - 0.003	0.001
	Ni	0.002 - 0.009	0.004	2.759 - 3.327	3.047	2.028 - 5.379	2.245
	Pb	0.000 - 0.000	0.000	0.290 - 0.340	0.302	53.687 - 58.755	56.100
	Se	0.068 - 0.116	0.080	0.109 - 0.135	0.118	1.544 - 1.920	1.615
	Zn	0.124 - 0.232	0.159	15.243 - 18.038	16.736	12.100 - 28.150	13.715
Soil 5	As	0.000 - 0.000	0.000	0.600 - 0.732	0.646	2.945 - 4.349	3.659
	Cd	0.000 - 0.000	0.000	0.200 - 0.231	0.216	0.000 - 0.000	0.000
	Cr	0.000 - 0.001	0.000	0.325 - 0.382	0.347	3.900 - 9.083	7.032
	Cu	0.000 - 0.000	0.000	12.187 - 14.558	13.209	1.825 - 2.550	2.113
	Hg	0.001 - 0.034	0.002	0.000 - 0.000	0.000	0.000 - 0.000	0.000
	Ni	0.000 - 0.000	0.000	3.272 - 3.777	3.530	1.583 - 5.488	3.898
	Pb	0.000 - 0.007	0.000	47.840 - 58.162	53.318	18.523 - 26.776	22.418
	Se	0.000 - 0.000	0.000	1.071 - 1.377	1.261	0.462 - 0.645	0.534
	Zn	0.001 - 0.031	0.002	18.681 - 21.512	20.115	9.521 - 22.601	17.437

Range of measurement between different sampling times (0, 6, and 12 months) and different conditions (Cond 1 - Cond 7)

Table 3.8: Pearson's correlation matrix for the heavy metals and metalloids concentrations (Soil 1).

	Cr	Ni	Cu	Zn	As	Cd	Hg	Pb	Se
Cr	1.000								
Ni	**0.980**	1.000							
Cu	0.566	0.572	1.000						
Zn	**0.910**	**0.877**	0.722	1.000					
As	**0.965**	**0.977**	0.618	**0.936**	1.000				
Cd	**0.926**	**0.933**	0.669	**0.973**	**0.968**	1.000			
Hg	0.279	0.427	-0.052	0.022	0.346	0.199	1.000		
Pb	0.837	0.800	0.800	**0.985**	**0.878**	**0.935**	-0.080	1.000	
Se	0.456	0.600	0.086	0.195	0.502	0.383	**0.959**	0.085	1.000

Table 3.9: Pearson's correlation matrix for the heavy metals and metalloids concentrations (Soil 2).

	Cr	Ni	Cu	Zn	As	Cd	Hg	Pb	Se
Cr	1.000								
Ni	0.742	1.000							
Cu	**0.890**	0.775	1.000						
Zn	**0.859**	**0.918**	**0.959**	1.000					
As	**0.911**	0.786	**0.995**	**0.956**	1.000				
Cd	**0.867**	0.716	**0.945**	**0.900**	**0.932**	1.000			
Hg	0.304	0.664	0.360	0.521	0.359	0.318	1.000		
Pb	**0.864**	0.738	**0.996**	**0.944**	**0.983**	**0.936**	0.334	1.000	
Se	**0.888**	0.718	**0.895**	**0.860**	**0.899**	**0.977**	0.307	**0.869**	1.000

Table 3.10: Pearson's correlation matrix for the heavy metals and metalloids concentrations (Soil 3).

	Cr	Ni	Cu	Zn	As	Cd	Hg	Pb	Se
Cr	1.000								
Ni	0.436	1.000							
Cu	**0.908**	0.773	1.000						
Zn	-0.424	0.630	-0.005	1.000					
As	**0.999**	0.424	**0.902**	-0.436	1.000				
Cd	-0.423	0.631	-0.005	**1.000**	-0.435	1.000			
Hg	-0.075	0.198	0.017	0.280	-0.111	0.275	1.000		
Pb	-0.160	0.818	0.268	**0.962**	-0.174	**0.961**	0.273	1.000	
Se	**0.902**	0.752	**0.985**	-0.022	**0.897**	-0.021	0.034	0.249	1.000

Table 3.11: Pearson's correlation matrix for the heavy metals and metalloids concentrations (Soil 4).

	Cr	Ni	Cu	Zn	As	Cd	Hg	Pb	Se
Cr	1.000								
Ni	0.719	1.000							
Cu	0.810	0.837	1.000						
Zn	0.706	**0.998**	**0.853**	1.000					
As	**0.960**	0.669	**0.885**	0.671	1.000				
Cd	-0.296	0.442	0.172	0.465	-0.270	1.000			
Hg	0.577	**0.893**	0.689	**0.890**	0.514	0.463	1.000		
Pb	**0.891**	0.528	**0.856**	0.535	**0.969**	-0.356	0.363	1.000	
Se	**0.952**	0.613	**0.863**	0.614	**0.992**	-0.337	0.461	**0.985**	1.000

Table 3.12: Pearson's correlation matrix for the heavy metals and metalloids concentrations (Soil 5).

	Cr	Ni	Cu	Zn	As	Cd	Hg	Pb	Se
Cr	1.000								
Ni	0.435	1.000							
Cu	0.125	**0.946**	1.000						
Zn	0.372	**0.997**	**0.966**	1.000					
As	**0.964**	0.644	0.371	0.592	1.000				
Cd	0.034	**0.914**	**0.995**	**0.940**	0.284	1.000			
Hg	-0.067	-0.068	-0.055	-0.042	-0.073	-0.040	1.000		
Pb	0.296	**0.986**	**0.985**	**0.995**	0.528	**0.963**	-0.064	1.000	
Se	0.332	**0.990**	**0.977**	**0.996**	0.560	**0.951**	-0.067	**0.999**	1.000

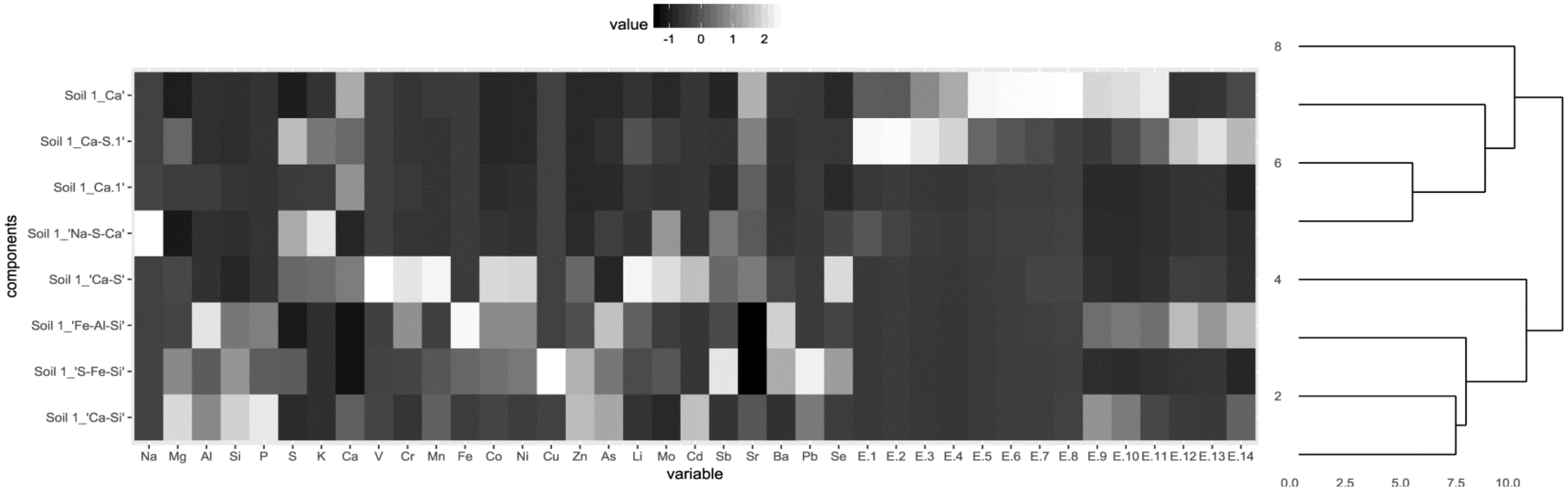

Figure 3.4: Heatmap and associated hierarchical cluster for the CISED extraction data for Soil 1.

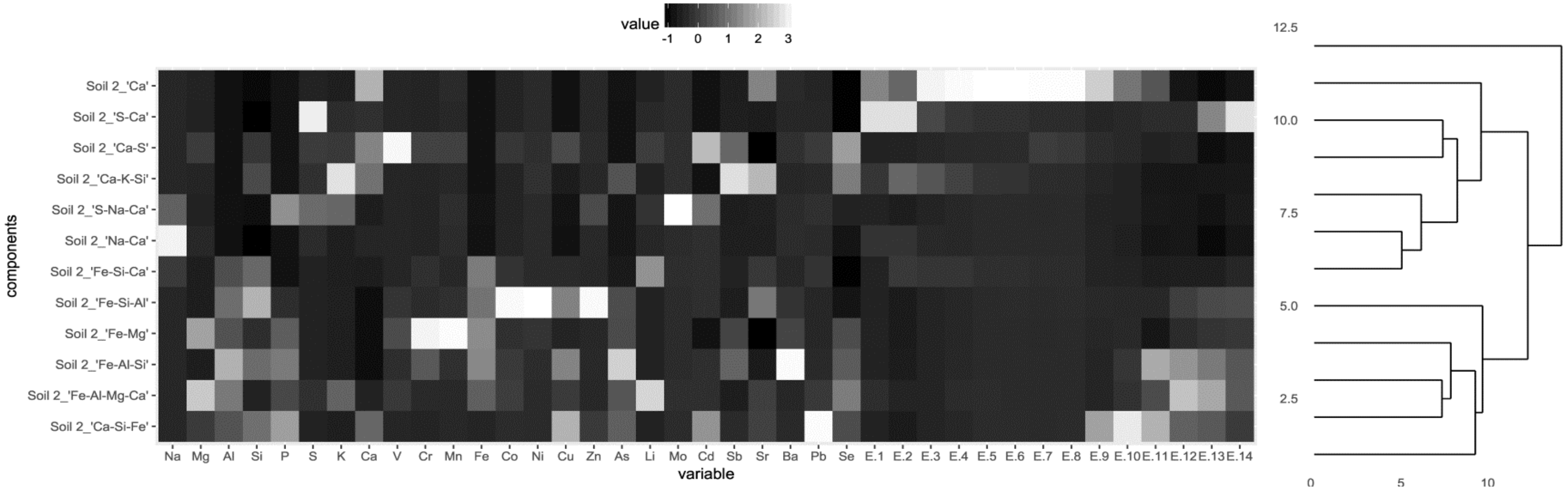

Figure 3.5: Heatmap and associated hierarchical cluster for the CISED extraction data for Soil 2.

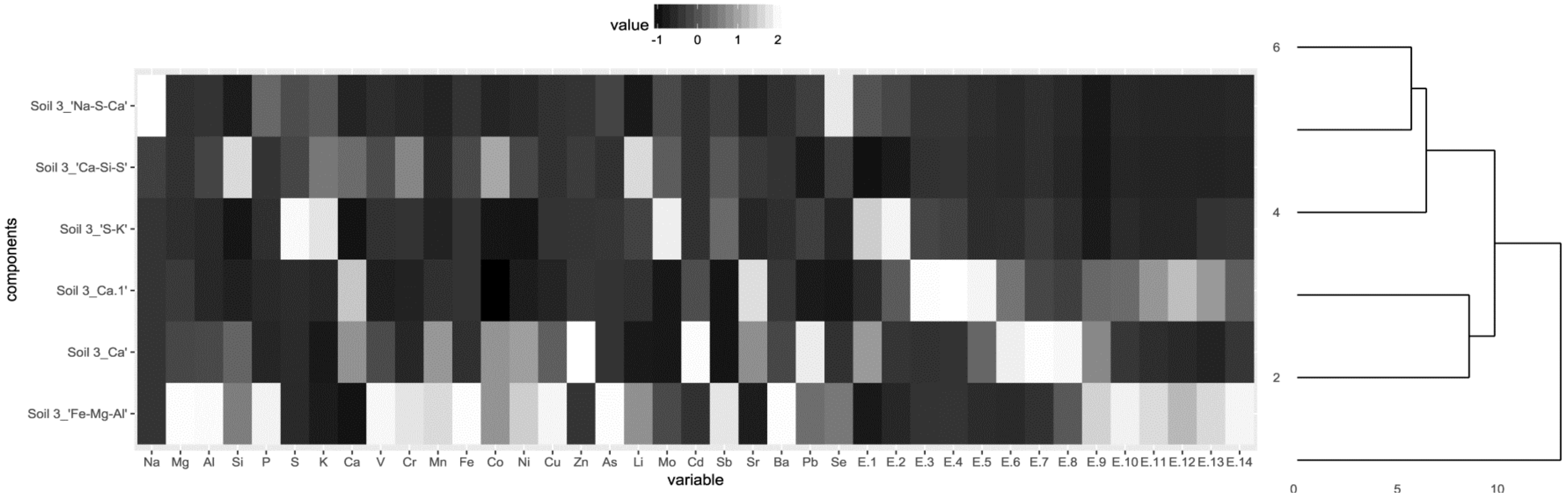

Figure 3.6: Heatmap and associated hierarchical cluster for the CISED extraction data for Soil 3.

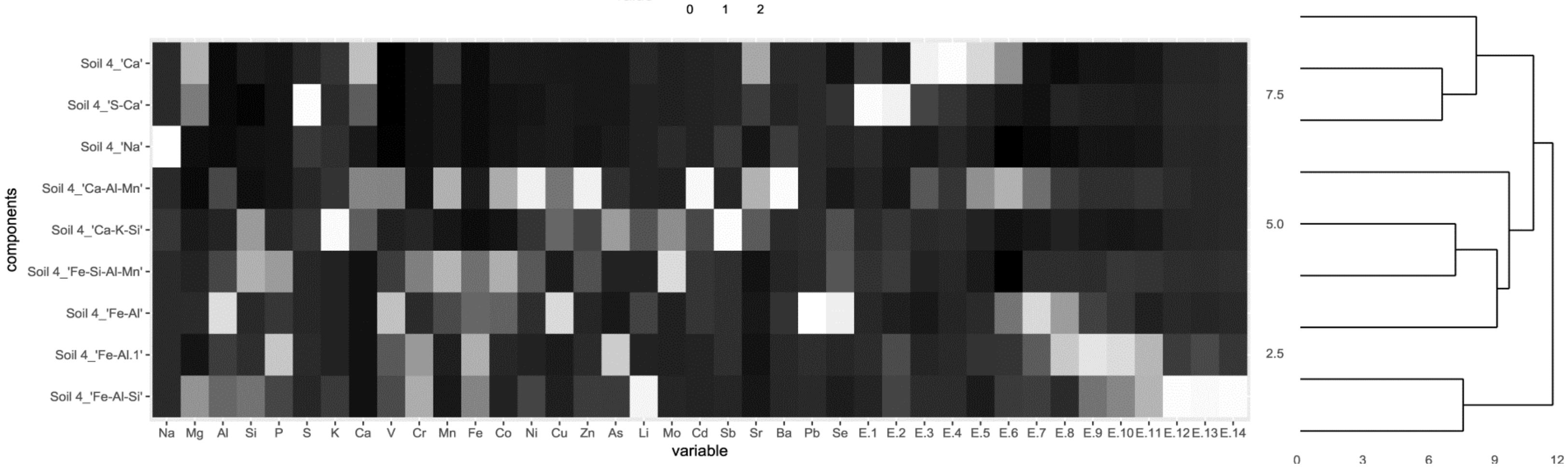

Figure 3.7: Heatmap and associated hierarchical cluster for the CISED extraction data for Soil 4.

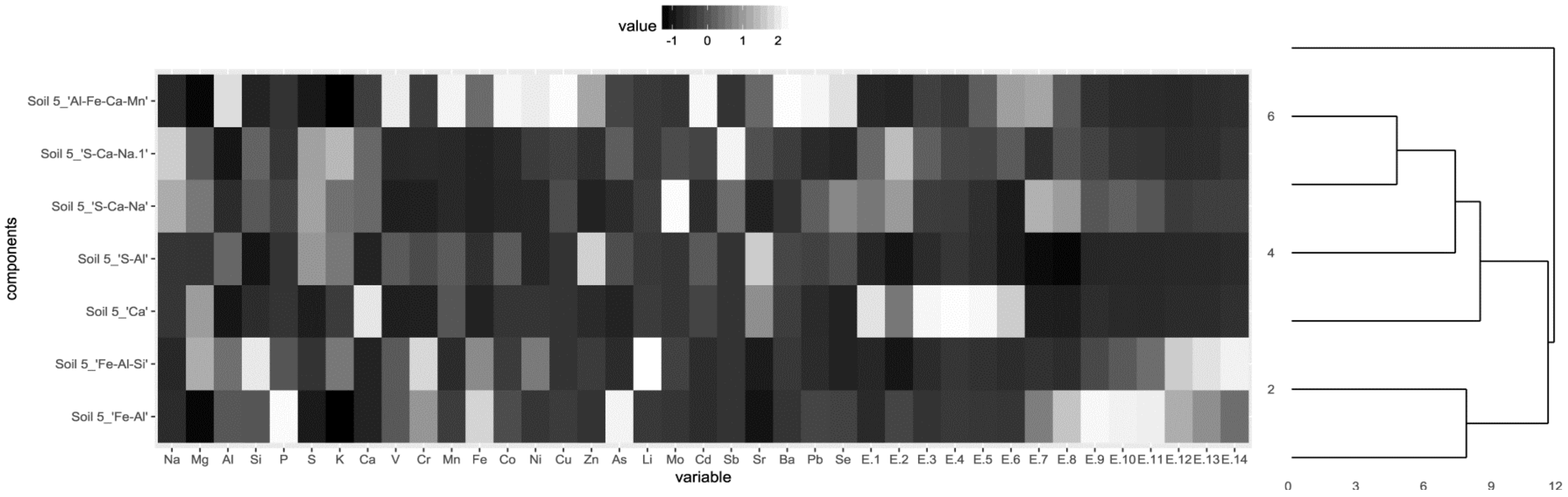

Figure 3.8: Heatmap and associated hierarchical cluster for the CISED extraction data for Soil 5.

3.7. Annex

Sequential extraction data processing and heatmap visualisation

The CISED non-specific sequential extraction, used in this study, assumes that the extraction media (14 extracts) contains a mixture of the soil target phases and apply mathematical modelling (chemometrics) based on principal component analysis (PCA) and factor analysis to resolve the mixture into their components.

The concentrations data obtained from the ICP-MS analysis, expressed in mg/kg, were arranged in a matrix (A) and scaled to its maximum value, to avoid features with large values to dominate. The first step was to identify the number of components in the extraction media; to do this the self-modelling mixture resolution (SMMR) algorithm was used to decompose this matrix (A) in the product of two matrices (B) and (C) (Figure 3.9). The matrix B contained the proportion of each components leached in the extraction media, while the matrix C contained information on the concentration (mg/kg) of the physico-chemical components (N-components) identified by the model.

From these two matrices further information can be obtained, such as: (1) the extraction profile of each modelled component (PRF), expressed in mg/kg, which is derived from the amount of each component found in the 14 extracts; (2) the relative proportions of each element in each modelled component, called distribution (DST) and expressed in mg/kg; (3) the chemical composition of each component (CMP) expressed as a percentage. Data from PRF and CMP were further used to calculate the individual element concentration (IEC) in each component identified by the model, an example of calculation is provided below:

$$\mathrm{IEC\ (mg/kg)} = \frac{\Sigma \mathrm{PRF}_{E1-E14}\ (\mathrm{Comp\ 1}) * \mathrm{CMP}_{\mathrm{element}}}{100}$$

In both Chapter 3 and Chapter 5 the SMMR algorithm was run separately for each of the soil sample, resulting in distinct sets of components for each soil investigated. From here, hierarchal clustering and heatmap were used to obtain a geochemical profile interpretation and to classify the components into the common distinct soil phases (i.e. pore water, exchangeable, non-exchangeable phases).

Briefly, for each soil sample, a matrix was created concatenating the transposed elements composition matrix (T)CMP (expressed as a percentage), and the transposed extraction profile matrix (T)PRF (expresses in mg/kg) of each soil under investigation (Figure 3.9). The concatenated matrix was then imported in RStudio (Version 1.1.423 –© 2009-2018 RStudio, Inc.) and subjected to hierarchical clustering where the data were mean centred and scaled with Euclidean distance and linkage using Ward's method (Ward, 1963) and the '*Agnes*' function in the cluster package (Maechler et al., 2018); details of the R script are provided in Figure 3.10. In the hierarchical clustering, initially each object (data point) is assigned to its own cluster; then the algorithm proceeds iteratively combining the two most similar clusters, continuing until there is just a single cluster (dendogram structure) (Vogt and Bajorath, 2017). In this study the aim of the clustering was to group the components (identified by the model) into clusters based on similarity, providing a means to corrrelate the clusters to their geochemical sources.

Clustering results (dendogram) were complemented by heatmap data visualisation created by using *ggplot2*, *reshape2*, *grid*, and *ggdendro* packages (Wickham, 2007; Kahle and Wickham, 2013), (Figure 3.10). In the heatmap each row represented a physico-chemical soil component found for a given soil. Soil name is indicated by the 'soil name' followed by the elements name (e.g. Ca, Ca-K-Si, Fe-Mg) that make up >10% of the physico-chemical component composition. The heatmap colour gradient was black-white, where black (low) and white (high) represented the mean-centered concentrations of elements (left) and the extraction profiles (right). The hierarchal clustering obtained was used in parallel with chemical profile to provide interpretations and classify the components into common distinct soil phases (e.g. pore water, carbonates, and aluminium-silicates/oxides) and to assess the partitioning and bioavailable concentrations of HM/metalloids in soil.

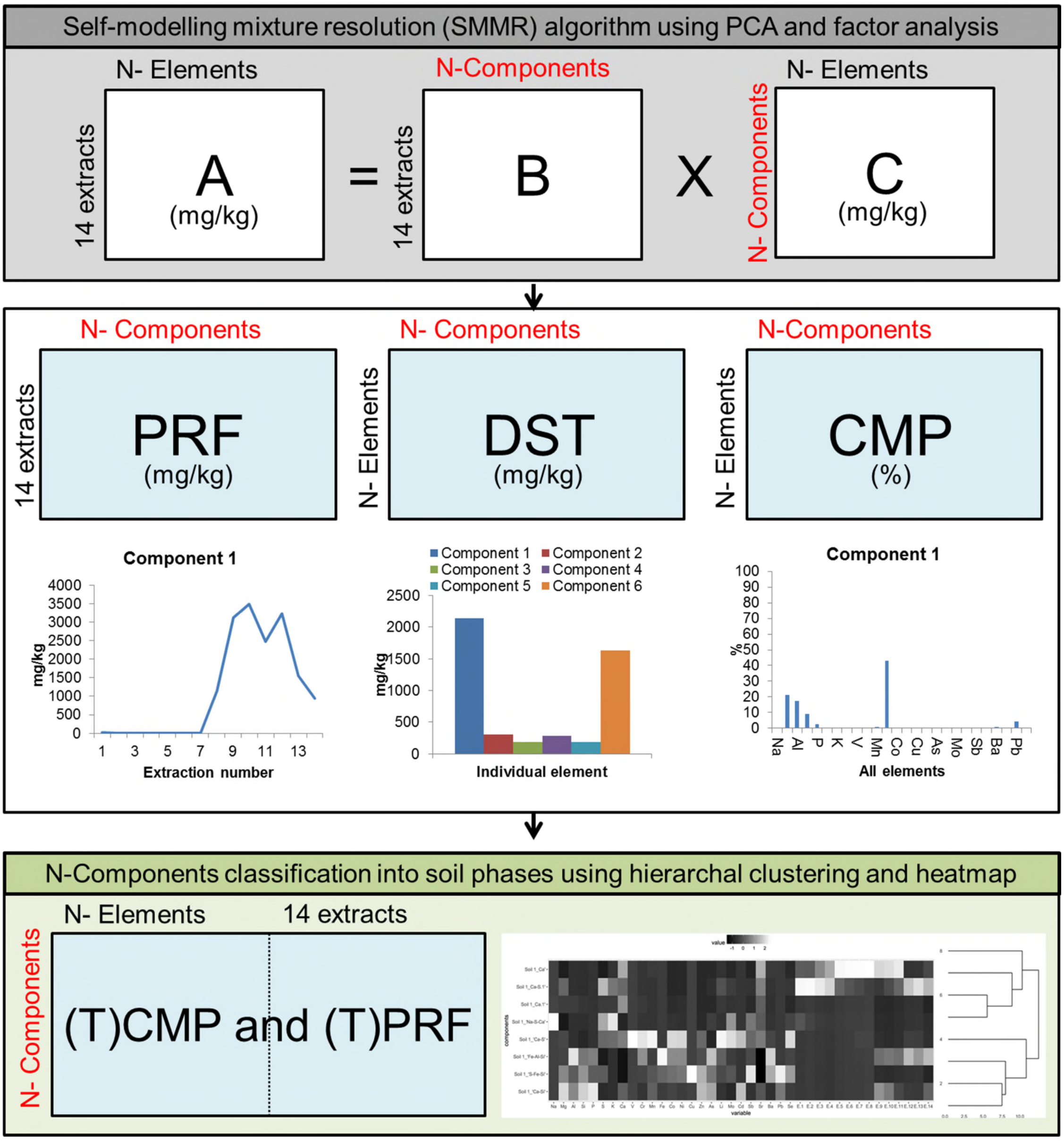

Figure 3.9: Illustrative scheme of the principal component (PCA) and factor analysis output (matrices B and C), the model outputs including profile (PRF), distribution (DST), and composition (CMP) matrices obtained through chemometric analysis, and the classification of N-components into soil phases.

```
#cluster analysis and heatmap

#Packages to upload
require(ggplot2)
require(cluster)
require(ggdendro)
require(vegan)
require(decostand)

#read table: Components as rows and in the columns: All Metals (%) + E1-E14 (mg/kg)
cised.components <-read.csv( "Z:\\mydata.csv")
rownames(cised.components) <- cised.components[,1]
cised.components<- cised.components[-c(1)]
View(cised.components)

#Cluster ward method using agnes
cised.components.scaled=decostand(cised.components,method="standardize")
cised.ward=agnes(dist(cised.components.scaled),method="ward")
plot(as.hclust(cised.ward),cex=0.6,hang=-1)

#Create dendrogram plot
dendro.agnes=dendro_data(cised.ward)
dendro.plot=ggdendrogram(dendro.agnes,rotate=TRUE,labels=FALSE)
print(dendro.plot)

#Create heatmap
library(reshape2)
new.cised<-cbind(rownames(cised.components.scaled),cised.components.scaled)
colnames(new.cised)[1]<-"components"
max(cised.long$value)

#Creating a long format for heatmap
cised.long<-(melt(new.cised, id= "components"))

#Heatmap
heatmap.plot <- ggplot(cised.long, aes(variable, components)) +
  geom_tile(aes(fill = value))+
  scale_fill_gradient2() +
  theme(axis.text.y = element_text(size = 7)
        )
print(heatmap.plot)

#Put dendrogram and heatmap in the same figure
require(grid)
grid.newpage()
print(heatmap.plot, vp = viewport(x = 0.4, y = 0.5, width = 0.8, height = 1.0))
print(dendro.plot, vp = viewport(x = 0.90, y = 0.445, width = 0.2, height = 1.0))

#Reorder heatmap with rows to amtch dendrogram
cised.dendro=as.dendrogram(cised.ward)
cised.order=order.dendrogram(cised.dendro)

cised.long$components <- factor(x = cised.long$components,
                               levels = cised.long$components[cised.order],
                               ordered = TRUE)

#New heatmap with ordered components by cluster
heatmap.plot <- ggplot(cised.long, aes(variable, components)) +
  geom_tile(aes(fill = value))+
  scale_fill_gradient(low="black",high="white") +
  theme(axis.text.y = element_text(size = 9),
  legend.position="top"
  )
grid.newpage()
print(heatmap.plot, vp = viewport(x = 0.4, y = 0.5, width = 0.8, height = 1.0))
print(dendro.plot, vp = viewport(x = 0.90, y = 0.44, width = 0.2, height = 1.0))
```

Figure 3.10: Screenshot of the RStudio script used to compute hierarchal clustering and heatmap.

4. Predicting bioavailability change of complex chemical mixtures in contaminated soils using visible and near-infrared spectroscopy and random forest regression

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Abstract: A number of studies have shown that visible and near infrared spectroscopy (Vis-NIRS) offers a rapid on-site measurement tool for the determination of total contaminant concentration of petroleum hydrocarbons compounds (PHC), heavy metals and metalloids (HM) in soil. However none of them have yet assessed the feasibility of using Vis-NIRS coupled to random forest (RF) regression for determining both the total and bioavailable concentrations of complex chemical mixtures. Results showed that the predictions of the total concentrations of polycyclic aromatic hydrocarbons (PAH), PHC, and alkanes (ALK) were very good, good and fair, and in contrast, the predictions of the bioavailable concentrations of the PAH and PHC were only fair, and poor for ALK. A large number of trace elements, mainly lead (Pb), aluminium (Al), nickel (Ni), chromium (Cr), cadmium (Cd), iron (Fe) and zinc (Zn) were predicted with very good or good accuracy. The prediction results of the total HMs were also better than those of the bioavailable concentrations. Overall, the results demonstrate that Vis-NIRS coupled to RF is a promising rapid measurement tool to inform both the distribution and bioavailability of complex chemical mixtures without the need of collecting soil samples and lengthy extraction for further analysis.

Keywords: visible and near infrared spectroscopy, random forest, bioavailability, contaminated land, rapid measurement tools.

4.1. Introduction

A number of anthropogenic activities such as waste disposal, mining activities, manufacturing, and petrochemical industries as well as poor environmental management practices have left a legacy of contaminated sites across Europe and worldwide (World Health Organization, 2012). Contaminants of concerns are often present on site as a complex mixture (Swartjes et al., 2012) and their co-occurrence and interactions can impact their adsorption behaviour in soil, and influence their availability (Ye et al., 2017). Recovery of brownfield sites is often challenging as hazards are very heterogeneous, reliable exposure data are lacking, and remediation often requires large investments and involves multiple stakeholders (Zhu et al., 2015).

Risk assessment is recognised as a robust process to support decision-making strategies for contaminated land, and to prevent further damage to the environment and human health (Doak et al., 2004). It has been further shown that measuring only the total concentration of contaminants in soil does not give a useful basis for the evaluation of the potential risks to human and the environment (Ortega-Calvo et al., 2015). In fact, in the United Kingdom, and increasingly across the world, over the last decade the end-point of remedial activity is defined by the concentration of the chemicals of concern likely to pose significant risk, the bioavailable concentration (Cipullo et al., 2018; Kuppusamy et al., 2017). Similarly, several risk-based frameworks for contaminated soils have been published under the auspices of national and international regulatory organizations each reflecting national legislation, a range of expert judgments and socioeconomic issues (Rodrigues et al., 2009). However they all typically adopt a three tiered approach with increasingly sophisticated levels of data collection and analysis as an assessor moves through the tiers.

The common steps include (1) developing a conceptual site model (CSM) of the site based on a-priori information and historical land use, (2) conducting a preliminary site assessment to refine the initial CSM, (3) deciding if further assessment (generic and detailed) are needed. Risk assessments generally require more data when moving from preliminary to generic (comparison with general contamination threshold) and to detailed risk assessments (comparison with site-specific contamination threshold). Therefore, in order to establish practical and sustainable criteria to achieve a reasonable level of clean-up for the intended land use, it is important to: 1) reduce uncertainties associated with sampling especially for large site, 2) deliver cost-effective approaches to support site investigation, 3) reduce analytical cost associated with complex-contaminant assessment, and 4) reduce significantly the time associated with sampling and subsequent laboratory analysis.

The preliminary site investigation plays a key role in the risk assessment process, as the accuracy of the information gathered at this step is fundamental to correctly manage the associated time and costs (Wan et al., 2016). Often, at this stage, sample collection is not included, and probability-based sampling strategies are mostly designed from conceptual site model information, combining random and selected sampling starting points (Horta et al., 2015). In this regards rapid-measurement tools (RMT), such as reflectance spectroscopy, including visible and near-infrared (Vis-NIR) or mid-infrared (MIR) spectroscopy, can support the decision making strategies, by improving quality and quantity of information collected during site investigation (Douglas et al., 2018). Additionally, the on-the-go instrument could be used to perform real-time monitoring and assessing on-site remediation efficacy or natural attenuation (O’Rourke et al., 2016).

The reflectance spectra of contaminated soils in the visible near-infrared and short wave infrared region (400–2500 nm) (Vis-NIR-SWIR) allows rapid and cost-effective acquisition of soil information based on the unique absorption spectra of specific chemical compounds (Douglas et al., 2018; Okparanma and Mouazen, 2013). Vis-NIRS has been successfully used to estimate both petroleum-derived compounds (Okparanma and Mouazen, 2013) and heavy metals (Shi et al., 2014) in genuine and spiked soil samples. In particular, Vis-NIRS coupled with random forest (RF) modelling has been previously shown to outperform other regression techniques such as partial least square regression (PLSR) as it is able to account for the non-linearity associated with the soil spectral responses (Douglas et al., 2018).

The principle of Vis-NIRS is based on the frequencies of which molecules rotates or vibrates generating discrete measurable energy levels (Horta et al., 2015). Infrared spectroscopy is mostly used for the estimation of organic compounds which allows the determination of a fixed-wavelength responding to the vibration caused by C-H and C-C bonds stretching and bending (Schwartz et al., 2011). However, Wu et al. (2010) showed that while there is no direct spectral response of HM within the NIR range, Vis-NIRS can detect HM due to vibrations of -OH bonds as a result of their association with Fe oxides, clays and organic matter. Therefore most of the trace elements can be easily detected at very high concentrations (i.e. Cr and Cu at >4000 mg/kg; (Wu, 2007) and with reasonable accuracy at low levels (O'Rourke et al., 2016).

In the past five years, several studies have shown that Vis-NIR can successfully predict in soil both total concentration of HM (Gholizadeh et al., 2015; Horta et al., 2015; Kemper and Sommer, 2002; Siebielec and McCarty, 2004; Todorova et al., 2014) and total concentration of PHC (Chakraborty et al., 2015; Douglas et al., 2018). However none have yet investigated the feasibility of using Vis-NIR as a RMT to predict on site the bioavailable concentration of HM and PHC, simultaneously.

In this study, the performance of Vis-NIR spectroscopy coupled to RF regression was therefore assessed for predicting the total and the bioavailable concentrations of heavy metals/metalloids and petroleum hydrocarbons mixtures in five genuinely-contaminated soils.

4.2. Materials and methods

4.2.1. Sample collection and preparation

Three genuinely contaminated soils, denoted as Soil 1, Soil 2, and Soil 3, were collected from a treatment site located in the United Kingdom. Two additional soil types were collected from a rural site contaminated by diesel (Soil 4), and mineral oil (Soil 5). Information regarding original location of the soil samples collected, and specific details regarding the treatment applied, were not disclosed by the treatment facility to maintain anonymity and confidentiality. All samples were collected randomly from the soil layer down to a depth of 30 cm and immediately stored at 4°C to minimise biological transformation and other chemical reactions. A total of 21 samples were collected for each soil type (e.g., for the 5 soil types a total of 105 samples) and split into five sub-samples; one of them was used for spectroscopic measurements and the other four for chemical analytical determinations of total and bioavailable (HM/metalloids and PHC) contents. An outline of experimental and analytical procedures used is presented in Figure 4.1.

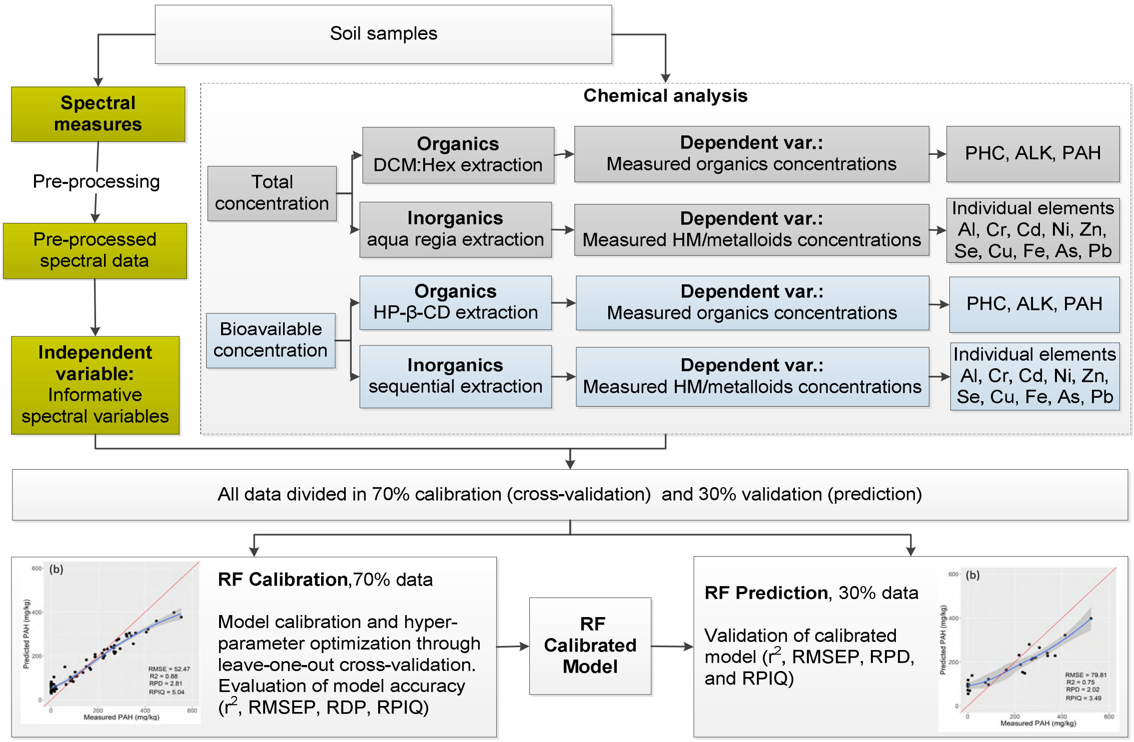

Figure 4.1: Illustrative block diagram showing the different steps for the estimation of complex chemical mixtures of total and bioavailable concentrations in soils using chemical methods and Vis-NIRS coupled with Random Forest (RF).

DCM: dichloromethane; Hex: hexane; HP-β-CD: hydroxypropyl-β-cyclodextrin; PHC: petroleum hydrocarbons compounds; HM: heavy metals; PAH: polycyclic aromatic hydrocarbons; ALK: alkanes; Al: aluminium; Cr: chromium, Cd: cadmium; Ni: nickel, Zn: zinc; Se: selenium, Cu: copper; Fe: iron; As: arsenic; Pb :lead, ML: machine learning; r^2:coefficient of determination; RMSEP : root mean square error of prediction; RPD: ratio of prediction deviation; RPIQ: ratio of the performance to interquartile distance.

4.2.2. Extraction and quantification of total and bioavailable PHC and HM and metalloids

The method used to determine total petroleum hydrocarbons compounds, including PAH and ALK fractions in soil, was adapted from the procedure described by Risdon et al. (2008). Briefly, PHC were extracted using solvent ultra-sonication from 2.5 g of soil mixed with 15 mL of a mixture of 1:1 dichloromethane: hexane. The bioavailable hydrocarbons content was instead extracted using 20 mL of a 50 mM solution of hydroxypropyl-β-cyclodextrin (HP-β-CD) as described by Cipullo et al. (2018 b). Extraction, identification, and quantification of total and bioavailable PHC, PAH, and ALK were performed by gas chromatography-mass spectrometry (GC-MS) as described by Cipullo et al. (2018 a, b).

The pseudo-total element digestion was performed according to the ISO 11047 method with aqua regia (ISO 11047:1998). The bioavailable heavy metals and metalloids content were determined using a modified procedure of the sequential extraction method of Cave et al. (2004). Briefly soil samples (2 g) were consecutively extracted by addition of 10 mL of a nitric acid solution of increasing concentration from 0 to 5 M. All total and sequential extracts were analysed by inductively coupled plasma mass spectrometry (NexION® 350D ICP-MS, Perkin Elmer) as described by Cipullo et al. (2018 a). In this work the HM bioavailable fraction was considered to be the amount of elements associated with pore water phase (readily available or bioavailable), and carbonates phases (potentially available with time).

4.2.3. Soil spectra analysis

4.2.3.1. Spectra collection

Soil samples were air-dried and sieved (2 mm) to get the fine earth and separate large particles like plant parts (roots, stem, and leave), cobbles, and pebbles (Roy et al., 2014). The fine earth was mixed well, before three sub-samples were made from each soil sample and packed into three plastic Petri dishes (1 cm height, and 5.6 cm in diameter). The sample surface was smoothened gently with a spatula to obtain optimal diffuse reflection, and hence, a good signal-to-noise ratio (Mouazen et al., 2005). The diffuse reflectance spectra of the soil samples were measured using an ASD LabSpec2500® VIS–NIR spectrophotometer (350 – 2500 nm). The spectral resolution varied from 3 nm in 700 nm and 6 nm in 1400-1200 nm (Analytical Spectral Devices Inc., CO, USA). A high-intensity probe that has a built-in light source made of a quartz-halogen bulb of 2727°K was placed in contact with soil sample to collect the spectra. Measurement was done under dark conditions, to control the artificial illumination and reduce the effects of stray light. Before scanning the ASD instrument was first warmed-up for at least 30 min, and then calibrated by a white Spectralon® disc (Diffuse Reflectance Standards, Labsphere, INC, US) of almost 99% reflectance. For each sample, 3 successive spectra were acquired at three equidistant positions approximately 120° apart *a*nd these were averaged in one representative spectrum of a soil sample.

4.2.3.2. **Spectra pre-treatment**

The raw average spectra of the 105 samples were subjected to pre-treatment including successively, noise cut, maximum normalization, first derivative and smoothing using *Prospectr-R package* (Stevens and Ramirez Lopez, 2014, 2013) in RStudio (Version 1.1.423 – © 2009-2018 RStudio, Inc.). Maximum normalisation was implemented to align all spectra to the same scale and to obtain even distribution of the variances and average values. Spectra were then subjected to first derivation using Gap–segment derivative (gapDer) algorithm (Norris, 2001) with a second-order polynomial approximation. Finally, the Savitzky-Golay (SG) algorithm with a window size of 11 and polynomial of order 2 was carried out to remove noise from spectra (Douglas et al., 2018 a).

4.2.4. Random forest regression analysis

4.2.4.1. Selection of Input variables

A two-dimensional data matrix was created by combining the reference values of chemical analyses of PHC, PAH, ALK, and HM/metalloids contents (dependent variables) and pre-treated spectra (independent variables) of 105 soil samples. Removal of outliers for each data set was based on principal components analysis (PCA). PCA was followed by randomly splitting the dataset into 70% for calibration (74 samples) and 30% for prediction (31 samples) (Figure 4.1).

4.2.4.2. Model calibration

The hyper-parameter optimisation and calibration of the model was done through leave-one-out cross-validation (LOOCV) (Reyna et al., 2017). For the calibration dataset of n = 74 samples, LOOCV means that n-1 samples are used to calibrate the model and 1 sample is used to assess the accuracy; this is repeated n times for each single sample in the calibration dataset (Niazi et al., 2015). Model accuracy (predicted vs measured PHC, PAH, ALK and HM contents) was evaluated using the coefficient of determination (r^2), the root mean square error of prediction (RMSEP), and the ratio of prediction deviation (RPD) (standard deviation of measured values divided by RMSEP) and the ratio of the performance to interquartile distance (RPIQ). In general, a good model prediction should correspond to high r^2, RPD and RPIQ, and low RMSEP values. In particular, model classification criterion adopted in this study were based on RPD values, which were divided into six classes: of excellent (RPD > 2.5), very good (RPD = 2.5 – 2.0), good (RPD = 2.0 – 1.8), fair (RPD = 1.8 – 1.4), poor (RPD = 1.4 – 1.0), and very poor model (RPD < 1.0) (Viscarra Rossel et al., 2006). The model hyper-parameters optimised during the LOOCV are the number of trees to be grown (*ntree*), number of predictor variables used to split the nodes at each partitioning (*mtry*), and the minimum size of the leaf (*node size*). The hyper-parameter optimization returned ntree = 500, mtry = 2 and note size = 3. All PHC, PAH, ALK, and HM models of both the total and bioavailable contents were developed with Random Forest-R package (Liaw and Wiener, 2015), utilising the Breiman and Cutler's Fortran code (Breiman, 2001).

4.2.4.3. Prediction

The calibrated models were then validated using the prediction data sets (31 samples) for both the total and bioavailable contents of PHC, PAH, ALK and each individual HM. Once again the accuracy of the prediction (predicted vs measured) was evaluated by r^2, RMSEP, RPD, RMSEP, and RPIQ and the outcome classified according to the criteria of Viscarra et al. (2006) as described above.

4.3. Results and discussions

4.3.1. Total and bioavailable PHC and HM contents in soils

The industrial soils (Soil 1 and 2) had the highest concentrations of total PHC with average of 445 mg/kg of which about 40% was found to be bioavailable (Figure 4.2). The PHC distribution was dominated by the EC_{21-35} PAH fraction which represented between 45% and 55% of the total PHC. The dominant ALK were within the EC_{16-35} fraction. These profiles are typical of aged contamination. The average HM content for both soils was 350 mg/kg and the bioavailable content was low (< 30%) especially for Al, Zn, Fe and Pb.

The other industrial contaminated soil (Soil 3) had an average concentration of HM 8 times higher (2800 mg/kg) and the PHC concentration was 3 times lower than Soil 1 and 2 (Figure 4.2). The EC_{21-35} PAH fraction contributes over 20% of the total PHC content. In contrast the bioavailable concentration were high for Zn and Pb (≥ 90, 70%), low for Cu and Se (29 and 33%), and very low for Al (6%), Fe (1%) and Cr (3%) (data not shown).

In the rural contaminated soils (Soil 4 and 5) the total average PHC content was two times lower compared to the industrial soils ranging between 230 and 180 mg/kg, of which about 50% was found to be bioavailable (Figure 4.2). As per the contaminated industrial soils, the PHC distribution was dominated by the EC_{21-35} PAH fraction and the ALK fraction EC_{16-35}.

The total average HM contents (< 200 mg/kg) were also 2 times lower than those found in the industrial contaminated soils (Figure 4.2). The average bioavailable concentrations of metals for rural soil samples were high for Cd only (≥ 90%), low for Cu and Se (38%, and 12%), and very low for Al, Fe, Pb and Cr (< 1%) in Soil 4. In Soil 5 HM were more available, in particular concentrations were high for Cu, Se, Cd and Pb (≥ 90%), medium for Zn and Ni (66% and 43%), and low for Al (8%), Fe (6%) and Cr (2%) in Soil 5.

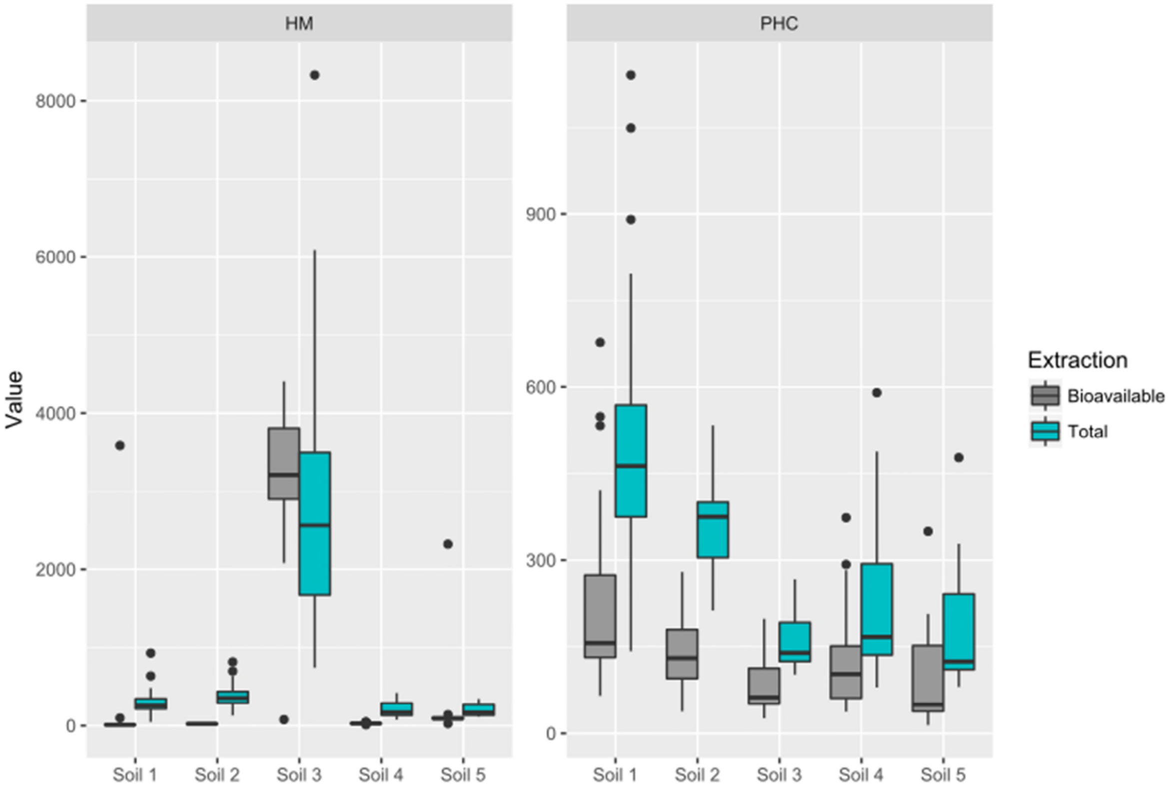

Figure 4.2: Total and bioavailable concentrations (mg/kg) of heavy metals/metalloids (HM) (left) and petroleum hydrocarbons compounds (PHC) (right) across the five soil types (n=105).

4.3.2. Model calibration and performance

Data obtained from soil spectral analysis and chemical analysis (total and bioavailable PHC, PAH, ALK and HM concentrations) were used in the calibration of the RF regression model; descriptive statistics of data used at this step are provided in Table 4.1.

The results of LOOCV of the ML model for total and bioavailability organic compounds are shown in Table 4.2 and Figure 4.5 (Appendix). The LOOCV results for both the total (r^2 = 0.88, RPD = 2.81, RPIQ = 5.04, and RMSEcv = 52.47 mg/kg) and bioavailable (r^2 = 0.82, RPD = 2.38, RPIQ = 3.62, and RMSEcv = 33.62) PAH were better than those for total and bioavailable PHC and ALK (Table 4.2 and Figure 4.5). The lowest accuracy was observed for ALK; however the LOOCV results of the total concentration were slightly better than those of the bioavailable concentration; r^2, RPD, RPIQ, and RMSEcv values of 0.82 and 0.77, 2.42 and 2.10, 1.75 and 1.62, and 30.74 and 18.74 mg/kg, respectively (Table 4.2).

As for the organics, the LOOCV results for HM were better for the total than for the bioavailable concentration. Descriptive statistics of HM concentrations used in calibration step are presented in Table 4.1, and parameters used to establish goodness of the model are presented in Table 4.2 and Figure 4.6 (Appendix). The highest LOOCV performance for the total concentration was obtained for Al (r^2 = 0.93, RPD = 4.05, RPIQ = 5.17, and RMSEcv = 2194.5 mg/kg) followed by Cr, Fe, Ni, and Cd, whereas the worst performance is obtained for Se (r^2 = 0.88, RPD = 2.99, RPIQ = 4.16, and RMSEcv = 0.36 mg/kg), followed by Pb, As, Zn and Cu (Table 4.2 and Figure 4.6). The models developed for the bioavailable concentration showed some similarities to those of the total concentrations, for the calibration model. Again Al model for bioavailable concentration was the highest performing in LOOCV (r^2 = 0.92, RPD = 3.77, RPIQ = 4.99, and RMSEcv = 96.67 mg/kg), followed by Cr, Cu, Cd and Fe, whereas the lowest performance was obtained for the Zn model (r^2 = 0.82, RPD = 2.41, RPIQ = 1.3, and RMSEcv = 257.87 mg/kg), followed by Se, As, Pb, Ni (Table 4.2 and Figure 4.6).

Table 4.1: Descriptive statistics of the calibration datasets of total and bioavailable contents of PHC, PAH, ALK and HM/metalloids used for the Random Forest (RF) modelling.

			N^{o}	Min	1st Q	Median	Mean	3rd Q	Max	SD
Organics	Total (mg/kg)	PHC	74	79	137	241	285	389	1049	188
		PAH	73	0.3	2.1	102	145	267	553	160
		ALK	73	49	109	126	146	163	496	74
	Bioavailable (mg/kg)	PHC	73	14	48	109	127	159	548	107
		PAH	73	0.2	1.2	60	76	131	326	82
		ALK	73	7.3	32	47	55	62	263	39
Inorganics	Total (mg/kg)	Al	74	2375	7289	12301	14409	18808	46195	9605
		Cr	73	5	17	25	29	37	85	16
		Cd	72	0.1	0.2	0.3	0.4	0.6	2	0.4
		Ni	74	2	11	15	18	26	49	10
		Zn	73	15	64	108	244	164	1964	393
		Se	72	0.4	1	2	2	3	6	1
		Cu	73	4	12	27	33	40	128	25
		Fe	74	787	10857	15300	17969	20955	57669	10822
		As	73	1	7	10	11	13	34	6
		Pb	74	9	31	61	288	131	2864	600
	Bioavailable (mg/kg)	Al	72	1	8	234	339	685	1037	355
		Cr	73	0.1	0.3	1	1	1	2	1
		Cd	73	0.1	0.2	0.2	0.3	0.2	2	0.4
		Ni	74	1	1	3	3	4	12	2
		Zn	72	4	9	15	314	26	1911	624
		Se	72	0.1	0.5	1	1	1	2	0.4
		Cu	72	0.2	2	6	7	12	18	6
		Fe	73	5	8	98	171	159	928	244
		As	72	0.3	0.5	1	1	1	1	0.2
		Pb	74	0.1	0.3	5	295	54	2463	690

PHC: petroleum hydrocarbons compounds; PAH: polycyclic aromatic hydrocarbons; ALK: alkanes; HM: heavy metals; Al: aluminium; Cr: chromium, Cd: cadmium; Ni: nickel, Zn: zinc; Se: selenium, Cu: copper; Fe: iron; As: arsenic; Pb :lead, Q: quartile, SD: standard deviation.

Table 4.2: Random Forest (RF) outputs for the calibration of the total and bioavailable concentrations of PHC, PAH, ALK and HM/metalloids in the contaminated soil samples.

		Compound	N°	r^2	RMSE(mg/kg)	RPD	RPIQ
Organics	Total (mg/kg)	PHC	74	0.83	78.2	2.4	3.2
		PAH	73	0.88	52.5	2.8	5.1
		ALK	74	0.82	30.7	2.4	1.8
	Bioavailable (mg/kg)	PHC	74	0.80	48.5	2.3	2.5
		PAH	73	0.82	33.6	2.4	3.6
		ALK	74	0.77	18.7	2.1	1.6
Inorganics	Total (mg/kg)	Al	73	0.93	2195	4.1	5.2
		Cr	73	0.93	4	3.7	4.8
		Cd	72	0.92	0.1	3.5	5.2
		Ni	74	0.92	3	3.6	5.6
		Zn	73	0.9	121	3.3	1.8
		Se	72	0.88	0.4	3	4.2
		Cu	73	0.9	8	3.3	3.5
		Fe	74	0.92	2967	3.6	3.4
		As	73	0.89	2	3.1	3.2
		Pb	74	0.88	198	3	2.6
	Bioavailable (mg/kg)	Al	72	0.92	97	3.8	5
		Cr	73	0.92	0.1	3.7	5.3
		Cd	73	0.91	0.1	3.3	3.4
		Ni	74	0.77	0.9	3.1	3.6
		Zn	72	0.82	258	2.4	1.3
		Se	72	0.86	0.1	2.7	3.2
		Cu	72	0.89	1.5	3.7	6.5
		Fe	73	0.89	78	3.1	1.9
		As	72	0.86	0.07	2.8	3.1
		Pb	74	0.86	199	2.8	2.8

PHC: petroleum hydrocarbons compounds; PAH: polycyclic aromatic hydrocarbons; ALK: alkanes; HM: heavy metals; Al: aluminium; Cr: chromium, Cd: cadmium; Ni: nickel, Zn: zinc; Se: selenium, Cu: copper; Fe: iron; As: arsenic; Pb: lead, r^2:coefficient of determination; RMSEP : root mean square error of prediction; RPD: ratio of prediction deviation; RPIQ: ratio of the performance to interquartile distance.

4.3.3. Model prediction: Estimation of total and bioavailable concentrations of complex chemical mixtures using RF regression

The RF calibration model developed was further validated using the prediction sets (30% of the data) of total and bioavailable complex chemical mixtures concentration. The descriptive statistics are provided in Table 4.3.

4.3.3.1. Prediction of total and bioavailable PHC

Based on the Viscarra et al. (2006) classification of RPD classes, the RF prediction performance trend for the total and bioavailable concentrations was PAH > PHC > ALK very good and fair for total and bioavailable PAH, good and fair for total and bioavailable PHC and fair and poor for total and bioavailable ALK (Table 4.4). The prediction of the total concentration of PAH was of better performance (r^2 = 0.75, RPD = 2.02, RPIQ = 3.49, and RMSEP = 79.81 mg/kg) than that for the bioavailable concentration (r^2 = 0.65, RPD = 1.72, RPIQ = 2.12, and RMSEP = 51.85 mg/kg) (Table 4.4 and Figure 4.3). Our prediction results are slightly better than the results reported by Douglas et al. (2018 a) for total PAH (r^2 = 0.71, RPD = 1.99, and RMSEP = 0.99 mg/kg), and comparable to those results reported by Okparanma et al. (2014) using partial least squares regression (PLSR) for oil contaminated soil samples collected from the Niger delta, Nigeria. The difference of results can be attributed to variation in the concentration range as well as the standard deviation (SD) between our study (range from 0.30 to 533 mg/kg, SD = 160) and those reported by Douglas et al. (2018 b) (range from 0.52 to 312.28 mg/kg, SD = 40.20). Statistical similarity between the calibration and prediction sets including the range as well as SD can be observed indicating positive impact of the models performance (Kuang and Mouazen, 2011).

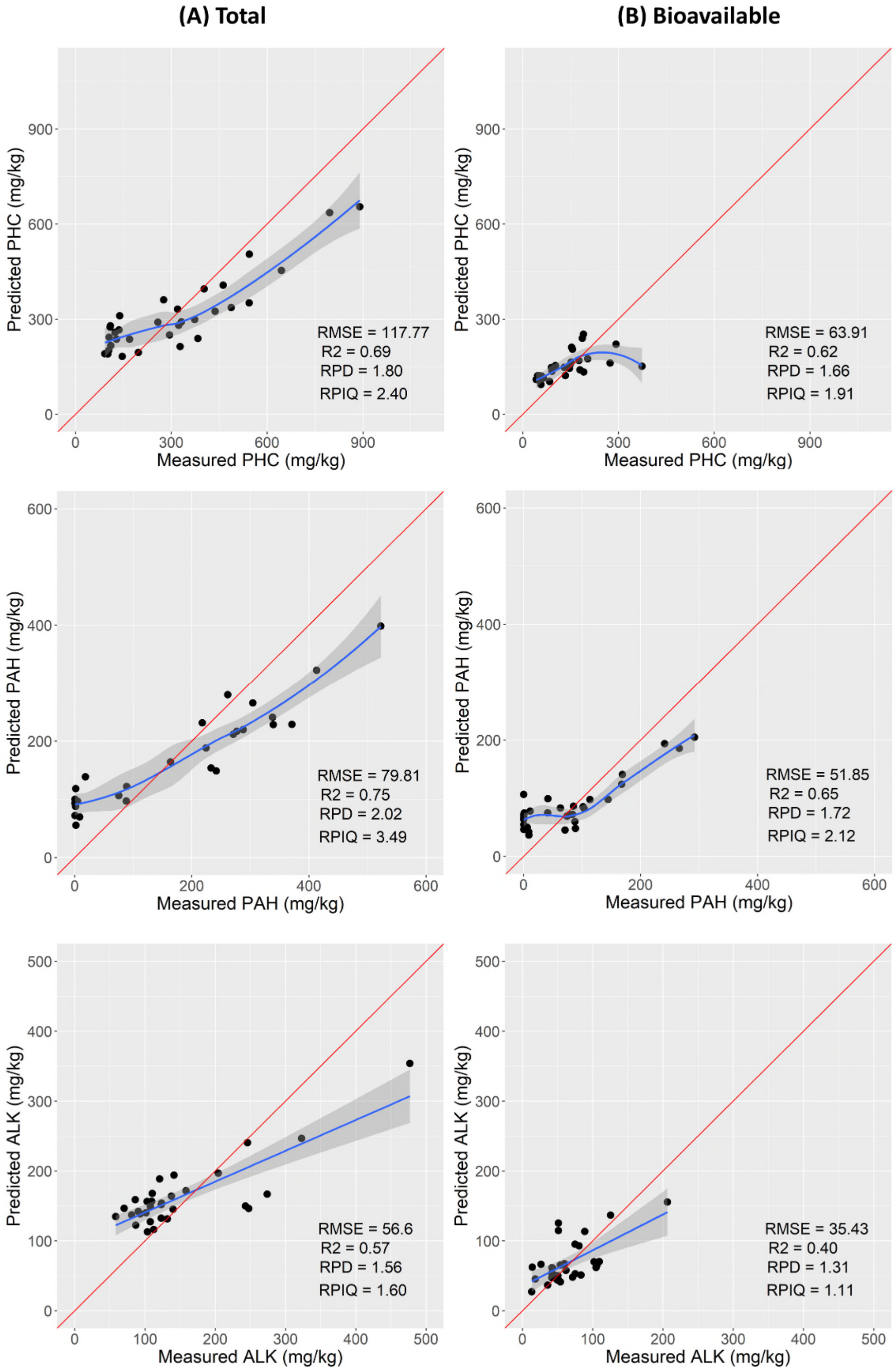

Figure 4.3: Scatter plots of the prediction datasets of total (A) and bioavailable (B) total petroleum hydrocarbons (PHC), aromatic (PAH), and alkanes (ALK), respectively.

4.3.3.2. Prediction of total and bioavailable HM/metalloids

Results of the prediction set (for 31 samples, descriptive statistics provided in Table 4.3) for HM/metalloids total concentration were rated as follow Pb > Al > Ni> Cr > Cd, where the highest performance was obtained for Pb (r^2 = 0.81, RPD = 2.35, RPIQ = 2.30, and RMSEP = 216.62 mg/kg). The lowest prediction performance is obtained for Cu (r^2 = 0.60, RPD = 1.59, RPIQ = 1.93, and RMSEP = 14.54 mg/kg), followed by Se, Zn, As and Fe (Table 4.4 and Figure 4.4). On the basis of the RPD values, predictions of the total content of Pb (RPD = 2.35) was the best, and can be classified as very good, as well as the prediction of Al, Ni, Cr, and Cd with RPD values of 2.21, 2.13, 2.10, 2.10, respectively; whereas the prediction of Fe, As, and Zn can be classified as good with RPD values of 1.95, 1.92, 1.89, respectively. The Se and Cu can be classified as fair predictions with RPD values of 1.77 and 1.59, respectively.

The prediction models developed for the bioavailable concentration showed the highest performance for Al (r^2 = 0.77, RPD = 2.13, RPIQ = 3.89, and RMSEP = 154.22 mg/kg), followed by Pb, Cr, Cd, and Ni, whereas the worst prediction was for As (r^2 = 0.45, RPD = 1.37, RPIQ = 1.74, and RMSEP = 0.15 mg/kg), followed by Se, Zn, Fe, and Cu (Table 4.4 and Figure 4.4). The prediction of the bioavailable concentrations shows differences of prediction quality, where Al, Pb, Cr, and Cd predictions are classified as very good with RPD values of 2.13, 2.10, 2.05, and 2.05, respectively. The prediction of Ni, Cu, Fe, Zn, and Se can be classified as fair with RPD values of 1.73, 1.63, 1.58, 1.55, and 1.44, respectively, whereas As prediction is of the worst accuracy (RPD = 1.37) and can be classified as poor. It can be confirmed that Al and Pb models showed the highest prediction performance for both the total and bioavailable concentrations, but with relatively high RMSEP values of 4101.3, and 154.2 mg/kg for Al, and 216.6 and 343.1 mg/kg for Pb, for total and bioavailable concentration, respectively.

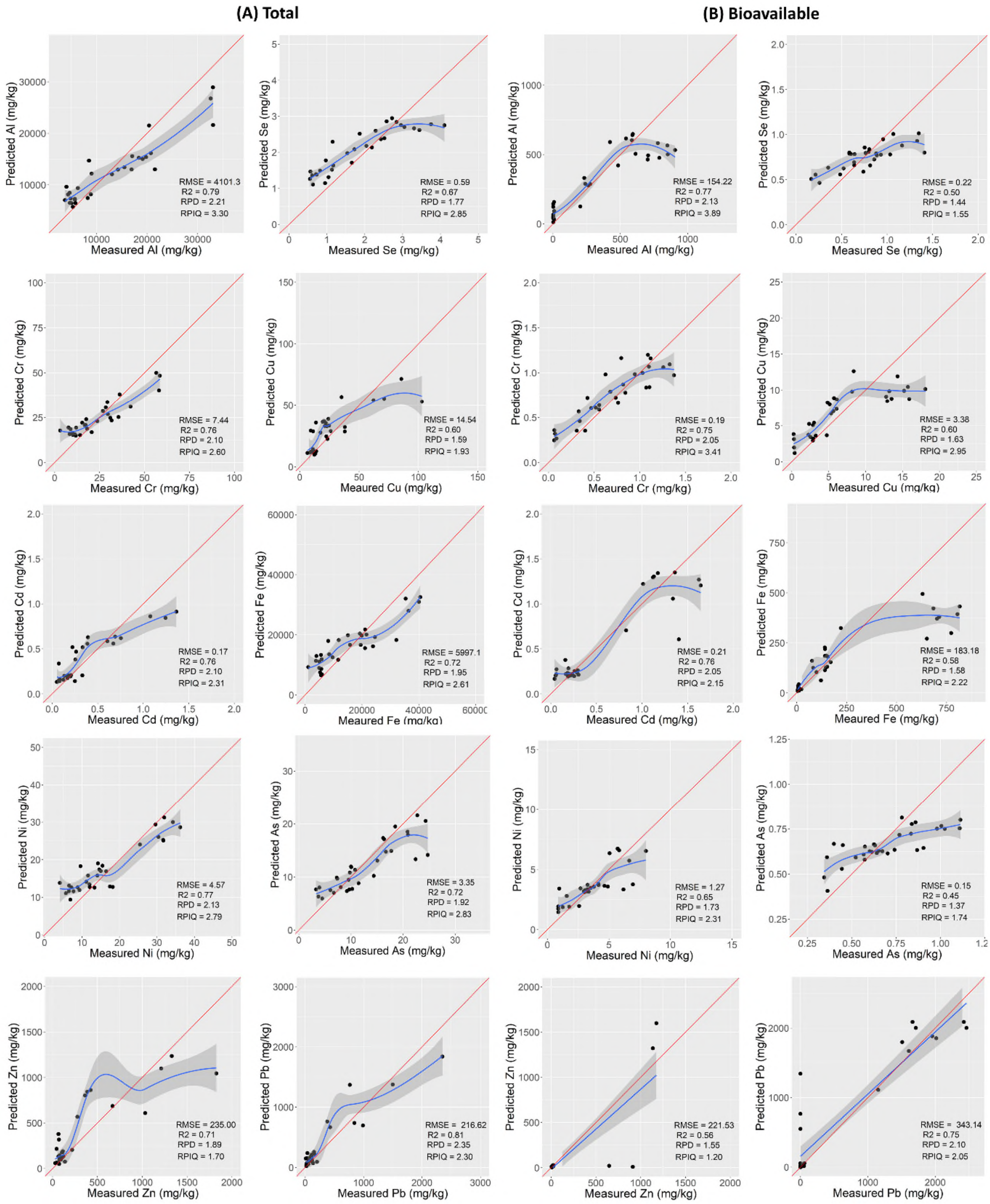

Figure 4.4: Scatter plots of the prediction datasets of total (A) and bioavailable (B) contents of HM/metalloids (Al, Cr, Cd, Ni, Zn, Se, Cu, Fe, As, and Pb).

Table 4.3: Descriptive statistics of the prediction datasets of total and bioavailable PHC, PAH, ALK and HM/metalloids used for the Random Forest (RF) modelling.

		Compound	Nº	Min	1st Q	Median	Mean	3rd Q	Max	SD
Organics	Total (mg/kg)	PHC	31	92	127	285	308	411	890	210
		PAH	31	0.6	2.9	190	172	285	522	160
		ALK	31	58	102	120	149	150	477	88
	Bioavailable (mg/kg)	PHC	31	42	58	130	131	177	374	80
		PAH	31	0.3	3.8	70	76	102	292	81
		ALK	31	35	13	54	65	82	206	38
Inorganics	Total (mg/kg)	Al	31	1543	5222	8920	12677	18772	33055	9113
		Cr	31	3	11	18	23	31	59	15
		Cd	31	0.1	0.1	0.2	0.4	0.4	1	0.4
		Ni	31	4	9	14	16	22	36	10
		Zn	31	30	66	105	303	320	1827	446
		Se	31	1	1	2	2	3	4	1
		Cu	31	6	12	21	27	25	103	23
		Fe	31	1109	5647	11825	15774	21352	40529	11672
		As	31	3	8	10	13	17	25	6
		Pb	31	11	40	106	314	291	2349	519
	Bioavailable (mg/kg)	Al	31	1	2	263	344	603	906	329
		Cr	31	0.1	0.4	1	1	1	1	0.4
		Cd	31	0.1	0.2	0.2	1	1	2	1
		Ni	31	1	2	3	4	5	8	2
		Zn	31	5	12	18	147	24	1176	343
		Se	31	0.2	1	1	1	1	1	0.4
		Cu	31	0.3	3	5	7	13	18	5
		Fe	31	6	18	142	252	426	816	290
		As	31	0.4	1	1	1	1	1	0
		Pb	31	0.1	0.3	5	577	1511	2408	888

PHC: petroleum hydrocarbons compounds; PAH: polycyclic aromatic hydrocarbons; ALK: alkanes; HM: heavy metals; Al: aluminium; Cr: chromium, Cd: cadmium; Ni: nickel, Zn: zinc; Se: selenium, Cu: copper; Fe: iron; As: arsenic; Pb: lead, Q: quartile, SD: standard deviation.

Table 4.4: Random Forest (RF) outputs for the prediction for total and bioavailable concentrations of PHC, PAH, ALK and HM/metalloids in contaminated soils.

		Compound	N°	r^2	RMSE(mg/kg)	RPD	RPIQ
Organics	Total (mg/kg)	PHC	31	0.69	117.8	1.8	2.4
		PAH	31	0.75	79.8	2.0	3.5
		ALK	31	0.57	56.6	1.6	1.6
	Bioavailable (mg/kg)	PHC	31	0.62	63.9	1.7	1.9
		PAH	31	0.65	51.9	1.7	2.1
		ALK	31	0.40	35.4	1.3	1.1
Inorganics	Total (mg/kg)	Al	31	0.79	4101	2.2	3.3
		Cr	31	0.76	7	2.1	2.6
		Cd	31	0.76	0.2	2.1	2.3
		Ni	31	0.77	5	2.1	2.8
		Zn	31	0.71	235	1.9	1.7
		Se	31	0.67	0.6	1.8	2.9
		Cu	31	0.6	15	1.6	1.9
		Fe	31	0.72	5997	1.9	2.6
		As	31	0.72	3	1.9	2.8
		Pb	31	0.81	217	2.4	2.3
	Bioavailable (mg/kg)	Al	31	0.77	154	2.1	3.9
		Cr	31	0.75	0.2	2.0	3.4
		Cd	31	0.76	0.2	2.0	2.2
		Ni	31	0.65	1.3	1.7	2.3
		Zn	31	0.56	222	1.6	1.2
		Se	31	0.5	0.2	1.4	1.6
		Cu	31	0.6	3	1.6	3
		Fe	31	0.58	183	1.6	2.2
		As	31	0.45	0.2	1.4	1.7
		Pb	31	0.75	343	2.1	2.1

PHC: petroleum hydrocarbons compounds; PAH: polycyclic aromatic hydrocarbons; ALK: alkanes; HM: heavy metals; Al: aluminium; Cr: chromium, Cd: cadmium; Ni: nickel, Zn: zinc; Se: selenium, Cu: copper; Fe: iron; As: arsenic; Pb: lead, r^2:coefficient of determination; RMSEP : root mean square error of prediction; RPD: ratio of prediction deviation; RPIQ: ratio of the performance to interquartile distance.

4.3.4. Applicability of Vis–NIRS to predict bioavailability of complex chemical mixtures

Although there are to date no other studies that used Vis-NIRS to predict bioavailable concentrations of complex chemical mixtures of hydrocarbons and HM in soils, some comparison can be drawn with previous studies. For example, Cave et al. (2015) showed that PAH bioaccessibility in soil samples can be successfully predicted using a combination of soil properties (measured by NIR and MIR spectra) and physico-chemical properties of the PAH. The accuracy (measured vs predicted bioaccessible PAH fraction (BPF)) of the RF model used in this study was found to be good (RMSEP = 0.038 mg/kg) and precise (normalised RMSEP < 15%). This confirms our findings that RF models which use infrared techniques in combination with organic contaminants and soil physico-chemical properties can be used to predict bioaccessible and bioavailable fractions with reasonable accuracy and precision.

Similarly, Chodak et al. (2007) used Vis–NIRS coupled with PLSR to determine the total and exchangeable concentrations of Zn and Pb in forest soil samples. However PLSR was found to be unsatisfactory for the prediction of both the total and exchangeable concentrations due to low RPD values (ranging between 1.1 - 1.3) and a tendency of underestimating both the total and the exchangeable HM at high concentrations. In contrast in our study, both the r^2 and RPD values for the bioavailable HM were much higher (Zn r^2 = 0.56 and RPD = 1.6; Pb r^2 = 0.75 and RDP = 1.6; average for all HM r^2 = 0.64 and RPD = 1.75) indicating that the RF model was better at predicting Pb bioavailable concentrations.

In another study, Li et al. (2011) showed a good prediction for the determination of metal ions in water samples using a pre-concentration step on a high capacity adsorbent material followed by NIR diffuse reflectance spectroscopy analysis. The r^2 values of the PLSR model were 0.92, 0.96, and 0.99 for Hg, Pb, and Cd, respectively. These values are higher than the one obtained in our study (r^2= n.a (Hg), r^2= 0.75 (Pb), r^2= 0.76 (Cd)). This could be attributed to (1) the use of a sorbent material rather than soil samples, (2) the homogeneous range of concentration obtained in the pre-concentration step where elements were uptaken from the aqueous solutions and transferred to the high capacity adsorbent (concentration range Hg = 4.3–50.4 mg/l, Pb = 4.93 – 48.8 mg/l and Cd = 5.9 – 48.8 mg/l). In contrast in our study genuine contaminated soil samples from 5 different locations have been used, creating a more heterogeneous dataset with different soil characteristics and different concentrations (Hg below detection limit; Pb = 0.03- 2463.4 mg/kg; Cd = 0.03 - 6.79 mg/kg).

4.4. Conclusion

This study demonstrated that Vis-NIRS can be used as a rapid measurement tool for discriminating and estimating complex chemical mixtures of heavy metals, metalloids and petroleum hydrocarbons in soils. The predictions for the total concentrations of the chemical mixtures were very good especially for the PAH and elements including Pb, Al, Cr, Cd, Fe, Ni, and Zn; good to fair for the PHC, As and Se and fair to poor for the ALK and Cu. In contrast the predictions of the bioavailable concentrations of both PHC and HM were generally weaker than the total concentrations probably due to the small data set used for the calibration and prediction and overall lower concentrations values (≤50% of the total concentration value). Nevertheless, the results are promising and better than other studies focusing only on total concentrations. Overall this study confirmed that coupling Vis-NIRS to machine learning model offers a promising way forward to speed-up site investigation, identify and discriminate contaminant (i.e. hydrocarbons vs heavy metals) and predict not only the total concentration of the chemical of concern but also the concentration likely to pose significant risk (bioavailable) and therefore inform the risk assessment and decision making for contaminated sites in a timely fashion.

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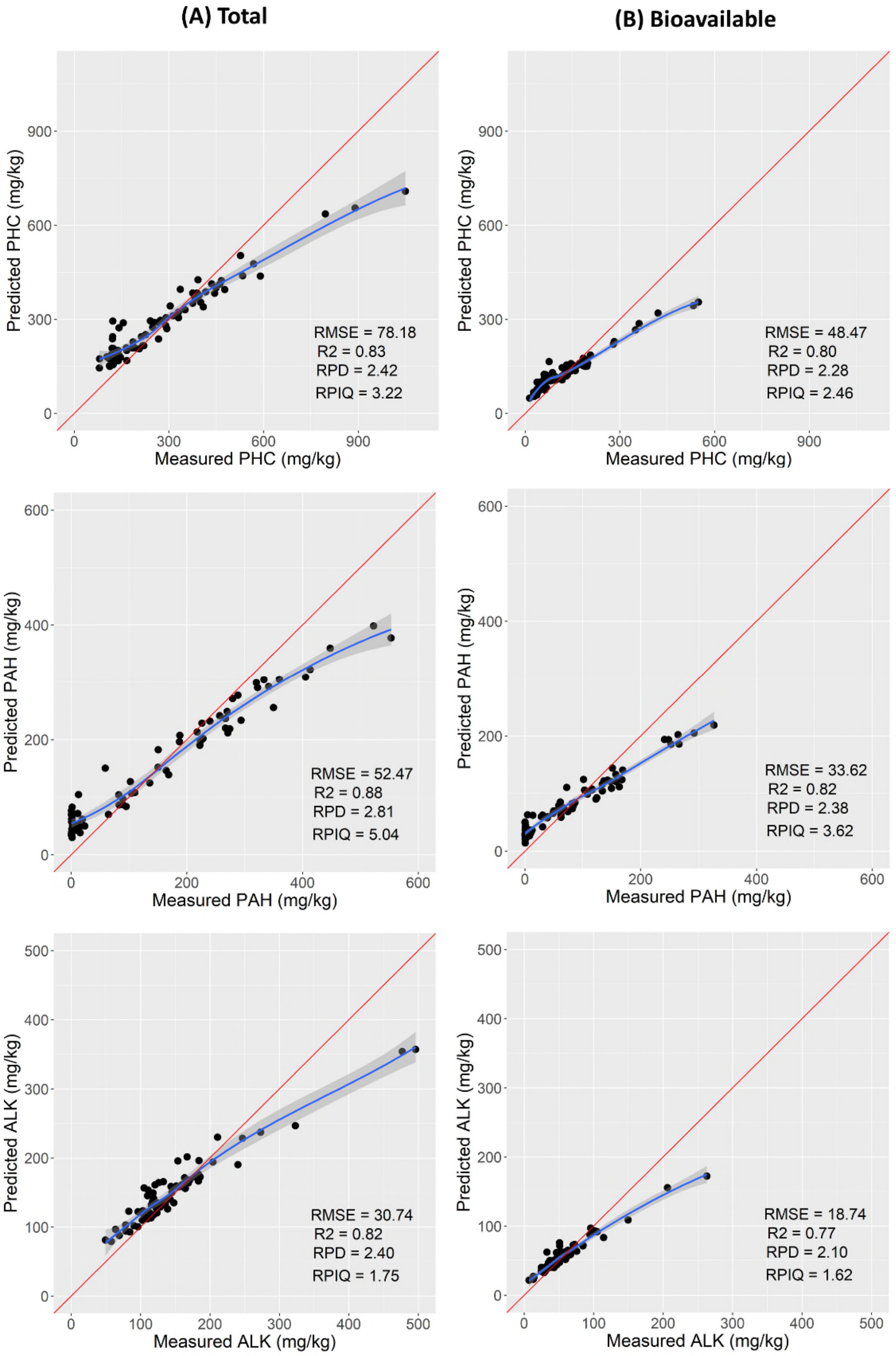
4.6. Appendix



(A) Total

(B) Bioavailable

Figure 4.5: Scatter plots of the calibration datasets of total (A) and bioavailable (B) contents of petroleum hydrocarbon compounds (PHC), polycyclic aromatic hydrocarbon (PAH), and alkanes (ALK).

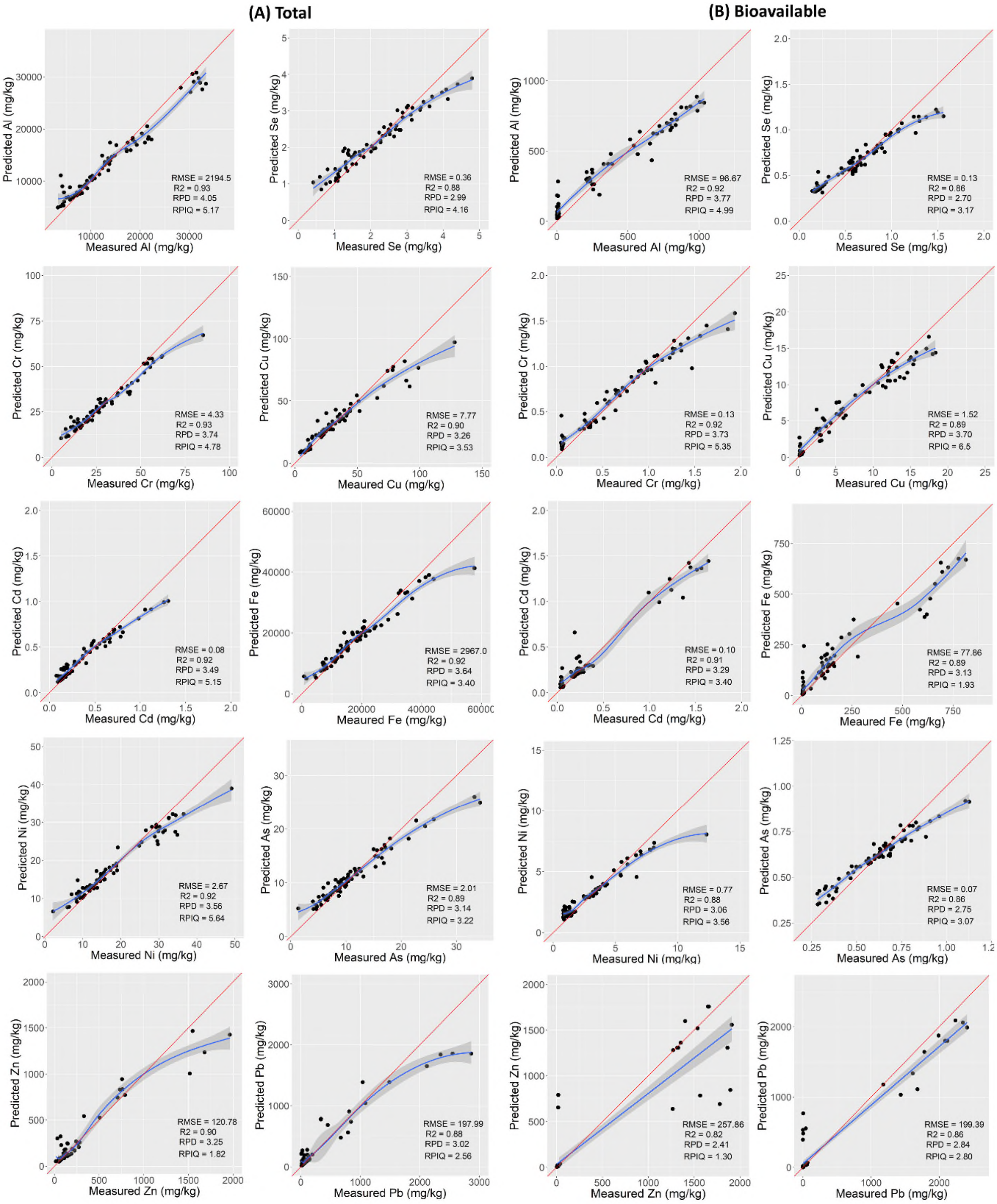


Figure 4.6: Scatter plots of the calibration datasets of total (A) and bioavailable (B) contents of HM/metalloids (Al, Cr, Cd, Ni, Zn, Se, Cu, Fe, As, and Pb).

5. Linking bioavailability and toxicity changes of complex chemicals mixture to support decision making for remediation endpoint of contaminated soils

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Abstract: A six-month laboratory scale study was carried out to investigate the effect of biochar and compost amendments on complex chemical mixtures of tar, heavy metals and metalloids in two genuine contaminated soils. An integrated approach, where organic and inorganic contaminants bioavailability and distribution changes, along with a range of microbiological indicators and ecotoxicological bioassays, was used to provide multiple lines of evidence to support the risk characterisation and assess the remediation end-point. Both compost and biochar amendment ($p = 0.005$) as well as incubation time ($p = 0.001$) significantly affected the total and bioavailable concentrations of the total petroleum hydrocarbons compounds (PHC) in the two soils. Specifically, PHC concentration decreased by 46% and 30% in Soil 1 and Soil 2 amended with compost. These decreases were accompanied by a reduction of 78% (Soil 1) and 6% (Soil 2) of the bioavailable hydrocarbons and the most significant decrease was observed for the medium to long chain aliphatic compounds (EC_{16}-$_{35}$) and medium molecular weight aromatic compounds (EC_{16}-$_{21}$). Compost amendment enhanced the degradation of both the aliphatic and aromatic fractions in the two soils, while biochar contributed to lock the hydrocarbons in the contaminated soils. Neither compost nor biochar affected the distribution and behaviour of the heavy metals (HM) and metalloids in the different soil phases, suggesting that the co-presence of heavy metals and metalloids posed a low risk. Strong negative correlations were observed between the bioavailable hydrocarbon fractions and the ecotoxicological assays suggesting that when bioavailable concentrations decreased, the toxicity also decreased. This study showed that adopting a combined diagnostic approach can significantly help to identify optimal remediation strategies and contribute to change the over-conservative nature of the current risk assessments thus reducing the costs associated with remediation endpoint.

Keywords : contaminated soils, mixtures, bioavailability, bioremediation, toxicity.

5.1. Introduction

Anthropogenic activities are the main cause of release of potentially toxic compounds in soil, among which heavy metals, metalloids, and petroleum hydrocarbon products are the ones mostly found at contaminated sites (Hou and Al-tabbaa, 2014). Remediation approaches at contaminated sites are promising strategies to mitigate the risks posed by the pollutants; in particular bio-stimulation and bio-addition are common practices aiming at improving soil quality; adding organic matter, delivering nutrients, balancing pH, and increasing water holding capacity, thus enhancing the degradation process (Wang et al., 2017). Commonly used amendment for soil remediation include composted agricultural by-products and coal combustion products (e.g. biochars) (U.S. Environmental Protection Agency, 2007), which have been widely studied due to their sustainable, efficient, and cost-effective approach (Ahmad et al., 2014). Compost amendment is a common remediation strategy where organic materials, such as manure or decomposed organic matter, are added to contaminated soil to stimulate soil microorganisms and thus promoting transformation of hydrocarbons into less toxic compounds (Davie-Martin et al., 2017). Apart from providing a carbon source for the existing pool of bacteria, compost addition can also introduce new microorganisms presenting different catabolic activities that could potentially enhance the remediation of polluted soils (Baldantoni et al., 2017).

Another common amendment strategy is adding biochar, a carbon-rich material obtained from the decomposition of biomass in absence (or low exposure) of oxygen (Liu et al., 2015). Due to its highly porous structure and alkaline nature, biochar is able to immobilise soil contaminants, hence its frequent usage in soil remediation (Egene et al., 2018). When added to the soil, biochar causes the release of carbonates, phosphates, and hydroxyl ions because of its alkaline pH value of 7–10, thus favouring metal stabilisation. Both electrostatic (surface adsorption) and non-electrostatic (functional groups complexation) interactions are responsible for a decrease in metals mobility and bioavailability (Van Poucke et al., 2018).

While organic amendments have been shown to effectively improve degradation of pollutants in soil, remediation success has often been defined by reduction of total contaminant concentration (Kuppusamy et al., 2017) rather than bioavailable concentrations. However, the extent to which contaminants are bioavailable has significant implications for the clean-up targets and risk assessment, as receptors respond to the fraction of contaminant that is bioavailable rather than the total fraction (National Research Council, 2003). Bioavailability, the freely available fraction of contaminants in soil (Semple et al., 2003), is nowadays regarded as an important feature to integrate in risk assessment as it can help to explain contaminants partitioning and degradation in the environment (Ortega-Calvo et al., 2015). In this regard, soil bioassays have been largely used to determine the ecological effects of complex chemicals or mixtures in environmental samples, since they provide a rapid characterization of the contaminants' bioavailable fraction (Mazzeo et al., 2014).

There is a need to find a pragmatic and practical integrated approach where biological and chemical measures of bioavailability are correlated rather than developed independently, supporting the necessity of several lines of evidence for robust and informed risk assessment (British Standards Institution, 2017). Ecotoxicological methods along with bioavailability have the potential to offer a cost-saving approach to contaminated land by applying relatively cheap bioassays to evaluate the potential effects of contaminants of concern, and to demonstrate that a contaminated site may not require further actions (Kim et al., 2014; Sarsby and Meggyes, 2009; Udovic et al., 2013). However, the majority of these approaches focus often on single contaminant rather than mixtures. Thus, assessing and implementing bioavailability of complex chemical mixtures in order to reduce conservativisms of the traditional chemical-based approach, remains a challenge (Cachada et al., 2016; Kienzler et al., 2016).

Therefore in the present study, we investigated the effect of soil amendments (compost or biochar) on the behaviour and bioavailability of a complex tar mixture containing aromatics, aliphatics, heavy metals, and metalloids. This work aims at assessing the relevance of a different range of biological indicators to understand the implications for risk assessment and identifying the end-point remediation. Moreover, bioavailability-proxy and the toxicity data were further correlated to provide the necessary evidence that these tools may be suitable for predicting site-specific bioavailability of complex chemical mixtures.

5.2. Materials and methods

5.2.1. Sample collection, physico-chemical characterisation

Two soils were collected from two UK brownfield sites occupied by former gasworks in Kent (51° 18' 39" N 0° 43' 17" E, Soil 1) and Northamptonshire (52° 20' 23" N 0° 39' 17" W, Soil 2). Prior analysis, soil samples were homogenised through 2 mm sieve to separate large particles such as plant parts (roots, stems, and leaves), cobbles and pebbles. Each soil samples was divided and processed for analysis, individual air-dried samples were used for: Soil pH analysis (10 mL), particle size distribution (10 mL), loss of ignition (5 g), total nitrogen (TN) and total carbon (TC) (0.001 mg), total phosphorous (TP) and available phosphorous (AP) (5 g). Additionally, fresh soil samples were used for dry matter and water content analysis (5 g).

TN and TC in soil material were determined by combustion at approximately 900°C in the presence of oxygen; the amount of nitrogen and carbon was then measured by a thermal conductivity detector (TCD) (BS EN 13654-2 2001) using a vario EL III Element Analyser (Elementar Analysensysteme GmbH,DE). Total phosphorous was measured with a hydrochloric/nitric acid mixture extraction and the phosphorus content was determined by a spectrometric measurement in solution (ISO 11047:1998) with a Spectronic Helios Gamma (Thermo Electron Corporation, UK). Available phosphorous was measured by treating the soil with a 0.5 mol/L sodium hydrogen carbonate solution at pH 8.5, the extract was then analysed by spectrometry (ISO 11263:1994). Ammonium-N, nitrate-N and were extracted from soil using a 2 mol/L solution of potassium chloride, the extract was analysed by spectrometry (Method 53 of the MAFF Reference Book RB427 1986).

Dry matter and water content in soil samples were measured by drying at 105°C ± 5°C for 24 h. The difference in mass of an amount of soil before and after the drying procedure was used to calculate the dry matter and water contents on a mass basis (ISO 11465:1993). Soil pH was measured with 1 part of soil per 5 parts of water after shaking for 60 min and subsequent equilibration period of 30 min (ISO 10390:2005). The soil organic content was determined by loss of ignition (LOI): air-dried soil was dehydrated at 105°C for 24 h then ashed at 450°C for 5 h and ignition loss was expressed as a percentage of the dehydrated sample (BS EN 13039:2000). Particle size distribution was determined by the sieving and sedimentation method. In short, soil organic matter was decomposed with hydrogen peroxide and the resulting slurry dispersed with a buffered sodium hexametaphosphate solution, then the different particle size fractions were determined by a combination of sieving and sedimentation (ISO 11277:2009). The corresponding soil texture classes were identified by using a soil texture calculator (Natural England Technical Information Note TIN037 2008).

To obtain the mass fraction, the compost and biochar samples were oven dried at 60°C for 24 hours to remove any residual water absorbed during storage (López et al., 2002). The samples were then sieved using a series of five sieves with mesh widths of 20 mm, 10 mm, coarse (0.6 - 2 mm), medium (0.2 – 0.6 mm), and fine (0.06 - 0.2 mm). The contribution of each mass fraction was expressed as percentage of the total.

5.2.2. Mesocosms experimental design

For each soil, duplicate mesocosms (i.e. 10-L polypropylene buckets) containing 5 kg of soil amended either with 15% w/w compost (Soil + Compost), with 5% w/w biochar (Soil + Biochar), or without amendment (Soil) were prepared. The biochar used in this study was a commercial enriched biochar purchased from Carbon Gold (UK). The 5% biochar to soil ratio was selected, as it is often reported as the most efficient application rate to reduce leachable contaminant concentrations in contaminated soils. For instance, in these studies, biochar was applied (loading rates at 5% w/w) and significantly reduced bioavailability (Wang et al., 2017) and leachability of HM concentrations, for both genuinely contaminated (Novak et al., 2018) and spiked (Park and Choppala, 2011) soils. Compost used in this study was multi-purpose enriched peat-based compost with nutrients purchased from Westland Horticulture Ltd (UK). Similarly, 15% compost to soil ratio was selected as a best criteria based on previous studies (Singh and Ward, 2004; Taccari et al., 2012; Adams et al., 2015; Speight and El-Gendy, 2017).

All the mesocosms were manually mixed to obtain homogenous samples and stored outdoor for the whole duration of the experiment. Samples were collected from each mesocosm at 0, 30, 90 and 180 days for chemical, microbiological, and toxicological analysis. All samples were collected randomly by disturbing the top 30 cm of the mesocosm' s content and immediately stored at 4°C to minimise biological transformation or other chemical reactions. Biological and ecotoxicological analyses were always carried out within 3 days of sampling.

5.2.3. Chemical analysis

5.2.3.1. Total and bioavailable PHC extraction

A modification of the method reported by Risdon et al. (2008) was used to determine total, readily-available, and bioavailable petroleum hydrocarbons including both aliphatic and aromatic fractions (See Table 5.1 for the fractions and PHC content). Briefly, 2.5 g of soil were mixed with either (i) 15 mL of 1:1 dichloromethane: hexane, (ii) 15 mL of methanol, or (iii) 20 mL of 4:1 mixture of hydroxypropyl-β-cyclodextrin (HP-β-CD): water solution to extract the total, readily-available, and bioavailable petroleum hydrocarbons content, respectively. For the estimation of the total and readily-available hydrocarbon content, the samples were sonicated for 20 min at room temperature, and shaken at 150 rpm for 16 h. On the following day, samples were again sonicated for 20 min at room temperature and centrifuged (2000 g for 10 min). The supernatant was then cleaned onto a 6 mL SPE DSC-Si silica tubes. From the 15 mL, 0.5 mL of sample was taken and mixed with 0.5 mL of internal standards comprised of a deuterated alkanes mix ($C10^{d22}$, $C19^{d40}$, and $C30^{d62}$) and deuterated polycyclic aromatic hydrocarbons mix (1,4-dichlorobenzene ^{d4}, naphthalene^{d8}, anthracene^{d10}, chrysene^{d12} and perylene^{d12}) at 10 µg/mL each, respectively.

For the estimation of the bioavailable hydrocarbon content, samples were mixed with HP-β-CD : water solution according to Reid et al. (2000). Following 20 h mixing, the samples were centrifuged at 2000 g for 30 min. The supernatant was discarded and the soil pellets were resuspended in 1:1 dichloromethane: hexane (exhaustive solvent extraction) to assess the residual amount of organic compound as described by Risdon et al. (2008). The compounds uptaken by the cyclodextrin molecule was then measured subtracting the residual amount of organic compound extracted by dichloromethane: hexane after the initial HP-β-CD wash, against the total amount extracted by dichloromethane: hexane (Papadopoulos et al., 2007).

Concentration of petroleum hydrocarbons were identified and quantified by gas chromatography-mass spectrometry (GC-MS) using an Agilent gas chromatograph coupled to a Turbomass Gold mass spectrometer operated at 70 eV in positive ion mode. The column used was a Restek fused silica capillary column (30 x 0.25 mm internal diameter) coated with RTX$^{®}$-5MS (0.25 µm film thickness). Splitless injection with a sample volume of 1 µL was applied. The oven temperature was increased from 60°C to 220°C at 20°C/min then to 310°C at 6°C/min and held at this temperature for 15 min; for a total run time of 38 min. The mass spectrometer was operated using the full scan mode (range m/z 50-500) for quantitative analysis of target aliphatic and aromatic hydrocarbons. For each compound, quantification was performed by integrating the peak at specific *m/z*. External multilevel calibrations were carried out using alkane (standard solution (C_8-C_{40}) Sigma Aldrich, Dorset, UK) and PAH (EPA 525 PAH Mix A; Sigma Aldrich, Dorset, UK) standards, the concentration of which ranged from 2.5 to 50 µg/mL respectively. For quality control, blank controls and a 500 µg/mL diesel standard solution (ASTM C_{12}-C_{60} quantitative, Supelco) were analyzed every 20 samples. The variation of the reproducibility of extraction and quantification of soil samples were determined by successive injections (n= 7) of the same sample and estimated to ± 8%. In addition, duplicate reagent control and reference material were systematically used. The reagent control was treated following the same procedure as the samples without adding soil sample. The reference material was an uncontaminated soil of known characteristics, and was spiked with a diesel and mineral oil standard at a concentration equivalent to 16,000 mg/kg. The relative standard deviation (RSD) value for all the soils was < 10%.

5.2.3.2. Pseudo-total metal and CISED sequential extractions

Pseudo-total metal digestion was performed according to the ISO 11047 method with aqua regia (ISO 11047:1998). Briefly, 0.5 g of air-dried and 2 mm sieved soil was extracted with 8 mL hydrochloric/nitric acid mixture in a microwave digestion system. The extract was then filtered through 0.45 µm nylon syringe filters and diluted to 50 mL with deionised water. A modification of the method reported by Cave et al. (2004) was used for the sequential extraction; approximately 2 g of soil was consecutively extracted each time with 10 mL of solutions with increasing concentrations of nitric acid from (0 to 5 M) and H_2O_2 (Cipullo et al., 2018).

All pseudo-total and sequential extracts were diluted 4 times with 1% HNO_3 before analysis using a NexION® 350D ICP-MS (Perkin Elmer) calibrated with a mixture of both major (Ca, Fe, K, Mg, Mn, Na, S, Si, P) and trace (Al, As, Ba, Cd, Co, Cr, Cu, Hg, Li, Mo, Ni, Pb, Sb, Se, Sr, V, Zn) elements ranging between 1 and 40 µg/mL and 0.01 and 2 µg/mL, respectively. In both cases, working standards were prepared in matching sample matrix solutions (nitric acid 1%). Calibration standards and samples extracts were spiked with the following mix of four internal standards: Sc, Ge, Rh, and Bi. ICP–MS was calibrated after each sample (14 sequential extracts) and the limit of detection was defined as concentration three times larger than the standard deviation of the acid blank. Additionally, acid blanks (1% nitric acid), digestion blank, and guidance materials (BGS_{102}) were analysed every batch of 7 samples along with an adequate rinse time programmed in between samples; to monitor blank contamination, sensitivity, operating conditions, and extraction's accuracy. Mean repeatability of BGS_{102} (expressed as relative standard deviation%) was lower than 6 and 8% for sequential and aqua regia digestion respectively. All elements' concentrations have been converted into mg/kg extracted from the soil-solid matrix.

Data obtained from sequential extraction were used in a chemometric self-modelling algorithm known as the Chemometric Identification of Substrates and Element Distributions (CISED). The CISED was performed with MatLab® (Version R2015a, 8.5.0.197613, 64-bit, Academic Licence) following the protocol developed by Cave et al. (2004) and Denys et al. (2012), and was used to derive information on the partitioning and bioavailable concentrations of HM and metalloids in soil. The self-modelling mixture resolution (SMMR) algorithm produced three key outputs (1) profile output: contains the modelled soil component with similar physical-chemical properties (mg/kg), (2) distribution output: contains the concentration of each element across the identified soil components (mg/kg), (3) composition output: contains element concentration in the identified component (expressed as percentage). Modelled soil components and element distribution data, obtained from the MatLab® algorithm, have been post-processed in RStudio® (Version 1.1.423 – © 2009-2018 RStudio, Inc.) to create a matrix, which has been further categorised using a clustering methodology, and visualised in a heatmap as previously described by Wragg et al. (2014) and Cox et al. (2013) (Appendix Figure 5.9). More details on the sequential extraction data processing and heatmap visualisationare provided in paragraph 3.7 Annex. Both profile and clustering outputs were then used to calculate HM/metalloids concentration and distribution in: (1) pore water fraction: HM/metalloids easily extractable with DI water, 0.01 and 0.05 M nitric acid, therefore highly mobile and potentially bioavailable (Giller et al., 2009; Ogundiran and Osibanjo, 2009), (2) exchangeable fraction: HM/metalloids associated with carbonates that can become available with time (Karbassi and Shankar, 2005; Sundaray et al., 2011), and (3) non-exchangeable fraction: HM/metalloids bounded with oxides therefore non-available and unlikely to pose risk for receptors (Hodson et al., 2011; Kim et al., 2015) (Appendix Figure 5.10).

5.2.4. Microbiological analysis

5.2.4.1. Determination of total bacteria count

Determination of culturable bacteria was performed according to Coulon et al. (2010). Briefly, 1 g of soil was weighed into a 50-mL centrifuge tube and 10 mL of Ringer’s solution (1/4 strength) added. Tubes were then vortexed for 30 s and sonicated twice for 30 s and allowed to stand for a further 2 min. A 1-mL aliquot of soil suspension was removed and serially diluted in Ringer’s solution to the appropriate dilution factor (10^{-6}). An aliquot sample of 100 µL of each dilution series was added in triplicate to Tryptone Soya Agar (TSA) medium for incubation at 25ºC for 24 - 48 h. Subsequently, colony-forming units (CFU) were enumerated.

5.2.4.2. Basal respiration

Two replicates (5 g) of each soil were placed in a 24 mL sterilised vial and sealed. An empty vial, with an ambient air sample from the laboratory was taken and analysed to account for background conditions. Vials were left to equilibrate for 5 h, incubated for 24 h at 20ºC, and the headspace analysed for CO_2 content (Paton et al., 2006). The composition of the headspace produced was recorded by CSi 200 Series GC (Cambridge Scientific Instruments Ltd., Witchford, UK), using helium as carrier gas at 20 psi (138 kPa). The Gas Chromatography with Thermal Conductivity Detector (GC-TCD) was equipped with a CTR1 concentric packed column (Alltech, USA). The column oven and injector temperature were 110ºC and 125ºC, respectively. The instrument was calibrated with CO_2 calibration standards (STG of Calgaz^{TM}, UK) in the range 1- 5% CO_2 balanced with $N_{2.}$

5.2.4.3. Phospholipid Fatty Acid Analysis (PLFA)

Phospholipid fatty acid (PLFA) analysis was used to identify and assess the community structure as reported by Frostegård et al. (1993). Phospholipids in approximately 7 g of freeze dried soil were extracted with chloroform, methanol and citrate buffer (1:2:0.8 by volume), separated by solid-phase extraction and then derivatised by mild alkaline methanolysis. Fatty acid methyl esters were analysed by Gas Chromatography with Flame Ionisation Detector (GC-FID) (Agilent Technologies 6890N) fitted with a HP-5 fused silica capillary column (30 m length, 0.32 mm ID, 0.25 µm film). Helium was used as a carrier gas at 1 mL/min flow rate. The initial oven temperature was 50°C hold for 1 min (splitless mode) and subsequently ramped to 160°C at 25°C/min, 240°C at 2°C/min and 310°C at 25°C/min. Three injection volumes were 1 µL and the injector temperature was set at 310°C. A total of 34 different PLFA were detected according to Pawlett et al. (2013) and Tunlid (1992). The relative abundance of individual PLFA was expressed as a percentage of the total of the target responses of all identified PLFA peaks and calculated from the subtraction between peak response of the sample and blank (solvent) response. PLFA containing fewer than 14 or more than 20 carbons were excluded, as this range is considered to be typical of microbial cellular membranes from the domains of bacteria and fungi (Quideau et al., 2016).

5.2.5. Ecotoxicological bioassays

5.2.5.1. Seeds germination assay

Mustard (*B. alba*), rye grass (*L. perenne*), and pea (*P. sativum*) species were used in the seed germination assays (Dawson et al., 2007). Five mustard, five rye grass, and three pea seeds were added separately in glass jars (triplicates) containing 20 g of soil re-wetted to 70% moisture. Lids were loosely screwed on to reduce evaporation but allowing aeration. Seeds were left to germinate in a controlled temperature chamber in the dark at 25°C and 70% humidity. A clean uncontaminated soil (control) was used to take into account the germination rate of the seeds. Germination incidence of above 90% was recorded in the uncontaminated soil for all seeds at all sampling times over the experimental period. When > 70% seeds in the uncontaminated soil germinated, the number of seeds germinated in all soil samples was recorded; this was after 4, 6, or 7 days exposure.

5.2.5.2. Earthworms acute toxicity assay

Tiger worm (*E. fetida)* was used to assess lethality and sub-lethal effects in earthworm acute toxicity assay. Adult worms, maintained in uncontaminated compost, weighing between 0.5 and 1.5 g were washed in tap water and depuriated overnight. Individual earthworms were placed in a pot containing 50 g of soil re-wetted to 70% moisture (Dawson et al., 2007). Five replicates were used for each soil sample. Lids were screwed onto the pot and perforated to allow aeration, but prevent water loss and worm escape. Pots were incubated at room temperature for 14 d. Specimens were examined on days 3, 7, and 14 for lethality and assigned a score (0, 1, or 2) from a Condition Index (CI) (Langdon et al., 1999). To assess sub-lethal effects worms were again washed with tap water on day 14, re-weighed on day 15 and the change in weight calculated.

5.2.5.3. Microtox® Basic Solid Phase Test

Each of the soil samples (Soil, Soil + Compost, and Soil + Biochar) were collected at 0, 30, 90, and 180 days and used to evaluate soil toxicity to bioluminescent bacteria (*Vibrio fischeri*) with Microtox® assay (Modern Water). The Basic Solid Phase Test procedure (BSPT) tested a sample at 12 dilutions with 99,000 $mg_{soil}/L_{diluent}$ being the highest concentration for highly contaminated soil (Soil 1), and at 5 dilutions with 396,000 $mg_{soil}/L_{diluent}$ being the highest concentration for low-contaminated soil (Soil 2). Briefly 3.5 g sample were mixed with either 17.5 mL (Soil 1) or 4 mL (Soil 2) of diluent respectively, shaken for 10 minutes, centrifuged 3 min at 1000 g and analysed according to Microtox® BSPT assay. The bacterial reagent is sensitive to pH, therefore samples with pH higher than 8.00 were adjusted using small aliquots of HCl (200 µL at 0.25 M). A 100 g/L zinc sulphate standard solution was used to check the performance of both operator and analytical system and the 95% confidence range was maintained below 15% variation throughout the study. The soil dilution that inhibits 50% (EC_{50}) of the light output relative to was calculated for each sample, note that Microtox® EC_{50} values decline as toxicity increases.

5.2.6. Data analysis

Data analysis was performed on independent mesocosms duplicates for each amendment at the time points described (0, 30, 90, and 180 days). Aromatic fractions were grouped as EC_{10}-EC_{12}, EC_{12}-EC_{16}, EC_{16}-EC_{21}, and EC_{21}-EC_{35}, aliphatic fractions were grouped as EC_{10}-EC_{12}, EC_{12}-EC_{16}, EC_{16}-EC_{35}, and EC_{35}-C_{40} according to Coulon et al. (2010). The overall aromatic (Σ PAH), overall aliphatic (Σ ALK) and total petroleum hydrocarbon compounds (ΣPHC) contents were also calculated (Table 5.1).

Repeated-measures ANOVA test was used to investigate the significance and relationship between soil amendment (biochar, compost, or un-amended) and incubation time on the toxicological response in model organisms (for *uni-variate datasets*, e.g. bacteria count, soil respiration, Microtox®). Permutational Multivariate Analysis of Variance (PERMANOVA) was used was used for multi-variate datasets, to investigate the significance and relationship between soil amendment (biochar, compost, or un-amended) and incubation time on: (1) the bioavailable and readily available fraction (organic contaminants) or the pore water and exchangeable fraction of inorganic contaminants (heavy metals/metalloids), (2) toxicological response in model organisms for *multi-variate datasets* (e.g. seeds germination, earthworm lethality, PLFA profiles). Both Repeated-measures ANOVA and PERMANOVA were performed in R Studio using the "*aov*" and "*adonis*" function of the vegan library respectively (Oksanen et al., 2011).

To establish correlation between the bioavailable fraction measured by chemical means and the toxicity response of the bioassays, univariate regression analysis was used by applying the "*corrplot*" package (Oksanen et al., 2011). Further to this, multivariate analyses were used to examine the combined relationships between bioavailable concentrations and each toxicity dataset. The Mantel test for dissimilarity matrices was used to evaluate the correlation between the overall bioavailable concentrations (multivariate dataset, e.g. whole bioavailable concentration of organic compounds) and the toxicological response in multiple bioassays. Mantel tests were performed on scaled matrix by using either Euclidean (all data) or Bray-Curtis distance (for community composition comparisons e.g. PLFA (Legendre and Legendre, 2012)) calculated with the "*vegdist*" function. Significance levels of each relationship were determined from the p value and recognised as significant where $p < 0.05$. All tests were computed with R Studio (Version 1.1.423 – © 2009-2018 RStudio, Inc.).

5.3. Results and discussion

5.3.1. Soil characteristics

The soil physicochemical properties of the two samples used in this study are summarised in Table 5.1. Textural soil analysis showed that Soil 1 was a coarse sand soil type while Soil 2 was fine-sandy-loam. The ammonium and nitrate were relatively low in Soil 1 and high in Soil 2. The phosphate concentration was similar in both soils, and C: N: P ratio was 254:5:1 and 78:3:1 for Soil 1 and Soil 2 respectively; where the ratio varied by orders of magnitude in particular in relation to TC content. Indeed C: N unbalanced ratio and nutrients deficiencies are often found in petroleum-contaminated soils along with a high carbon-to-nitrogen ratio (Saum et al., 2018). Available P was within the range 16 - 45 mg/kg established by soil quality UK framework (Griffiths et al., 2018) for both soil samples, as well as TN; which even though measured quite low, was in the range 0.14 - 0.70 mg/kg measured by Bhogal et al. (2015) across seven experimental sites in the UK.

The pH content of Soil 1 was alkaline (pH > 8), while for Soil 2 pH ranged between 5.5 - 7.9 which is similar to a previous study evaluating physical properties of nine UK soils (Mcgeough et al., 2016), and within the average range of 5.50 - 6.49 of over 200,000 UK arable and grassland soils as measured by the Soil Analysis Report of Professional Agricultural Analysis Group (PAAG, UK) (2016) and Goulding and Systems (2016). These conditions suggest that Soil 1 in particular could benefit from biostimulation with addition of compost. According to US EPA (2002) the appropriate C: N: P ratio for an active microbial population able to successfully bio remediate a contaminated soil is 100:10:1. The compost exhibited the following physicochemical composition and characteristics: 1.0% TN, 328 mg/kg available phosphorous, 300 mg-N/kg nitrate, and pH 6. The biochar nutrient content was 0.9% TN, 74 mg/kg available phosphorous, and pH 10. The overall PHC content of Soil 1 was 5 times higher than Soil 2. The GC-MS fingerprint was typical of weathered PHC with a predominance of low to medium chain aliphatic compounds (EC_{16-35}) and low to medium molecular weight aromatic compounds (EC_{16-21}) (Table 5.1). Other relevant soil and amendment properties are shown Table 5.1.

Table 5.1: Physicochemical characteristics of the genuine contaminated soil samples collected in Kent (Soil 1) and Northamptonshire (Soil 2) UK, and the biochar (Carbon Gold UK) and compost (Westland Horticulture Ltd UK) treatment materials used in the mesocosms setup.

Characteristics	Analysis	Soil 1	Soil 2	Biochar	Compost
Elements	Total N (%)	0.4	0.2	0.9	1
	Total C (%)	18.3	5.8	59	40.2
	Total P (%)	0.07	0.07	n.a	n.a
	C:N:P	254:05:01	78:03:01	n.a	n.a
	C:N	49.6	23.7	64.7	42.3
	Total P (mg/kg)	727.5	750.1	n.a	n.a
	Available P (mg/kg)	35	33.7	74.1	328.1
	Ammonium (mg-N/kg)	9.5	71	0	0
	Nitrate (mg-N/kg)	2	18	0	310
Physical properties	Dry matter content W_{dm} (%)	93.2	85.4	79.4	31.3
	Water content (%)	7.3	17.2	25.9	219.3
Chemical properties	pH	9.8	7.4	10	6
	LOI (%)	19.1	8.8	76	78.7
Particle size	Organic fractions:				
	% 10 - 20 mm	n.a	n.a	22.8	54.5
	% 2 - 10 mm	n.a	n.a	22.2	24.7
	% 0.6 - 2 mm (Coarse)	n.a	n.a	15.5	7.5
	% 0.2 – 0.6 mm (Medium)	n.a	n.a	37.7	9.0
	% 0.06 - 0.2 mm (Fine)	n.a	n.a	1.7	4.5
	Mineral fractions:				
	% 0.6 - 2 mm (Coarse sand)	46	7.8	n.a	n.a
	% 0.2 – 0.6 mm (Medium sand)	26.7	21.7	n.a	n.a
	% 0.06 - 0.2 mm (Fine sand)	15.4	23.2	n.a	n.a

Characteristics	Analysis	Soil 1	Soil 2	Biochar	Compost
	% 0.002 mm - 0.06 mm (Silt)	11	32.8	n.a	n.a
	% < 0.002 mm (Clay)	1	14.6	n.a	n.a
	Soil type	Coarse Sand	Fine Sandy Loam	n.a	n.a
Average* heavy metals and metalloids content (mg/kg)	As	29.6 ± 2.6	47.8±5.9	n.a	n.a
	Cd	<d.l	<d.l	n.a	n.a
	Cr	29.6 ± 2.0	48.2±1.7	n.a	n.a
	Cu	54.2 ± 3.1	17.8±2.2	n.a	n.a
	Hg	<d.l	<d.l	n.a	n.a
	Ni	28.2 ± 6.9	20.7±5.3	n.a	n.a
	Pb	78.5 ± 0.6	188.8±26.1	n.a	n.a
	Se	4.1 ± 0.2	4.2±0.7	n.a	n.a
	Zn	243.9 ± 37.8	162.2±10.3	n.a	n.a
Average* petroleum hydrocarbons content (mg/kg)	EC_{10-12}	23.9±8.3	15.2±1.2	n.a	n.a
	EC_{12-16}	86.1±3.0	24.4±2.3	n.a	n.a
	EC_{16-35}	1002.0±18.3	21.3±1.5	n.a	n.a
	EC_{35-40}	<d.l	<d.l	n.a	n.a
	Σ ALK	1112.4±52.9	95.9±6.3	n.a	n.a
	EC_{10-12}	<d.l	<d.l	n.a	n.a
	EC_{12-16}	599±20.09	717.2±4.7	n.a	n.a
	EC_{16-21}	4249.1±135.4	1026.6±70.3	n.a	n.a
	EC_{21-35}	3201.9±69.8	61.2±2.8	n.a	n.a
	Σ PAH	8050.2±226.1	1839.7±81.3	n.a	n.a
	Σ PHC	9162.7±278.9	1900.9±78.5	n.a	n.a

N: nitrogen, C: carbon, P: phosphorous, W_{dm}: dry matter, LOI: loss of ignition, n.a: not available, d.l: detection limit, ALK: alkanes, PAH: polycyclic aromatic hydrocarbons; PHC: petroleum hydrocarbons compounds.

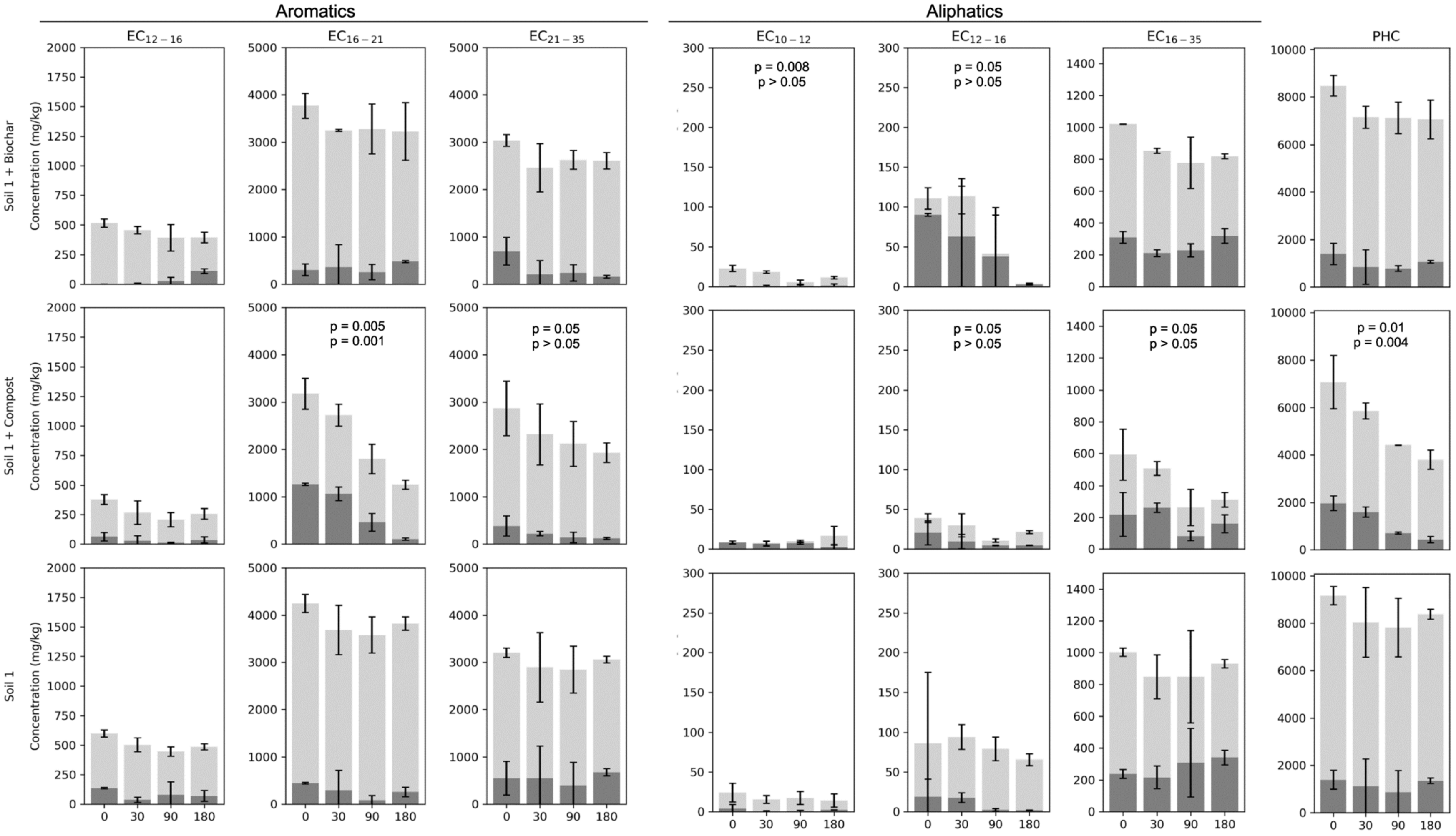
*Values for heavy metals/metalloids and petroleum hydrocarbons are provided based on average of duplicate measurement ± standard deviation.

5.3.2. Chemical mixture fractions behaviour and distribution changes overtime

5.3.2.1. Extractable total, bioavailable and readily available PHC concentrations

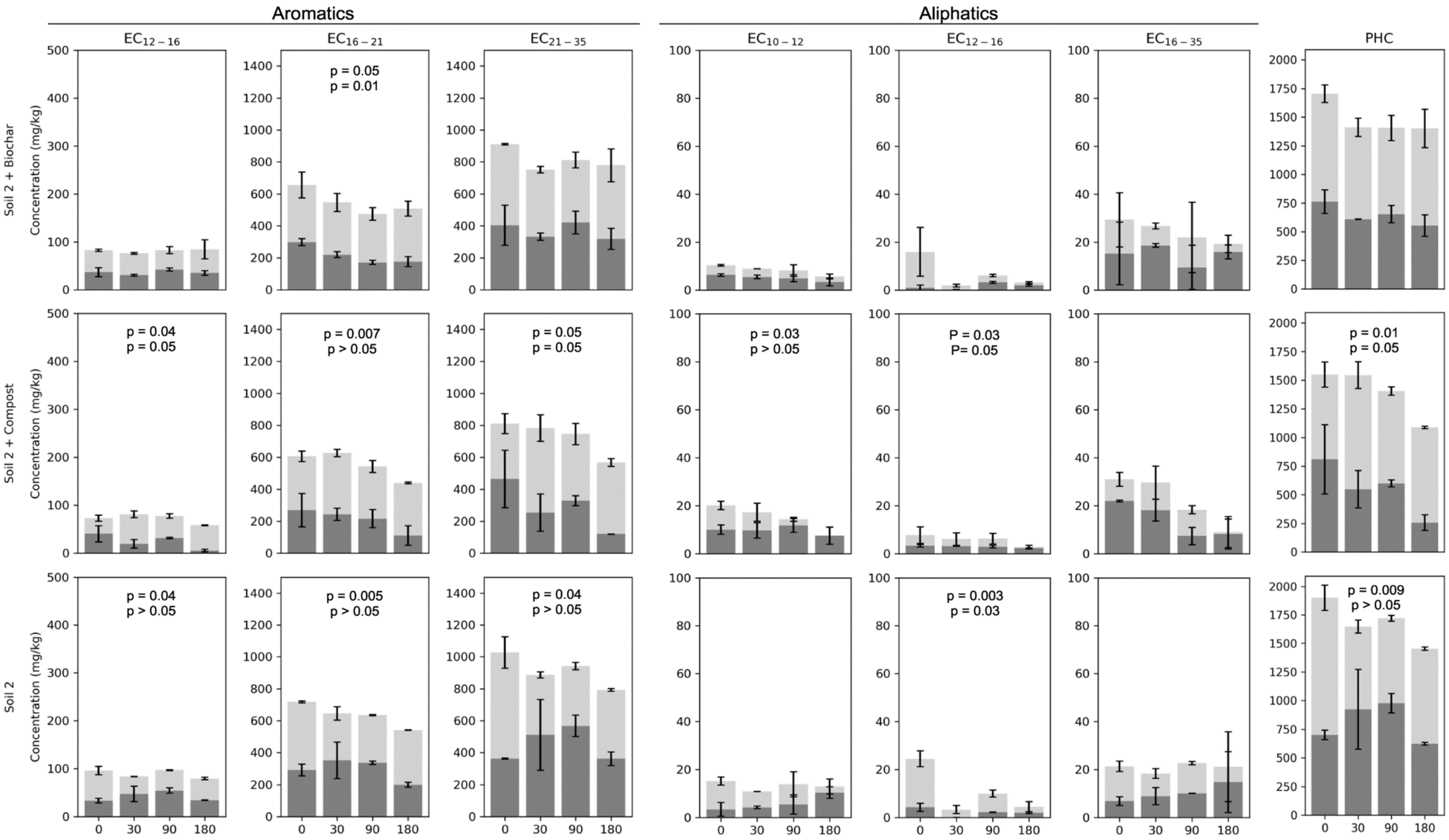
After 180 days incubation the PHC total content decreased on average by 46 and 30% in Soil 1 + Compost and Soil 2 + Compost. The decrease can be explained by biodegradation, as the readily available and bioavailable PHC contents significantly decreased overtime (Figure 5.1 and Figure 5.2, and Appendix Figure 5.7 and Figure 5.8). The most significant changes were observed for the aromatics EC_{16-21} and aliphatic EC_{16-35} fractions where their total concentration decreased by 60% and 48% in Soil 1 + Compost, and by 28% and 70% in (Soil 2 + Compost). The bioavailable concentrations of the aromatics $EC_{16-21,}$ and the aliphatic EC_{16-35} fractions decreased by 92% and 27%, respectively in Soil 1 + Compost, and by 59% and 62% in Soil 2 + Compost. The degradation of the medium and long chain aliphatic compounds (EC_{16-35}) and medium molecular weight aromatic compounds (EC_{16-21}) could be attributed to the biological activity which is supported by an increase in the viable microbial abundance and higher respiration rate (see section 5.3.3).

In contrast, in soils amended with biochar, PHC total content decreased by 18% in both samples, along with a decrease in PHC bioavailable concentration of 24 and 28% in Soil 1 and Soil 2 respectively. Additionally, in biochar amended soils, the PHC concentration was found to be 15-10% lower compared to un-amended soil samples, confirming that biochar was able to effectively lock the organic contaminants. Comparable to previous studies, compost addition was overall more efficient in promoting biodegradation than biochar (Cai et al., 2010; Han et al., 2016; Zhang et al., 2010). After amendment, the most prominent residual hydrocarbon fractions were the high molecular weight aromatic fractions (EC_{21-35}) for both soils. This recalcitrant behaviour is often observed for larger molecules in aged contaminated soils, where residual petroleum hydrocarbons compounds tend to bind tightly to the soil matrix, forming soil aggregates able to entrap and retain the compounds and therefore limiting their bioavailability (Huesemann et al., 2004).



Aromatic EC_{10-12} and aliphatic EC_{35-40} fractions are not shown (below detection limit for all soil samples). p-value for total and bioavailable concentrations, statistically significant at $p > 0.05$ (ANOVA test). Error bars correspond to the standard deviation across replicates for each grouped compound.

Figure 5.1: Soil 1, total (light grey) and bioavailable from hydroxypropyl-β-cyclodextrin extraction (dark grey) concentrations (expressed in mg/kg) of aromatics (EC_{12-16}, EC_{16-21}, and EC_{21-35}) and aliphatics (EC_{10-12}, EC_{12-16}, and EC_{16-35}) across the sampling time (0, 30, 90, and 180 days).



Aromatic EC_{10-12} and aliphatic EC_{35-40} are not shown (below detection limit for all soil samples). p-value for total and bioavailable concentrations, statistically significant at $p > 0.05$ (ANOVA test). Error bars correspond to the standard deviation across replicates for each grouped compound.

Figure 5.2: Soil 2, total (light grey) and bioavailable from hydroxypropyl-β-cyclodextrin extraction (dark grey) concentrations (expressed in mg/kg) of aromatics (EC_{12-16}, EC_{16-21}, and EC_{21-35}) and aliphatics (EC_{10-12}, EC_{12-16}, and EC_{16-35}) across the sampling time (0, 30, 90, and 180 days).

5.3.2.2. HM and metalloids pseudo-total content, solid phase distribution, and availability

HM and metalloids were almost entirely found in the non-exchangeable fraction (Appendix Figure 5.9), and no major changes in their distribution were observed with incubation time (time effect $p > 0.05$), suggesting that these HM are unlikely to become available with time (Appendix Figure 5.10). The most mobilised elements were Cu > As > Cr > Zn > Ni > Pb for Soil 1 and Zn > Ni > Pb > As > Cr > Cu for Soil 2, suggesting a common anthropogenic source for these elements. Cu, As, Cr, Zn, Ni and Pb are often found at gaswork site, as a result of the manufacturing process (CL:AIRE, 2015). The principal waste types at gasworks sites responsible for HM/metalloids contaminations includes: residual spent oxides from gas purification, by-products of carbonisation (ash, clinker residues), furnace residues (coke, cokebreeze), and residuals from batteries, pipelines, and paint (CL:AIRE, 2015; Wong, 2012). Additionally the pseudo-total concentrations of these elements was relatively low (Table 5.1 and Appendix Table 5.4, Table 5.5), where only As, Pb, and Zn (Soil 2) and Zn (Soil 1) were found to exceed guideline values (As = 32 mg/kg, Pb = 450 mg/kg, Zn = 150-200 mg/kg) (UK CLEA Soil Guideline Values, 2009; EC Directive 86/278/EEC, 1986).

5.3.3. Indices of hydrocarbon fractions biodegradation

5.3.3.1. Microbial counts and respiration rate

Changes in microbial community, biomass, and CO_2 production can be used as indices of degradation rates (Chi and Hieu, 2017). In this study the positive effect of compost amendment on the microbial community was observed for both soils, while the effect of biochar addition was limited (Figure 5.3). This finding supports the idea that besides supplying nutrients, compost can provide additional microorganisms able to enhance the biodegradation process (Gandolfi et al., 2010). In addition, the higher specific surface area, associated with compost amendment (Table 5.1), provided a greater surface for interaction, thus potentially increasing the number of microorganisms attached to it, which may also lead to a higher degree of degradation (Ge et al., 2015).

Such effect seems to be more pronounced where initial nutrients level was low, and soil microbial activity and communities were initially distressed by high contaminants concentrations, as in Soil 1. Both incubation time ($p = 0.0004$) and amendment ($p = 0.0007$) significantly influenced soil respiration rate in Soil 1, while only Soil 2 + Compost was found to be highly significant ($p = 0.00002$) (Figure 5.3).

At the onset Soil 1 had no measurable CO_2 production (below detection limits) for most samples. Over the 90 days monitoring Soil 1 + Compost increased by two orders of magnitude the numbers of culturable bacteria (data not shown) compared to Soil 1 un-amended, this translated into enhanced CO_2 production with a steady increase in net mineralized CO_2 (Figure 5.3). The positive effect of compost addition on the total number of culturable microorganisms has been previously observed in soil contaminated with diesel oil (Gandolfi et al., 2010) and heavy crude oil (Trejo-Hernández et al., 2007). Similar findings showed that bioavailable and readily available concentrations may be reduced when applying compost amendment without hindering biodegradation (Bielská et al., 2017; Marchal et al., 2013). The beneficial effect observed for compost-amended soil can be attributed to (1) the increase in nutrients content, (2) the enrichment of the microbial community, (3) a positive effect of pH adjustment (slightly acidic pH of compost) towards a more neutral pH value (Kästner and Miltner, 2016).

Soil 2 demonstrated a slight decrease in net mineralized CO_2 rate during the first 30 days, followed by an increase over the next 60 days (Figure 5.3); however compost and biochar amendments did not significantly affect the numbers of culturable bacteria (data not shown). Soil 2 + Biochar showed a lower CO_2 production, compared to Soil 2 + Compost, and a similar trend to un-amended soil, suggesting that the addition of biochar did not produce a significant advantage for the microbial community, in the sample with lower PHC contamination. While many studies demonstrated the ability of biochar amendment to increase the population of microorganisms (Douds et al., 2014; Hua et al., 2011; Yoshizawa et al., 2005; Zhang and Sun, 2014), no significant increase of the number of culturable bacteria was observed in this study. In particular, all the above mentioned studies involve the use of a primary source of nutrients (green waste or sludge) along with biochar addition. Thus biochar used in combination with traditional composting can increase the quality of treatment (increasing particle-size distribution, creating free air space, improving cation exchange capacity); however, when applied on its own, it does not bring any benefit for the bacterial and fungal community. Moreover, the ineffectiveness of biochar in regards of reducing toxicity for microorganisms can have multiple explanations including: (1) biochar, may still contains PAH due to the pyrolysis (Hale et al., 2012) causing toxicity for the microorganisms (Oleszczuk et al., 2012; Quilliam et al., 2013); (2) biochar strong sorption capacity (Joseph et al., 2010) may reduce nutrients availability (Oleszczuk et al., 2013); (3) the lack of nutrients in the soil sample, was addressed in compost- amended mesocosms but not in biochar-amended ones.

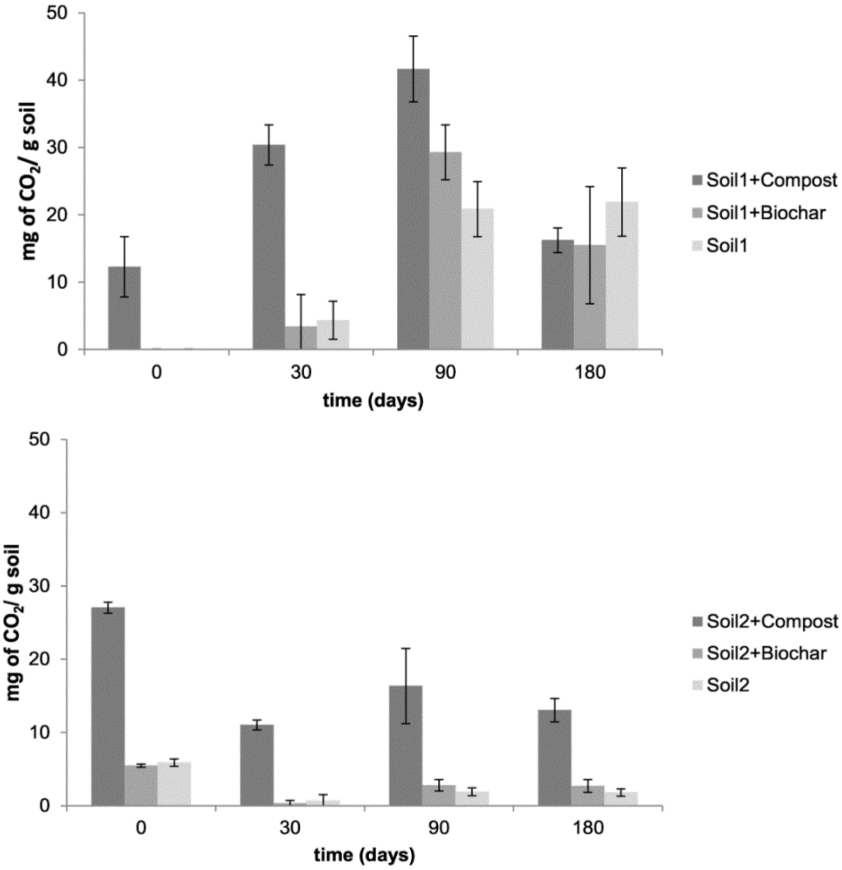


Figure 5.3: Soil respiration expressed as CO_2 production (mg CO_2 /g soil) for treated with compost, biochar, or un-amended (Soil 1 and Soil 2), tested at 0, 30, 90 and 180 days. Error bars correspond to the standard deviation across replicates.

5.3.3.2. Phospholipid-derived fatty acids analysis

As previously highlighted, compost amendment consistently generated an increase in viable microbial community and CO_2 production for both soils; this finding was also supported by a significant shift in the microbial community composition over time, thus clearly differentiating compost amendment from un-amended soils (Figure 5.4). For Soil 1 and Soil 1 + Biochar, a threefold increase of Gram negative and a net decrease of fungi (over 70%) were observed between T0 (onset) and T180. Previous studies correlated petroleum hydrocarbons loss and PLFA specific for the total bacterial community of Gram-negative bacteria, and soil fungi (Al-Hawash et al., 2018; Bell et al., 2013; Margesin et al., 2007).

For the least contaminated soil, the microbial community composition for Soil 2 and Soil 2 + Biochar was more subjected to changes during incubation, compared to Soil 2 + Compost. We hypothesised that, in this soil sample, the biodiversity of indigenous microorganisms (provided by compost amendment) may have acted as a barrier to exogenous microorganisms (Kennedy et al., 2002) reducing population shifts. Both incubation time ($p = 0.001$) and amendment applied ($p = 0.002$) were found to be equally significantly affecting microbial community composition in both soil samples.

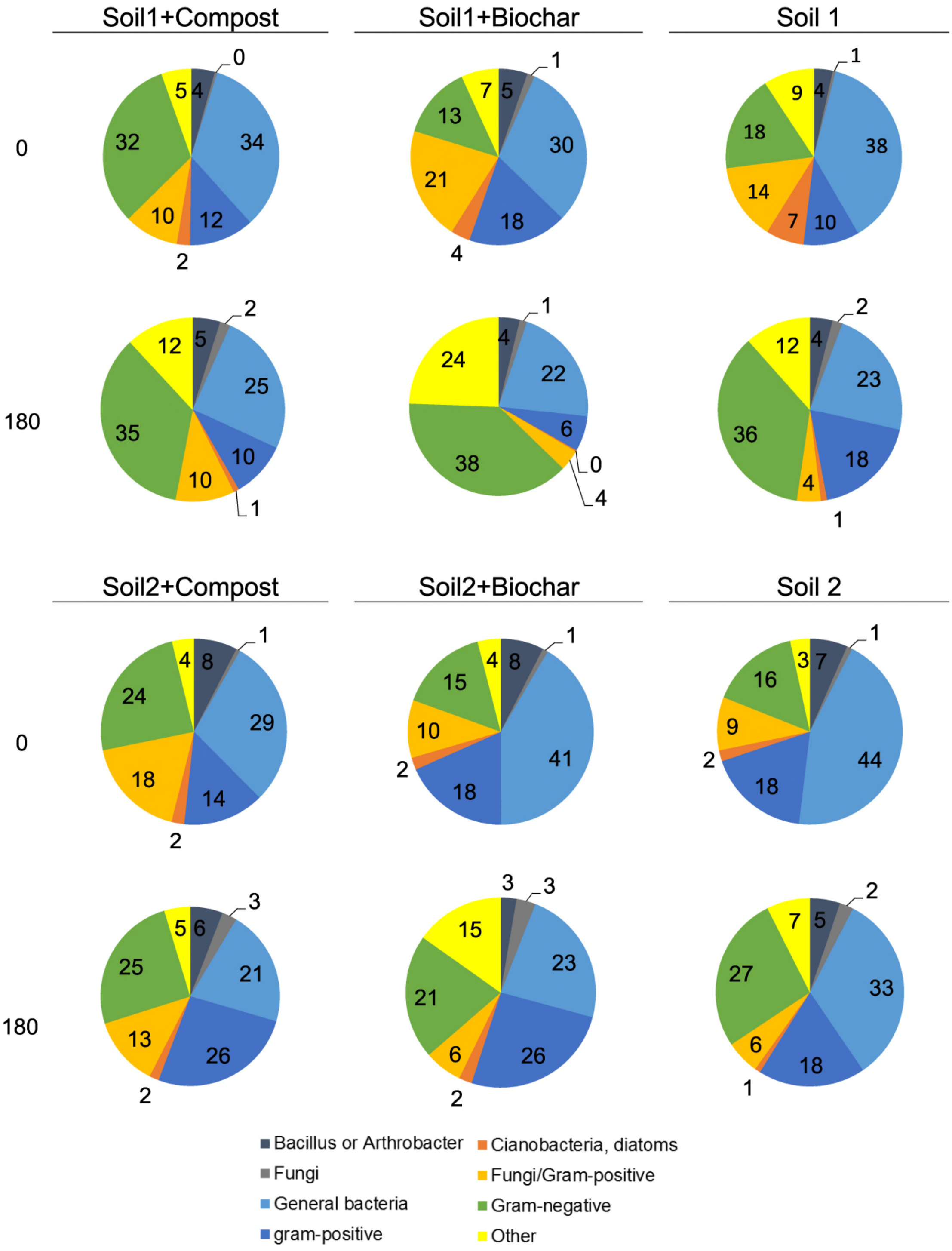


Figure 5.4: Classification of PLFA identified in soil treated with compost, biochar, or un-amended (Soil 1 and Soil 2) comparing onset (0 days) and end of incubation period (180 days). The relative abundance of individual PLFA was expressed as a percentage of the total of the target responses.

5.3.4. Soil ecotoxicology

5.3.4.1. Seeds germination

Both soils amended with compost had the highest percentage of seeds germination (> 90%), indicating that the toxic effect was lower compared to un-amended and biochar amended samples (Appendix Figure 5.11). For Soil 1 both incubation time and soil amendment applied were found to significantly affect germination rate ($p = 0.001$). Although a significant increase (> 40%) in seed germination was still recorded for Soil 1 and Soil 1 + Biochar in peas (after 30 days Appendix Figure 5.11 C) mustard and rye grass (after 90 days Appendix Figure 5.11 A and B), the rate and degree of growth were slower compared to uncontaminated soil (control), suggesting some time lag ecotoxicological effect.

In Soil 2 and Soil 2 + Biochar the germination rate decreased at day 30, but remained constant for Soil 2 + Compost. This can be explained by the fact that Soil 2 amended with compost relied on an established and a more stable microbial community (as previously highlighted in PLFA composition), which was able to degrade organic contaminations, along with a significant reduction in bioavailability, thus reducing toxicity. On the contrary Soil 2 and Soil 2 + Biochar had a more dynamic microbial population along with a less effective microbial degradation (decrease in net mineralized CO_2); therefore changes in contaminant concentration were likely to be mainly depending on sorption/desorption processes rather than microbial degradation. Indeed, changes in sorption-desorption equilibrium can drive the release of organic contaminants which were previously encapsulated in soil aggregates (Jiang et al., 2016), hence increasing compounds bioavailability and toxicity. This was observed for Soil 2 where bioavailable concentrations of $EC_{16\text{-}21}$ and $EC_{21\text{-}35}$ were higher at 30 days compared to the onset of the experiment (Figure 5.2) along with a lower germination rate recorded for mustard, rye grass, and pea seeds at 30 days (Appendix Figure 5.11 A, B, and C).

5.3.4.2. Earthworms lethality

Earthworm acute toxicity assay was more sensitive compared to seed germination, as both dermal absorption and feeding can impact *E. fetida* (Korte, 2003; Vijver et al., 2003). Condition index (CI) for Soil 1 and Soil 1 + Biochar remained 0 (mortality) at all sampling points, along with a significant decrease in weight (on average up to 50% reduction) (Table 5.2). In Soil 1 + Compost, the CI consistently increased across the sampling time. *E. fetida* was also less affected by changes in weight loss. Similar to the other bioassays, Soil 2 was seen as having a lower impact on *E. fetida* (Table 5.2). Neither amendment applied nor mesocosms incubation time were significantly affecting condition index in Soil 2 ($p > 0.05$). This can be explained by the fact that Soil 2 was overall less contaminated, and perhaps this specific ecotoxicological test may not be adequate (low sensitivity) to highlight the relationship between the two variables (toxicity/contamination). In this case the use of a chronic test, such as inhibition of earthworm reproduction, (OECD, 2004) could have provided a more ecologically relevant endpoint rather than acute toxicity (Lionetto et al., 2012).

Table 5.2: Average Condition Index (CI) of *E. Fetida* and weight loss (percentages) in earthworm acute toxicity bioassay, at the four sampling times (0,30 ,90, and 180 days) for Soil 1 and Soil 2 treated with compost, biochar, or un-amended. Condition index ranged from 0-2, where 0= mortality.

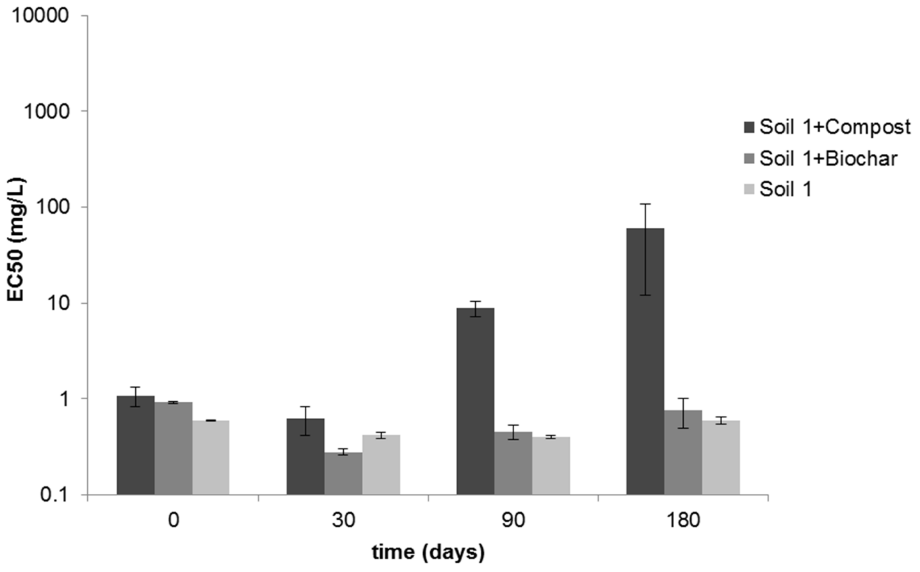
		Compost				Biochar				Un-amended			
		0	30	90	180	0	30	90	180	0	30	90	180
Soil 1	CI 3 days	0.0	0.3	1.5	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CI 7 days	0.0	0.0	1.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CI 14 days	0.0	0.0	1.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	% weight loss/gain	-34.9	-20.1	-14.3	7.4	-53.0	-50.3	-64.0	-50.0	-55.5	-37.0	-57.1	-50.0
	stdev	2.0	40.5	54.5	4.2	6.2	1.8	23.4	0.0	1.1	0.4	4.0	0.0

		Compost				Biochar				Un-amended			
		0	30	90	180	0	30	90	180	0	30	90	180
Soil 2	CI 3 days	1.4	2.0	2.0	1.8	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
	CI 7 days	1.5	1.8	1.3	1.8	1.3	1.8	2.0	2.0	2.0	2.0	2.0	2.0
	CI 14 days	1.4	1.8	1.5	1.7	1.9	1.8	1.7	1.7	1.8	2.0	2.0	2.0
	% weight loss/gain	-1.4	22.5	46.2	11.9	19.0	34.7	2.2	22.5	14.1	12.9	5.4	25.5
	stdev	36.0	0.1	1.9	7.6	3.8	0.8	3.0	5.9	9.6	4.4	7.7	4.8

CI : condition index, stdev : standard deviation

5.3.4.3. Microtox® Basic Solid Phase Test

The use of Microtox® bioassay, in combination with the other ecotoxicological tests, provide a supplementary tool for a real-time assessment of toxicity associated not only with the presence of contaminants in a mixture, but also with their potential mutual interactions (Kuczyńska et al., 2005). Previous findings highlight the positive effect of bioremediation in reducing toxicity of organic contaminants to *V. fischeri* (Khan et al., 2012, 2013; Macken et al., 2008). At the onset of the experiment, the toxicity levels were similar for all samples due to the co-presence of organic and inorganic pollutants, however after 180 days the overall acute toxicity significantly decreased (EC_{50} increase), in particular for Soil 1 + Compost (Figure 5.5). In comparison for Soil 2 changes in toxicity to *V. fischeri* were limited, as this sample was overall less contaminated. In particular in Soil + Biochar toxicity was halved due to a combination of bonding between contaminants and organic sorbent (biochar), and a reduction of the compounds bioavailability. This can provide an explanation for the difference observed in toxicity among the samples studied in spite of similar levels of PHC. Amendment alone ($p = 0.00004$), incubation time ($p = 0.004$) and combined effect of time and amendment ($p = 0.003$) were found to be significantly affecting changes in EC_{50} for Soil 1 and Soil 2.



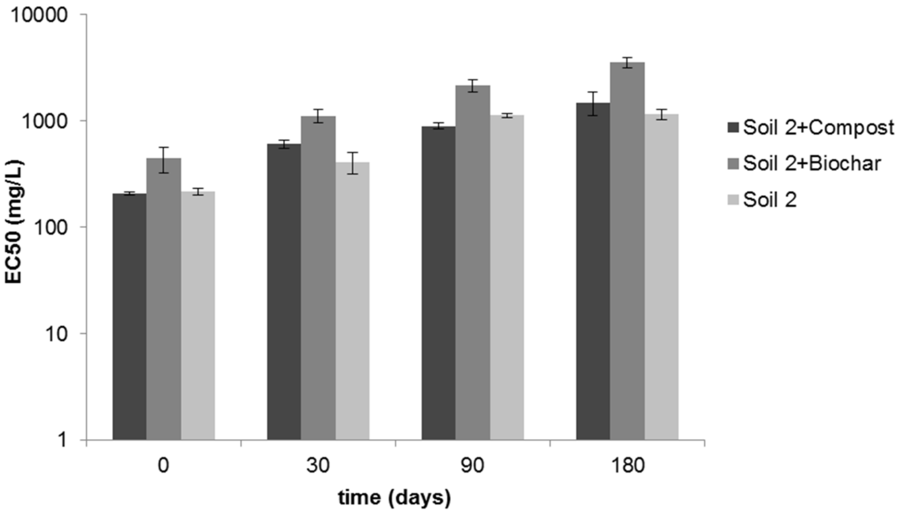
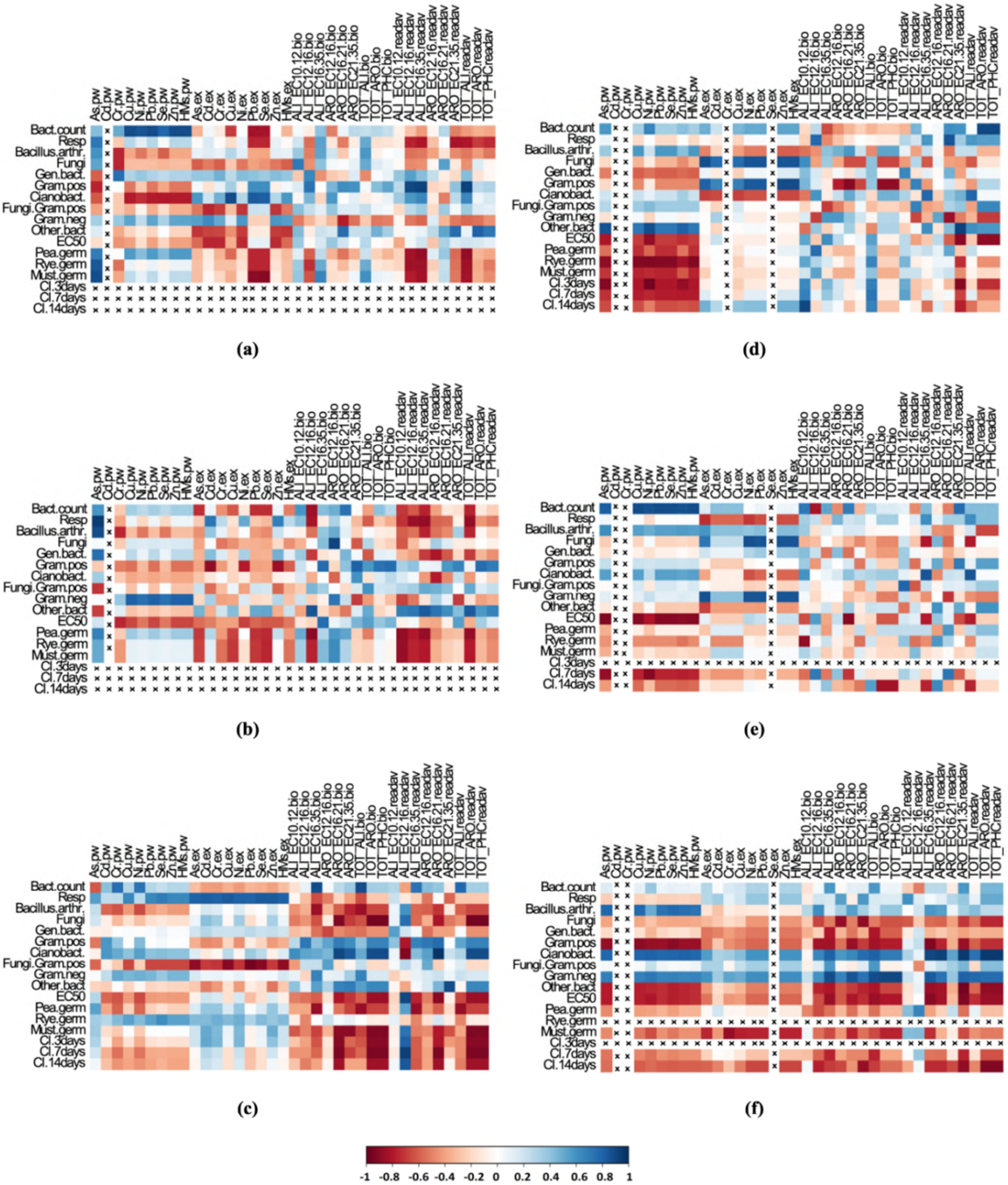


Figure 5.5: Microtox® Basic Solid phase Test (BSPT) assay average results expressed as EC_{50} concentration (mg/L) (y-axis in log scale) for light decrease values at the onset and after 30, 90, and 180 days for treated with compost, biochar, or un-amended (Soil 1 and Soil 2). Error bars indicate standard error of the repeated measures (toxicity decreases when the EC_{50} value increases).

5.3.5. Correlation between bioavailable fraction and bioassays

The bioavailable (HP-β-CD extracted) and readily-available (methanol extracted) concentrations of the main hydrocarbon groups, and HM/metalloids fractions were plotted along with the toxicological responses of the multiple bioassays (Figure 5.6). The strength of the correlations between the bioavailability-proxy and the toxicity data can provide an indication of which technique is more suitable for predicting site-specific bioavailability of complex chemical mixtures. Strong negative correlations were observed between bioavailable/readily available aromatic and aliphatic concentrations and the ecotoxicological assays (e.g. bacteria count, soil respiration, seeds germination, and condition index) in particular in Soil 1 + Compost and Soil 2 + Compost (Figure 5.6 c-f). Results indicate that when bioavailable/readily available concentrations decrease, the toxicity also decrease, thus diversity of microbial community increase along with soil respiration, condition index, and EC_{50} (Figure 5.6).



(a) (d)

(b) (e)

(c) (f)

x rows = all values equal, no correlation
x columns = all values below detection limits

Pw: pore water, Ex: exchangeable, ALI: aliphatics hydrocarbons, ARO: aromatics hydrocarbons, PHC: petroleum hydrocarbons compounds, Bio: bioavailable, Readav: readily available, Bact.Count: bacteria count, Resp: respiration, Bacillus.Arthr.: *Bacillus* or *Arthrobacter*, Gen.Bac :general bacteria, Gram.Pos: gram positive, Cianobact: cyanobacteria, Gram Neg.: gram negative, Other Bac: other bacteria, EC_{50}: Microtox® EC_{50} values, Germ: germination, CI: condition index (at 3, 7, and 14 days).

Figure 5.6: Correlation (based on Spearman coefficient) between organic and inorganic bioavailable concentrations and toxicological responses in multiple

bioassays. Soil 1 (a), Soil 1+ Biochar (b), Soil 1 + Compost (c), Soil 2 (d), Soil 2 + Biochar (e), and Soil 2 + Compost (f). Positive correlations were displayed in blue and negative correlations in red colour, were intensity were proportional to the correlation coefficients (scale).

Even though, for both soils, un-amended and biochar amended samples often displayed a similar pattern in their biological responses for microbial count, soil respiration, PLFA profile, seeds germination and earthworm lethality, the correlation between bioavailability and ecotoxicology was different (Figure 5.6 a and b). Less correlation was observed in Soil 1 + Biochar highlighting that toxicity changes can be driven by multiple factors (combined effects) which may not be accounted for in univariate linear regression analysis (correlation).

Interestingly, for both Soil 1 and Soil 2, the HM/metalloids pore water concentrations were often found to be positively correlated (dark blue); meaning that when bioavailable concentrations is high, toxicity is low for the microbial bioassays (bacteria count, respiration, and in some cases PLFA). Indeed moderate concentrations of HM have previously been reported to have a beneficial effect to microbial growth (Chen et al., 2015).

Multivariate analyses (Table 5.3) highlighted a statistical significant relationship ($p = 0.008$, $p = 0.007$) between bioavailable concentrations of complex mixtures of PHC and seed germination assay, microbial growth (CFU), in Soil 1 + Compost and Soil 1 + Biochar. No significant relationships identified through the Mantel test were observed for the low contaminated soil (Soil 2) where the overall, readily available and bioavailable PHC concentrations were not strongly correlated ($r^2 < 0.75$ and $p > 0.05$) with the bioassays. As previously highlighted the effect of HM, in particular the dissolved elements present in the pore water fraction, was significant for the bacterial count in Soil 1 + Compost ($p = 0.028$) and Soil 2 + Biochar ($p = 0.049$).

In this study we observed that toxicity can be highly variable in relation to the type of assay applied, suggesting that toxic effect can be driven by multiple different sources. This is consistent with the fact that various organisms, used for the ecotoxicological assays, are characterised by various levels of sensitivity to complex chemical mixtures (Isidori et al., 2003). Other studies also highlighted the challenge of establishing direct relationship between organics content and ecotoxicity parameters (Oleszczuk et al., 2014). For example Buss and Mašek (2014) show the significant effect of volatile organic compounds rather than bioavailable PAH concentrations on seed germination assay. However, the significant uni- and multivariate relationships ($p < 0.05$) observed between the bioavailability-proxy and the toxicity data provides the necessary evidence that this integrated approach is suitable for predicting site-specific bioavailability of complex chemical mixtures and could potentially be implemented with confidence in a stepwise tiered approach.

Table 5.3: Multivariate analyses between bioavailable concentrations of organic compounds (hydroxypropyl-β-cyclodextrin extractions), readily available (methanol extractions), heavy metals and metalloids bioavailable concentrations (pore water and exchangeable fraction), and toxicological response in multiple bioassays performed with Mantel test for dissimilarity matrices.

Treatment	Soil	Bioassay	PHC bioavailable (HP-β-CD)		PHC readily available (MeOH)		HM pore water		HM exchange able		All	
			r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Compost	Soil 1	bact. count	-0.272	0.837	-0.260	0.766	**0.790**	**0.028**	-0.071	0.385	-0.116	0.495
		soil resp.	0.050	0.375	-0.027	0.524	0.357	0.095	0.616	*0.007*	0.052	0.368
		PLFA	0.386	0.090	0.289	0.217	-0.104	0.608	0.287	0.123	0.309	0.186
		seeds	**0.789**	***0.008***	0.635	*0.030*	0.141	0.267	-0.105	0.712	**0.752**	**0.009**
		worms	0.405	*0.028*	0.420	*0.010*	-0.151	0.816	0.240	0.152	0.421	*0.010*
		EC_{50}	0.224	0.059	0.047	0.271	-0.192	0.720	0.170	0.141	0.098	0.241
Compost	Soil 2	bact. count	0.037	0.575	0.136	0.314	-0.105	0.710	-0.076	0.556	0.155	0.281
		soil resp.	-0.065	0.630	0.203	0.217	-0.028	0.535	-0.087	0.632	0.159	0.240
		PLFA	-0.353	0.729	0.171	0.363	0.046	0.189	-0.124	0.477	-0.211	0.460
		seeds	0.042	0.583	0.271	0.167	0.204	0.083	0.374	0.229	0.338	0.104
		worms	0.214	0.333	-0.05	0.611	0.120	0.250	-0.036	0.604	0.349	0.094
		EC_{50}	0.382	*0.026*	0.054	0.432	0.324	0.109	-0.125	0.682	0.412	*0.010*
Biochar	Soil 1	bact. count	**0.750**	**0.007**	0.013	0.484	-0.106	0.622	0.301	0.064	0.272	0.281
		soil resp.	-0.095	0.587	0.176	0.160	0.008	0.434	0.132	0.274	0.131	0.247
		PLFA	0.096	0.313	0.236	0.224	0.144	0.248	0.015	0.467	0.217	0.236
		seeds	**0.775**	**0.017**	0.176	0.222	-0.157	0.740	0.471	*0.045*	*0.480*	*0.003*
		worms	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*
		EC_{50}	0.257	0.099	0.159	0.184	0.290	0.108	0.130	0.250	0.247	0.115

Treatment	Soil	Bioassay	PHC bioavailable (HP-β-CD)		PHC readily available (MeOH)		HM pore water		HM exchange able		All	
			r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Biochar	Soil 2	bact. count	-0.140	0.384	0.591	*0.014*	**0.871**	**0.049**	0.328	0.314	0.387	*0.019*
		soil resp.	-0.020	0.526	0.454	0.007	0.245	0.130	0.209	0.139	0.553	*0.012*
		PLFA	-0.011	0.821	0.003	0.436	-0.278	0.868	-0.081	0.349	-0.076	0.833
		seeds	0.050	0.972	0.153	0.175	-0.202	0.679	-0.305	0.622	0.073	0.689
		worms	0.224	0.510	0.153	0.175	0.088	0.094	-0.185	0.500	0.151	0.396
		EC_{50}	0.320	*0.045*	0.211	0.201	**0.759**	**0.005**	-0.120	0.738	0.510	*0.019*
Un-amended	Soil 1	bact. count	0.109	0.306	-0.118	0.606	0.521	*0.023*	0.061	0.411	0.062	0.363
		soil resp.	0.203	0.104	0.449	*0.003*	0.117	0.231	0.199	0.106	0.449	*0.002*
		PLFA	0.247	0.167	0.497	*0.017*	-0.247	0.892	0.185	0.220	0.428	*0.036*
		seeds	0.279	0.193	0.333	0.080	0.039	0.392	0.693	*0.007*	0.320	0.064
		worms	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*	*n.a*
		EC_{50}	-0.149	0.833	0.127	0.149	-0.019	0.563	0.276	0.063	-0.045	0.594
Un-amended	Soil 2	bact. count	-0.279	0.955	0.502	0.052	**0.005**	**0.524**	0.003	0.488	0.115	0.307
		soil resp.	-0.293	0.873	0.511	0.050	0.015	0.333	-0.376	0.917	0.148	0.149
		PLFA	-0.229	0.892	0.589	*0.026*	0.125	0.253	-0.100	0.597	0.347	0.076
		seeds	-0.068	0.576	-0.169	0.701	0.478	*0.021*	-0.356	0.972	0.001	0.465
		worms	-0.057	0.635	0.378	0.165	0.265	0.146	-0.254	0.747	0.371	0.146
		EC_{50}	0.123	0.236	0.284	*0.042*	**0.764**	**0.038**	0.150	0.226	0.466	*0.010*

PHC: petroleum hydrocarbons compounds, HP-β-CD: hydroxypropyl-β-cyclodextrin, HM: heavy metals (and metalloids), all: bioavailable measures combined (bioavailable, readily available pore water, and exchangeable), bact count: bacteria count (CFU), soil resp: respiration (mg CO_2/ g soil), PLFAs: phospholipid fatty acids analysis, seeds: seed germination assay (% germinated/total), worms: earthworm acute toxicity assay (condition index), EC_{50}: Microtox® EC_{50} values. Statistically significant at p-value > 0.05. Spearman correlation coefficient (r >0.75).

5.4. Conclusion

In this study the effect of compost and biochar addition on two soils contaminated with complex chemical mixtures was evaluated with a particular attention to their influence on the chemical behaviour, bioavailability, and degradation of the chemical mixtures. In parallel the effect of bioavailability of complex chemical mixtures on the microbial community composition and soil ecotoxicology were assessed. The addition of compost was effective in enhancing PHC degradation with a reduction of ≥ 30%, and reducing significantly soil toxicity (e.g. EC_{50} increased 60 and 7 times in Soil 1 and Soil 2, respectively). While biochar amendment was less effective in reducing total PHC (≤ 19% decrease), the PHC concentration was still 15 - 10% lower compared to un-amended samples suggesting that biochar was able to effectively lock organic contaminants in soil. This was evidenced by a significant decrease in bioavailability of the aromatic EC_{16-21} and aliphatic EC_{16-35} compounds in both amended soils (≥ 80%); yet the high molecular weight (HMW) aromatic compounds were not posing any risk, as none of the fractions were found to be significantly bioavailable in any of the soil samples tested. Heavy metals and metalloids were almost entirely found in the non-exchangeable fraction, and no major changes in their distribution were observed with incubation time, suggesting that there HM are unlikely to become available with time, thus not posing risk. In our study, the soil pH for both samples was found to be neutral or alkaline; this condition is responsible for reducing HM and metalloids mobility due to adsorption, desorption, and co-precipitation processes. This study shows that the concentration of low to medium chain aliphatic compounds and low to medium molecular weight aromatic compounds can be effectively reduced through degradation by compost amendment and to a lower extend stabilised by biochar amendment. Thus, these fractions should be considered and monitored when defining remediation end-points, as they are easily degraded by microorganisms and potentially constitute the drivers for toxicity reduction. Since a valid ecotoxicological assessment should reflect the changes of contaminant concentrations, toxicity, and bioavailability of the complex mixtures; in this study we attempted to combine the complexity of the biological indicators with the chemical analysis. The bioassays were selected based on ease of execution and environmental relevance, and were used to provide information on remediation effectiveness. Overall, this study highlighted that there was a significant relationship ($p < 0.05$) between the bioavailable/readily available fraction of the chemical mixtures and the ecotoxicological bioassays. *E. fetida* (CI at 14 days), and the seed germination assay were the best at discriminating between the amended and un-amended soils (in particular for Soil 1). These assays are expected to be more reliable to be used in the risk assessment, and could significantly help to identify optimal remediation strategies, and contribute to change the over-conservative nature of the current risk assessments.

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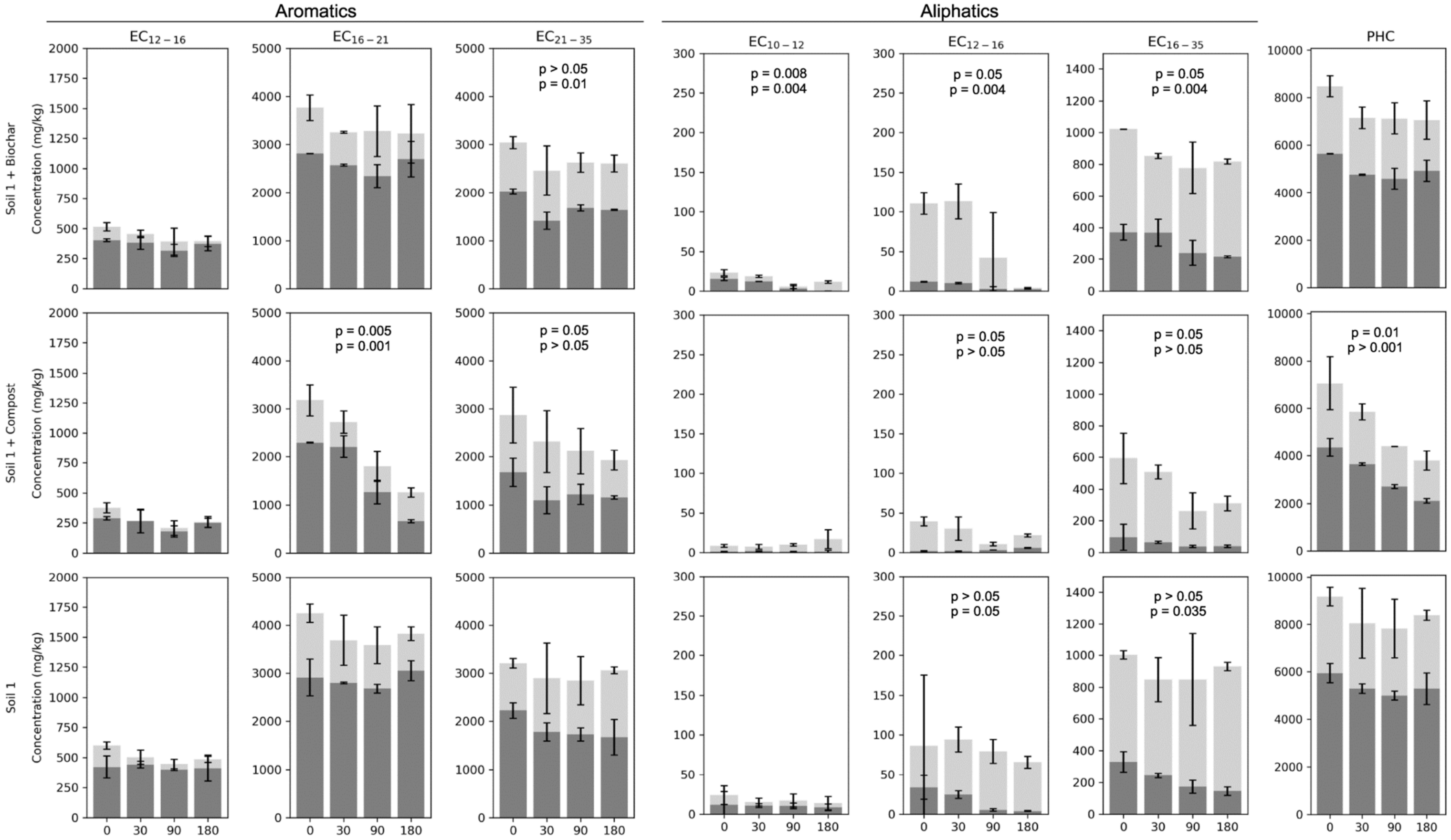
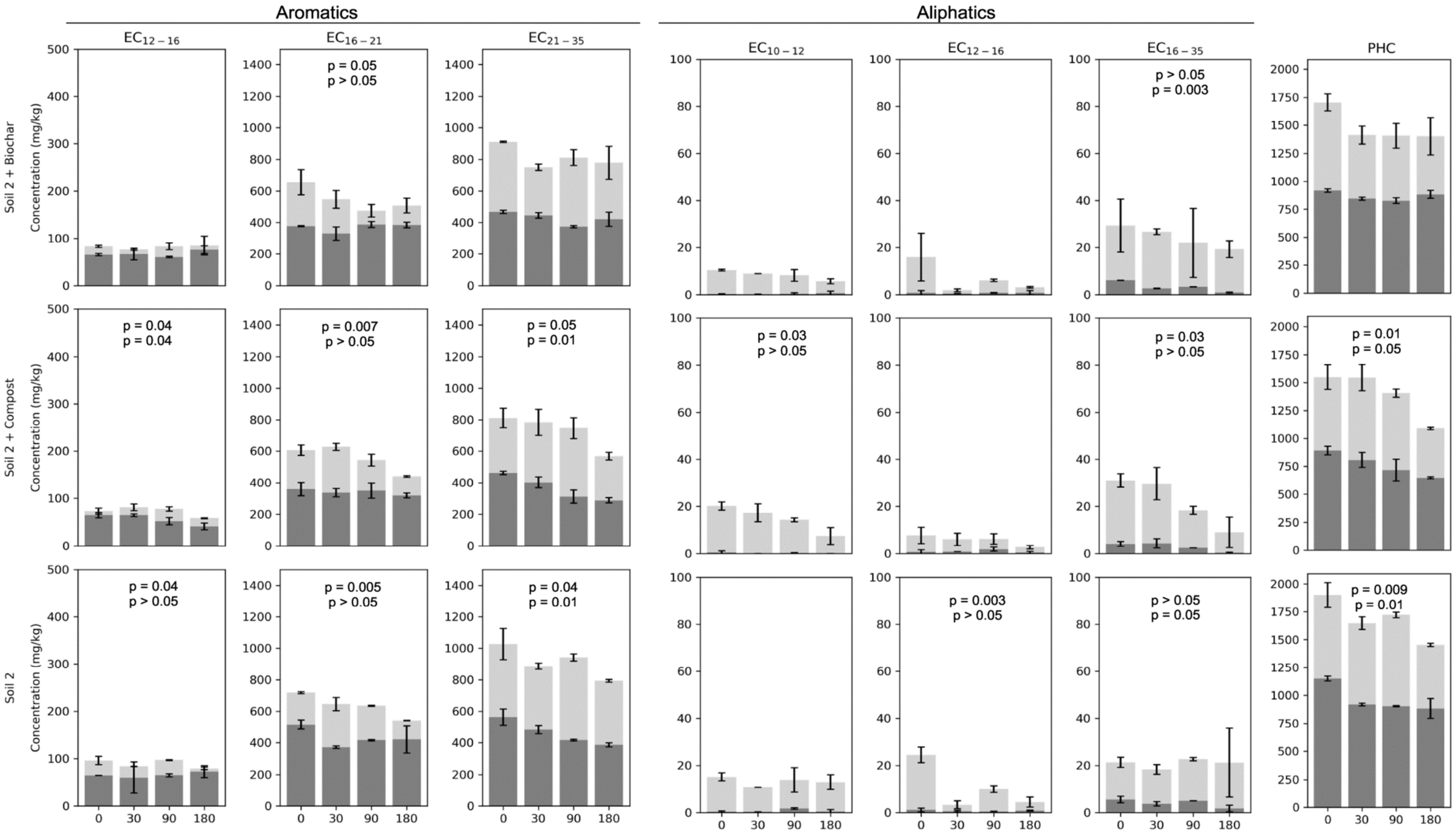
5.6. Appendix



Aromatic EC_{10-12} and aliphatic EC_{35-40}, fractions are not shown (below detection limit for all soil samples). Error bars correspond to std. deviation.

Figure 5.7: Soil 1, total (light grey) and bioavailable from methanol extraction (dark grey) concentrations (expressed in mg/kg) of aromatics (EC_{12-16}, EC_{16-21}, and EC_{21-35}) and aliphatics (EC_{10-12}, EC_{12-16}, and EC_{16-35}) across the sampling time (0, 30, 90, and 180 days), p-value significance $p > 0.05$ (ANOVA Test).



Aromatic EC_{10-12} and aliphatic EC_{35-40} fractions are not shown (below detection limit for all soil samples). Error bars correspond to std. deviation.

Figure 5.8: Soil 2, total (light grey) and bioavailable from methanol extraction (dark grey) concentrations (expressed in mg/kg) of aromatics (EC_{12-16}, EC_{16-21}, and EC_{21-35}) and aliphatics (EC_{10-12}, EC_{12-16}, and EC_{16-35}) across the sampling time (0, 30, 90, and 180 days), p-value significance $p > 0.05$ (ANOVA Test).

Table 5.4: Pseudo-total elements concentration (aqua regia extraction), for Soil 1 treated with compost, biochar, or un-amended. Values are expressed in mg/kg and averaged across sampling times.

Element	Soil1+Compost				Soil1+ Biochar				Soil 1			
	Min.		Max.	Median	Min.		Max.	Median	Min.		Max.	Median
Al	6146.94	-	12460.33	10000.56	1609.58	-	12975.65	10648.62	2794.92	-	12896.52	10844.83
As	15.41	-	34.85	26.69	12.42	-	46.29	31.09	27.03	-	39.87	32.54
Ba	91.08	-	167.01	156.74	95.97	-	197.99	161.20	155.05	-	207.05	171.08
Ca	17186.74	-	37755.48	33665.62	22088.72	-	40643.36	37304.06	19851.48	-	40367.05	37475.94
Cd	0.78	-	2.11	0.81	0.73	-	2.57	0.86	0.92	-	3.17	1.04
Co	4.62	-	13.49	6.48	4.16	-	12.61	7.11	3.54	-	14.29	7.50
Cr	17.97	-	42.82	22.75	22.32	-	38.78	25.92	23.36	-	41.97	28.62
Cu	31.88	-	58.46	47.44	37.68	-	59.79	52.07	51.02	-	62.19	56.79
Fe	11275.80	-	31248.09	18790.43	9459.48	-	28517.16	21154.18	15622.58	-	30092.68	19650.83
Hg	0.07	-	0.43	0.23	0.07	-	0.40	0.15	0.07	-	0.67	0.23
K	1865.34	-	3610.84	2717.94	961.67	-	3504.76	2474.43	2099.33	-	3119.73	2509.84
Li	10.37	-	15.08	11.63	4.59	-	15.63	13.03	13.23	-	16.49	14.58
Mg	1350.69	-	2272.04	1834.62	945.59	-	2189.44	1785.87	1183.37	-	2233.06	1849.72
Mn	181.85	-	338.23	281.97	196.23	-	316.48	273.35	200.68	-	350.03	303.87
Mo	0.77	-	1.53	1.02	0.20	-	1.60	1.11	0.32	-	1.80	1.20
Na	388.00	-	615.45	456.15	382.75	-	605.93	414.27	345.00	-	453.18	411.87
Ni	11.79	-	33.39	21.40	9.59	-	28.81	21.77	17.90	-	35.10	24.02
P	476.84	-	1265.03	802.10	353.69	-	1173.00	793.70	404.29	-	1012.59	755.82
Pb	38.59	-	87.04	60.67	45.64	-	122.52	77.05	45.47	-	79.11	62.00
S	4380.49	-	7813.52	6715.96	3706.56	-	8867.50	7040.18	2398.27	-	7869.69	6474.71
Sb	1.76	-	2.89	2.41	0.09	-	3.16	2.57	0.21	-	3.68	2.90
Se	2.34	-	4.23	3.70	0.99	-	4.07	3.88	1.46	-	4.39	3.84
Si	595.45	-	1584.63	1367.55	94.81	-	1294.14	1013.08	137.09	-	1148.71	884.69
Sr	63.36	-	112.38	103.92	75.41	-	125.55	112.40	103.69	-	124.64	107.17
V	20.17	-	35.15	28.56	2.28	-	40.20	31.16	28.98	-	40.79	32.72
Zn	119.77	-	225.88	167.31	155.67	-	247.82	191.08	113.47	-	281.67	202.83

Table 5.5: Pseudo-total elements concentration (aqua regia extraction), for Soil 2 treated with compost, biochar, or un-amended. Values are expressed in mg/kg and averaged across sampling times.

Element	Soil2+Compost				Soil2+Biochar				Soil2			
	Min.		Max.	Median	Min.		Max.	Median	Min.		Max.	Median
Al	18456.86	-	22020.17	20636.38	19445.05	-	26004.06	23268.89	4979.88	-	25555.68	22382.98
As	40.60	-	60.61	48.77	43.19	-	67.66	56.06	41.95	-	66.03	58.02
Ba	94.80	-	230.29	118.87	109.82	-	132.32	128.61	84.39	-	157.15	132.52
Ca	14272.25	-	23481.43	18753.55	16684.37	-	25273.96	21207.72	17097.67	-	24528.51	20349.74
Cd	0.16	-	0.60	0.21	0.16	-	0.38	0.17	0.15	-	0.22	0.19
Co	8.55	-	15.36	11.90	9.31	-	15.67	12.51	10.21	-	16.99	12.27
Cr	44.51	-	69.19	58.77	44.31	-	74.30	57.67	46.48	-	75.85	63.32
Cu	14.23	-	27.34	19.98	18.52	-	27.88	23.53	15.57	-	27.23	23.84
Fe	37574.01	-	59631.47	49589.09	43487.70	-	67169.16	53936.24	31614.50	-	68257.18	57294.26
Hg	0.06	-	0.39	0.31	0.06	-	0.49	0.35	0.06	-	0.40	0.35
K	3350.01	-	4585.49	3922.12	3449.97	-	4987.05	4304.63	1564.07	-	5090.93	4151.42
Li	24.37	-	31.85	28.28	26.17	-	37.83	32.29	10.20	-	38.23	31.42
Mg	1812.92	-	2451.26	2107.87	1821.60	-	2446.65	2254.30	1354.14	-	2442.97	2163.91
Mn	379.02	-	863.46	475.30	379.79	-	606.31	471.69	373.28	-	563.74	507.11
Mo	0.73	-	1.26	1.18	0.88	-	1.46	1.17	0.17	-	2.78	1.33
Na	328.22	-	472.02	419.01	297.66	-	509.63	404.58	176.03	-	452.05	381.29
Ni	20.44	-	34.63	26.69	22.65	-	34.43	28.41	15.37	-	33.44	30.44
P	781.12	-	1087.28	876.32	680.12	-	1095.66	1045.18	337.06	-	1098.17	954.72
Pb	49.92	-	455.62	75.00	67.47	-	167.88	82.73	71.03	-	214.95	76.92
S	5344.44	-	8583.54	6308.99	5240.50	-	10146.32	7518.39	4765.50	-	8624.88	7883.46
Sb	0.40	-	1.84	1.21	0.94	-	3.09	1.41	0.04	-	2.96	1.48
Se	5.09	-	8.70	5.83	5.19	-	10.15	6.18	3.50	-	11.62	6.33
Si	553.29	-	946.11	725.54	434.27	-	1044.67	753.57	89.12	-	1066.08	640.46
Sr	43.02	-	65.99	52.59	44.69	-	65.48	62.20	44.08	-	63.06	53.76
V	75.78	-	109.99	84.47	78.05	-	116.16	91.66	81.19	-	114.80	101.17
Zn	144.45	-	481.76	200.25	147.42	-	255.94	195.80	151.98	-	278.97	201.92

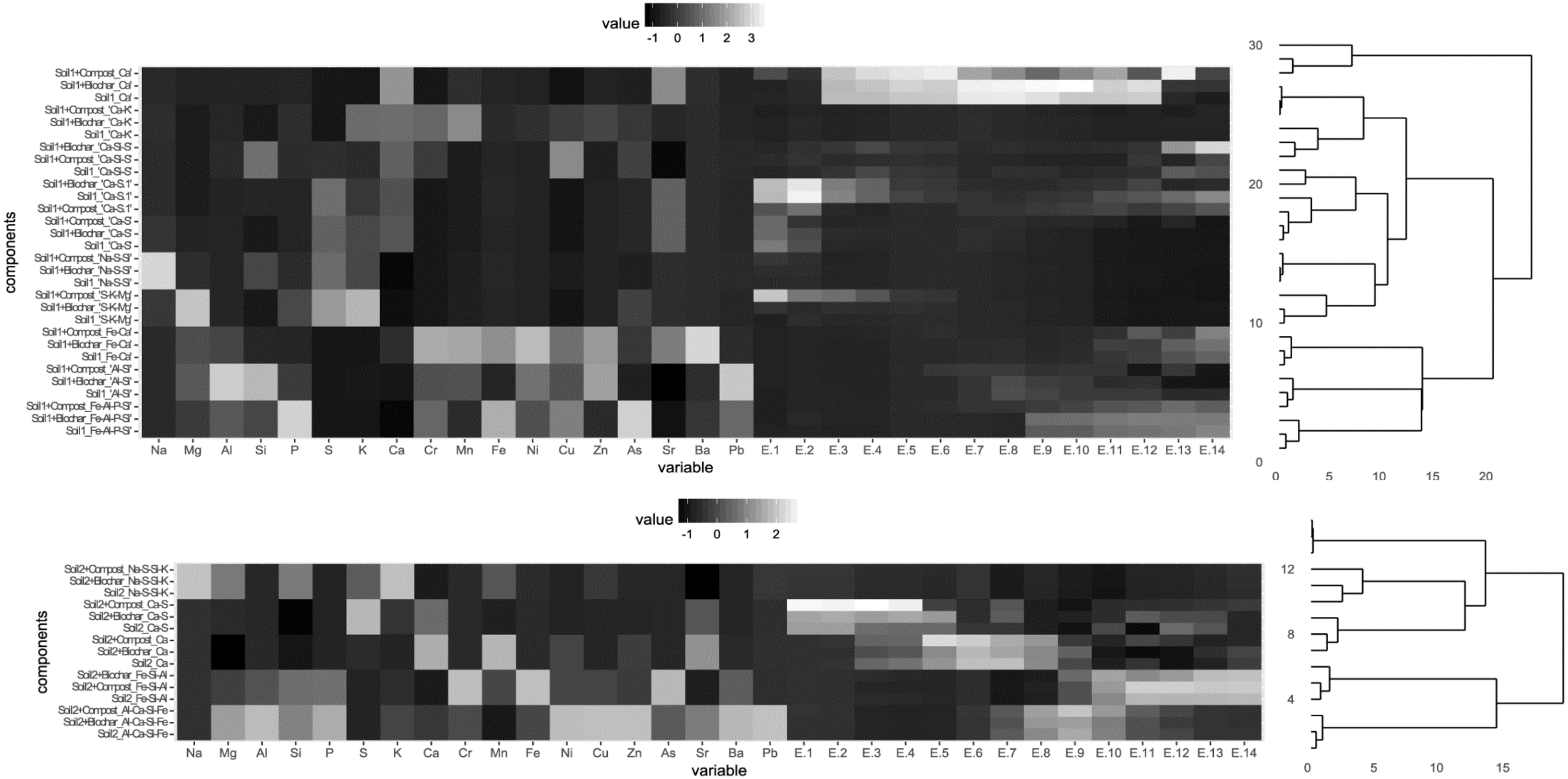


Figure 5.9: Heatmap and associated clustergram for CISED extraction data for the Soil 1 (top) and Soil 2 (bottom) treated with compost, biochar, or un-amended. On the left side the elements composition (e.g. Na, Mg, Al) on the right side the extraction number data (E_1 to E_{14}). The heatmap colour gradient represents the mean-centered concentrations. Cd, Hg, and Se were removed as concentration values were below detection limits.

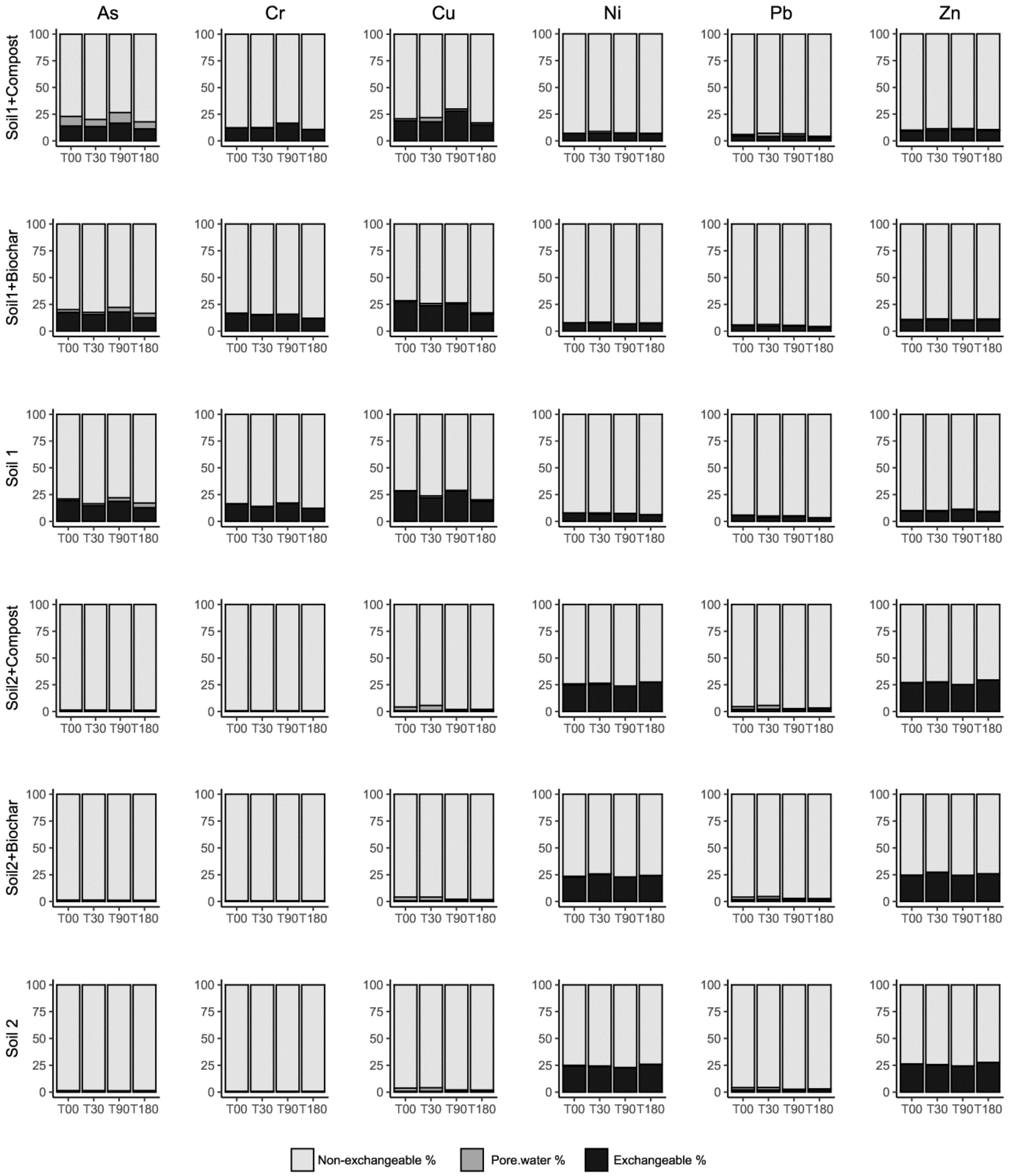
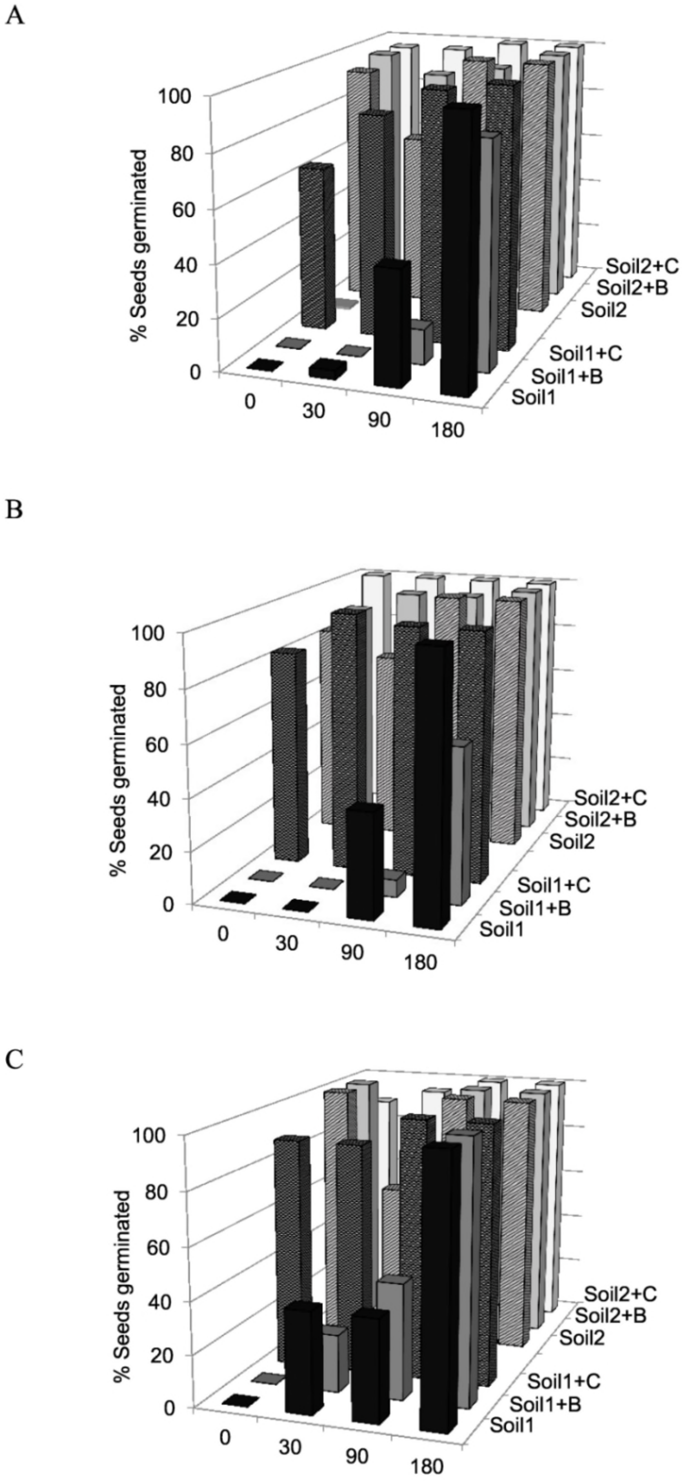


Figure 5.10: Heavy metals and metalloids distribution expressed as percentage (pore water, exchangeable, and non-exchangeable), for both soils (Soil1 and Soil 2) treated with compost, biochar, or un-amended, at 0, 30, 90 and 180 days. Cd, Hg, and Se were removed as concentration values were below detection limits.



C: compost, B: biochar

Figure 5.11: Seed germination assay for (A) Mustard (*B. alba*), (B) rye grass (*L. perenne*), and (C) pea (*P. sativum*) seeds. Germination rate (expressed as a percentage of the total) at the four sampling time (onset, 30, 90, and 180 days) for Soil 1 and Soil 2 treated with compost, biochar, or un-amended.

6. Prediction of bioavailability and toxicity of complex chemical mixtures through machine learning models

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Abstract: Empirical data from a 6-month mesocosms experiment were used to assess the ability and performance of two machine learning (ML) models, including artificial neural network (NN) and random forest (RF), to predict temporal bioavailability changes of complex chemical mixtures in contaminated soils amended with compost or biochar. From the predicted bioavailability data, toxicity response for relevant ecological receptors was then forecasted to establish environmental risk implications and determine acceptable end-point remediation. The dataset corresponds to replicate samples collected over 180 days and analysed for total and bioavailable petroleum hydrocarbons and heavy metals/metalloids content. Further to this, a range of biological indicators including bacteria count, soil respiration, microbial community fingerprint, seeds germination, earthworm's lethality, and bioluminescent bacteria were evaluated to inform the environmental risk assessment. Parameters such as soil type, amendment (biochar and compost), initial concentration of individual compounds, and incubation time were used as inputs of the ML models. The relative importance of the input variables was also analysed to better understand the drivers of temporal changes in bioavailability and toxicity. It showed that toxicity changes can be driven by multiple factors (combined effects), which may not be accounted for in classical linear regression analysis (correlation). The use of ML models could improve our understanding of rate-limiting processes affecting the freely available fraction (bioavailable) of contaminants in soil, therefore contributing to mitigate potential risks and to inform appropriate response and recovery methods.

Keywords: risk assessment; machine learning; bioavailability; complex chemical mixtures; compost; biochar.

6.1. Introduction

Contaminants in the environment are found as a combination of multiple chemicals, often including both organic and inorganic compounds, such as petroleum hydrocarbons and heavy metals/metalloids (European Environment Agency, 2012). The contaminants’ fraction potentially available to receptors (bioavailable fraction) depends on both chemical properties of pollutants and soil properties (Semple et al., 2003), and is a better proxy than the total concentration of contaminant (Guo et al., 2016) when predicting the actual exposure to organisms and the ecosystem (Gourlay-Francé and Tusseau-Vuillemin, 2013). Bioavailability provides a basis to make robust decision regarding appropriate risk assessment, predicting contaminants’ fate, transport, and potential environmental impact (Lehmann and Joseph, 2015). While greater attention has been given to the role of bioavailability of complex contaminants in regard to remediation end points (Brand et al., 2012; Harmsen and Naidu, 2013; Kördel et al., 2013; Ortega-Calvo et al., 2015) as well as its implication for regulatory frameworks (Umeh et al., 2017), its recognition and effective application by European-based environmental regulators is still limited (Harmsen and Naidu, 2013).

The recent shift toward sustainable remediation approaches is spawning a growing interest towards biological amendments such as composting, land farming, bioventing, and biopiling (Bardos et al., 2011). Biochar and compost amendments have been largely applied as effective bioremediation approaches for enhancing recovery of soil contaminated with petroleum hydrocarbons and heavy metals (Lyu et al., 2016; Wu et al., 2014). Even though bioremediation is regarded as a sustainable and economical approach, with minimal disruption on site (often applicable in-situ) and great public acceptance, it has some limitations (Boopathy, 2000). For instance, some chemicals may not be degradable, or their degradation may yield by-products, such as oxy-PAH, which can exhibit greater toxicity than the parent compounds (Hu et al., 2012) and therefore slow down the degradation and reduce remediation efficiency. Because of these challenges, our mechanistic understanding of complex chemical mixtures degradation, the associated toxicity, and the subsequent implication for risk assessment and remediation end-point are still limited.

Accurately monitoring the bioremediation process and supporting risk evaluation and degradation assessment often involve long-term experiments with multiple soil sampling which are labour intensive, time consuming, and expensive. Therefore, the use of machine learning (ML) methods trained on empirical data could be advantageous to make predictions on the potential degradation and reduction in toxicity occurring during remediation. ML models are able to learn the relationships between input variables (e.g. soil amendment, soil type) and output variables (e.g. long term changes in contaminants' bioavailability) from a training dataset, these relationships can then be generalised to make informed decisions in new cases (Wu et al., 2013). The application of ML to environmental issues, such as waste recovery and degradation studies, have been widely investigated in the literature (Abbasi and El Hanandeh, 2016; Heshmati et al., 2014; Khamforoush et al., 2011; Mason, 2006; Petric and Selimbašić, 2008; Wu et al., 2013), however their implementation is still limited, as often these methods are data-specific, or even variable-specific and their performance depends on many factors (Li et al., 2011). In particular, previous research highlighted the potential of ML to determine remediation end-points based on bioavailability predictions (Wu et al., 2013) and showed that a better understanding of the impacts of bioavailability is necessary to fully comprehend the extent of efficacy of bioremediation and manage the associated risks.

Therefore, in this study, we use ML to predict bioavailability but also the actual toxicity of tar-contaminated soils from two former manufactured-gas plant sites before and after lab-based bioremediation with biochar and compost amendments. Specifically, the objectives were to use empirical data from a bioremediation experiment to predict (1) change of complex chemical mixtures bioavailability, (2) change in the associated toxicity, and (3) to assess the input variables which are the most important for the estimation of toxicity.

6.2. Material and methods

6.2.1. Mesocosms setup ad data collection

A 6-month mesocosms experiment was carried out using two contaminated soils collected from two former gasworks sites (Soil 1 and 2) located in the United Kingdom (UK). The soil physicochemical properties of the two samples used in this study are discussed and summarised elsewhere (Chapter 5, Paragraph 5.3.1,Table 5.1). Briefly, the soils were amended in duplicate with either 5% w/w biochar (Soil + Biochar) or 15% w/w compost (Soil + Compost). A set of duplicate samples were also left with no physical remedial action (Soil). All samples were stored outdoor for 180 days. Soil samples were collected at day 0, 30, 90, and 180 and subsequently processed for chemical, microbiological, and toxicological analysis. For the chemical analysis, total and bioavailable petroleum hydrocarbons and heavy metals were extracted and analysed for both soil samples at the 4 sampling times. Details of extraction technique are provided in paragraph 6.2.2.

A battery of biological and eco-toxicological indicators, representing different trophic levels, has been used to assess the ecological health change of the soils undergoing bioremediation treatments (Coulon et al., 2004). The indicators included soil respiration as described previously by Paton et al. (2006), phospholipid fatty acids analysis (PLFA) as per Frostegård et al. (1993) , seeds germination (mustard, rye grass, and pea), earthworm’s lethality as described by Dawson et al. (2007) and Coulon et al. (2010), and Microtox® basic soil phase assay as described by Coulon et al. (2004). Information on the determination of the toxicological responses of the model organisms are summarised in Table 6.4 (Appendix).

6.2.2. Chemical analysis

A modification of the method reported by Risdon et al. (2008) was used for the analysis of the total PHC concentration using a dichloromethane: hexane mixture as explained below. For the estimation of the bioavailable concentration of organic compounds soil subsamples were extracted with mild solvent (methanol) as this has been previously found to be a good representation of the bioavailable fraction, and has been correlated with both the accessibility to earth-worms and bacterial mineralization assays (Kelsey et al., 1997; Reichenberg and Mayer, 2006; Yu et al., 2011; Wu and Zhu, 2016).

Briefly, 2.5 g of soil were mixed with either 15 mL of 1:1 dichloromethane: hexane (for total content) or 15 mL of methanol (for bioavailable content), sonicated for 20 min at room temperature, and shaken at 150 g for 16h. On the following day, samples were again sonicated for 20 min at room temperature and centrifuged (2000 g for 10 min). The supernatant was then cleaned onto a 6 mL SPE DSC-Si silica tubes, and 0.5 mL of sample was taken and mixed with 0.5 mL of internal standards and analysed by gas chromatography-mass spectrometry (GC-MS) as described by Cipullo et al. (2018 a). For the data analysis aromatic fractions were grouped as EC_{10}-EC_{12}, EC_{12}-EC_{16}, EC_{16}-EC_{21}, and EC_{21}-EC_{35}, and aliphatic fractions were grouped as EC_{10}-EC_{12}, EC_{12}-EC_{16}, EC_{16}-EC_{35}, and EC_{35}-EC_{40} according to Coulon et al. (2010 a) to provide a summary of the contaminant composition. The overall aromatic (Σ Aromatics), overall aliphatic (Σ Aliphatics) and total petroleum hydrocarbon compounds (Σ PHC) contents were also calculated and are presented in Table 6.2 (Appendix).

The pseudo-total and bioavailable heavy metals and metalloids content were determined using either aqua regia extraction (ISO 11047:1998) or a modified procedure for sequential extraction as described in Cave et.al. (2004). Briefly, for pseudo-total content 0.5 g of soil was extracted with 8 mL hydrochloric/nitric acid mixture in a microwave digestion system then diluted to 50 mL with DI water. For bioavailable heavy metals and metalloids content 2 g of soil samples were consecutively extracted by addition of 10 mL of an extraction solution which contained an increasing concentration of nitric acid and hydrogen peroxide (i.e. from 0 to 5 M, and 100 volumes). All total and sequential solutions extracted were filtered with 0.45 µm 25 mm nylon syringe filters and diluted 4 times with 1% HNO_3 before analysis by inductively coupled plasma mass spectrometry (NexION® 350D ICP-MS, Perkin Elmer). Data from the sequential extraction were then analysed using MatLab® (Version R2015a) and R Studio (Version 1.1.423 – © 2009-2018 RStudio, Inc.) as described by Cipullo et al.

(2018) to derive information on the partitioning and bioavailable concentrations of HM and metalloids in soil. Summary of the soils chemical concentrations are shown in Table 6.2 and Table 6.3.

6.2.3. Modelling methods and variables input

The ML prediction of soil toxicity for a given time t is done in two stages (Figure 6.1); (a) first we use ML to predict the bioavailable concentration of some hydrocarbons at time t, then (b) we use these predictions to estimate the toxicity at time t. Our implementation relies on *scikit-learn*, a machine learning library for Python (Pedregosa et al., 2011). We tested Neural Network (NN) and Random Forest (RF) which are two ML techniques often used to model complex and nonlinear environmental problems (Rajaee et al., 2009; Sahoo et al., 2006; Wu et al., 2013).

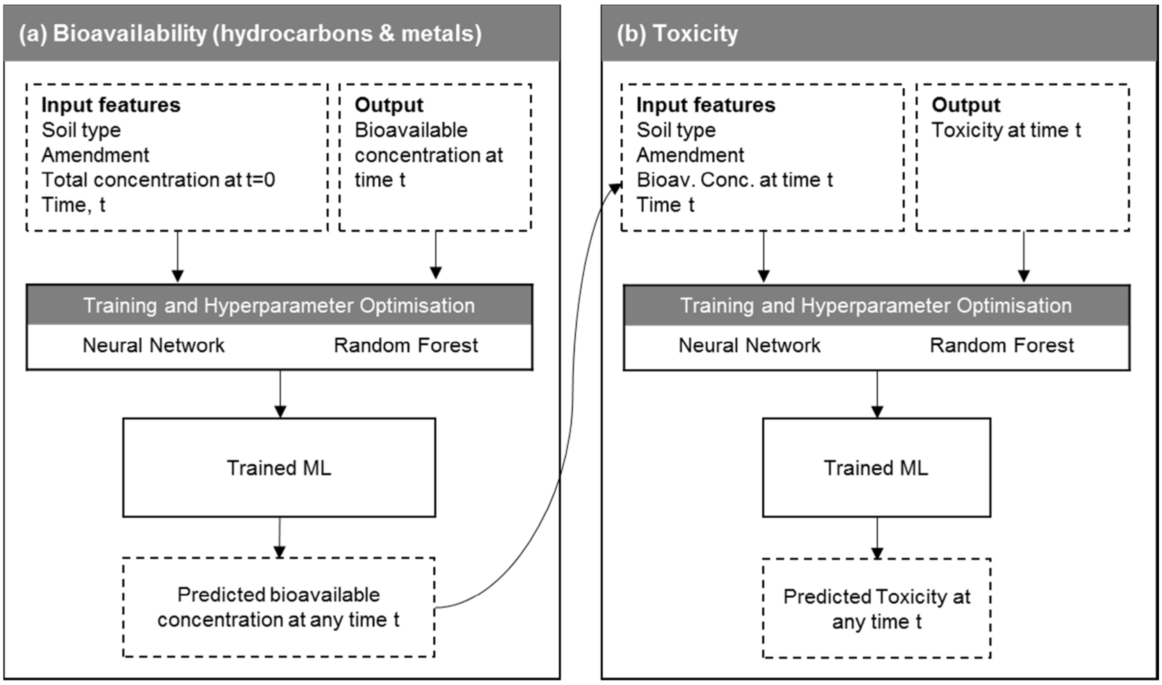


Figure 6.1: Two-stage approach to predict soil toxicity. (a) A machine learning (ML) algorithm is used to predict the bioavailable concentration of hydrocarbons and heavy metals/metalloids at time t. (b) The predicted concentrations are used as inputs of a second ML algorithm to predict soil toxicity.

For both the NN and RF methods, each input feature was first scaled by removing its average value and dividing by its standard deviation. This was done to avoid features with large values to dominate in the training phase. For the NN, we used a Multi-Layer Perceptron (MLP) which is a category of NN with at least three layers of neurons fully connected (an input layer, an output layer, and one or more hidden layers). NN models require several hyper-parameters to be tuned (Table 6.1), and are often criticised for being black boxes, as their behaviour cannot easily be explained by inspecting their internal structure (Sturm et al., 2016). That is why Random Forest (RF) is also used in this study. RF can be considered as a grey box (Prasad et al., 2006), because it allows exploring the relative importance of the different input features. RF also has fewer hyper-parameters than NN (mainly the number of trees and the maximum depth of a tree; Table 6.1). More information on the behaviour of NN and RF can be found in the Appendix (Paragraph 6.6.1).

Table 6.1: Hyper-parameters of the artificial neural network (NN) and random forest (RF) models.

NN model		RF model	
Hyper-parameters	Values	Hyper-parameters	Values
Activation function	Identity, Relu	Number of trees	20,30,40
Number of hidden layers and neurons	3 layers with 100 neurons, 4 layers with 100 neurons, 5 layers with 100 neurons	Maximum depth	20,30,40
Regularisation term	0.0001, 0.001, 0.01, 0.1, 1, 10		
Learning rate (constant)	0.001,0.01,0.1		

Relu: rectified linear unit

6.2.4. Hyper-parameters optimisation of ML models

For both models, the tuning of the hyper-parameters was done automatically using an exhaustive grid search where all the combinations of hyper-parameters are tested to find the best combination. The best combination is found through a 4-fold cross-validation. The dataset is split into 4 subsets. Then for each of the 4 folds, the model is trained using 3 of the folds and tested on the 4th fold. The performance of the regression is measured as the mean r^2 value obtained across the 4 folds. After finding the best set of hyper-parameters (Table 6.5 and Table 6.6), the model is retrained on the whole dataset.

6.2.5. Estimation of bioavailable concentrations and toxicity

ML algorithms were trained and tested for each metal (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, and Zn) and each of the following hydrocarbon: (1) aliphatic fraction including all individual compounds in the range of EC_{10} to EC_{40}, (2) aromatic fraction including: Acenapthene (AE), Fluorene (F), Anthracene (A), Phenantrene (P), Pyrene (PY), Chrysene (C), Benzo(a)anthracene (BA), Benzo(a)pyrene (BaP), Benzo(b)fluoranthrene (BB), Benzo(k)fluoranthrene (BK), Dibenzo(a,h)anthracene (DA), Benzo[g,h,i]perylene (BP), and Indeno[1,2,3-c,d]pyrene (IP). To estimate the concentration of a hydrocarbon/metal at time t, the inputs of the ML models were the soil type (Soil 1 or Soil 2), the amendment (un-amended, biochar, or compost), and the initial (t=0) total concentration of the hydrocarbon/metal. After training/testing via the 4-fold cross-validation, the NN models with $r^2 > 0.7$ (good model fitting) were selected to generate the inputs for the estimation of the toxicity (Figure 6.1, b). Using models with a lower r^2 may generate inaccurate inputs which would confuse the estimation of the toxicity.

NN was used instead of RF, because RF provides a discrete output dictated by the finite number of split points in each tree as explained in paragraph 6.2.3. Since measurements are only available at time t=0, 30, 60, and 180 days, the RF output does not vary continuously in between these data points which may lead to unrealistic variations in bioavailable concentrations. Here, RF is mainly used (i) as a benchmark against NN for which finding an appropriate combination of hyper-parameters is less straightforward, and (ii) because it provides access to the relative importance of the input variables.

6.2.6. Estimation of toxicity

NN and RF algorithms were then trained and tested for each biological and eco-toxicological indicators listed in Table 6.4 (Appendix). To estimate a given indicator at time t, the ML models take again as inputs the soil type (Soil 1 or Soil 2), the amendment (un-amended, biochar, or compost), but also the bioavailable concentration of the hydrocarbons/metals predicted with NN having $r^2>0.7$. The latter is anticipated to be a valuable input for the toxicity estimation, as bioavailable concentration is reported to be a good proxy for toxicity estimation (Gourlay-Francé and Tusseau-Vuillemin, 2013).

6.2.7. Drivers of bioavailability and toxicity

To investigate which of the input features are driving the estimation of the bioavailability/toxicity variables, we rely on the RF model which can output the relative importance of each input feature. To do so, the skicit-learn library implements the method based on the Mean Decrease in Impurity (MDI) described by Breiman (1984). In a decision tree, every node corresponds to a logical *if-then* condition on a single input feature, which splits the input dataset into 2 subsets. The choice of the feature and its splitting condition is based on a measure called impurity. For regression trees, the impurity in the data is quantified by the variance, and the training phase corresponds to finding the splits which lead to the greatest reduction in variance. To reflect the feature importance, the MDI accounts for the number of times a feature is selected to split the data and how many data samples it splits (i.e. the importance of the splits). In practice, an input feature is important if it is often used by the trees to make decisions and if these decisions are concerning many data samples.

6.3. Results and discussion

6.3.1. Prediction of bioavailable concentration of PHC and HM

The accuracy of the ML models (r^2 value) for the prediction of bioavailability is shown in Figure 6.2. A thorough inspection of the data revealed that noisy measurements, i.e. when two replicates are different from each other, are one of the main reasons for the lower r^2 values obtained for some hydrocarbons and elements. The corresponding combinations of hyper-parameters returned by the grid search are shown in Table 6.5 (Appendix). The models with high r^2 can be used to estimate bioavailability at any time step. For example, Figure 6.3 shows the predicted bioavailable concentration of Fluorene (F) (Figure 6.3, a and b), benzo[a]anthracene (BA) (Figure 6.3, c and d), benzo[a]pyrene (BaP) (Figure 6.3, e and f), and Copper (Cu) (Figure 6.3, g and h) with RF and NN, respectively. The corresponding r^2 values obtained from prediction were r^2 =

0.93 (RF) and r^2 = 0.89 (NN) for F, r^2 = 0.97 (RF) and r^2 = 0.91 (NN) for BA, r^2 = 0.96 (RF) and r^2 = 0.90 (NN) for BaP, and values of r^2 = 0.96 (both RF and NN) for Cu.

As explained in the methodology section, RF generates an output with discrete values which may lead to unrealistic variations due to the measurements being available only at a few time steps. In comparison, NN generates a continuous output which can be used to estimate the concentration at intermediate time steps, but also to forecast the potential evolution of the concentration for time steps beyond 180 days. Therefore, in the next section, we use NN, and not RF, to generate the estimated bioavailable concentrations used as inputs of the toxicity models. The selected hydrocarbons are EC_{12}, EC_{14}, EC_{16}, EC_{17}, EC_{19}, EC_{21}, EC_{23}, EC_{25}, EC_{26}, AE, F, A, P, PY, C, BA, BaP, BB, BK, BP, DA, IP, P, and the selected elements are As, Cd, Cr, Cu, Hg, Ni, Se, Pb, and Zn (Figure 6.2; NN $r^2 > 0.7$).

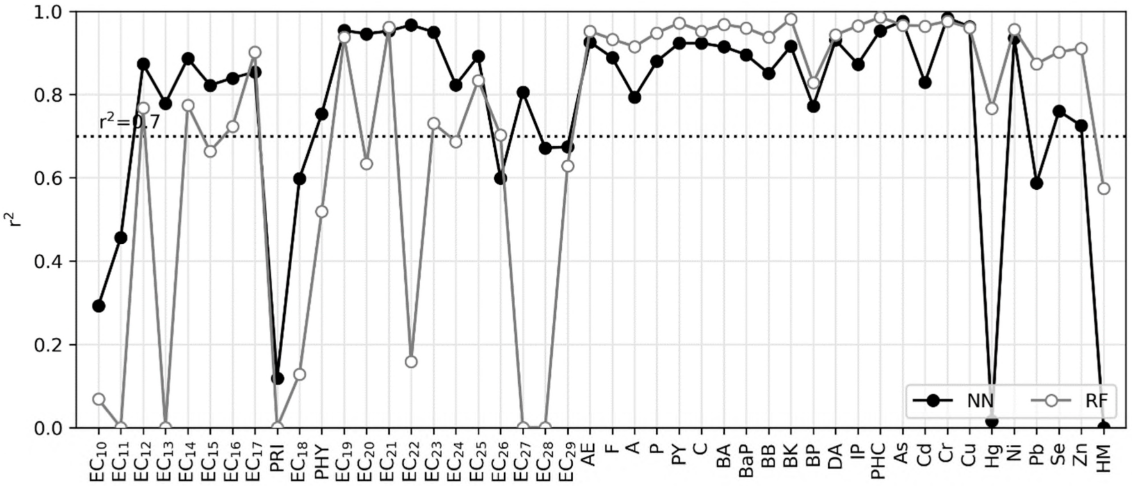
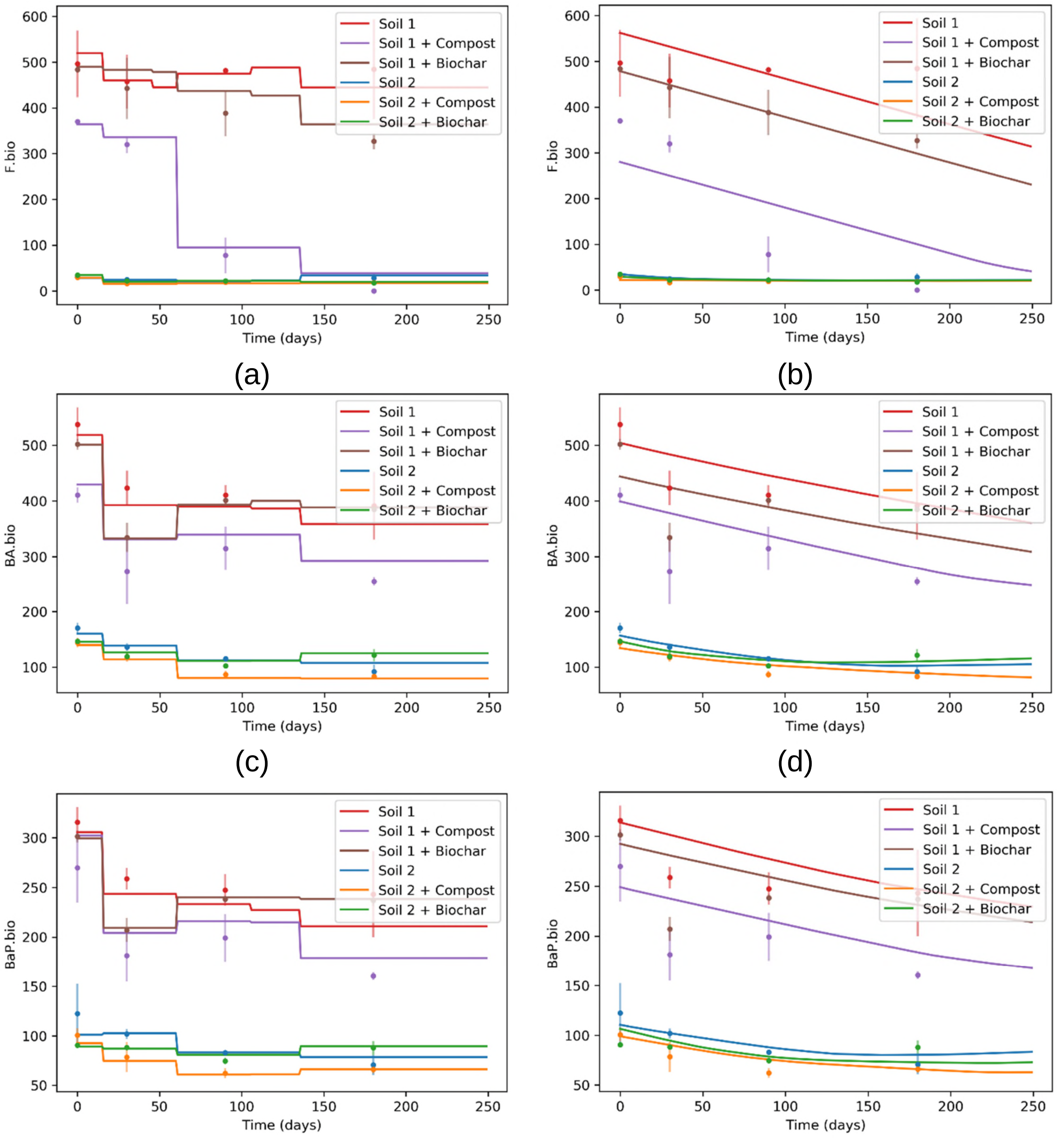


Figure 6.2: Prediction accuracy (r^2) for the bioavailable concentration of hydrocarbons and heavy metals/metalloids using neural network (black) and random forest (grey).

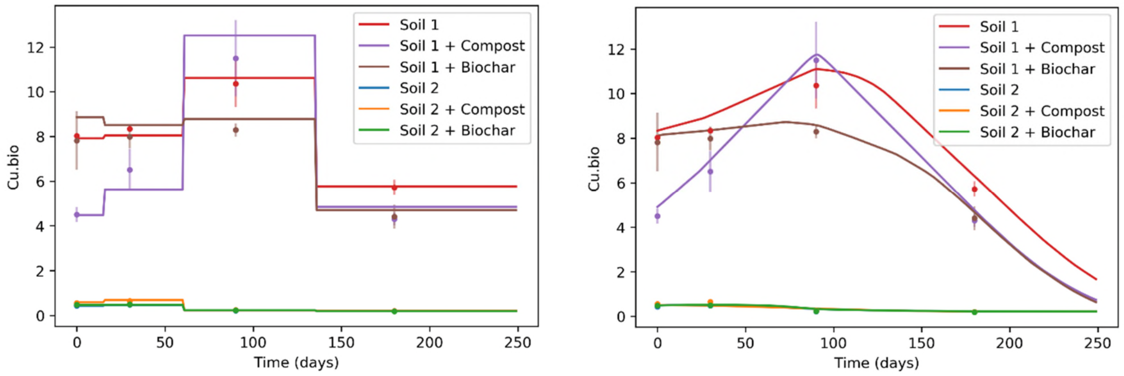
In this mesocosms experimental setup the decrease in hydrocarbons bioavailable concentration was 2 times higher in Soil 1 (high contamination) than in Soil 2 (low contamination). The most significant changes were observed for the aromatics $EC_{16\text{-}21}$ and aliphatic $EC_{16\text{-}35}$ fractions; where readily available/bioavailable (methanol extracted) concentration decreased by 71, 58% (Soil 1 + Compost) and by 11, 91% (Soil 2 + Compost). Fewer changes in bioavailable concentration were observed in Soil 2, which overall had lower concentrations of contaminants. This is clearly highlighted in Figure 6.3 where Fluorene (F) was plotted as representative aromatic compound for the EC_{16}-EC_{21} fraction. The concentration of F significantly decreased after 180 days incubation for Soil 1 + Compost. It was also clear that the soils amended with compost had F concentration 10 times lower than the non-treated (Soil 1) soil at the end of the simulation period.



(a) (b)

(c) (d)

(e) (f)



(g) (h)

Figure 6.3: Prediction of the bioavailable concentration (mg/kg) of Fluorene (F), benzo[a]anthracene (BA), benzo[a]pyrene (BaP), and Copper (Cu) using random forest (left) and neural network (right). The continuous lines are the predicted concentrations for the different soils (Soil 1 and Soil 2), amendments (biochar, compost) and un-amended. The dots are the available measurements after averaging the replicates. The error bars are the standard deviation across the replicates.

The relative feature importance when estimating bioavailability with RF for compounds modelled with r^2> 0.7 is presented in Figure 6.7 (Appendix). The bar chart shows that for each compound different features (time, soil, or amendment) dominate. For example, the prediction of the AE, P, PY, BA, BaP, and DA were mainly driven by soil properties and the total concentration of the compounds at onset. The importance of the soil feature reflects the differences observed in bioavailable concentrations between Soil 1 (heavily contaminated) and Soil 2 (low contamination). For most of the HMs/metalloids (As, Cd, Cr, Cu, Ni, Zn) time (ageing) and amendment (compost or biochar) were not important variables influencing bioavailability prediction (Figure 6.7, Appendix). Indeed HMs/metalloids bioavailable concentration did not change significantly over incubation time.

6.3.2. Prediction of the toxicity

Overall, RF performed slightly better than NN to predict the toxicity (Figure 6.4), and was particularly good at predicting seed germination, condition index, and EC$_{50}$. Lower r^2 values were obtained for the PLFA, which can be explained by different factors including: (1) the use of quantitative data (concentrations) over qualitative data (relative abundance of individual PLFA) could have provided a better input for r^2 estimation; (2) it was not possible to conclusively attribute these PLFA changes based on the input parameters of this model as there are multiple factors (or additional factors) affecting community composition. Further to this, we did not evaluate the effects of other chemicals (e.g. metabolites) which may have impacted the microbial community at early experimental stages (e.g. 2, 5, 10, 20 days), thus sampling regime established (30, 90, 180 days) was not significant for assessing smaller but significant toxic effects on the microbial community. The corresponding combinations of hyper-parameters returned by the grid search are shown in Table 6.6 (Appendix).

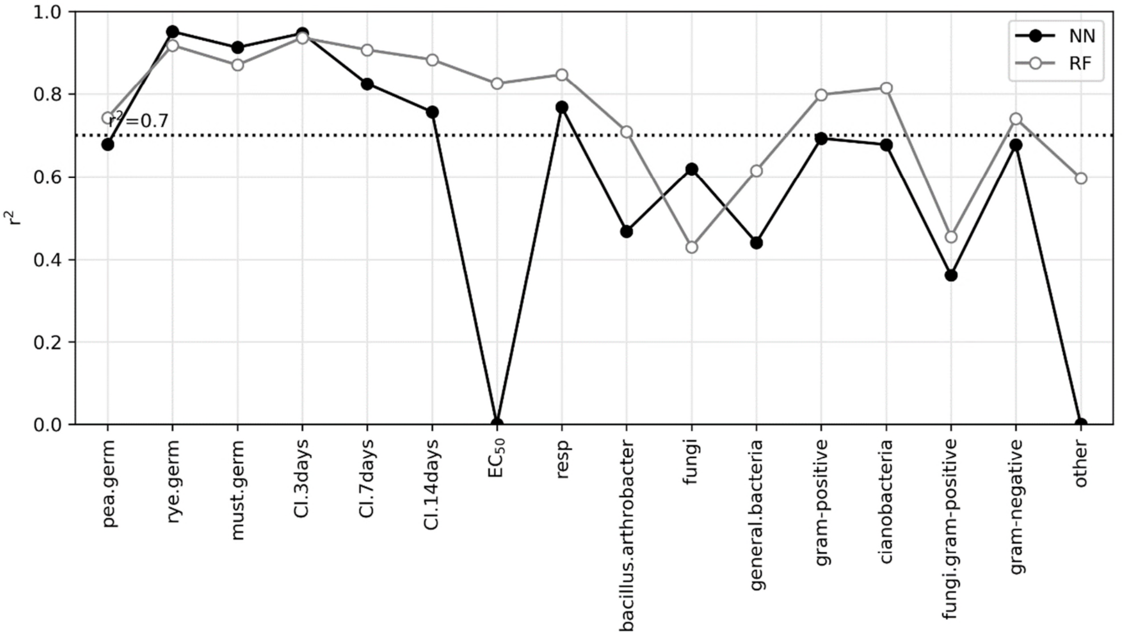
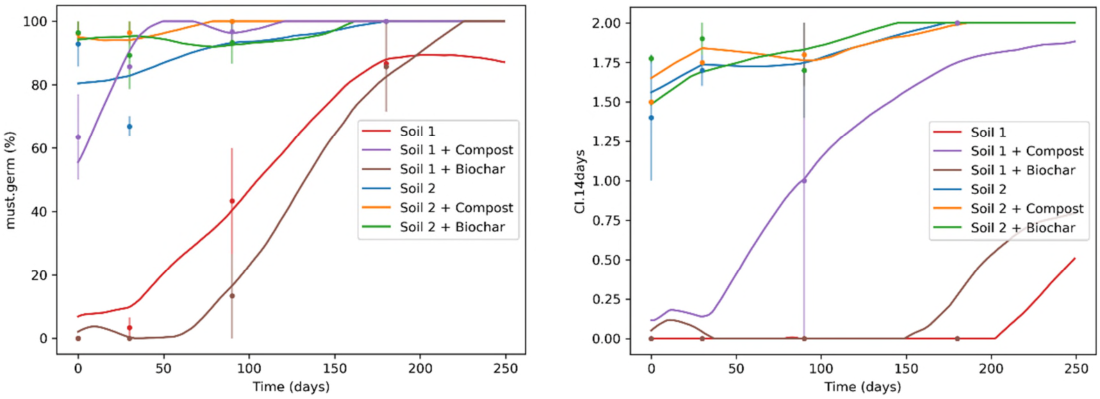


Figure 6.4: Prediction accuracy for the toxicity using neural network (NN) (black) and random forest (RF) (grey).

Germ: germination, CI: condition index, EC_{50}: Half maximal effective concentration, resp: soil respiration (mg CO_2/g soil).

As done for the hydrocarbons, metals and metalloids, ML models with $r^2 > 0.7$ were successfully used to predict the toxicity at intermediate time step; for example Figure 6.5 shows the NN prediction obtained for (a) mustard germination and (b) the earthworm toxicity assay (14-day condition index CI). Good predictions were also obtained for CI 3 days ($r^2 = 0.95$), CI 7 days ($r^2 = 0.83$), CI 14 days ($r^2 = 0.76$), mustard germination ($r^2 = 0.91$), ryegrass germination ($r^2 = 0.95$), and soil respiration ($r^2 = 0.77$).



(a) (b)

Figure 6.5: Prediction of (a) mustard seeds germination (r^2 = 0.91) and (b) earthworms 14-day condition index (r^2 = 0.75) using neural network. The continuous lines are the predicted values for the different soils and amendments, the dots are the available measurements after averaging the replicates. The error bars are the standard deviation across the replicates. The minimum value allowed is 0, and the maximum values allowed are 100 and 2 for mustard germination and condition index, respectively.

Empirical data, obtained from the mesocosms experimental setup showed that Soil 1 had a greater toxic effect on mustard seed germination than Soil 2, likely because concentration of contaminants was 4 times lower in Soil 2 (PHC > 2000 mg/kg) compared to Soil 1 (PHC > 8000 mg/kg). Interestingly, seed germination showed a good ecological recovery for Soil 1 + Compost and even for Soil 1 which reached similar recovery after 180 days. The model predictions highlighted the potential for all soil samples to achieve 100% germination after 250 days (Figure 6.5, a).

Similar ecological recovery for Soil 1 was observed from empirical data obtained for earthworm lethality assay (*E fetida*); while the CI was similar at the onset of the experiment (CI = 0, mortality), and only Soil 1 + Compost showed greater ecological recovery (CI was 2 times higher than biochar and un-amended conditions). The predicted ecotoxicity of Soil 1 + Biochar and Soil 1 significantly decreased after 250 days (CI = 0.4 - 1) (Figure 6.5, b). The progressive positive effect of Soil 1 + Compost was also reflected by the prediction where at 250 days the sample ceased to be lethal (CI = 2) to earthworms assay. For the less contaminated samples, a higher CI was recorded during the experimental setup for both Soil 2 + Biochar and Soil 2 +

Compost compared with Soil 2 (Figure 6.5, b). The earthworms in Soil 2 + Compost and Soil 2 + Biochar remained in the healthiest condition throughout the 14 days assay as no significant differences were observed in the empirical data which was also reflected by the model.

6.3.3. Drivers for toxicity

The most important input features for the estimation of the toxicity are shown in Figure 6.6. They were identified by looking at the relative feature importance from the RF model with $r^2 > 0.7$. This allows assessing the influence of each feature on the prediction.

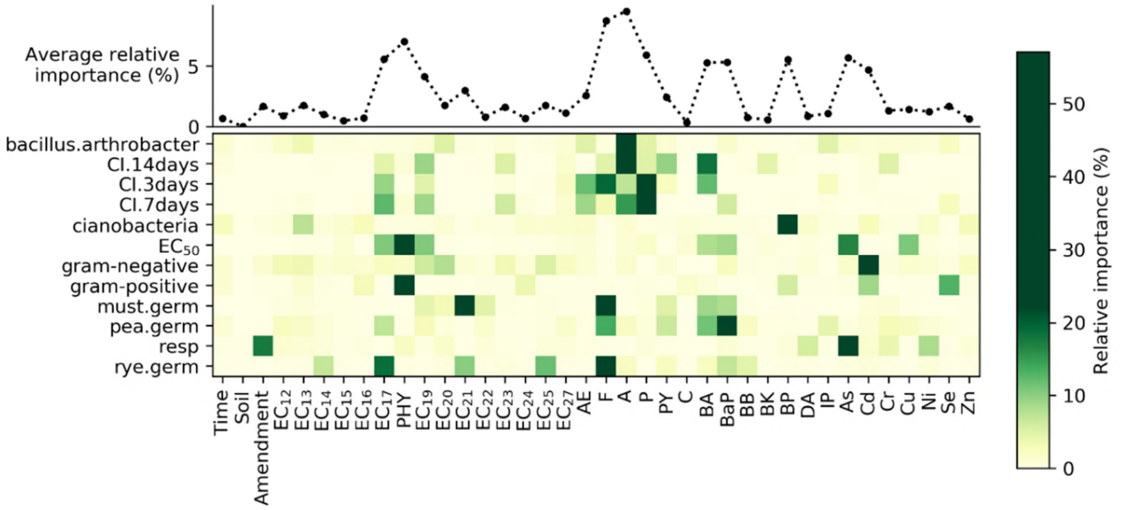


Figure 6.6: Relative feature importance when estimating toxicity with random forest. The heatmap shows the relative feature importance for all the toxicity variables which were modelled with an r^2 value above 0.7. The top graph shows the relative feature importance averaged across the toxicity variables.

The heatmap shows that, for each toxicity variable, different features dominate. For example, the prediction of the CI (earthworm acute toxicity) was mainly driven by EC_{17}-EC_{19}, AE, F, A, P, and PY. A previous study highlighted the key role of the mid-chain length aliphatic compounds in toxicity to earthworms (Dorn and Salanitro, 2000). In particular, these compounds are the most volatile, soluble, and biodegradable constituents of the tar fraction remaining after the initial removal of the more soluble/volatile components (e.g. low molecular weight compounds). Thus, mid-chain length aliphatic compounds can be a valuable indicator of acute toxicity to soil dwelling organisms (e.g., earthworms).

Furthermore, this fraction, along with small aromatic compounds such as P and AE, represented the least aged fraction (more bioavailable) with the highest toxicity potential, and should therefore be of special concern with respect to remediation/natural attenuation monitoring (Yang et al., 2016).

In comparison, the heatmap suggests that bacterial respiration was mainly related to arsenic (As) and soil amendment (biochar or compost). Toxicity to As is caused by its ability to inhibit basic cellular functions, and disrupt microbial metabolism (Walker et al., 2000). A number of studies previously highlighted the adverse effects of As contamination on microbial biomass C and respiration rates (Edvantoro et al., 2003; Van Zwieten et al., 2003) and in particular bioavailable arsenic exerted greater inhibitory effect rather than total arsenic concentration (Ghosh et al., 2004). On the opposite, the model showed that EC_{15}-EC_{20}, Cd, Cr, Cu, Ni, Se, and Zn ranked among the least important features for respiration. Overall, Time, soil type, amendment (biochar and compost) were not identified as important features. This is probably because these features were already used as inputs of the ML models to estimate the bioavailable compounds, thus their effects may be embedded in the variations of bioavailable hydrocarbons and metals. In summary, these findings reinforce the idea that the bioavailability of multiple hydrocarbons and metals drives the soil toxicity.

6.3.4. Implications and limitations of machine learning for risk assessment and remediation end-point evaluation

The two ML models used in this study were trained using a limited dataset. The models are a piori only valid for the values of the input variables which are captured by the training dataset. For example, the models may not accurately predict the toxicity for soils which are too different from Soil 1 and Soil 2. Similarly, the accuracy of the models may decrease when predicting toxicity at time steps larger than 180 days. This remark also applies to the analysis of feature importance. For example, our results suggest that the bioavailable concentration of arsenic is important to predict respiration. Although, this is true for our dataset, applying our method to a different soil type and contamination may suggest that other variables are also driving changes in respiration.

Nonetheless, our study indicates that ML models can help us understand complex chemical mixtures fate, and identify the key variables affecting their behaviour and the environmental risks posed by the various pools of contaminants. Future studies should indeed investigate the importance of other input variables such as pH, soil organic matter, and dissolved organic carbon on contaminant bioavailability and toxicity changes. Additionally, to provide a modelling tool suitable for multiple stake-holders, future work should also focus on (1) giving easier access to national datasets (e.g. national repository of ground investigation, geological maps, contamination record, former site investigation reports, and site-specific historical data), (2) increasing awareness and enhance understanding and utility of ML among non-specialists, via the development of ML tools with accessible graphical interfaces.

6.4. Conclusions

Empirical data from a 6-month mesocosms experiment were used to assess the ability and performance of two ML models to predict temporal bioavailability changes of polycyclic aromatic hydrocarbons, aliphatic hydrocarbons, and heavy metals/metalloids in contaminated soils amended with compost or biochar. In addition, ML was used to predict the toxicity changes, mainly based on the knowledge of some of the bioavailable concentrations. Results obtained showed that both NN and RF were able to model the bioavailability of various contaminants and should be used in combination as: NN model provides a realistic continuous output, while RF can explain which input measurements are actually important to predict the toxicity. This study suggests that ML models are good candidate tools to support remediation monitoring of multi-contaminated sites, in a cost-effective manner. ML capabilities should be further investigated with larger datasets encompassing a representative range of soil types and contaminations.

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6.6. Appendix

6.6.1. Neural network and random forest

Multi-Layer Perceptron (MLP) is a category of NN with at least three layers of neurons fully connected (an input layer, an output layer, and one or more hidden layers). Each neuron in the hidden layers does the same operation: (i) it computes the weighted sum of all its inputs and adds a bias, then (ii) the result goes through an activation function which maps the weighted sum to a standard output value (Grus, 2015). The value of the weights and bias of each neuron is learnt during the training step. The number of hidden layers, the number neurons in each hidden layer, and the type of activation function are among the hyper-parameters of the model which need to be tuned (Table 6.1). NN are often criticised for being black boxes, because inspecting all the internal weights and bias does not provide much understanding of how NN predicts the output (Sturm et al., 2016).

In comparison, RF can be considered as a grey box (Prasad et al., 2006), because it allows exploring the relative importance of the different input features. RF also has fewer hyper-parameters than NN (mainly the number of trees and the maximum depth of a tree; see Table 6.1). The regression trees which form the RF are constructed using the training dataset as follows. The first node of the tree splits the data samples into two subsets, based on a logical if-then condition on one input variable (Hastie et al., 2001). This partitioning is repeated until there is less than a given number of data sample at each final node, or until the maximum depth of tree has been reached. The predicted output at each final node corresponds to a simple constant model equal to the mean of the output samples in that node. RF provides discrete output values because the final prediction is given by a constant model and because of the finite number of splitting nodes. In comparison, the output neuron of the NN provides the predicted output as a continuous value.

Table 6.2: Descriptive statistics for aliphatic (ALK) and aromatic (PAH) total (dichloromethane:hexane) and bioavailable (methanol extracted) concentrations expressed in mg/kg in Soil 1 and Soil 2 (un-amended, compost, and biochar).

		Aliphatic					**Aromatic**				
		EC_{10-12}	**EC_{12-16}**	**EC_{16-35}**	**EC_{35-40}**	**ΣALK**	**EC_{10-12}**	**EC_{12-16}**	**EC_{16-21}**	**EC_{21-35}**	**ΣPAH**
Total concentration (mg/kg)	**Min.**	3.8	0.9	4.5	< d.l	14.9	< d.l	57.9	435.4	550.1	1050.6
	Max.	32.3	149.1	1053.8	< d.l	1166.8	< d.l	620.1	4384.5	3413.2	8276.3
	Median	10.9	10.6	109.0	< d.l	134.6	< d.l	133.4	957.7	1438.9	2562.6
Bioavailable concentration (mg/kg)	**Min.**	0.0	0.1	0.1	< d.l	0.3	< d.l	36.4	300.4	275.3	640.6
	Max.	23.6	44.5	428.3	< d.l	449.1	< d.l	488.8	3194.0	2341.1	5772.0
	Median	0.7	1.5	20.4	< d.l	23.4	< d.l	115.1	586.5	752.0	1577.1

d.l: detection limit

Table 6.3: Descriptive statistics for heavy metals and metalloids (HM), total (aqua regia extraction) and bioavailable (sequential extraction) concentrations expressed in mg/kg in Soil 1 and Soil 2 (un-amended, compost, and biochar).

		As	**Cd**	**Cr**	**Cu**	**Hg**	**Ni**	**Pb**	**Se**	**Zn**
Total concentration (mg/kg)	**Min.**	12.4	0.2	18.0	14.2	0.1	9.6	38.6	1.0	113.5
	Max.	67.7	3.2	75.9	62.2	0.7	35.1	455.6	11.6	481.8
	Median	41.3	0.7	43.6	29.9	0.3	26.6	74.9	4.4	199.6
Bioavailable concentration (mg/kg)	**Min.**	0.2	0.1	<d.l	0.2	<d.l	0.3	4.5	0.1	9.9
	Max.	4.9	0.2	1.3	13.2	<d.l	1.4	13.0	0.8	32.9
	Median	1.2	0.1	0.3	2.3	<d.l	0.8	7.9	0.5	20.8

d.l: detection limit

Table 6.4: Overview of the biological and eco-toxicological parameters used in the experimental study.

	Microbiological analysis		**Eco-toxicological analysis**		
Experimental parameters	**Basal respiration**	**Phospholipid Fatty Acid Analysis (PLFA)**	**Seeds germination assay**	**Earthworms acute toxicity assay**	**Biosensor assay**
Species	Multiple species present in the soil	Multiple species (*bacillus* or *arthrobacter*, fungi, general bacteria, gram+, cyanobacteria, fungi/gram+, gram- , unknown-unclassified)	Pea (*P. Sativum*) Mustard (*B. Alba*) Rye (*L. Perenne*)	*E. Fetida*	*Vibrio fischeri*
Mean of assessment	Soil replicates were incubated for 24 h at 20ºC, and the headspace analysed for CO$_2$ concentration with Gas Chromatography with Thermal Conductivity Detector (GC-TCD)	Soil replicates were freeze dried and solvent extracted, then and analysed By Gas Chromatography with Flame Ionisation Detector (GC-FID)	Replicate of seeds were exposed to contaminated soil and germination incidence assessed after 4-day exposure	Replicates specimens were exposed to contaminated soil and examined for mortality on days 3, 7, and 14	Soil replicates were mixed with 4 mL of diluent and bioluminescent bacteria and analysed with Microtox® assay (Modern Water)
Unity of measure	Expressed as mg of CO$_2$ /g soil	Relative abundance of individual PLFAs expressed as a percentage of the total	Germination incidence 0-100%	condition index (CI) expressed as a score from 0 to 2 where: 0 = mortality, 1= affected, 2=non-affected	EC$_{50}$: Soil dilution that inhibits 50% of the light output relative to control soil calculated for each sample
Reference	(Paton et al., 2006)	(Frostegård et al., 1993)	(Dawson et al., 2007)	(Langdon et al., 1999)	(Jiang et al., 2016)

Table 6.5:: Values of the hyper-parameters found for the bioavailability estimation models with r^2 >0.7. Empty cells (n.a) correspond to r^2 values below 0.7.

	Neural Network				**Random Forest**	
	Activation	**Hidden layer sizes**	**Learning rate**	**Alpha**	**Max depth**	**N trees**
EC$_{12}$	relu	(100, 100, 100, 100, 100)	0.001	10	40	20
EC$_{13}$	relu	(100, 100, 100, 100, 100)	0.001	1	30	20
EC$_{14}$	relu	(100, 100, 100, 100)	0.001	0.0001	20	30
EC$_{15}$	relu	(100, 100, 100, 100)	0.01	1	20	30
EC$_{16}$	relu	(100, 100, 100, 100)	0.01	0.1	30	40
EC$_{17}$	relu	(100, 100, 100, 100)	0.001	1	30	40
PHY	relu	(100, 100, 100)	0.01	0.1	40	40
EC$_{19}$	relu	(100, 100, 100)	0.001	10	40	30
EC$_{20}$	relu	(100, 100, 100, 100, 100)	0.001	0.0001	40	40
EC$_{21}$	relu	(100, 100, 100)	0.001	0.01	20	20
EC$_{22}$	relu	(100, 100, 100, 100, 100)	0.001	10	30	20
EC$_{23}$	relu	(100, 100, 100, 100, 100)	0.001	0.01	20	40
EC$_{24}$	relu	(100, 100, 100)	0.001	0.0001	20	30
EC$_{25}$	relu	100	0.1	10	40	40
EC$_{26}$	n.a	n.a	n.a	n.a	20	40
EC$_{27}$	relu	(100, 100, 100)	0.001	0.1	30	30
AE	relu	100	0.01	0.001	20	30
F	identity	(100, 100, 100, 100)	0.001	1	40	20
A	relu	100	0.01	10	30	40
P	relu	(100, 100, 100)	0.001	0.1	30	40
PY	relu	100	0.01	0.0001	40	40
C	relu	100	0.01	1	30	40
BA	relu	100	0.01	0.01	20	20
BaP	relu	100	0.01	0.0001	20	40
BB	relu	100	0.01	1	30	20
BK	relu	100	0.01	0.0001	20	30
BP	relu	(100, 100, 100)	0.01	0.001	20	40
DA	relu	(100, 100, 100, 100)	0.01	10	20	30

	Neural Network				**Random Forest**	
	Activation	**Hidden layer sizes**	**Learning rate**	**Alpha**	**Max depth**	**N trees**
IP	relu	100	0.01	1	20	30
PHC	relu	(100, 100, 100)	0.001	0.0001	40	20
As	relu	(100, 100, 100, 100)	0.01	0.1	30	20
Cd	relu	(100, 100, 100)	0.001	0.1	40	40
Cr	relu	(100, 100, 100, 100, 100)	0.001	0.1	40	40
Cu	relu	(100, 100, 100, 100)	0.01	1	20	20
Hg	identity	(100, 100, 100)	0.01	0.0001	20	20
Ni	relu	(100, 100, 100, 100)	0.01	10	40	30
Pb	n.a	n.a	n.a	n.a	40	40
Se	relu	(100, 100, 100, 100, 100)	0.01	0.1	30	30
Zn	identity	100	0.01	0.1	20	30

Relu: rectified linear unit

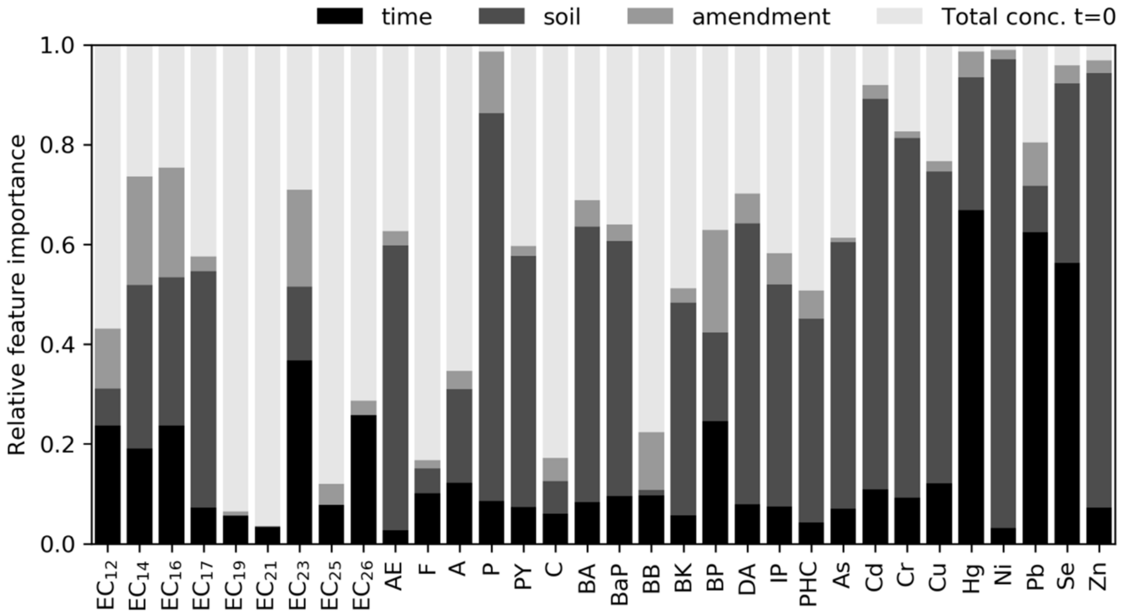


Figure 6.7: Relative feature importance (time, soil, amendment, and initial individual contaminant concentration) when estimating bioavailability with random forest, for all the compounds modelled with an r^2 value above 0.7. Time: (0, 30, 90, 180 days); soil (Soil 1, Soil 2); amendment: compost, biochar or un-amended; total: total concentration of the compound at onset (t=0).

Table 6.6: Values of the hyper-parameters found for the toxicity estimation models with r^2 >0.7. Empty cells (n.a) correspond to r^2 values below 0.7.

	Neural Network				**Random Forest**	
	Activation	**Hidden layer sizes**	**Learning rate init**	**Alpha**	**Max depth**	**N trees**
Pea.germ	n.a	n.a	n.a	n.a	30	40
Rye.germ	identity	(100, 100)	0.001	0.0001	20	40
Must.germ	identity	(100, 100)	0.001	0.001	20	20
CI.3days	identity	100	0.001	0.0001	20	40
CI.7days	relu	100	0.001	0.01	20	30
CI.14days	identity	(100, 100)	0.001	0.001	40	20
EC_{50}	n.a	n.a	n.a	n.a	40	20
Resp	relu	(100, 100)	0.001	0.0001	40	40
Bacillus, Arthrobacter	n.a	n.a	n.a	n.a	20	30
Gram-positive	n.a	n.a	n.a	n.a	30	30
Cianobacteria	n.a	n.a	n.a	n.a	20	40
Gram-negative	n.a	n.a	n.a	n.a	40	20

Relu: rectified linear unit

7. Overall discussion and implications of the PhD study findings

7.1. Introduction

This PhD research addressed a complex issue such as understanding bioavailability of chemical mixtures, with the aim of integrating this information it in the current framework to support and inform risk-based contaminated land management.

To achieve this aim, two laboratory studies were designed to (i) investigate the influence of the physico-chemical factors on the bioavailability of complex mixtures of petroleum hydrocarbons, heavy metals and metalloids, and to (ii) link bioavailability measuraments to toxicity changes. In addition, two modelling studies were implemented using the empirical data obtained from the two mesocosm studies to (i) assess the ability of Visible and Near Infrared spectroscopy (Vis-NIRS) to predict total and bioavailable concentrations of PHC, HM and metalloids in soils and to (ii) evaluate the performance of machine learning (ML) tools to predict changes of complex chemical mixtures bioavailability and the associated toxicity. This chapter provides a summary of the key findings, and an overview of how the different objectives contributed to achieve the aim of the PhD study (Figure 7.1).

Overall, this research provided valuable knowledge concerning distribution, behaviour and ageing of complex chemical mixtures in soil on a range of conditions not previously investigated (Chapter 3). Further findings (Chapter 5) can support the understanding of the likely effects of exposure (toxicity) to multi-contaminated environments by providing multiple lines of evidence and highlighting the need for a successful integration of effective analytical techniques. Moreover, the use of rapid-measurement tools, such as Vis-NIRS (Chapter 4), and the implementation of ML models (Chapter 6) can further improve site investigation and assessment by providing real-time information on contaminants fate and behaviour therefore allowing the establishment of realistic and achievable remediation end-points and speeding up decision making. Such findings can be implemented in the current framework (see *7.3* research implication, Figure 7.2) and will lead to a more accurate and rapid assessment of risk, and cost-effective land management.

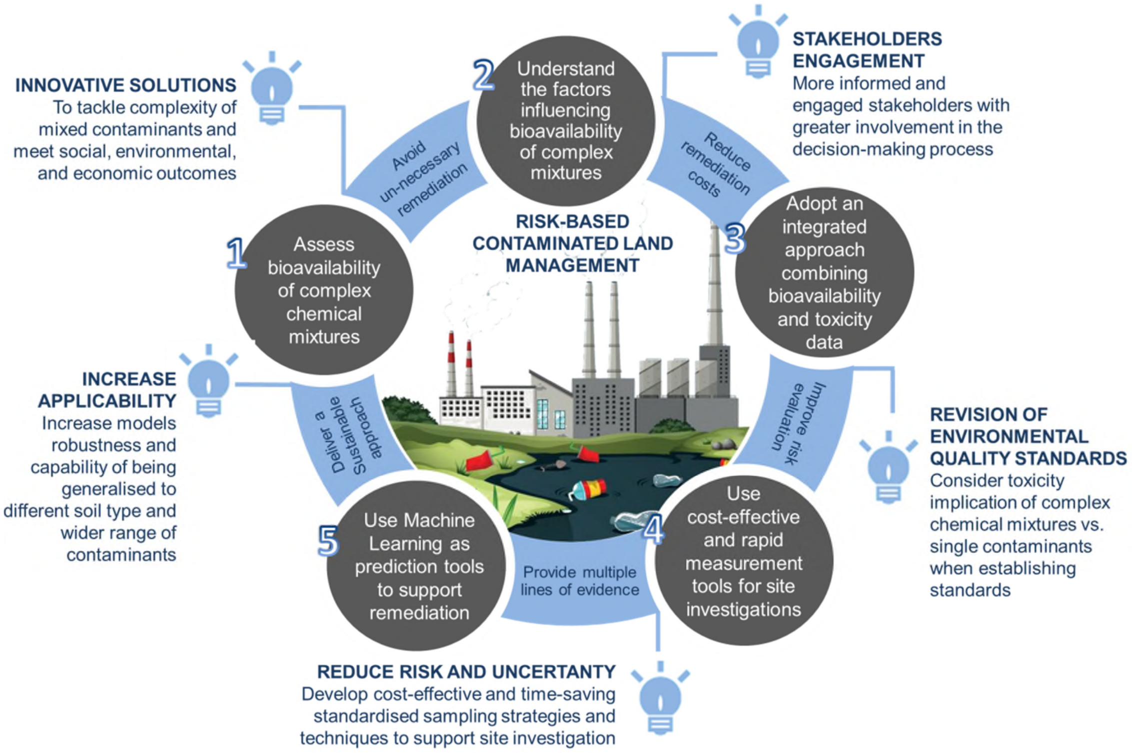


Figure 7.1: Schematic interrelations between the objectives (1 to 5) of the study and overall impact on the land remediation sector.

7.2. Key findings and knowledge gaps filled by this research

As the European Union aims to develop a common framework for the management of contaminated sites (Pérez and Sánchez, 2015), increasing our understanding of complex chemical mixtures behaviour and its implication for bioavailability can provide a better assessment of risk, thus delivering more sustainable remedial solutions (Bardos et al., 2018; Breure et al., 2018).

Chapter 1 identified the research gaps that motivated the aim and objectives of this study, In particular it highlighted the need for: (1) improving understanding of the science underpinning complex chemical mixtures bioavailability; (2) promoting a better implementation of multidisciplinary approaches to contaminated land risk-based management; (3) developing reliable rapid measurement tools for on-site assessment and contaminant’s fate and behaviour modelling.

Based on the critical review of the current literature, **Chapter 2** discusses the challenges of assessing complex chemical mixtures bioavailability and its implementation in the contaminated land risk assessment framework. At present there is no general concept or unified method able to estimate accurately the risk of complex chemical mixtures, and integrate the bioavailability in the risk assessment process (Naidu et al., 2015). While a number of physical, chemical and biological techniques to estimate bioavailable fraction have been developed (Kuppusamy et al., 2017), and successfully applied on case-by-case scenarios (Ortega-Calvo et al., 2015), doubts have been casted on their applicability, due to lack of standardisation (Cipullo et al., 2018). The review highlighted the role of biotic and abiotic factors affecting bioavailability measurament, and how different mechanisms, partitioning, can affect the risk estimation (**Objective 1**). Moreover this review provided an in-depth evaluation of advantages and disadvantages of different extraction techniques. Thus, in order to provide increased confidence for applying bioavailability concepts into risk assessment more information are needed on the influence and interactions of multi-factors on bioavailability and how these affect the toxicity response in ecological receptors.

In **Chapter 3**, we applied a sequential extraction technique to five genuine-multi-contaminated soil samples in order to evaluate the spatial distribution and bioavailability of heavy metals (HM) and metalloids. Metal partitioning was assessed through the non-specific sequential extraction and Chemometric Identification of Substrates and Element Distributions (CISED) using MatLab$^{®}$ (Version R2015a) following the protocol developed by Cave et al. (2004).

Further the study assessed the influence of different physico-chemical factors on HM/metalloids solid phase distribution and bioavailability, and also evaluated the effect of co-occurrence of petroleum hydrocarbons (**Objective 2**). Different temperature conditions were simulated by storing samples in controlled temperature rooms at 20°C and 4°C, or placed outside and subject to seasonal

UK temperature variations. Soil samples were amended with a mixture of sulphuric acid and water to achieve different pH conditions. Moisture content was maintained by adding deionized water up to 20 and 70% of the soils' maximum water holding capacity (WHC) and moisture content was re-assessed bi-monthly.

This 12-month lab-based study showed that while for some of the soils investigated, the pseudo-total metal concentrations of Pb and Zn exceeded 8 times and 4 times the UK Soil Guideline Values (SGVs) and the European Directive 86/278/EE; only a negligible fraction of these elements were dissolved in pore water, confirming that these metals were not readily-available and risk was low. Thus potential of re-using soil that has been treated or remediated is a viable and sustainable strategy (Mehta et al., 2018). Moreover, no significant effects of total petroleum hydrocarbons compounds (PHC) on HM bioavailable fraction were observed for most of the soil samples under investigation. Lastly re-partitioning among soil phases did not occur during the 12-month weathering and under the different physico-chemical treatment applied. HM/metalloids showed little or no difference in distribution across the three sampling events (T0, T6, and T12). The novelty of this study lies in the fact that it highlights the importance of considering the effects of a range of environmental simulated stressors (pH, moisture, and temperature), along with ageing (incubation time) on bioavailability of complex chemical mixtures. This study highlights that sequential extraction can allow site specific assessment criteria providing a better estimate of the HM/metalloids potential bioavailable concentration in multi-contaminated soil.

In **Chapter 4** the empirical data obtained from the 12-month lab-based study (Chapter 3) were used in combination with spectra obtained from visible and near infrared spectroscopy (Vis-NIRS). A number of studies have previously investigated the application of Vis-NIRS to on-site investigations; however assessment of bioavailable concentrations of complex chemical mixtures remains unexplored. In this study we showed that random forest (RF) has a potential to predict the total and bioavailable concentrations of petroleum hydrocarbons compounds (PHC) and heavy metals/metalloids (HM) in genuine multi-contaminated soil samples. The RF models developed could be used for fast screening of PHC, (including polycyclic aromatic hydrocarbons and alkanes), and HM/metalloids concentrations with appreciable accuracy. Results showed that RF model of total concentration outperformed those of bioavailable concentration in cross-validation and prediction.

Results showed that the predictions of the total concentrations of PAH, PHC, and ALK were very good, good and fair, with residual prediction deviation (RPD) values of 2.02, 1.80, and 1.56, respectively; whereas the bioavailable models predictions were of less accuracy of fair (RPD = 1.72 and 1.66), for PAH and PHC, and poor (RPD = 1.31) for ALK. The prediction results of the total HM were also better than those of the bioavailable concentration. The best results were obtained for the total Pb (RPD = 2.35) followed by Al, Ni, Cr, and Cd, whereas the best prediction for bioavailable was for Al (RPD = 2.13) followed by Pb, Cr, Cd, and Ni, whereas the poorest prediction was for As (RPD = 1.37). Most notably a large number of trace elements (Pb, Al, Ni, Cr, Cd, Fe, and Zn) were predicted with very good or good accuracy with RF model. Results obtained indicate that the Vis-NIR spectroscopy coupled with RF algorithm can be a promising approach for screening of complex chemical mixture in genuine-contaminated soil.

In **Chapter 5**, a 6-month laboratory scale study was carried out to investigate the effect of biochar and compost amendments on complex chemical mixtures of tar, heavy metals and metalloids in two genuine contaminated soils. The overall PHC content of Soil 1 was 5 times higher than Soil 2. The GC-MS fingerprint was typical of weathered PHC with a predominance of low to medium chain aliphatic compounds (EC_{16-35}) and low to medium molecular weight aromatic compounds (EC_{16-21}). For each soil, duplicate mesocosms containing 5 kg of soil amended either with 15% w/w compost (Soil + Compost), with 5% w/w biochar (Soil + Biochar), or without amendment (Soil) were prepared. An integrated approach, where complex chemical mixtures bioavailability along with a range of microbiological indicators and ecotoxicological bioassays, were used to provide multiple lines of evidence to support the risk characterisation and assess the remediation end-point.

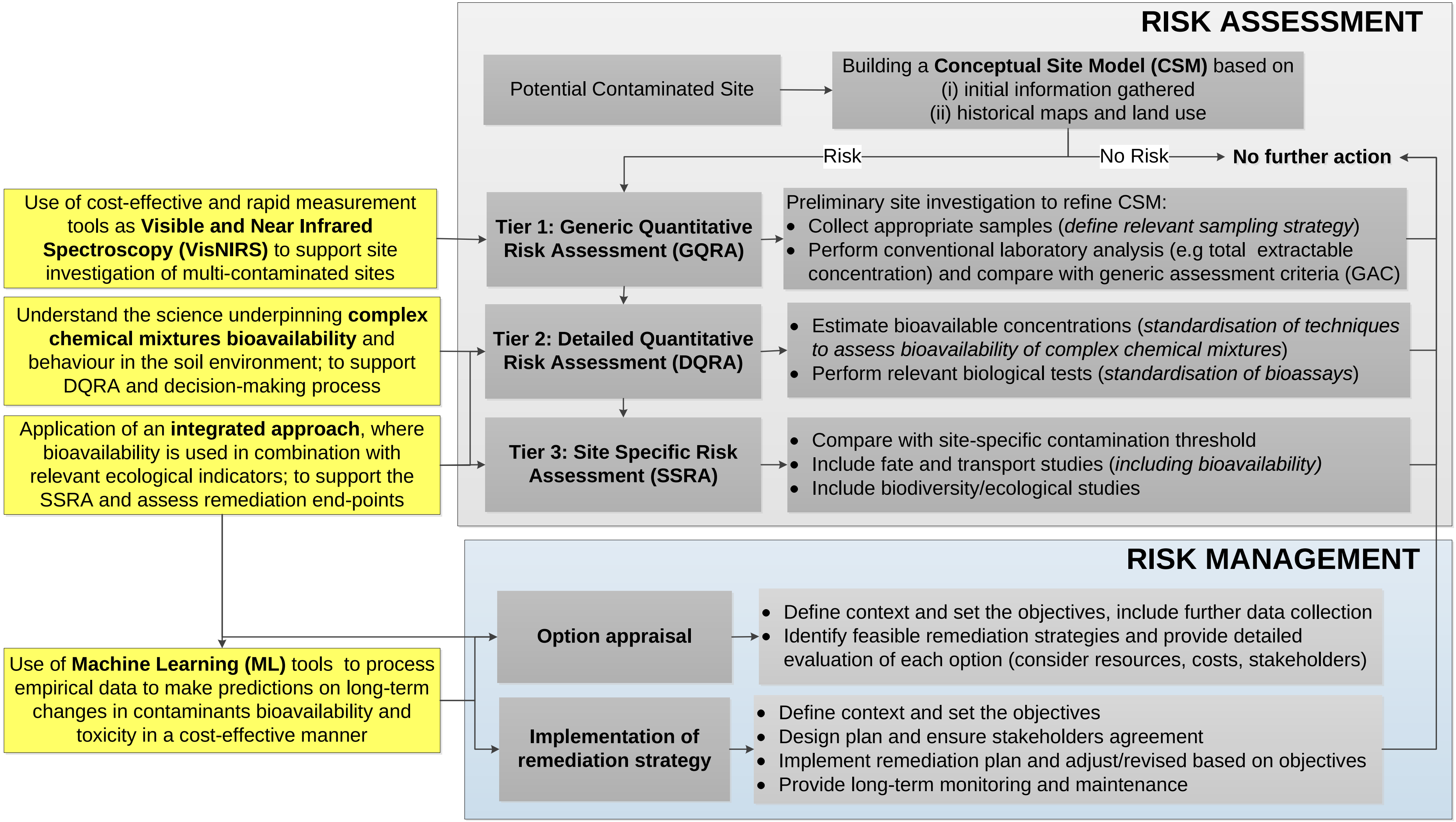
In this lab-based setup, both compost and biochar amendment ($p = 0.005$) as well as incubation time ($p = 0.001$) significantly affected the total and bioavailable concentrations of the petroleum hydrocarbons in the two soils. The total petroleum hydrocarbons (PHC) concentration decreased by 46 and 30% in Soil 1 and Soil 2 amended with compost. This decrease was accompanied by a reduction of 78% (Soil 1) and 68% (Soil 2) of the bioavailable hydrocarbons; where the most significant decrease was observed for low to medium chain aliphatic compounds (EC_{16-35}) and low to medium molecular weight aromatic compounds (EC_{16-21}). HM and metalloids were almost entirely found in the non-exchangeable fraction, with no changes during incubation time, thus posing low risk.

Toxicity to bioassays was reduced due to both the addition of compost, which was effective in enhancing PHC degradation, and the addition of biochar which was able to stabilise contaminants in the soil. Lastly a significant relationship ($p < 0.05$) between the bioavailable fraction of the chemical mixtures and the ecotoxicological bioassays was found. Strong negative correlations were observed between bioavailable/readily available aromatic and aliphatic concentrations and the ecotoxicological assays (e.g. bacteria count, soil respiration, seeds germination, and condition index) in particular in Soil 1 + Compost and Soil 2 + Compost. Results indicates that when bioavailable/readily available concentrations decrease, the toxicity also decrease, thus diversity of microbial community increases along with soil respiration, and other relevant parameters (condition index and EC_{50}). This work suggests that a combined diagnostic approach is fundamental to identify optimal remediation strategies and contribute to change the over-conservative nature of the current risk assessments.

In **Chapter 6** the empirical chemical and toxicological data obtained from the 6-month lab-based study (**Chapter 5**) were used to assess the ability and performance of two machine learning (ML) models, namely artificial neural network (NN) and random forest (RF), to predict temporal bioavailability changes of complex chemical mixtures and toxicity. Parameters such as soil type, amendment (biochar and compost), initial concentration of individual compounds, and incubation time were used as inputs of the ML models. The relative importance of the input variables was also analysed to better understand the drivers of temporal changes in bioavailability and toxicity. Results showed that both ML models were able to model the bioavailability of various contaminants. In particular r^2 values above 0.7 were obtained with NN for the following hydrocarbons EC_{12}, EC_{14}, EC_{16}, EC_{17}, EC_{19}, EC_{21}, EC_{23}, EC_{25}, EC_{26}, AE, F, A, P, PY, C, BA, BaP, BB, BK, BP, DA, IP, P, and the following elements As, Cd, Cr, Cu, Hg, Ni, Se, Pb, and Zn, and therefore bioavailability could be estimated at un-known time steps. Bioavailable data were then used as input to predict toxicity. RF performed slightly better than NN to predict the toxicity, and was particularly good at predicting seed germination, condition index, and EC_{50}. Lower r^2 values were obtained for the PLFA. This chapter highlights the potential of ML tools to reduce costs associated with chemical analysis to support decision making and for remediation monitoring of multi-contaminated sites.

7.3. Research implications

The presented framework relates to an integrated and harmonized approach where the bioavailability of complex chemical mixtures is taken into account in the tiered risk approach adapted from UK CLR11 (Environment Agency, 2004) and ISO 19204:2017 (Figure 7.2). In this work, a number of techniques have been identified and utilised to provide a transparent and scientifically-sound evaluation of contaminated site risk-characterisation and support the establishment of sustainable clean-up objectives.



Building a **Conceptual Site Model (CSM)** based on
(i) initial information gathered
(ii) historical maps and land use

Preliminary site investigation to refine CSM:
- Collect appropriate samples (*define relevant sampling strategy*)
- Perform conventional laboratory analysis (e.g total extractable concentration) and compare with generic assessment criteria (GAC)

- Estimate bioavailable concentrations (*standardisation of techniques to assess bioavailability of complex chemical mixtures*)
- Perform relevant biological tests (*standardisation of bioassays*)

- Compare with site-specific contamination threshold
- Include fate and transport studies (*including bioavailability)*
- Include biodiversity/ecological studies

- Define context and set the objectives, include further data collection
- Identify feasible remediation strategies and provide detailed evaluation of each option (consider resources, costs, stakeholders)

- Define context and set the objectives
- Design plan and ensure stakeholders agreement
- Implement remediation plan and adjust/revised based on objectives
- Provide long-term monitoring and maintenance

Use of cost-effective and rapid measurement tools as **Visible and Near Infrared Spectroscopy (VisNIRS)** to support site investigation of multi-contaminated sites

Understand the science underpinning **complex chemical mixtures bioavailability** and behaviour in the soil environment; to support DQRA and decision-making process

Application of an **integrated approach**, where bioavailability is used in combination with relevant ecological indicators; to support the SSRA and assess remediation end-points

Use of **Machine Learning (ML)** tools to process empirical data to make predictions on long-term changes in contaminants bioavailability and toxicity in a cost-effective manner

Figure 7.2: Proposed framework for including bioavailability in risk assessment based on UK CLR11 (Environment Agency, 2004) and adapted from ISO 19204:2017 and Ortega-Calvo et al. (2015). Yellow boxes represent research contribution to the framework.

In the initial step (Tier 1), a preliminary site investigation is performed to gather information to support the initial risk-evaluation based on the conceptual site model (CSM) (Umeh et al., 2017). While on-site data collection at this stage is critical to address uncertainties, it is often challenging to obtain sufficient and representative samples due to heterogeneity of the soil matrix, the potential presence of un-identified hot-spots, and the limited accessibility to the sampling locations (Ajmone-Marsan and Biasioli, 2010; Liu et al., 2010). Additionally, costs associated with laboratory analytical measurements are often prohibitive for large sites, thus the need to balance between reasonable accuracy and affordable costs implies that often estimates for un-sampled locations are generalised through statistical models (Horta et al., 2015). Such limitations could be overcome by improving the quality and quantity of information obtained at this step of the RA, with the implementation of Vis-NIRS measurements (**Chapter 4**). Vis-NIRS has been previously used successfully to predict petroleum-derived compounds (Okparanma and Mouazen, 2013) and heavy metals (Shi et al., 2014) concentrations in soil. Vis-NIRS has also found application in the discrimination of weathered contaminated soils (Douglas et al., 2018). Further, this technique has the advantage of being relatively cheap, portable, easy, and ready-to-use in the field requiring minimal sample preparation (Conforti et al., 2015). Therefore, data obtained during the preliminary site investigation can speed up the decision-making process and help to prioritise swiftly site/location posing a significant risk during risk-assessment and remediation planning.

Moving along the tiered-approach, after each tier, a decision is made whether risk is still present for the receptors and further investigation is still required. At the initial stage (Tier 1), bulk contaminant concentration (total) in soil is measured and compared with the available environmental quality standards (e.g. Generic Assessment Criteria (GAC) or Soil Screening Levels (SSL)) (Ortega-Calvo et al., 2015). While this approach is currently applied by several European countries such as the Netherlands, UK, Denmark, and Spain (Cachada et al., 2016), it has been recognised that it can underestimate or overestimate risks of complex contaminants (Cipullo et al., 2018). Moreover the comparison of field-data with such guidelines present other limitations, as often the GAC are: (a) based on freshly spiked soils (with higher bioavailability compared with weathered samples), (b) derived for single chemicals or using single-species toxicity data, (c) lacking information on complex mixture effects, (e) un-related to relevant soil properties; thus their application and generalisation may be limited.

In this regards, bioavailability measurements could be included at a higher-tier to provide further information and refine the detailed quantitative risk assessment (DQRA) (Ortega-Calvo et al., 2015). The inclusion of bioavailability of complex chemical mixtures in higher-tier risk evaluation (Figure 7.2, Tier 2) provides an opportunity to develop a more detailed assessment addressing barriers associated with uncertainty, risk perception, and lack of transparency. Further implementations suggested in the TRIAD approach (ISO 19204:2017) include the use, in this tier, of a range of simple ecological assays which can provide information on biological activity of the soils (e.g. microbial biomass, soil respiration, and phospholipid-derived fatty acids (PLFA) profile) (Jensen and Mesman, 2006) (Figure 7.2, Tier 2).

The knowledge provided by the literature review on bioavailability of complex chemical mixtures (**Chapter 2**) and our lab-based studies (**Chapter 3**, and **Chapter 5**) further support the proposed implementation. However, to produce reliable guidelines and to effectively implement bioavailability, it is necessary to standardise and validate the techniques to assess both bioavailability and toxicity (bioassays) (see Paragraph 7.5, further research recommendations).

Following the detailed quantitative risk assessment, if a significant risk is still present, further tests can be performed in Tier 3, to obtain more detailed and case-specific evaluation (Figure 7.2, Tier 3). At this step, in-situ bioassays can be used (Lourenço et al., 2012) including monitoring of biodiversity, and implementing site-specific chemical fate modelling (incorporating bioavailability). The proposed approach (**Chapter 5**) applied multi-disciplinary techniques (linking chemistry with eco-toxicology) developing new intra-disciplinary fields for site investigations and risk assessment.

At the end of the risk assessment evaluation if the risk is deemed unacceptable, then risk management approaches (e.g., remedial actions) are implemented (Figure 7.2, risk management). At this stage, aligning risk assessment with risk management is fundamental in order to maximize benefits (e.g. matching the established clean-up requirement), while at the same time minimizing costs associated with the remediation. It is often the case that a detailed evaluation of feasible remedial options is performed prior the application of field-scale treatments. Thus, lab-based study or on-site mesocosms are often used to establish efficacy and the approximate time-scale of the selected technology (Kuppusamy et al., 2017). At this stage the use of Machine Learning tools (ML) can be fundamental to predict the results of bioavailability and consequently toxicity in order to define the most appropriate remedial action for a particular contaminated site. ML techniques can learn and recognise the patterns among empirical data (Mitchell, 1997) during ‘training’, and applying it (generalisation) to un-known (independent) data sets. As highlighted in **Chapter 6** there is potential to capture the non-linear relationship between multiple variables and forecast complex contaminants’ bioavailability changes. However, for use and implementation of ML models in the contaminated land community, further work is required to make these models more user-friendly and easy to generalise for different soil type and different applications (see 7.5 further research recommendations).

7.4. Limitations of the research

- The soil samples obtained for the first experimental study (**Chapter 3**) were provided anonymously from a treatment facility and no further information on the geology, location or origins of the contamination were provided; thus limiting our understanding of the effects of geogenic or (anthropogenic) contribution on HM/metalloids bioavailability. Additionally no information were available on the type of cement stabiliser used in Soil 2 (post-treatment), therefore it was not possible to draw further conclusion on the mechanism dominating the fixation of HM/metalloids for this particular soil sample.
- The data sets used in the calibration and prediction, obtained from the 12-month lab-based study and Vis-NIR spectra (**Chapter 4**) were relatively small (~100 samples); thus it might have affected the prediction performance of the developed models.
- The results of correlation between bioavailable fraction and toxicity response (**Chapter 5**) were based on a single study where only a small number of substances have been considered. It should therefore be treated with a degree of caution; as any apparent observed toxicity could be also caused by other substances, or degradation by-products that have not been measured in our study, or by compounds that occurred below the analytical detection limit.
- The two ML models used in this study (**Chapter 6**) were trained using site-specific data (e.g. soil type). Therefore, these models can be applied with confidence only for the range of the values of the inputs which are captured by the training dataset.

General comments on limitations associated with modelling:

- Despite the large amount of information that sequential extraction can provide (**Chapter 3**), the method of Chemometric Identification of Substrates and Element Distributions (CISED) requires considerable amount of multiple expertise (analytical chemistry, geochemistry, data analysis and modelling). In addition the significant amount of personal judgment required in order to make assumptions about metal-soil phase association, and the case-by-case approach are the main factor potentially limiting its standardisation and applicability in the framework.

- In order to make predictions with ML models on total and bioavailable multi-contaminants concentrations (**Chapter 4** and **Chapter 6**), it is necessary to explore correlation among input/output data: (1) chemical data and soil reflectance spectra obtained with Vis–NIR reflectance spectroscopy, or (2) chemical data and remediation treatment applied (biochar, compost). In both cases the application of chemometrics techniques and multivariate analysis require complex mathematical analysis, such as principal components analysis (PCA), artificial neural networks (NN), and random forest (RF), which require expert and highly-qualified operators.

7.5. Further research recommendations

While methodologies for assessing bioavailability and the combination effects of chemicals are being developed and used by scientists and regulators in specific circumstances, as yet there is no systematic, comprehensive and integrated approach. Future work should focus on:

- Defining relevant and standardised sampling strategies, and providing field-measurement tools to support on-site investigation producing more reliable and accurate information, thus reducing uncertainties.
- Providing convincing evidence that contaminants left behind in the soil do not pose a risk (low bioavailability = low harm), through establishment of standardised techniques (in vivo and in vitro methods) to assess bioavailability of complex mixtures.
- Investigating a methodology to assess bioavailability of petroleum hydrocarbon degradation by-products (metabolites) generated during remediation, and their potential toxic effect to receptors.

- Considering toxicity implications of complex chemical mixtures vs. single contaminants for the estimation and application of revised environmental quality standards.
- Promoting better and more coordinated effort to implement multidisciplinary approach to contaminated land risk-based management; where different lines of evidence (chemistry, toxicology, and ecology) are combined rather than developed independently.
- Increasing ML models robustness and capability of being generalised, using larger data sets (increase sample number), and wider soil variability (different soil type) and concentration ranges.

7.6. References

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