



## Bioengineering remediation of former industrial sites contaminated with chemical mixtures

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### ABSTRACT

Former gasworks sites are known to be contaminated with complex chemical mixtures that require remediation before redevelopment. Bioamendments such as biochar and spent mushroom compost (SMC) offer a green and sustainable remediation approach to help tackle this issue. However, the effectiveness of different biochar types and their interactions with the soil microbial community is still not well understood. To address this, a full factorial microcosm experiment was carried out using biochar derived from rice husk (RHB) and wheat straw (WSB) mixed with soil from a former gasworks site at varying concentrations (0%, 2.5%, and 5%), with and without SMC. The experiment aimed to evaluate the fate of contaminants including alkanes, PAHs, and metals, and their effect on the soil microbial community, as well as the implications for remediation endpoints. The results showed that the bioamendments had an average TPH reduction of 92%, with SMC and WSB-SMC having the highest degradation rates at 93%. While the bioamendments did not significantly affect the extent of TPH removal compared to the control, they did improve the degradation of high molecular weight (HMW) PAHs, particularly in RHB-SMC for EC17-20 (60%) and EC21-35 (62%) of total PAH concentration, and in WSB-SMC for HMW bioavailable PAH concentration (89%). The bioamendments also affected the partitioning and distribution of metals after 120 days of treatment, leading to decreased available phase fractions. The treatments increased microbial abundance in the soil, with Gram positives, Gram negatives, and fungi increasing by 4%, 8%, and 38%, respectively, after 120 days, particularly in SMC and mixed treatments. This was mirrored in increased microbial soil respiration. After 120 days, low metal ( $178 \pm 5$  mg/kg) and TPH ( $21 \pm 7$  mg/kg) bioavailability translated into higher  $EC_{50}$  ( $10624 \pm 710$  mg/L), indicating lower toxicity. There was a strong correlation between bioavailability and toxicity of TPH and metals with microbial relative abundance and activity. In summary, while green and sustainable remediation may accelerate the remediation process, monitored natural attenuation may be sufficient for site reclamation. However, this strategy, as demonstrated here, can reduce metal bioavailability, and promote the biodegradation of HMW PAHs.

### 1. Introduction

Former industrial sites are often contaminated with complex chemical mixtures, mainly hydrocarbon-derived products, polycyclic aromatic hydrocarbons (PAHs), metals and metalloids such as lead, chromium, arsenic, zinc, cadmium, copper, mercury, and nickel (CL:AIRE, 2015; Zhao et al., 2022). Co-contamination of metals and PAHs increases toxicity in the environment and may make remediation of polluted soil more difficult (Li et al., 2020). A high metal content in soil, for example, was reported to inhibit PAH degradation (Obuekwe & Semple, 2013). The most typical technique of remediating former gasworks sites has been excavation and transport to prescribed hazardous land-fill locations, which has recently become less desirable due to rising

costs among other disadvantages (Baylis & Allenby, 2010; Haleyr et al., 2018). The use of bioremediation has been proposed as a cost-effective and ecologically beneficial solution, even though the approach has shown less efficiency when co-contamination and high concentration of contaminants are present (Zhang et al., 2020). Organic residues like biowaste and composts are being used more and more in land remediation since they can enhance the physical, chemical, and biochemical properties of soil and reduce the need for inorganic fertilisation (Alvarenga et al., 2009). Additionally, their use supports an integrated approach to waste management by encouraging nutrient recycling and reducing issues with final disposal (Wang et al., 2021). Consequently, biochar and spent mushroom compost (SMC) are two examples of organic residue products that can improve biodegradation of chemical pol-

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lutants. And this could be a useful way to improve the bioremediation process.

Biochar, a low-cost carbon material, is emerging as a cost-effective alternative to activated carbon in the removal of organic and inorganic contaminants from the environment (Ahmad et al., 2014). Biochar has several remarkable properties, such as a high internal specific surface area, microporosity, surface negative charge, and durability against degradation, and has been used in a variety of applications (Guo et al., 2015). For instance, in environmental remediation, where biochar can promote high contaminant complexation, immobilisation, sorption and partitioning, as well as high carbon sequestration (Oliveira et al., 2017). Although the application of biochar has been demonstrated to be conceptually and experimentally successful, its success is dependent on the type of biomass feedstock material, carbonisation process, pyrolysis conditions, and biochar dose (Bian et al., 2013; Hassan et al., 2020). The availability of low-cost, easily grown biochar biomass feedstock would increase biochar's sustainability and efficiency in remediation. Also, combining biochar with other low-cost adsorbents or degradation materials/organisms may also improve remediation performance (Ane et al., 2021). Spent mushroom compost is an important material that, when combined with biochar, may help in the remediation process.

Spent mushroom compost (SMC) is a by-product of mushroom production that is generated in large quantities and has also been reported to enhance the bioremediation of polluted soils (Asemoloye et al., 2020). It has also been reported to serve as soil conditioner, thereby improving soil nutrient (Cai et al., 2021). Furthermore, SMC can bind and immobilise metals in soils, reducing their toxicity to the soil microbial community and plants (Wei et al., 2020). It has these effects because, after mushroom harvesting, SMC is likely to contain not only a large and diverse group of microorganisms, but also a diverse range of extracellular enzymes, such as cellulase, hemicellulose,  $\beta$ -glucosidase, lignin peroxidases, and laccase (Chang et al., 2021), all of which help in breaking down and transformation of the contaminants. Additionally, it has a high organic content (Gouma et al., 2014), which is another contribution to its effectiveness in remediation. Equally, for every 1 kg of edible mushrooms produced, 5 kg of SMC is generated (Chang et al., 2021). This gives rise to millions of tonnes of SMC generated over time whose disposal becomes a major problem for mushroom farmers. Hence, since SMC is considered a good biologically reactive material and has great potential for bioremediation of many toxic chemical contaminants, there is need for this alternative use (Sadiq et al., 2018).

The recent global environmental consciousness, stringent legislation, and a shift in research toward the application of sustainable and circular processes has led to the scientific community's interest in innovative and environmentally friendly waste-stream utilisation systems (Ferronato & Torretta, 2019). Therefore, the use of both materials (biochar and SMC) enables a sustainable remediation technique that makes use of industrial and agricultural wastes, leading to a large decrease in environmental footprint (Hu et al., 2021). Also as biochar is made from carbonaceous waste biomass and mushroom production is the world's largest solid-state fermentation industry (Letti et al., 2018) which releases large amount of SMC as waste, the application of these materials for PAHs and metal (loids) bioremediation could be regarded as an efficient low-cost bioremediation method. Furthermore, due to the properties of these materials elucidated here, their use provides an opportunity to overcome soil nutrient limitation, increase sorption/decrease bioavailability of the chemicals, and increase surface contact of contaminants with the soil microbial community (Zhu et al., 2017a), all of which have implications for improving soil microbial remediation of the hydrocarbons and metals. For example, biochar has been used alone (Carlini et al., 2023; Cipullo et al., 2019; Jia et al., 2023), bioaugmented with bacteria consortia (Guirado et al., 2021; Wang et al., 2018; Wei et al., 2021), or with bacteria immobilized on the biochar (Guo, Liu, & Tang, 2022; Zhou et al., 2023). Studies have shown that biochar can effectively

degrade both low- and high-molecular-weight polycyclic aromatic hydrocarbons (PAHs) (Kong, Song, Zhang, Gao, & Liu, 2021; Zhou et al., 2023). Additionally, biochar has been activated to form a composite material such Fe<sub>3</sub>O<sub>4</sub>-BB for improved remediation (Dong, Chen, & Hung, 2017). SMC has also been found to be effective for contaminants removal (Asemoloye, Jonathan, Jayeola, & Ahmad, 2017; Sun et al., 2021; Udume et al., 2023), with *Agaricus bisporus* SMC demonstrated particularly high (72 %) degradation ability (Mohammadi-Sichani et al., 2019).

However, while biochar can induce changes in soil microbial activities that lead to contaminants transformations, the mechanisms underlying these processes are still not yet fully understood (Zhang et al., 2019). As a result, understanding biochar-microbe interactions is essential for recognising the link between biochar characteristics and a variety of soil processes, particularly contaminant degradation (Yuan et al., 2019; Zhu et al., 2017b). Also, as previously stated, because biochar success among other factors is dependent on the type of biomass feedstock material and application dose, it is important to compare biochar from different feedstock and different application rates. In the context of using biochar and SMC for bioremediation, it is critical to evaluate the bioavailable fraction of pollutants, which is the fraction that has been reported to be able to permeate organisms cellular membranes and cause toxicological impacts (Cipullo et al., 2019; Yuan et al., 2019). Similarly, while there have been studies on biochar with compost or bacteria sources, few have examined the use of biochar with a fungal bio-addition material, such as SMC, to study the fate and behaviour of mixed contaminants in bioremediation. As a result, the objective of this study is to examine how biochar and/or spent mushroom compost affect the fate and behaviour of hydrocarbons and metals(loids), promote biodegradation of hydrocarbons, and how the amendments affect the soil microbial community, as well as draw implications for remediation endpoint.

## 2. Materials and methods

### 2.1. Sample collection: soil, biochar and spent mushroom compost

The soil was collected from a former gasworks site based in the UK. The site's background: Until 1896, the location was known as an "Old Freestone Quarry." Following the construction of the "Riverbank Gasworks" in May 1904, coal gas production commenced at the location. Between 1910 and 1938, various expansions were carried out. The gas works ceased in 1961, and the site was deemed suitable for conversion into a high-pressure gas reforming plant in 1962. Gas production began in 1966, with facilities being expanded in 1967. The factory discontinued operations in 1973. The soil samples were collected when the site was undergoing a supplementary investigation to further assess the significance of historic residual Non aqueous phase liquid (NAPL) impacts in some areas of the site. Soil samples were taken from three trial pits sunk between 1.0 and 2.0 metres below ground level. The soil was sieved with 5.60 mm in the lab to remove large particles and stones. It was then sieved through a 2 mm sieve and kept at 4°C until soil characterisation analyses and the setup of microcosms.

Rice Husk Biochar and Wheat Straw Biochar derived from rice husk and wheat straw pellets, both produced by the UK Biochar Research Centre, School of Geosciences, University of Edinburgh, were used in this study. The biochars production was done in a pilot-scale rotary kiln pyrolysis unit with a nominal peak temperature of 550°C, a pH of 9.94, and a total carbon content of 68.3 wt %. Both are biochars that have been thoroughly characterised (UK Biochar Research Centre, 2014)

Littleport Mushrooms LLP, which is owned by G's Fresh Ltd, UK, provided spent mushroom compost and the major basidiomycete present was *Agaricus bisporus*, also known as the cultivated white button mushroom.

**Table 1**  
Overview of the soil microcosm bioamendments conditions

Experiment Design	Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5
	150 g soil				150 g soil	150 g soil
	No Rice husk biochar (RHB)				Amended with 2.5 %	Amended with 2.5 %
	No Wheat straw biochar (WSB)	150 g soil	150 g soil	150 g soil	Rice husk biochar (RHB)	Wheat straw biochar (WSB)
	No Spent mushroom compost (SMC)	Amended with 5 %	Amended with 5 %	Amended with 5 %	Amended with 2.5 %	Amended with 2.5 %
		Rice husk biochar (RHB)	Wheat straw biochar (WSB)	Spent mushroom compost (SMC)	Spent mushroom compost (SMC)	Spent mushroom compost (SMC)
((1 Control + 5 treatments) = 6) × 3 replicates × 2 Sampling time = 6 × 3 × 2 = 36 microcosms						

## 2.2. Microcosms experimental design

Microcosms were set up in 10cm X 8cm plant pots (sealed bottom) with the sieved stored soil, each containing 150 g of soil. Soils were amended with either rice husk biochar, wheat straw biochar, or spent mushroom compost at 2.5 % or 5 % (Table 1).

The 5 % biochar to soil ratio used in this work was chosen because it is frequently reported as the most effective application rate for reducing mobile contaminant concentrations in contaminated soils (Cipullo et al., 2019; Novak et al., 2018; Wang et al., 2017). All the microcosms were mixed manually to obtain homogenous samples and kept in 20 °C constant temperature room for the 120 days of the experiment. The soil moisture was adjusted twice a week by adding deionised water equivalent of the microcosms' weight loss within the range of the soil moisture content (29 %). Samples were taken for chemical, microbiological, and toxicological analyses after 60 and 120 days to determine the effect of the treatment variables on the fate of contaminants present in the oil contaminated soils (PAH and metal(loid)s), the influence on the soil microbial community and its implication for remediation end points.

## 2.3. Physico-chemical characterisation

Air-dried soil samples were analysed based on BS EN 13654-2:2001. Total nitrogen (TN) (0.001 mg), Total Carbon (TC) (0.001 mg) and Total Organic Carbon (TOC) (following the removal of carbonates with 4 mol/L hydrochloric acid dropwise until visible reaction stops) were based on BS 7755 Section 3.8:1995. They were analysed using a vario Element Analyzer EL 3 (Elementar Analysensysteme GmbH, DE). Total phosphorous was determined by extracting with acid mixture (6ml 11.65mol/L hydrochloric and 2ml 15.8mol/L nitric) and determining the phosphorus content of the extract (ISO 11047, 1998) using a NexION® 350 D ICP-MS (Perkin Elmer). A 0.5 mol/L sodium hydrogen carbonate solution at pH 8.5 was used to extract available phosphorous (AP) (5 g) from the soil. The extract was then analysed using spectrometre (ISO 11263, 1994). Soil pH was determined according to ISO 10390 (2005) using a soil:water ratio of 1:5 (Jenway 3540 pH Meter, Keison Products, UK). The organic content of the soil (%) was calculated using loss of ignition (LOI): (BS EN 13039, 2000). Based on BS ISO 11277:2009, the particle size distribution was determined using the sieve and sedimentation method, and the associated soil texture classes were identified using a soil texture calculator (Natural England Technical Information Note TIN037, 2008) and eventual sieving using 0.6mm, 0.212mm, 0.063mm sieves. Gravimetric soil moisture and dry matter (%) was determined by drying at 105 °C (ISO 11465, 1993).

## 2.4. Chemical analyses

### 2.4.1. Total and bioavailable hydrocarbons

A modified version of Risdon et al. (2008) was used to determine the total and bioavailable petroleum hydrocarbon, which included both aliphatic and aromatic compounds. A 2.5 g of soil were mixed with 15 mL of 1:1 dichloromethane:hexane solvent, and 50 mL of 50 mM

hydroxypropyl- $\beta$ -cyclodextrin solution, respectively, to extract the total and bioavailable petroleum hydrocarbon content.

Total hydrocarbon content was determined by sonicating the samples (20 minutes) at room temperature (Ultrasonic Bath, U2500H, Ultrawave (UW), UK), shaken for 16 hours at 150 rpm (Multi Reax Shaker, Heidolph Instruments GmbH & CO. KG). On the second day, samples were sonicated for 20 minutes at room temperature before being centrifuged (2000g for 10 minutes) (Thermo Scientific™, Sorvall™ ST 40 Centrifuge Series). Following that, the supernatant was transferred to 6 mL SPE DSC-Si silica tubes for cleaning. A 0.5 mL sample of clean extract was combined with 0.5 mL of internal standards, including a deuterated alkane mix (C10<sup>d22</sup>, C19<sup>d40</sup> and C30<sup>d62</sup>) and deuterated PAH mix (naphthalene<sup>d8</sup>, anthracene<sup>d10</sup>, chrysene<sup>d12</sup> and perylene<sup>d12</sup>).

Samples were mixed with a cyclodextrin:water solution to determine the bioavailable hydrocarbon content. After 20 hours of shaking, the sample was centrifuged at 2000 g for 30 minutes. The supernatant was discarded, and the soil pellets were resuspended in a 1:1 dichloromethane:hexane solution and processed as described in the total hydrocarbon section above. After the initial cyclodextrin wash, the amount of organic component extracted by dichloromethane:hexane was subtracted from the total amount extracted by dichloromethane:hexane. Concentration of petroleum hydrocarbons present in the sample were detected and quantified by gas chromatography–mass spectrometry (GC–MS) using the Shimadzu GCMS-TQ8040 following the GC method described in Cipullo et al. (2019),

### 2.4.2. Total and bioavailable metals

Total metal digestion was carried out using aqua regia and the ISO 11047 method (ISO 11047, 1998). In brief, 0.5 g of air-dried soil was extracted in a microwave digestion system by adding 6 ml of hydrochloric acid (11.65 mol/L) and 2 ml of nitric acid (15.8 mol/L) (Multiwave 3000 microwave oven, Anton Paar/Perkin Elmer, UK). After filtering through Whatman 542, the extract was diluted to 50 mL with deionized water.

For the determination of the bioavailable fractions of the metals, a single solvent extraction involving water soluble using: water; exchangeable: 0.01M CaCl<sub>2</sub>; organically complexed: 0.05 M EDTA; and acid extractible: 0.43 M HNO<sub>3</sub> (Gupta & Sinha, 2007; ISO 17586:2016(E), 2016; Neel et al., 2007; Ure et al., 1995). In brief, the extracting vessel and contents were shaken at 150 rpm (Multi Reax Shaker, Heidolph Instruments GmbH & CO. KG) for 4 h and centrifuged at 2000 g for 10 min (Thermo Scientific™, Sorvall™ ST 40 Centrifuge Series). After that, the extract was filtered through 0.45 m nylon syringe filters.

All total and single solvent extracts were diluted four times with 1 % HNO<sub>3</sub> before analysis with a NexION® 350D ICP-MS (Perkin Elmer) calibrated with a mixture of 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. Working standards in matching sample matrix solutions (1 % nitric acid) were created in both cases. A mixture of four internal standards was used to spike the calibration standards and sample extracts (Sc, Ge, Rh, and Bi). After each sample, the ICP-MS was calibrated, and the limit of detection was set at three times the variance of the acid

**Table 2**  
Physiochemical characteristics of the contaminated soil sample collected from former gasworks site in UK.

Characteristics	Analysis	Soil
Elements	Total C (%)	13.62
	Total N (%)	0.15
	Total P (%)	0.07
	C:N:P	100:1.1:0.5
Physical properties	Total P (mg/kg)	718
	Available phosphorus (mg/kg)	5.39
	Dry matter content (%)	77.72
	Water content (%)	28.67
	Water potential (MPa)	0.86
Particle size distribution	% 0.6-2 mm (coarse sand)	37.02
	% 0.2-0.6 mm (medium sand)	18.20
	% 0.06-0.2 mm (fine sand)	11.66
	% 0.002-0.06 mm (silt)	21.59
	% <0.002 mm (clay)	11.54
Chemical properties	pH	8.19
	Loss on ignition (%)	11.14
Average heavy metal(oids) (mg/kg) <sup>a</sup>	Cr	40.4±2.7
	Ni	136.0±5.9
	Cu	107.0±3.2
	Zn	248.5±1.4
	As	18.1±0.5
	Se	7.0±2.0
	Cd	0.1±0.0
	Pb	78.2±7.2
	Hg	7.5±7.5
	Average petroleum hydrocarbon content (mg/kg) <sup>b</sup>	EC10-12
EC12-16		111.48
EC16-21		155.76
EC21-35		73.14
EC>35		1.56
<b>Σ Aliphatic</b>		<b>409.98</b>
EC10-12		15.24
EC12-16		186.12
EC16-21		710.82
EC21-35		171.18
<b>Σ Aromatic</b>		<b>1083.36</b>
TPH		1493.34

<sup>a</sup> These are the average of duplicate measurement ± standard deviation of the pseudo-total concentration (Aqua regia extraction)

<sup>b</sup> There is no replication and so no standard deviation not available. C: carbon, N: nitrogen, P: phosphorus, Cr: chromium, Ni: nickel, Cu: copper, Zn: zinc, As: arsenic, Se: selenium, Cd: cadmium, Pb: lead, Hg: mercury, EC: equivalent carbon number, TPH: total petroleum hydrocarbon

blank. In each batch of seven samples, acid blanks (1 % nitric acid), digestion blanks, and guidance materials (BGS102) were also analysed. A sufficient rinse time was programmed in between samples to assess the accuracy of the extraction and the sensitivity and contamination of the blanks.

## 2.5. Microbiological analysis

### 2.5.1. Respiration

MicroResp™ colorimetric microplate-based respiration system for measuring CO<sub>2</sub> evolved from soil which water or carbon substrates have been added is based on Campbell *et al.* (2003). The method gives responses to these substrates and reflects activity by measuring responses (CO<sub>2</sub> production) after 6 hours. Briefly: The detection plates – microplate plates with purified agar and indicator solution (cresol red, KCl, NaHCO<sub>3</sub>) are added in a 1:2 ratio – were prepared and stored in sealed desiccator prior to use to avoid absorbing CO<sub>2</sub> from the environment. In the deepwell plates, 0.32 g of soil samples and 93.6 mg/ml substrates solution were added into it. Detection plate were read at 570 nm (Microplate readers, SpectraMax® Plus384, Molecular Devices) and assembled onto the deepwell plate with the MicroResp™ seal, secured in metal clamp and incubated at 25°C for 6 hours and re-reading the detection plate at 570 nm. Substrates (alanine, citric acid, glucose, gamma-aminobutyric acid, α-

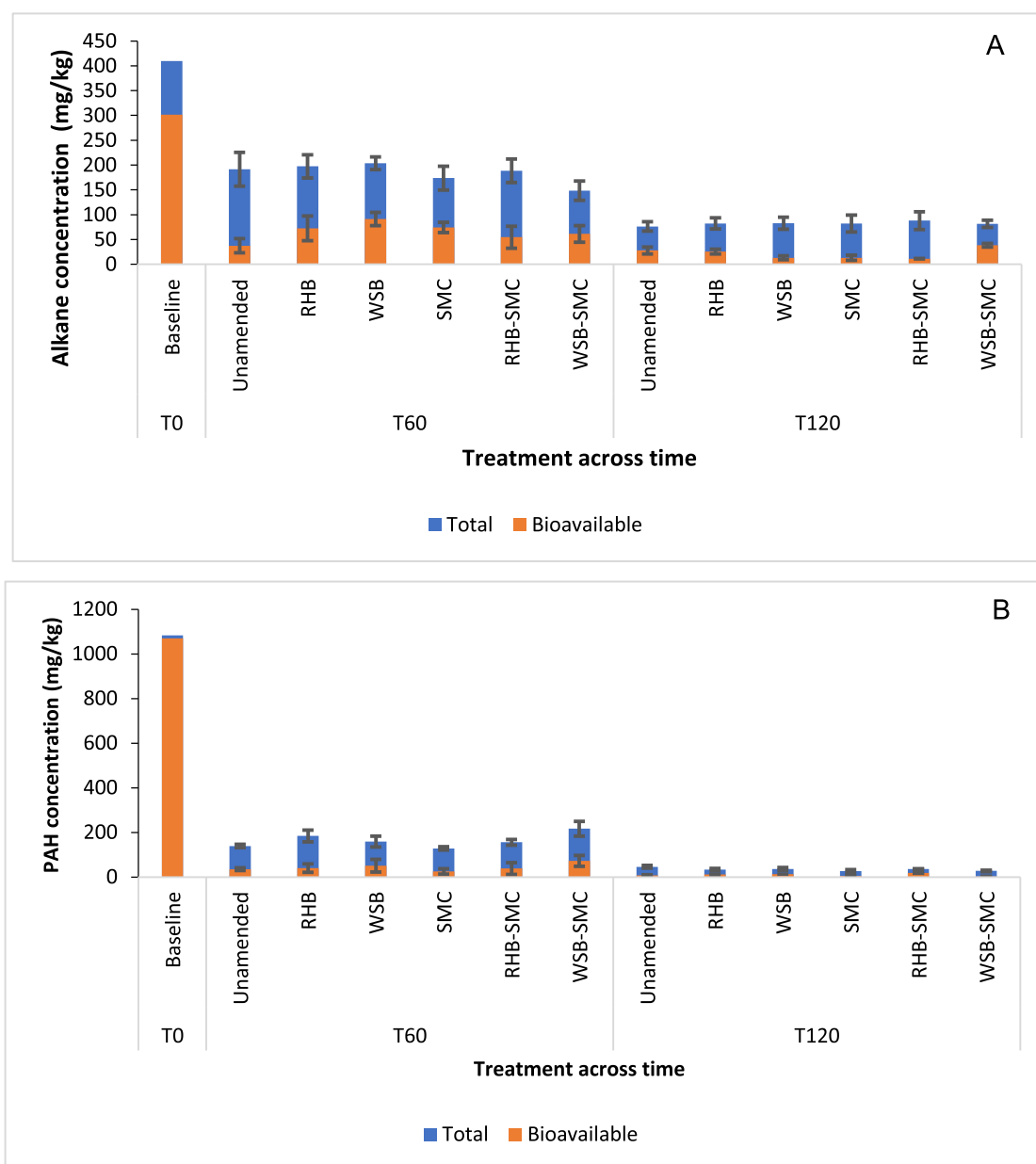
ketoglutaric acid, malic acid) were selected considering Creamer *et al.* (2016); lignin was added as a complex carbon source based on availability in the lab. The basal respiration rate was calculated using the CO<sub>2</sub> generated by the wells in which water other than substrates were added.

### 2.5.2. Phospholipid fatty acid analysis (PLFA)

Using Phospholipid fatty acid (PLFA) analysis based on Frostegard, Tunlid and Baath (1993), the microbial community structure was examined. In brief, from the freeze dried (Christ Alpha 1–2 LD plus –80 °C Freeze Dryer) soil samples, solid-phase soil extraction using 10 g of each sample was performed using Bligh and Dyer solution (chloroform, methanol, and citrate buffer in 1:2:0.8 by volume). The extract was further derivatised by mild alkaline methanolysis. By using a GC-FID (Agilent Technologies 6890N) equipped with an HP-5 (Agilent Technologies) fused silica capillary column (30 m length, 0.32 mm ID, 0.25 μm film), fatty acid methyl esters were analysed. GC conditions were as described by Pawlett *et al.* (2013). The target responses of all discovered PLFA peaks were sum up to determine the relative abundance of each unique PLFA, which was reported as a percentage (mol %).

### 2.5.3. Ecotoxicological bioassay

Soil toxicity of biotreatments was evaluated using the Solid Phase Microtox® assay (Modern Water Monitoring Ltd). The assay was car-



**Fig. 1.** Total and bioavailable aliphatic and aromatic hydrocarbons fractions change overtime. A: Aliphatic (alkanes); B: Aromatic (PAH). The error bars represent the standard error for each treatment's replicates.

ried out as directed by the manufacturer (ModernWater Microtox Acute Toxicity). Briefly, 3.5 g soil was transferred into the diluent, shaken for 10 minutes (Multi Reax Shaker, Heidolph Instruments GmbH & CO. KG), centrifuged for 3 minutes at 1000 g (Thermo Scientific™, Sorvall™ ST 40 Centrifuge Series), and the sample was transferred into SPT tubes in the incubator block (Microtox® Model 500 (M500) analyser) and serial dilutions made. The tubes were read after the Microtox Acute Toxicity Reagent was reconstituted and added. The performance of both the operator and the analytical system was checked using a 100 g/L zinc sulphate standard solution, and the 95 percent confidence range was kept below 15 % variance throughout the investigation. For each sample, the soil dilution that inhibits 50 % ( $EC_{50}$ ) of the light output compared to the light output before soil addition was computed. As toxicity increases, Microtox®  $EC_{50}$  values decrease.

## 2.6. Data analysis

The significance and relationship between soil amendments (rice husk biochar (RHB), wheat straw biochar (WSB), spent mushroom compost (SMC), RHB+SMC, WSB+SMC, or un-amended) and incubation time on the alkanes, PAHs, metals, and microbial PLFA profiles triplicate datasets, were investigated using Repeated-measures ANOVA test. Principal component analysis was used for multi-variate datasets, to evaluate the variations between soil amendment and incubation time on microbial community dynamics and respiration profiles from multiple substrates induced respiration. Both Repeated-measures ANOVA and PCA were performed using Statistica (TIBCO Statistica® 13.3 June 2017).

Pearson correlation in SPSS (IBM SPSS Statistics for Windows, Version 21.0. Apr 2019) was used to establish correlation between the bioavailable fractions and the toxicity response of the Microtox®, PLFA profile and microbial soil activity dataset.



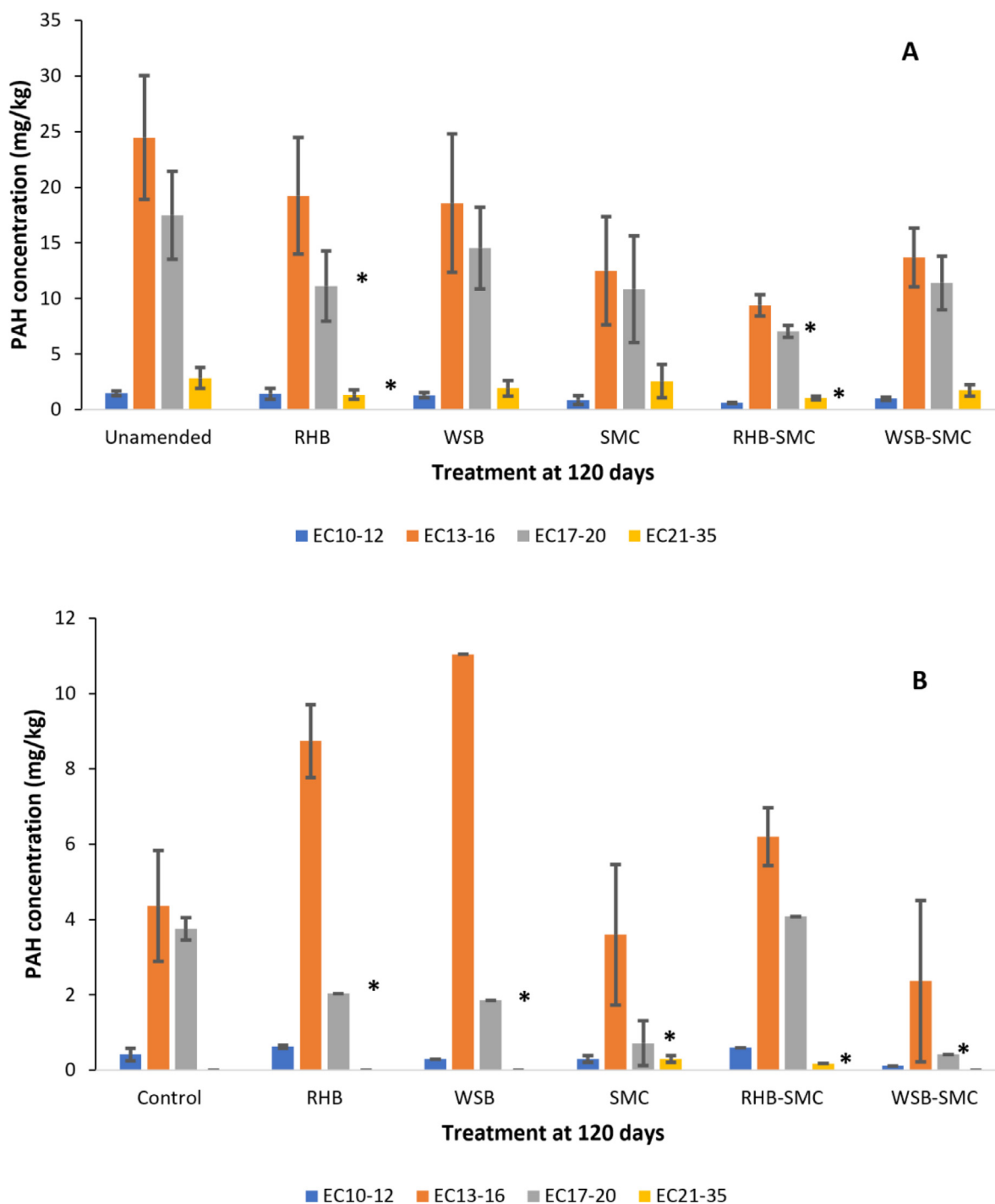


Fig. 2. Total and bioavailable aromatic (PAH) fractions at 120 days based on equivalent carbon (EC) number. A: Total; B: Bioavailable. The error bars represent the standard error for each treatment's replicates. \*Significant difference between the amendment and the control (unamended) at  $p < 0.05$

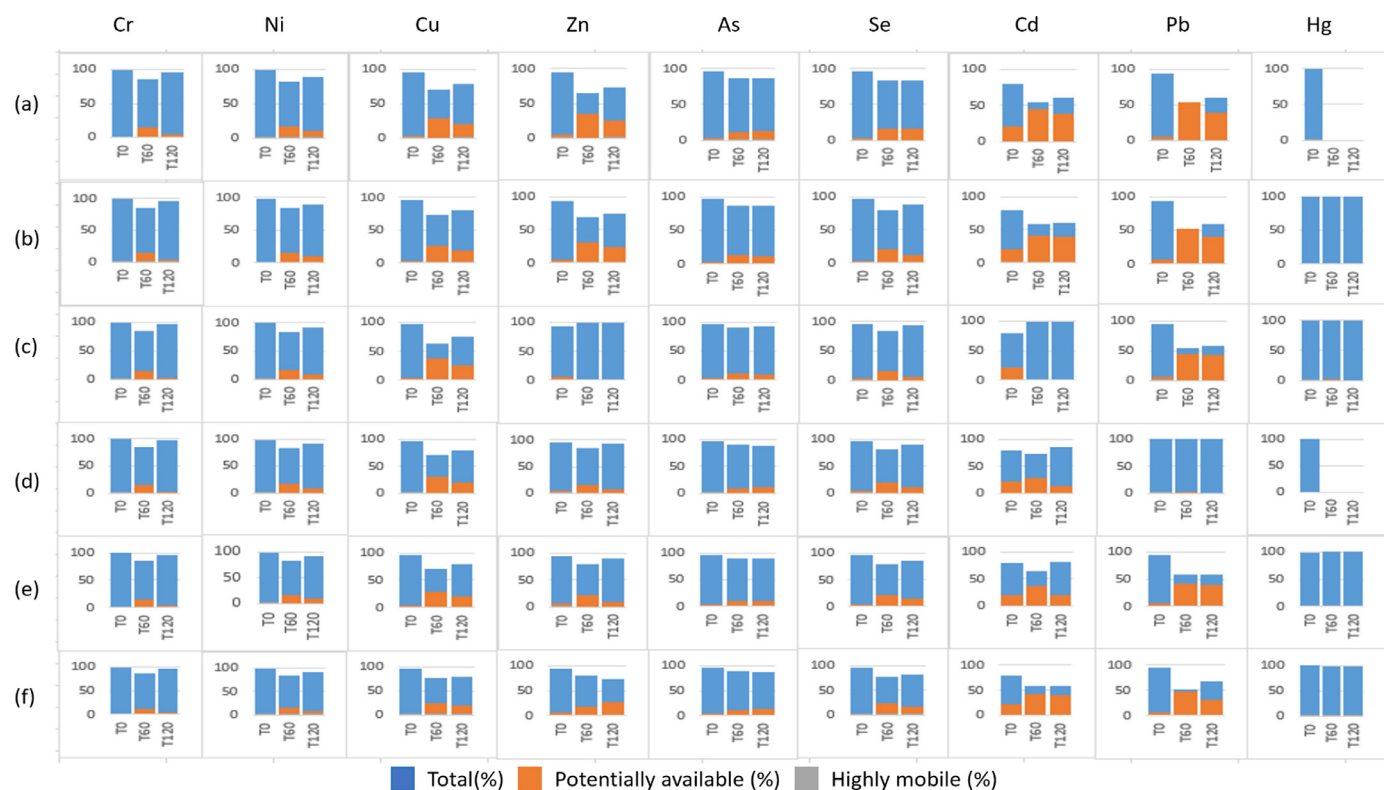
### 3. Results and discussion

#### 3.1. Soil sample and physicochemical properties

Total petroleum hydrocarbons (alkanes and PAHs) concentration was 1493 mg/kg at the onset and PAH accounting for 72 % of the TPH. The total concentration of metal(loid)s was 642.8 mg/kg. The aliphatic fraction was characterised by the dominance of lower molecular weight fractions while the aromatic fraction was dominated by heavy molecular PAH compounds with 4 or more aromatic rings (Table 3.2)

The soil has a high moisture content (29 %) and relatively moderate organic matter (11 %) (Table 3.2). These levels have been reported to favour microbial activity (Griffiths et al., 2018). The alkaline pH of the soil (8.2) is higher than the values obtained from a previous study evaluating physical properties of nine UK soils (McGeough et al., 2016). This pH value also could be the reason for the low phosphorus level since pH >8.0 have been shown to have a potential for nutrient interaction issues (Griffiths et al., 2018).

The concentration of metals/metalloids was determined using Inductively coupled plasma mass spectrometry (ICP-MS). The Aqua Regia extraction allowed the pseudo-total metal(loid)s to be quantified (Table 2).



**Fig. 3.** Metals and metalloids total, solid phase distribution and available content in the soil of the treatments and un-amended soil from 60 to 120 days. Rice husk biochar RHB (a), Wheat straw biochar WSB (b), Spent mushroom compost SMC (c), Rice husk biochar + Spent mushroom compost RHB-SMC (d), Wheat straw biochar+ Spent mushroom compost WSB-SMC (e), unamended (f).

Metals/metalloids were almost entirely present in both the pseudo-total and bioavailable fractions, indicating soil contamination at the gasworks site. This contamination could be the result of residual spent oxides from gas purification, carbonisation by-products, furnace residues, and residuals from batteries, pipelines, and paint (CL:AIRE, 2015). Some of the potentially toxic elements examined, such as Cr, Ni, Hg, and Se, were found to exceed soil guideline values (ALS Environment, 2009). Also, at pH less than 7, Zn and Cu values will be considered above limits based on the ALS guideline. If the risk framework were based on the bioavailable fractions, this soil will be considered safe since all the elements here are below the guideline values. While moisture and organic properties are considerable, the pH level and the heavy metals/metalloids content of the soil might render it unsuitable for crops and other applications.

### 3.2. Behaviour and fate of chemical mixture fractions over time

#### 3.2.1. Total and bioavailable hydrocarbon fractions concentrations

After 60 and 120 days, the total extractable aliphatic hydrocarbons fraction in all treatments was reduced to 53% and 80% of its initial concentration, respectively. The extent of degradation was mostly explained by the extent of degradation of the bioavailable fraction as shown in Figure 1a. No significant difference was observed between treatments ( $p=0.103$ ), except temporal effects from day 0 to day 120 ( $p=0.001$ ). With regards to aromatic hydrocarbons fraction, more than 98 % of the PAH were bioavailable, and their concentrations were reduced by 84 % and 97 % after 60 and 120 days, respectively (Figure 1b). Again, there was no significant difference between the treatments and control ( $p=0.6313$ ). Only temporal effect was seen as significant on the loss of PAH ( $p=0.006$ ). The absence of significant differences between the treatments and control group suggests that incorporating biologically active matrices did not provide any observable benefits in this study. This outcome may be attributed to the fact that the hydrocarbon level was below 15,000 mg/kg, most of the lighter fractions were already

degraded, and the nutrient level was sufficiently high (as indicated in Table 2). Therefore, the use of biochar or SMC had minimal impact on the light fractions. However, both bioadmentment types had positive effect on the extent of degradation of the high molecular weight (HMW) PAHs such as chrysene, Benzo[a]pyrene, and Benzo[ghi]perylene (on average 37% for the EC17–20 and 39% for the EC21–35 compared to the control). For the bioavailable HMW PAHs ranging between EC17-20, the extent of degradation was 66% more effective than the control. This was observed for both the total and bioavailable PAH concentrations at 120 days (Figure 2) The RHB-SMC exhibited the most favourable results in terms of Total PAH concentration for both EC17-20 (60%) and EC21-35 (62 %). On the other hand, WSB-SMC performed better in terms of HMW bioavailable PAH concentration (89%). Typically, high molecular weight PAHs tend to be sorbed, leading to their presence in the total extraction phase (Total extractable TPH) but less prevalent in the bioavailable phase. Given that HMW PAHs are potent carcinogens and mutagens that pose a significant threat to human health, their elimination from the environment is vital (Pandey et al., 2021). Thus, the study demonstrates that the bioadmentments have enabled the degradation of these persistent PAHs to a certain extent, even if the overall concentration did not show a significant change.

Legacy sites that have a history of contamination often harbour microbial communities that have adapted to the presence of contaminants and have developed enzymatic and metabolic pathways for degradation (Li et al., 2017). However, laboratory studies are typically carried out at a small scale, and under stable conditions of temperature and moisture, which may not fully represent the complexity of interactions between organisms and environmental factors in field situations (Calisi & Bentley, 2009; Mazzocchi, 2008). For instance, Zhang et al. (2020) reported the abundance of microbial communities with functional genes related to xenobiotic biodegradation and metabolism in a long-term industrial contamination site. The application rate and timing of biochar and spent

mushroom compost can also affect their effectiveness in reducing hydrocarbon concentration in soil. If the application rate was too low, 5% in this study, or if the timing (120 days) of the application was not optimal, it could have resulted in reduced efficacy of the treatments compared to the control. Soil characteristics can also influence it. For example, in the CNP ratio of the soil, it appeared to be low on nitrogen. While the use of biochar and SMC has been shown to improve TPH biodegradation due to the functional properties of these materials, there have also been reports of potential drawbacks in using biochar, such as nutrient immobilization, sorption of contaminants rendering them unavailable for degradation, and biochar toxicity (Anasonye, 2017; Dike et al., 2021; Gouma et al., 2014; Novak et al., 2018; Wang et al., 2017; Zhu et al., 2017a). These factors could have influenced the results of this study.

### 3.2.2. Metals and metalloids behaviour and fate during biotreatment

Metals including, Cr, Ni, Cu, Zn, As, Se, Cd, Pb, and Hg were among the key elements investigated (Table 3). The bioamendments changed the behaviour of the metals over time, resulting in changes in distribution and partitioning, particularly in the bioavailable phase (potentially available and highly mobile) ( $p=0.014$ ;  $0.001$ ). The most significant changes are observed in Cu, Zn, Cd, and Pb, and to a lesser extent Se, Ni, As, and the least Cr in all amendments. The highest changes were observed in WSB and SMC treatments. The bioavailable concentration of the metals at the end of the study were all below UK Environment Agency soil guideline values (ALS Environment, 2009). The amendment however, had no effect on how Hg was partitioned and distributed across all treatments. The results demonstrate how the use of bioamendments can reduce the bioavailability of toxic metals in soils.

Studies have shown that biochar soil amendment at 4 % and 5 % stabilise Cd, Cu, Ni, Pb, and Zn and reduce their bioavailability due to biochar's ability to enhance sorption and cause chemical precipitation, which is heavily influenced by biochar cation exchange capacity, pH, and ash content (Guo et al., 2020). Rice straw biochar has been shown to significantly reduce soil heavy metal solubility, with a maximum 35% reduction in root uptake of Cu and Pb (Wang et al., 2019). Also, soil application of fine rice straw biochar resulted in 97.3 and 62.2 percent reductions in extractable Cu and Zn (Yang et al., 2016).

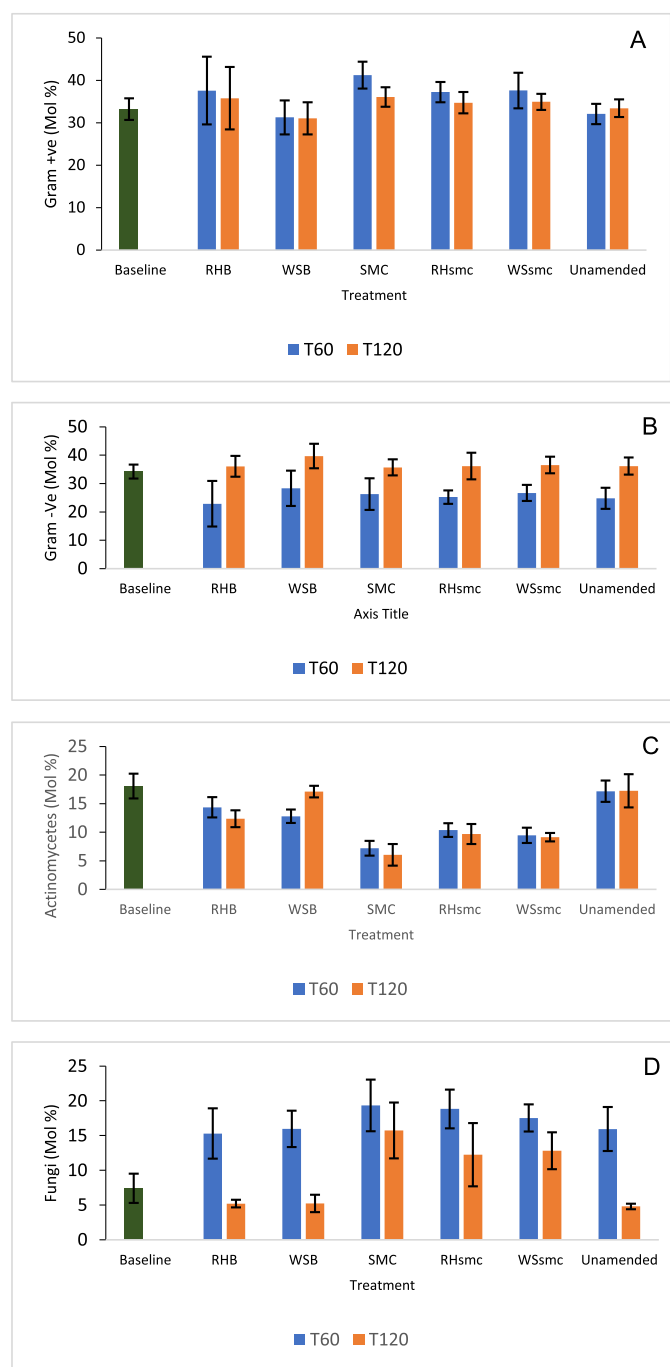
Similarly, SMC mixed with 30% ochre, 40% steel slag, and 10% limestone was effective in removing metals with more than 90 % removal efficiencies (Molahid et al., 2019). Furthermore, SMC amended soil has been demonstrated to reduce plant uptake of toxic metals. For example, soil amended with SMC caused decrease in Pb, Cd and Cu levels in *Atriplex halimus* shoot by 23.3 %, 51.3 % and 53 %, respectively (Frutos et al., 2010).

When metals occur in contaminated soils in their pure or mixed solid forms in inert or slowly reactive phases, these phases are unlikely to control ion activity in soil solution (Degryse et al., 2009). However, it has been demonstrated that the use of bioamendment, such as biochar, can alter the distribution and partitioning of these metals in soil (Cipullo et al., 2019). As a result, bioamendments with biochar and spent mushroom compost can change the total extractable metals concentration in the soil resulting in the release or immobilisation of bioavailable concentrations of the metals.

### 3.3. Hydrocarbon biodegradation indicators

#### 3.3.1. Microbial community relative abundance and dynamics

When the bioamendments were applied, the relative abundance of microbial biomass increased, and a microbial community shift was observed (Figure 4). In general, except for actinomycetes, the microbial groups increased by an average of 34% and 12% at day 60 and 120, respectively. Specifically, for the Gram positives, all the treatments performed uniquely ( $p=0.0432$ ) but there were no time effects ( $p=0.1064$ ) (Figure 3.4a). On Gram negatives, the time impact was significant ( $p=0.0004$ ) while the treatment effect was not ( $0.6653$ ) (Figure 3.4b). In the fungi group (Figure 3.4d), there were significant differences in



**Fig. 4.** Influence of bioamendments on the relative abundance of the different microbial groups at day 60 and 120. The error bars represent the standard error for each treatment's replicates. (a) Gram positive, (b) Gram negative, (c) Actinomycetes, (d) Fungi

time and treatments ( $p=0.0001$ ). Actinomycetes were significantly reduced in the SMC, RHB-SMC, and WSB-SMC (Figure 3.4c) with both time ( $p=0.03165$ ) and treatment ( $p=0.0001$ ) effects observed.

Increased in Gram negative populations are consistent with degradation activities occurring in petroleum impacted soils (Al-Hawash et al., 2018; Cipullo et al., 2019). The fungal population increases significantly ( $p=0.0001$ ) from the baseline at day 60, followed by a decline at day 120, as is typical of fungi, which flourish in complex environments and may have been replaced by other microbial groups as the medium becomes less complex. (Dai et al., 2022; Gouma et al., 2014; Tesei et al., 2019).



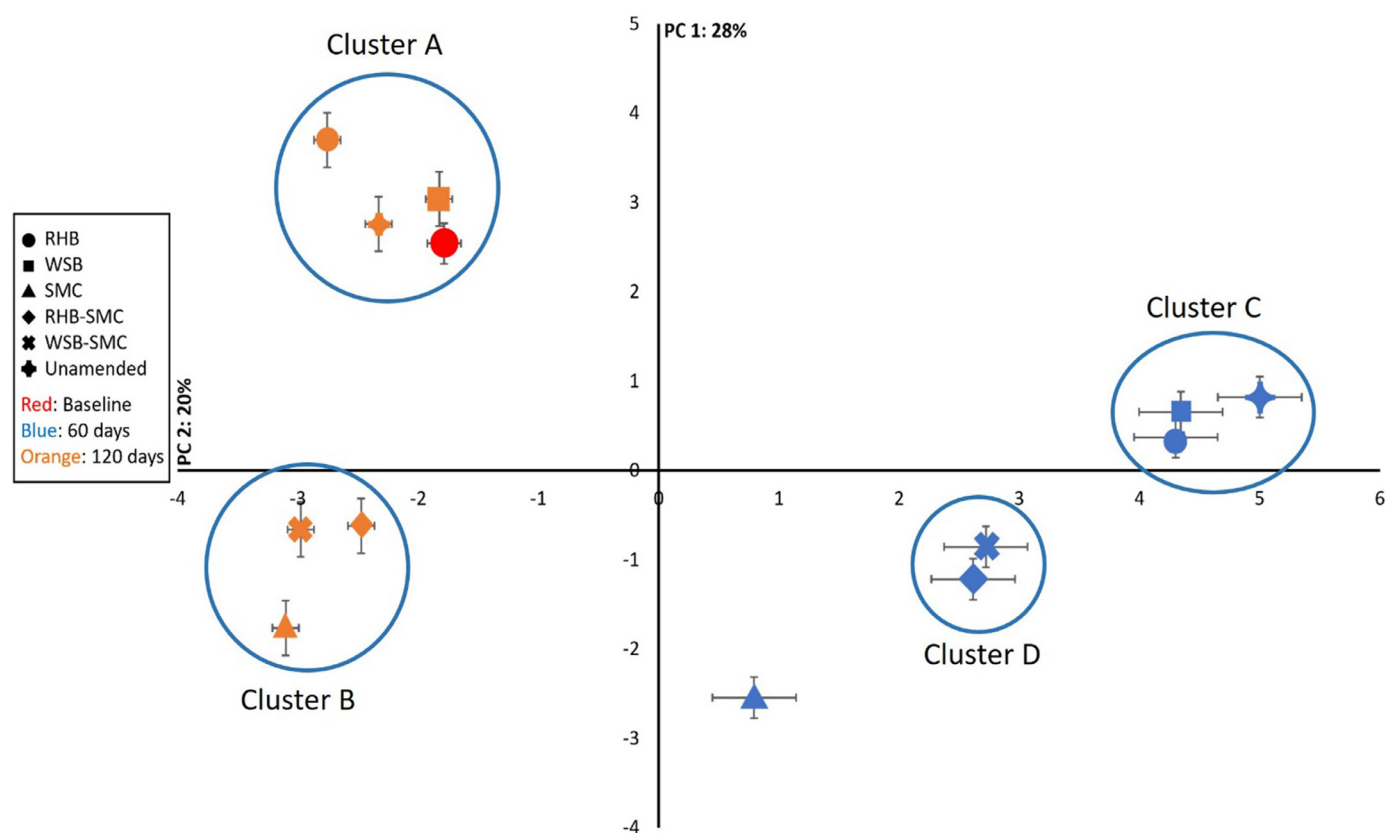


Fig. 5. Microbial community dynamics extracted from the treatments and unamended soil across incubation time from the onset (T0) to the end of incubation (T120). The error bars represent the standard error for each treatment's replicates. (RHB: rice husk biochar; WSB: Wheat straw biochar; SMC: Spent mushroom compost).

The bioamendments influenced the microbial community and induced a community shift, as illustrated by cluster C, D and Cluster A, B at day 60 and 120, respectively (Figure 5). The convergence of the microbial community between day 60 and day 120 indicates that the microbial groups are merging, most likely due to the recovery of the contamination in the soils over time. The similarities in the behaviour of the microbial communities may explain the non-significant difference in the treatments' performance on the TPH degradation (Figure 1). Incubation time has been shown to have a significant effect on the microbial community composition in the treatments ( $p=0.001$ ). There is also a significant difference in the treatments, particularly in the SMC, RHB-SMC, and WSB-SMC, which differ from the unamended soil ( $p=0.001$ ). Previously, soil amendment with biochar and spent mushroom compost has been shown to influence microbiological characteristics required for remediation (Cipullo et al., 2019; Dike et al., 2021; Lu Zhang & Sun, 2014).

### 3.3.2. Microbial catabolic profile and activity

Preservation of soil function is one of the important components in sustainable remediation. Microbial decomposition and substrate conversion are important soil functions that are frequently observed using respiration proxy data (Kaurin and Lestan, 2018). The influence of the bioamendments on the microbial community function ( $\text{CO}_2$  production rate) was observed in all the treatments. The function was influenced by both biochar and, to a large extent, spent mushroom compost. Hence, in addition to nutrients, spent mushroom compost contains additional microbes that can improve the degradation process (Gouma et al., 2014). The amendments ( $p=0.0001$ ) significantly influenced soil respiration, but time did not ( $p=0.2114$ ).

At day 60, there was a 2-, 2.4-, and 5-fold increase in  $\text{CO}_2$  production in the RHB, WSB, and SMC treatments, respectively, compared to the control (Figure 6). The biochar-SMC mixture showed the least effect. Similar trends were observed at day 120, with the WSB-SMC treatment

showing over a 2.8-fold increase. The observed significant effect of the SMC-amended soil can be attributed to the high nutrient content, improvement of the microbial community, and enhancement of important soil properties such as aeration and pH adjustment towards neutrality (Kästner and Miltner, 2016). Additionally, the combination of biochar with SMC can improve the quality of treatment, as observed in the WSB-SMC treatment at 120 days, where the biochar improves particle-size distribution, enhances aeration, and improves cation exchange capacity, in addition to the effects of the SMC. (Cipullo et al., 2019).

### 3.3.3. Ecotoxicity

It is critical to evaluate soil ecotoxicology before, during, and after any remediation treatment, because a decrease in contaminants does not always imply a decrease in toxicity (Coulon et al., 2005). The simplest form of soil ecological organisms (bacteria cell) was used as the basis of the toxicity test, which was the Microtox® in vitro test (Morden Water, 2012). In Figure 7, TPH concentration (1493 mg/kg) before treatment corresponded to high  $\text{EC}_{50}$  value of 2224mg/L. It should be noted that the higher the  $\text{EC}_{50}$  concentration, the lower the soil's toxicity. Hence, the continuous decrease in TPH observed from 60 to 120 days corresponded to a higher  $\text{EC}_{50}$  value, indicating decreasing toxicity. This implies that the amendment effects of decreasing contamination corresponded to lower soil ecotoxicity. Except for the RHB, all bioamendments showed decreased toxicity, with the greatest improvement seen in  $\text{SMC} < \text{WSB-SMC} < \text{WSB} < \text{RHB-SMC}$ . While this is expected as reported in Cipullo et al. (2019), other research have found that toxicity increases as TPH decreases, with one of the most likely causes being the production of toxic intermediates during biodegradation, such as reactive oxygen species, epoxides, certain aldehydes, and ketones (Jiang et al., 2016; Mamindy-Pajany et al., 2012; Xu & Lu, 2010).

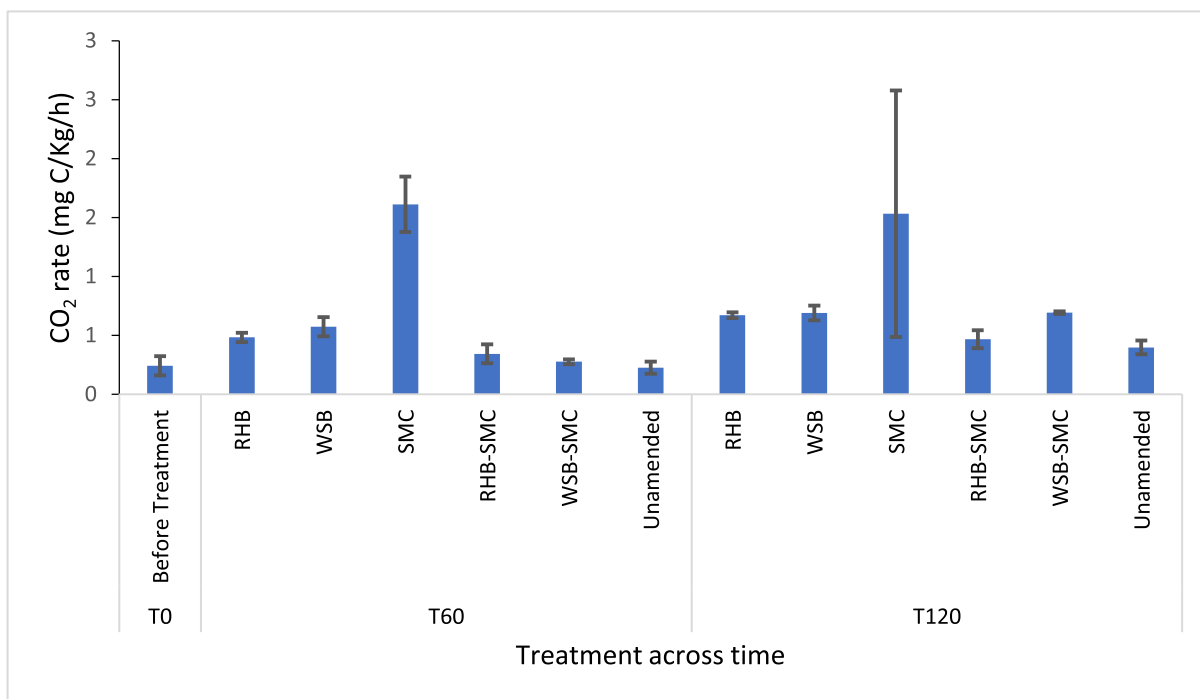


Fig. 6. Soil respiration expressed as CO<sub>2</sub> production (mg C/kg/h) for treatment with RHB: rice husk biochar; WSB: Wheat straw biochar; SMC: Spent mushroom compost; Unamended soil tested at 0, 60 and 120 days. The error bars represent the standard error for each treatment replicates.

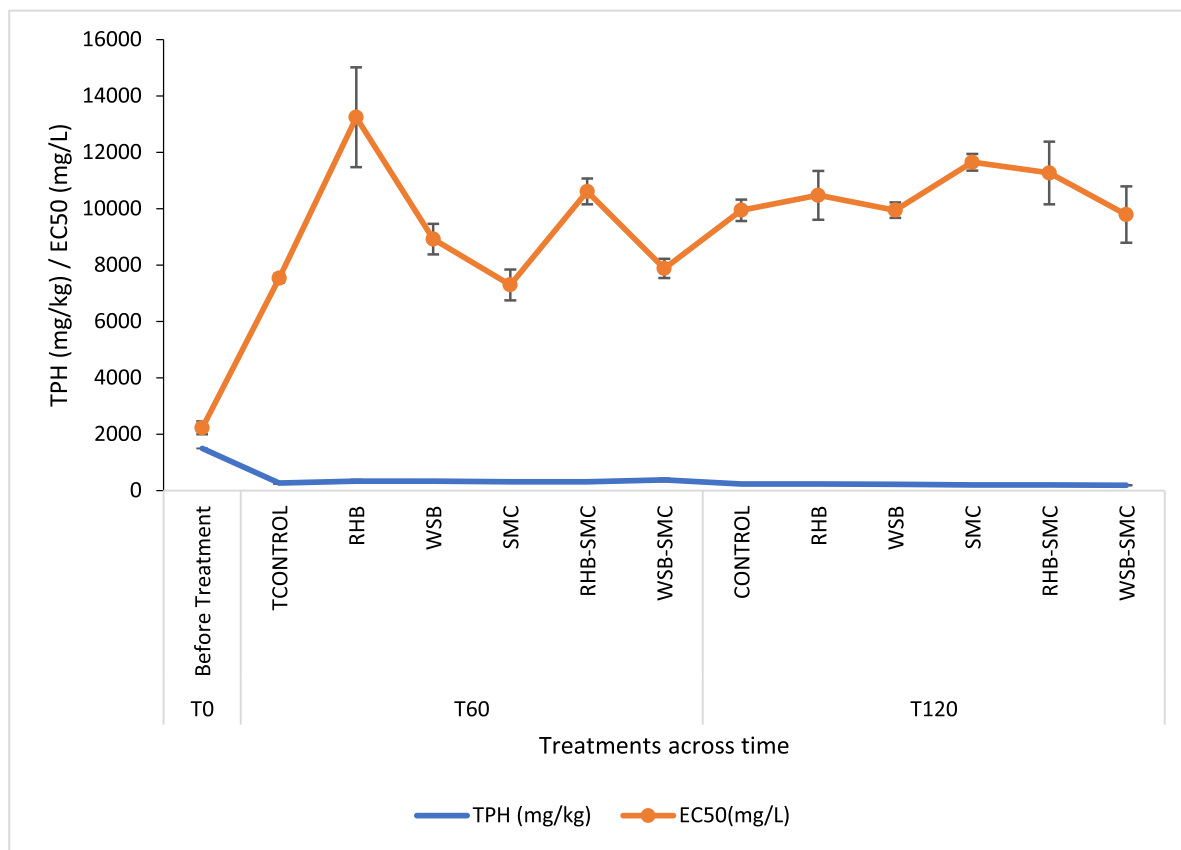
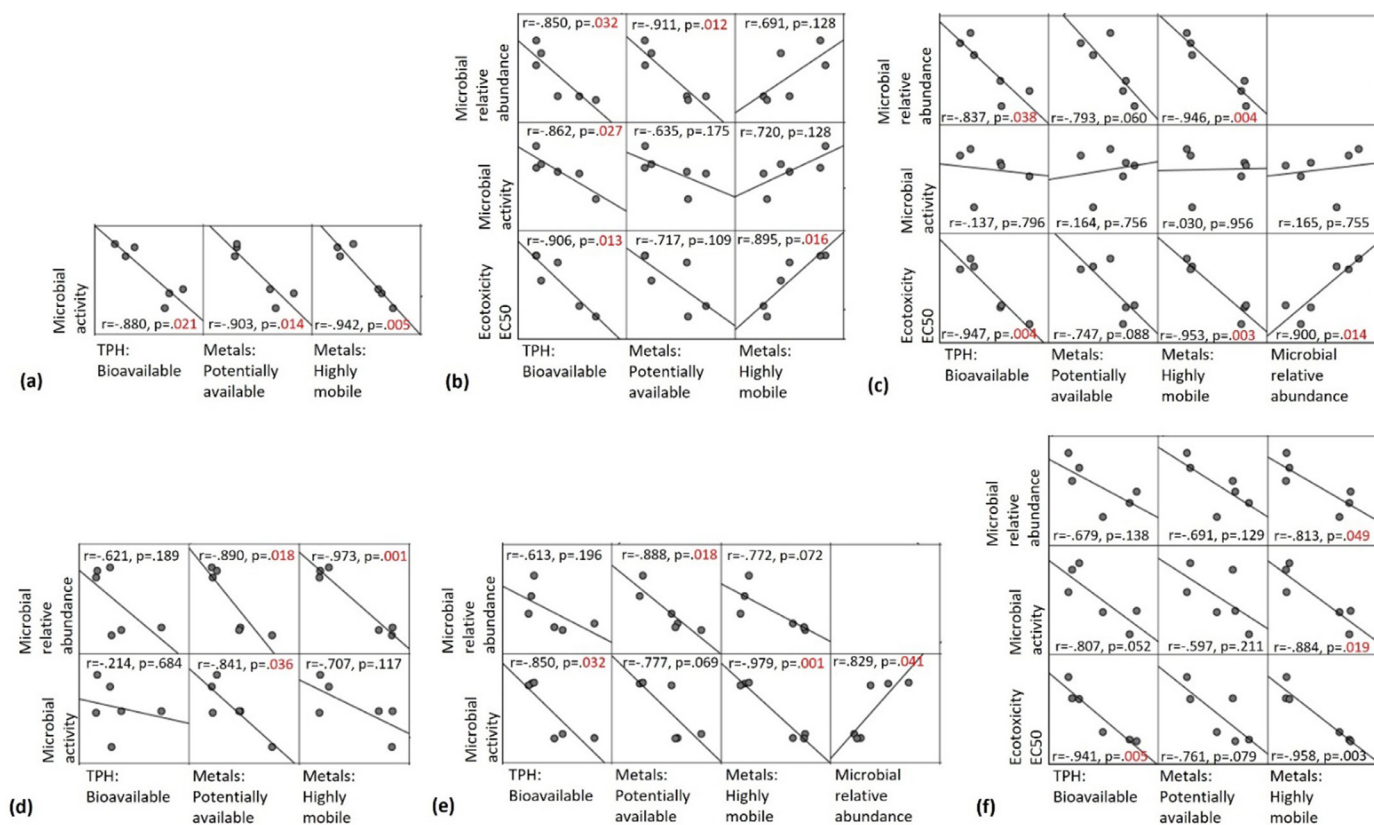


Fig. 7. Microtox Basic Solid Phase Test (BSPT) assay shown as EC<sub>50</sub> concentration (mg/L) for light decrease values at the onset and at 60, 120 days. The error bars represent the standard error for each treatment's replicates.



**Fig. 8.** Linear correlation (based on Pearson coefficient) between organic and inorganic bioavailable concentrations, toxicity data and the microbiological responses (Microbial relative abundance, and soil respiration). The various soil treatments, Rice husk biochar (a), Wheat straw biochar (b), Spent mushroom compost (c), Rice husk biochar+Spent mushroom compost (d), Wheat straw biochar+ Spent mushroom compost (e), unamended (f).  $g =$ Correlation is significant at the  $p < 0.05$

### 3.4. Correlation of the bioindicators for determining remediation endpoints

The study findings demonstrate that the amendment used had an impact on the relationships between the bioavailability of TPH and metal(loids), soil toxicity, microbial abundance, and respiration, providing insights into the effects of chemical mixtures on microbial communities (Figure 8). The negative correlation observed for bioavailable data and the positive correlation for toxicity data showed that lower concentrations of bioavailable hydrocarbons and metals resulted in an increase in the microbial community and soil respiration function, most notably in the WSB and WSB-SMC treatments (Figure 8b and d). In contrast for the RHB (Figure 8a), a negative correlation was observed between soil toxicity ( $EC_{50}$ ) concentrations and microbial responses (relative abundance and activity). Similarly, in SMC and RHB-SMC, only microbial activity correlated negatively with soil toxicity (Figure 8c and d). This could be due to increased environmental stress increasing the demand for energy to thrive, causing microbes to increase activity to overcome the stress's limiting effect, or in some cases, the compounds are being utilised by the microbes for growth (Gouma et al., 2014; Rogiers et al., 2021; Zhang, Lixun & Guan 2022; Zhu et al., 2017a).

This study demonstrated a correlation between bioavailability and toxicity and their impact on the soil microbial community. The findings suggested that lowering the bioavailable levels of contaminants led to a reduction in toxicity to the microbial community and its function. As a result, it indicated that if the only concern is the assessment of environmental risk receptors, then the remediation process could potentially be stopped once the bioavailable concentrations of contaminants have been reduced to a safe level. In the present study, nearly a 5-fold reduction in toxicity was observed, suggesting that a remediation endpoint below an  $EC_{50}$  value of 2225 mg/kg could be considered as appropriate.

## 4. Conclusion

The study investigated the effectiveness of two types of biochar, namely rice husk biochar (RHB) and wheat straw biochar (WSB), as well as spent mushroom compost (SMC), in reducing the concentration of total petroleum hydrocarbons (TPH) and metals in soil. The results showed that all three bioamendments significantly increased the reduction of TPH by at least 92%, as evidenced by a decrease in the bioavailable concentration of TPH. Moreover, the bioamendments stabilised and lowered the toxicity of metals in the soil by changing their distribution and partitioning, leading to a drop in their bioavailable fractions below the UK CLEA soil guideline limits. In terms of the reduction of TPH containing both aliphatic and aromatic compounds, both RHB and WSB performed equally well, but WSB was more effective in reducing the bioavailable concentration of hydrocarbons. On the other hand, RHB was more effective in influencing the distribution and partitioning of metals. Interestingly, there was no significant difference in the effectiveness of the biochars and SMC in total and bioavailable compound recovery at the 2.5% and 5% rates applied, suggesting that SMC may be preferred for remediation due to its lower cost compared to biochar. However, combining SMC with biochar, such as RHB+SMC, can enhance their effectiveness in reducing metal available phase fractions. The study also found that the bioamendments, particularly SMC, positively influenced microbial abundance and activity, resulting in increased soil respiration function. Furthermore, the lower bioavailable and toxicity concentrations of the chemical mixtures because of the bioamendments reduced the level of contamination to below remediation endpoint for the soil. Overall, the study's findings provide valuable insights into the potential of bioamendments for soil remediation and the importance of considering soil microbial communities in soil remediation strategies. This knowledge can help advance the field by promoting the use of more

sustainable and effective soil remediation approaches that consider the ecological implications of soil contamination in remediation.

### Data availability statement

Data supporting this study are openly available from Cranfield Online Research Data repository at 10.17862/cranfield.rd.21707273

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### CRedit authorship contribution statement

**Emmanuel Atai:** Conceptualization, Investigation, Resources, Formal analysis, Writing – original draft. **Raphael Butler Jumbo:** Resources, Investigation. **Richard Andrews:** Investigation. **Tamazon Cowley:** Investigation. **Ikeabiana Azuazu:** Resources, Investigation. **Frederic Coulon:** Conceptualization, Supervision, Resources, Writing – review & editing. **Mark Pawlett:** Conceptualization, Supervision, Resources, Writing – review & editing.

### Data availability

Data will be made available on request.

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# Bioengineering remediation of former industrial sites contaminated with chemical mixtures

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