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The impact of organic amendments on the fate and behaviour of phenanthrene in soil

By

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Declaration

I hereby declare that this thesis is my original research, and it has not been submitted elsewhere in part or whole for the award of any higher degree.

Chisom Ejileugha

Authorship statement

This thesis contains yet to be published articles in various chapters as shown below.

Chapter 3 (paper 1) will be submitted to Environmental Research as: Effect of soil-PAH contact time and organic amendment on ^{14}C–phenanthrene mineralisation in soil.

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants that pose significant health risks to humans and to the environment. Due to their hydrophobic nature and strong affinity to organic matter, soils often act as long-term sinks for PAHs, leading to their persistence and negative impacts on soil health and function. Conventional physical and chemical remediation methods are often not environmentally friendly nor sustainable. Consequently, organic amendments offer a more sustainable, environmentally friendly, and cost-effective alternative for mitigating adverse impacts of phenanthrene in soil. This thesis investigated the effects of biochar, spent mushroom compost (SMC), SMC:biochar blends, particle size variations, and the interaction between amendment dose/type and contact time on phenanthrene extractability and/or mineralisation in soil. Additionally, it examined the influence of dimethyl sulfoxide (DMSO) on phenanthrene mineralisation. Findings revealed that both organic amendments and DMSO enhanced mineralisation kinetics by shortening lag phases, accelerating mineralisation rates, and increasing overall mineralisation extents. Among the factors studied, contact time and its interaction with amendment dose had a greater impact on mineralisation kinetics than amendment dose alone. Biochar application at concentrations ≥1% negatively affected mineralisation kinetics due to its strong sorption capacity. However, fine biochar particles, which possess higher surface area and shorter diffusion pathway, may facilitate faster desorption compared to coarse biochar. SMC, when applied in its unprocessed form containing a mixture of particle sizes, led to greater cumulative mineralisation than any single particle size fraction. Nevertheless, fine-particle SMC enhanced mineralisation rates and shortened lag phases more effectively than whole SMC, likely due to its faster decomposition and readily available nutrients. Blending SMC with biochar improved cumulative mineralisation compared to using either amendment alone, with the highest mineralisation observed in soils treated with a 5:1 SMC-to-biochar blend. DMSO treatment enhanced mineralisation kinetics, though higher concentrations (≥1.0%) could potentially pose toxicity risks, likely due to excessive PAH solubilisation and adverse effects on soil microbial communities. Additionally, available phosphorus, ammonium nitrogen, and total organic carbon were strongly correlated with mineralisation kinetics, highlighting their crucial role in PAH biodegradation. Overall, this thesis provides evidence-based insights into the efficacy of biochar, SMC, their blends, and particle size variations as effective amendments for mitigating PAH contamination in soil. It also underscores the potential of DMSO in enhancing PAH biodegradation.

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Abbreviations

Al = Aluminium

ARGs = Antibiotic resistant genes

BDE-15 = 4,4'-dibromodiphenyl ether

BDE-47 = 2,2',4,4'-tetrabrominated diphenyl ether

C:N = Carbon to nitrogen ratio

CEC = Cation exchange capacity

Cu = Copper

DCM = Dichloromethane

DMSO = Dimethyl sulphoxide

DOC = Dissolved organic carbon

DOM = Dissolved organic matter

EC = Electrical conductivity

Fe = Iron

HMW = High molecular weight

HOCs = Hydrophobic organic contaminants

HP-β-CD = Hydroxypropyl-beta-cyclodextrin

HT = High temperature

K = Potassium

K_{OC} = Water partitioning coefficient

K_{OW} = Octanol water coefficient

LMW = Low molecular weight

LSC = Liquid scintillation counter

LSD = Least significant difference

LT = Low temperature

MANOVA = Multivariate analysis of variance

MBS = Minimal basal salt

MMW = Medium molecular weight

N = Nitrogen

NaOH = Sodium hydroxide

NERs = Non-extractible residues

NEETs = Non-exhaustive extraction techniques

NH^{+}_{4}-N = Ammonium nitrogen

OLSEN P = Available phosphorus

P = Phosphorus

PAH = Polycyclic aromatic hydrocarbon

PAHs = Polycyclic aromatic hydrocarbons

PCBs = Polychlorinated biphenyls

PTEs = Potential toxic elements

RPM = Rotation per minute

SEM = Standard error of mean

SMC = Spent mushroom compost

SOC = Soil organic carbon

SOM = Soil organic matter

SD = Standard deviation

TOC = Total organic carbon

TPH = Total petroleum hydrocarbon

VOCs = Volatile organic carbons

Zn = Zinc

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Chapter 1 Introduction to the research

Soil is one of the most valuable natural resources, essential for supporting ecosystems, sustaining agriculture, and maintaining biodiversity (Lal, 2008). However, this vital resource is increasingly contaminated by human activities (FAO and UNEP, 2021). Growing concerns over soil contamination, particularly from polycyclic aromatic hydrocarbons (PAHs), have provoked extensive research into soil remediation to mitigate toxicity and promote soil health and function. PAHs are organic chemicals with multiple fused aromatic rings, known for their toxicity and environmental persistence (Lawal, 2017; Wu et al., 2019). They exhibit genotoxic, carcinogenic, and teratogenic properties and can irritate the skin and respiratory tract (Lawal, 2017; Patel et al., 2020). Therefore, remediating PAH-contaminated soil is crucial to protect human health and ecosystems from the toxic, carcinogenic, and persistent effects of PAHs while restoring soil functionality and productivity.

Found in air, soil, water, and sediment, PAHs arise from both natural and human activities, with exposure occurring through skin contact, ingestion, inhalation, or contaminated food (Singh et al., 2016; Lawal, 2017; Patel et al., 2020). Their molecular weight, ring number, and structure influence their hydrophobicity, bioaccessibility, persistence, and biodegradation (Papadopoulos et al., 2007). High molecular weight (HMW) PAHs are more hydrophobic, persistent, and toxic, accumulating in organic matter and fatty tissues (Okere & Semple, 2012; Zheng et al., 2012; Patel et al., 2020). Due to the affinity of PAHs to organic matter, soil acts as a sink for PAHs as a high percentage of PAHs in the environment exist in soil (Semple et al., 2003; Okere & Semple, 2012).

Among all possible PAHs, sixteen (16) have been recognised by the United States Environmental Protection Agency (USEPA) as priority pollutants. The 16 PAHs in USEPA list include naphthalene, acenaphthene, acenaphthylene, phenanthrene, anthracene, pyrene, fluorene, fluoranthene, chrysene, benzo(a)pyrene, benzo(a)anthracene, benzo(a)fluoranthene, benzo(k)fluoranthene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, and indeno(1,2,3-cd)pyrene (Keith, 2015; Patel et al., 2020).

Biochar is a carbon-rich material produced through biomass pyrolysis, and compost is produced through aerobic degradation of organic waste materials to recover valuable nutrients and organic matter (Ejileugha, 2022). Biochar and compost are used as soil amendments to improve the quality of degraded soil (Mensah & Frimpong, 2018; Frimpong et al., 2021; Alkharabsheh et al., 2023; Howell et al., 2024), and mitigate the adverse impacts of hydrophobic organic contaminants (HOCs) in soils (Kästner & Miltner, 2016; Baldantoni et al., 2017; Xiang et al., 2019; Saeed et al., 2021; Kaur & Sharma, 2021). Both of these organic materials enhance soil microbial activity and improve soil structure and fertility, but biochar is spectacular in its ability to sorb HOCs (Patel et al., 2022). The sorption efficiency of biochar is through its porous structure which can also act as a habitat for soil microbes. Also, the neutral–alkaline pH of biochar is favourable for biodegradation and can bring a liming effect in acidic soils (Ahmad et al., 2014). By improving soil health and fertility, biochar and compost enhance soil microbial activity and present suitable soil conditions that stimulate the microbial catabolism of organic contaminants.

Mushroom cultivation requires thrice the volume of compost to the quantity of mushrooms produced (Horizon 2020, 2019). This compost can only be re-used for two to three harvests and then discarded as spent mushroom compost (SMC). Disposing of the SMC presents logistic and financial challenges to farmers, and if not properly managed or disposed, may present environmental challenges. The need to manage and tackle the challenge of the rising SMC in Europe can be seen in the government-funded project aimed at SMC valorisation (e.g., BIOrescue EU-funded project). Studies have demonstrated the effectiveness of SMC as a soil amendment for improving soil properties and crop productivity (e.g., Gümüş & Şeker, 2017; Afagh et al., 2019; Muchena et al., 2021; Umor et al., 2021; Chaudhary et al., 2022; Rajavat et al., 2022). SMC contains valuable nutrients (Becher et al., 2021) and farmers are encouraged to use SMC as a soil amendment as it contains similar properties as compost (Umor et al., 2021). Amendments that can enhance soil properties have the potential to enhance soil microbial activities and subsequent microbial degradation of organic contaminants (Zhu et al., 2017), making SMC a potential amendment for PAH-contaminated soil.

1.1 The rationale for the study

Existing chemical and physical methods for remediating PAH-contaminated soil are often costly, environmentally unfriendly, subject to regulatory challenges, and may not achieve complete PAH degradation (Patel et al., 2020). The use of organic amendments provides a sustainable, cost-effective, and environment-friendly alternative. The use of biochar and compost has shown promise in mitigating the adverse effects of PAHs in contaminated soils (Baldantoni et al., 2017; Song et al., 2017; Sigmund et al., 2018; Guo et al., 2021). However, the effectiveness of these

amendments is influenced by several factors, including contact time, amendment type, soil properties, and amendment amount. The interaction between contact time and amendment amount is critical to understanding the long-term effectiveness of biochar and compost in PAH remediation. While prolonged contact time generally reduces PAH bioaccessibility and mineralisation (Macleod and Semple, 2000; Macleod and Semple, 2003), the effect of amendment amount is less straightforward, with some studies suggesting that higher amounts do not always enhance PAH mineralisation (e.g., Namkoong et al., 2002; Puglisi et al., 2007; Scelza et al., 2007; Rhodes et al., 2008; Ogbonnaya et al., 2016; Omoni et al., 2020a; Omoni et al., 2020b; Tran et al., 2021). However, the combined effect of these two factors remains poorly understood. Evaluating their interaction effects while considering their individual effects will help quantify their relative influence and determine whether they act synergistically, additively, or antagonistically in shaping PAH mineralisation kinetics. Addressing this gap will lead to more evidence-based, optimised amendment strategies for sustainable PAH remediation in contaminated soils.

Biochar and SMC have individually demonstrated their ability to enhance soil health and functions (Saeed et al., 2021; Kaur & Sharma, 2021; Chaudhary et al., 2022; Rajavat et al., 2022; Alkharabsheh et al., 2023). A combined application of these amendments could offer synergistic benefits. SMC introduces nutrients, organic matter, and beneficial microbes, while biochar sorbs PAHs and improves soil structure, potentially enhancing microbial activity. Previous studies have demonstrated the superior impact of biochar-compost mixtures on soil properties and crop yields compared to either amendment alone (e.g., Agegnehu et al., 2015; Agegnehu et al., 2016;

Abideen et al., 2020; Al-Omran et al., 2019; Al-Omran et al., 2021). Furthermore, combined application of biochar and compost has shown a greater capacity to reduce PAH toxicity in soils compared to their individual applications (Bielska et al., 2017; Sigmund et al., 2018). Research has demonstrated that the combined application of biochar and compost is more effective in improving soil properties and stimulating microbial activity (Frimpong et al., 2021; Bong et al., 2021; Bello et al., 2023). However, the potential of biochar – SMC blends for improving the mineralisation kinetics of PAHs in soil remains largely unexplored, presenting a significant research gap.

Biochar's ability to sorb PAHs is influenced by its surface area, with finer particles offering greater sorption capacity due to increased surface area and shorter diffusion pathways (Zand & Grathwohl, 2016; Kang et al., 2018; Xiang et al., 2020; Saeed et al., 2021). However, fine-particle biochar may exhibit lower sorption stability, potentially leading to faster desorption of PAHs. Understanding the impact of biochar particle size on PAH bioaccessibility and biodegradation is critical, yet studies addressing this aspect are limited. Existing research indicates that biochar particle size influences soil properties, microbial activity, and plant growth (e.g., Liao & Thomas, 2019; Billah et al., 2019; Piew et al., 2020; Edeh & Mašek, 2021; Ahmad et al., 2023). Fine biochar particles have been shown to enhance soil pH, water-holding capacity, and microbial abundance (Chen et al., 2017; Sarfraz et al., 2020; Zhao et al., 2020; Özenç et al., 2023), which could improve PAH bioaccessibility and biodegradation. However, the role of biochar particle size in modulating PAH bioaccessibility and mineralisation in soil remains poorly understood.

The mineralisation of most PAHs in soil is primarily constrained by limited desorption and bioavailability rather than microbial catabolic capacity (Semple et al., 2006; Allan et al., 2007). Enhancing PAH solubility has shown promise in overcoming these limitations. For example, surfactant application has been demonstrated to increase PAH biodegradation (e.g., Rathankumar et al., 2022; Li et al., 2023; Liu et al., 2023; Zhang et al., 2024). The addition of solvents such as methanol, acetone, ethanol, and acetonitrile has also been reported to enhance PAH mineralisation (Caldini et al., 1995; White & Alexander, 1996; Bonten et al., 1999; Lee et al., 2001). Dimethyl sulfoxide (DMSO), with its high solvent and penetrative properties, has the potential to solubilize PAHs and facilitate their transfer across microbial membranes. While DMSO is widely used in drug delivery systems for its ability to enhance solubility and permeability (Pashynska et al., 2022; Ramkar et al., 2023; Sun et al., 2023), its application in improving PAH bioavailability and microbial degradation in soil has not been explored. This represents a novel approach with significant implications for advancing PAH remediation strategies.

Therefore, to address the gaps identified in the literature, the following hypotheses were developed and tested in this thesis

1. The kinetics of phenanthrene mineralisation in soil are influenced by the extent to which organic amendments and contact time affect microbial activity and PAH bioavailability, with both amendment amount and soil-PAH contact time modulating the accessibility of phenanthrene to microbial degraders through changes caused by soil-phenanthrene-amendment interactions.

2. Phenanthrene extractability and mineralisation kinetics are mediated by the particle size of biochar and SMC, which influences the soil organic matter and physicochemical properties, surface area available for sorption, pore structure accessibility, diffusion pathway, and suitable conditions for degrading microbes.
3. Application of SMC-biochar blend enhances phenanthrene mineralisation by synergistically improving microbial proliferation (via nutrient-rich SMC) and modulating sorption–desorption equilibria (via porous biochar), thereby supporting sustained microbial degradation.
4. The addition of DMSO enhances phenanthrene extractability and biodegradation kinetics by acting as a co-solvent that disrupts hydrophobic interactions between phenanthrene and soil organic matter, thereby increasing its aqueous phase concentration and facilitating microbial degradation.

1.2 Aims and objectives of the study.

The aim of this thesis is to investigate environmentally friendly, sustainable, and cost-effective approaches to stimulate biodegradation of PAHs in soil, using phenanthrene as a model PAH. By addressing the identified gaps, this thesis seeks to advance our understanding of how amendment type, amount, contact time, and particle size influence the reduction of PAH-related risks in soil. Additionally, it sought to pioneer the use of DMSO to enhance PAH bioavailability and mineralisation in soil, thereby offering innovative solutions to the challenges of PAH soil contamination. These were achieved through the following objectives:

1. To evaluate the effects of the amounts of biochar and SMC, their interaction with contact time, and their comparative impacts on the lag phases, fastest rates, and extents of mineralisation of ^{14}C-phenanthrene in soil
2. To compare the impact of SMC:biochar blends on the lag phases, fastest rates, and extents of mineralisation of ^{14}C-phenanthrene in soil compared to that of individual biochar and SMC amendments.
3. To investigate the effect of different SMC particle size fractions on the lag phases, fastest rates, and extents of mineralisation of ^{14}C-phenanthrene in soil
4. To examine the impact of biochar particle size fractions on ^{14}C-residual activity, extractability, and mineralisation kinetics of ^{14}C-phenanthrene in soil.
5. To explore the effect of different amounts of DMSO on the residual ^{14}C-activity, extractability, and mineralisation kinetics of ^{14}C-phenanthrene in soil.

1.3 References

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Chapter 2 Literature Review

2.1 Bioaccessibility, biodegradation, and persistence of PAHs in soil

PAHs, a group of toxic organic chemicals with fused benzene rings, are ubiquitous in our environment (Okere and Semple, 2012; Patel et al., 2020). The bioaccessibility of PAHs to biological entities in soils is a factor of the properties of the soil, PAHs, and environmental conditions (Chung and Alexander, 1998; Umeh et al., 2017). The concept and meaning of bioaccessibility and bioavailability have been explained in earlier articles (e.g., Semple et al., 2003; Semple et al., 2004; Semple et al., 2007; Semple et al., 2013; Riding et al., 2013; Umeh et al., 2017). Bioaccessible fraction of a contaminant is the amount of the contaminant which is available now and that which will be possibly available in the future to cross cell barriers, while the bioavailable fraction is the fraction of the contaminant that is readily available at a given time to cross cell barriers (Riding et al., 2013; Semple et al., 2013; Umeh et al., 2017). Biodegradation of PAHs in soil depends on the PAHs' bioavailability and potential of the microbes. The interaction of PAHs with soil and soil components affects its bioaccessibility through sorption, aging, and sequestration (Semple et al., 2013; Riding et al., 2013; Umeh et al., 2017). Aging is the decrease in bioaccessibility of a contaminant in soil over time (Semple et al., 2013). Sequestration relates to taking away the contaminants by soil matrix/colloids such that it is hidden (no longer accessible or available) from the microbes in the soil (Umeh et al., 2020). Sorption is the binding or adsorption of the contaminant to soil and soil components, and this can limit the availability of the contaminants to the microbes in the soil (Semple et al., 2013; Umeh et al., 2020). In soil, the sorption of PAHs to soil and its mineral and organic components controls its mobility, desorption,

and bioaccessibility (Semple et al., 2003). A reduction in the bioaccessibility of a contaminant in soil equates to a decrease in potential toxicity (Semple et al., 2013; Riding et al., 2013; Umeh et al., 2017; Umeh et al., 2020). Therefore, bioaccessibility is regarded as an important factor in the risk assessment of contaminated soils.

Biodegradation for most PAHs is not limited by microbial catabolic activity or microbial population but rather by contaminant desorption and bioavailability (Semple et al., 2006; Allan et al., 2007). Soil autochthonous microbes can catabolise PAHs but an increase in soil-PAH contact time will reduce biodegradation due to decreased bioavailability (Reid et al., 2001). Figure 1 shows the impact of soil-PAH contact time on possible extractability and bioavailability. Bioavailability is a limiting factor in the biodegradation of PAHs as only the bioavailable fraction of a contaminant can be biodegraded. Therefore, understanding of bioavailability of contaminants in soil is relevant for estimating mineralisation and associated contamination risks (Allan et al., 2006). Non-exhaustive solvent extraction can predict PAH bioaccessibility in contaminated soils (Patterson et al., 2004; Papadopoulos et al., 2007). Studies have shown that non-exhaustive extraction techniques (NEETs) like the use of Tenax (Breedveld & Karlsen, 2000; Cornelissen et al., 2001; Bari et al., 2010), cyclodextrin (Reid et al., 2000; Allan et al., 2007), selective superficial fluid extraction (Cajthaml & Šašek, 2005; Bielská et al., 2013), and amberlite XAD (Northcott & Jones, 2001; Adedigba et al., 2018) can predict PAH bioaccessibility. Among the NEETs, the use of hydroxypropyl-β-cyclodextrin (HP-β-CD) has received much acceptance with several studies demonstrating its suitability for predicting microbial degradation of PAHs in soil (e.g., Reid et al., 2000; Allan et al., 2006; Duan et al., 2015; Vázquez-Cuevas et al., 2021; Posada-

Baquero et al., 2022; Jin et al., 2023). Tenax and HP-β-CD extractions are the most promising and accepted techniques but a comparison by Bernhardt et al. (2013) found HP-β-CD extraction to be more time efficient. HP-β-CD extraction adequately predicted PAHs microbial availability and mineralisation in dissimilar laboratory spiked soils (Duan et al., 2015), field contaminated soils (Papadopoulos et al., 2007), black carbon amended soils (Oyelami et al., 2014; Ogbonnaya et al., 2016), and in the presence of co-contaminants (Allan et al., 2006). However, HP-β-CD may overpredict the bioavailability and biodegradation of high molecular weight (HMW) PAHs in soils due to the low mineralisation of HMW PAHs (Huesemann et al., 2004; Papadopoulos et al., 2007). In black carbon amended soils HP-β-CD may underpredict PAH mineralisation at higher amendment amount due to sorption (Rhodes et al., 2008; Rhodes et al., 2012).

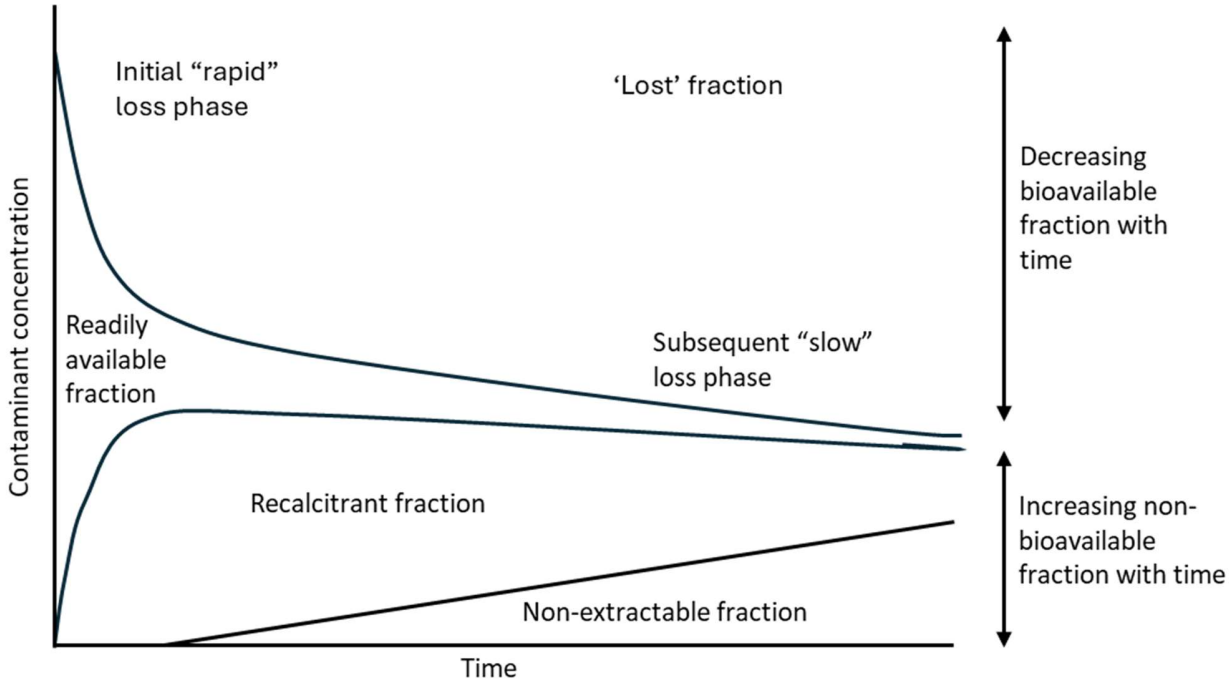


Figure 1: The impact of contact time on bioavailability and extractability of PAHs in soil (Sources: Semple et al., 2003; Riding et al., 2013).

2.1.1 Impact of soil properties and related factors on PAHs' fate and behaviour in soil

The PAHs in soils are influenced by environmental, biological, and physico-chemical processes (Umeh et al., 2017). The outcome of this influence is highly dependent on the properties of the PAHs and the soils. Figure 2 shows the fate and behaviour of PAHs in soil. PAHs in soil can volatilize, biodegrade, accumulate in organisms, transport through runoffs, sorb/bind to soil or leach down to aquifer/groundwater. Environmental factors include temperature, precipitation (rainfall), sunlight, wind, wetting and drying, and freezing and thawing. These environmental factors will determine the volatilisation, deposition, diffusion, photodegradation, aging, mixing, leaching, and runoffs of the PAHs in soils. Increase in temperature enhanced ^{14}C-phenanthrene bioavailability and mineralisation in Antarctic soils (Okere et al., 2017). Elevated temperature can enhance PAH microbial degradation through improved water availability, aqueous phase dissolution, molecular mobility, and temperature-dependent-improved microbial metabolism (Okere et al., 2017). Hence, the biodegradation of PAHs may be faster in warmer climates than in cold regions. Wetting and drying of soil increased aging and this is due to changes in the soil structure that occurred through changes in arrangement of soil particles and pores after drying (White et al., 1997). Freezing and thawing increased the naphthalene, phenanthrene, and pyrene extractability, and this is through destruction of compact soil structure and increase in soil pore size and pore water, which led to increased desorption of sorbed PAHs (Mahjoub and Gourdon, 1999; Zhao et al., 2013).

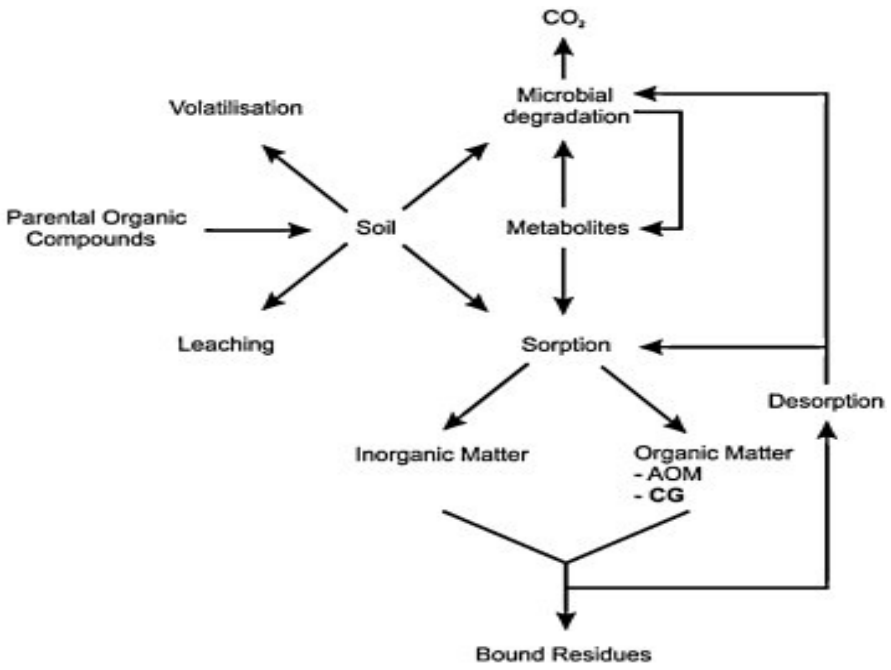


Figure 2: Fate and behaviour of PAHs in soil. AOM = amorphous organic matter; CG = carbonaceous geosorbents (Source: Semple et al., 2013)

The biological factors include the type and ability of the indigenous microbes in the soil to degrade, transform, detoxify, and catabolise the PAHs through their various enzymes, biosurfactants, and metabolic pathways (Umeh et al., 2017). This also include the ability of the available plants to accumulate and phytoremediate the PAHs, and in stimulating the rhizosphere microbes through root exudates to metabolise or transform the PAHs (Ma et al., 2010). Soil microorganisms can biodegrade PAHs in soils (Kastner and Mahro, 1996; Reid et al., 2001; Puglisi et al., 2007). Plant root exudates and biomass increases PAHs desorption and mineralisation in soil which increases with increase in exudates concentration (Gao et al., 2010; Wei et al., 2017; Vazquez-Cuevas et al., 2021). The desorption reduced with aging and the organic acids in

exudates may be responsible for the desorption effect. Malic and citric acid enhanced the desorption of ^{14}C-phenanthrene thereby enhancing bioaccessibility though there was no evidence to support their effect on biodegradation (Vazquez-Cuevas et al., 2020). The dissipation and biodegradation of PAHs in amended soils was significant in vegetated soil than non-vegetated soils indicating the influence of plants on the fate of PAHs in soils (Cheng et al., 2008; Feng et al., 2014; Oleszczuk et al., 2019).

In multifactorial consideration of PAH bioavailability in soils, soil type and soil-PAH contact time are the most significant (Wu et al., 2014). Amendment type and soil–amendment ratio are less significant though they can significantly interact with other factors and affect PAH bioavailability (Wu et al., 2013; Wu et al., 2014). This could be due to the influence of aging on PAH sequestration and sorption in soil, and the influence of soil properties. In addition, soil amendments decomposed faster in sandy soil than in clayey soil thereby showing the influence of soil type on nutrient decomposition and release (Liao et al., 2021). This could be attributed to differences in soil texture, SOM, water holding capacity, and porosity of sandy and clayey soils. The increase in pH and electrical conductivity, increased benzo(a)pyrene extractability in sandy (>55%) and clayey (>15%) soils (Meng et al., 2019). The difference could be due to a difference in soil texture and porosity as clay soil is less porous with finer soil particles. Similarly, high pH has been reported to improved phenanthrene desorption in soil due to enhanced aqueous phase solubility (Yu et al., 2016; Yu et al., 2018).

A recent study demonstrated that soil chemical and physical properties are the major soil properties affecting residual PAHs in soil (Chen et al., 2023). Studies have demonstrated that available P favoured microbial growth which could improve the microbial degradation of organic contaminants (Wu et al., 2021; Martinez-Toledo et al., 2022). The application of N and P significantly increased PAH removal (Betancur-Galvis et al., 2006; Yang et al., 2018), and the addition of nitrate and ammonium nitrogen resulted in improved PAH removal (Wang et al., 2022). Improved nutrient availability enhanced the biodegradation of 3-ring PAHs while the biodegradation >4-ring PAHs was mainly dependent on bioavailability irrespective of nutrient availability (Oyelami et al., 2013). This demonstrates the constraints caused by PAH molecular weight and ring number. Soil texture, SOM, mineral matter, redox potential, cation exchange capacity (CEC), porosity, and soil structure affect PAHs diffusion, sorption, aging, sequestration, and desorption in soils (White et al., 1997; Yang et al., 2009; Scelza et al., 2010; Zheng et al., 2012; Ren et al., 2018a). Soil structure, texture, and porosity affect contaminant diffusion within the soil matrix in relation to tortuosity. The redox potential, mineral content, and CEC of the soil chemically affect contaminants transformation in soil. PAHs sorbed to soil surface and labile matter (mineral and organic matter) are likely more susceptible to desorption, while those sorbed to non-labile matter (or that formed complex with soil minerals) are most likely to be irreversibly sorbed and can form non-extractable residues (NERs) (Yang et al., 2011). Humic and fulvic acid can sorb PAHs but humin is mainly responsible for slow PAH sorption (Yang et al., 2011). The biphasic sorption of PAHs is more pronounced in humin and this can explain why desorption reduces with aging as humin-bound PAHs may be irreversibly sorbed. This is consistent with the findings of Ukalska-Jaruga & Smreczak (2020) that humic and fulvic acid

positively influence PAH availability whereas humin affects their persistence. Sorption to humic-fulvic acid is readily reversible through desorption and these acids can modify PAHs and increase their bioaccessibility and mobility (Yang et al., 2011; Yu et al., 2015; Xie et al., 2017).

2.1.2 Impact of aging and soil microbes on PAH bioaccessibility and biodegradation

The increase in soil-PAH contact time increases aging and reduces the extractability of PAHs in soil (Macleod and Semple, 2000). Soil indigenous microbes can degrade PAHs, but aging reduces mineralisation due to reduced bioavailability (Reid et al., 2001). Figure 3 and Figure 4 show the behaviour of PAHs in soil and possible interaction with soil microbes. Soil microbes may contribute to the aging and fate of PAHs in soils as sterilisation was observed to reduce aging effect (Smidova et al., 2012). Soil microbes may contribute to the formation of NERs from PAHs in soils through the release of non-extractable metabolites as the amount of NERs was higher in nonsterile soil than in sterile soil (Macleod and Semple, 2003). NERs can also be formed by sequestering parent compounds in soil or through the incorporation of metabolites into humic acids, as humic acids can also bind PAH metabolites (Kobayashi and Sumida, 2015). Though bacteria prefer contaminants in an aqueous solution, attached/bound contaminants can be degraded by free and attached bacterial communities. Humin bound phenanthrene was degraded by two bacteria species (*Sphingobium* sp. and *Micrococcus* sp.) (Zhang et al., 2012). The bacteria could attach to humin and interact with humin-bound phenanthrene thereby eliminating bioaccessibility and desorption constraint. This could also be possible under biochar amendment considering its porous nature. HP-β-CD extraction underpredicted mineralisation in black carbon amended soils (Rhodes et al., 2008) which could be attributed to attached microbes

degrading bound contaminants which apparently were not extractable. A complete degradation of ^{13}C-pyrene was observed in compost-amended soil within 3 – 5 months (Adam et al., 2015). The remaining pyrene was found in microbial biomass as ^{13}C carbon indicating that soil microbes were responsible for the observed degradation. Similarly, actinomycetes and fungi were demonstrated to be involved in PAH degradation in contaminated soils (Bellino et al., 2019).

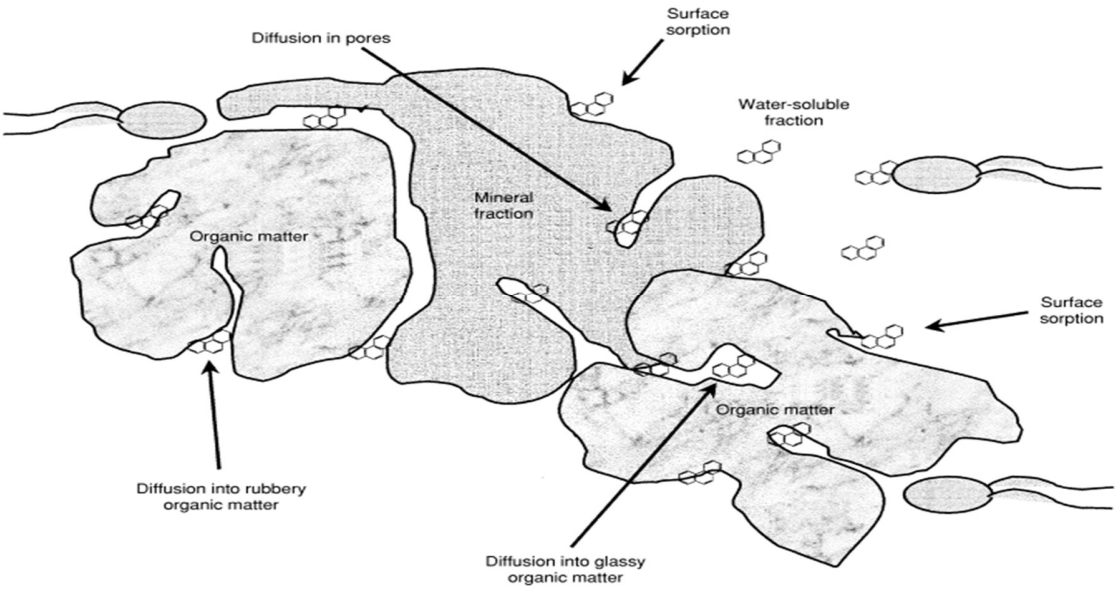


Figure 3: Behaviour of PAHs in soil showing possible sorption, sequestration, and microbial contact (Source: Semple et al., 2003)

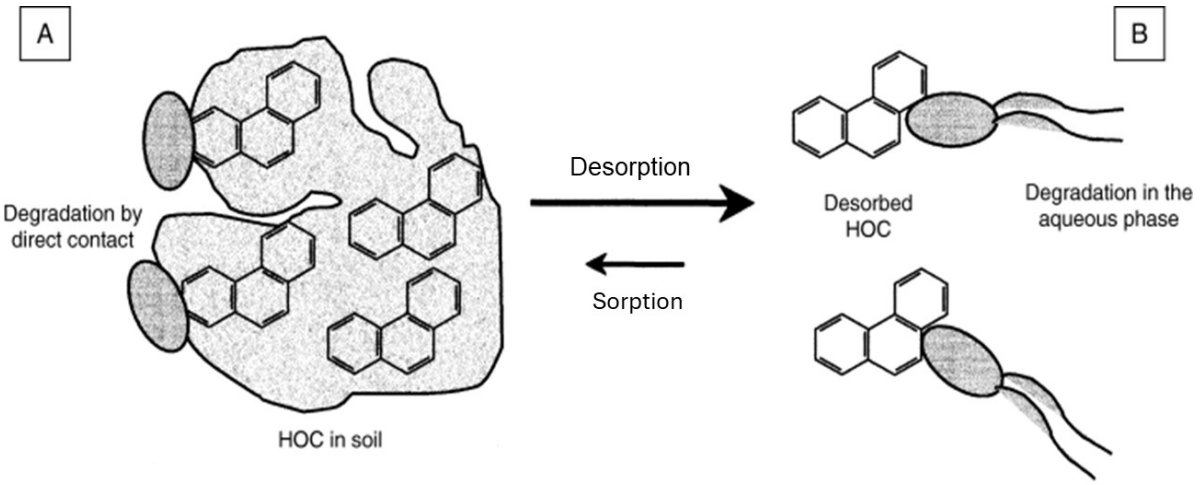


Figure 4: Microbial degradation of PAHs in soil showing A (degradation by direct contact) and B (degradation in aqueous phase) (Adapted from Semple et al., 2003)

The microbial degradation of phenanthrene has been widely investigated under both aerobic and anaerobic conditions. While complete mineralisation to CO_2 is often used as a benchmark for successful bioremediation, it represents only a fraction of the phenanthrene carbon fate in soil. Increasingly, research highlights that substantial portions of phenanthrene carbon are either assimilated into microbial biomass or converted into NERs, many of which persist in soil (Lee et al., 2009; Wang et al., 2017). Anabolic assimilation of phenanthrene into microbial biomass contributes to degradation processes but is transient, with biomass eventually decaying and turning over, as cells reach the end of their life cycle and biomass becomes necromass. In contrast, NERs (comprising bound and recalcitrant residues) are more persistent. Bound residues form through physical entrapment or covalent binding of phenanthrene or phenanthrene metabolites to soil organic matter such as humin, and this accounts for up to 70% of the parent compound (Wang et al., 2017). Some of these phenanthrene metabolites, like *cis*-phenanthrene dihydrodiol, are incorporated into stable soil matrices (Lee et al., 2009), while others form

desorption-resistant fractions that exhibit markedly reduced biodegradability even under enriched microbial conditions (White & Alexander, 1996).

The degradation of phenanthrene involves complex microbial enzymatic systems. Under aerobic conditions, initial dioxygenation at various ring positions leads to diol formation, which undergoes meta-cleavage to produce hydroxynaphthoic acids and other intermediates like trans-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid and 2,2-dicarboxychromene, which are further catabolized to salicylic acid and ultimately to CO_2 via the tricarboxylic acid (TCA) cycle (Mallick et al., 2007; Roy et al., 2012). The key enzymes for phenanthrene degradation include ring-cleavage dioxygenases (Zhang et al., 2010). In anaerobic environments, carboxylation has been proposed as the initial step, catalysed by UbiD-like carboxylase, leading to the formation of phenanthroic acid intermediates (Davidova et al., 2007; Sun et al., 2021). Additionally, multiple sulphate-reducing bacteria (SRB), including strains from the Desulfobacteraceae family and Desulfotomaculum genus, have been identified as capable of degrading phenanthrene (Himmelberg et al., 2018; Kraiselburd et al., 2019; Zhang et al., 2021). The initial step in the degradation pathway by SRB is carboxylation, producing 2-phenanthroic acid as a key intermediate (Himmelberg et al., 2018; Zhang et al., 2021). Subsequent steps involve ring reduction and cleavage, leading to the formation of benzene compounds and cyclohexane derivatives (Zhang et al., 2021). Genome analyses have identified genes encoding carboxylases and reductases potentially involved in the degradation process (Kraiselburd et al., 2019; Zhang et al., 2021).

Several microorganisms exhibit potent phenanthrene-degrading capabilities. *Enterobacter cloacae*, *Bacillus* sp., and *Bacillus thuringiensis* were shown to degrade phenanthrene into non-cytotoxic intermediates (Abdel-Razek et al., 2020). The filamentous fungus *Trichoderma* sp. CNSC-2 effectively degraded phenanthrene through the production of extracellular lignolytic enzymes, particularly laccase. The fungus was able to degrade 64% of 50 mg/L of phenanthrene in 10 d (Behera et al., 2023). Phenanthrene biodegradation by the ligninolytic fungus *Phanerochaete chrysosporium* involves oxidation to phenanthrene-9,10-quinone and ring-fission to 2,2'-diphenic acid, with incomplete mineralisation to CO_2. The fungus oxidizes phenanthrene at the C-9 and C-10 positions to form the ring-fission product 2,2'-diphenic acid (DPA), which is the major metabolite, through a pathway involving both ligninolytic and non-ligninolytic enzymes but not the extracellular lignin peroxidases (Hammel et al., 1992).

NERs include both bound residues (typically covalently attached to humic substances or physically sequestered in organic matter) and recalcitrant residues, which are inherently resistant to microbial transformation. The latter often consist of electronically stabilized or sterically hindered hydroxylated, carboxylated, or quinonoid aromatic structures. These compounds may be shielded within soil micropores or strongly sorbed to mineral surfaces like clays or iron oxides, rendering them environmentally persistent (Semple et al., 2003). Although NERs are generally considered as low risk due to limited mobility and bioavailability, under certain conditions such as microbial co-metabolism or soil disturbance, some reactivation of NERs may occur. The practical distinction between bound (reversibly sorbed) and recalcitrant (irreversibly sorbed) residues is significant, particularly for defining remediation endpoints and informing risk

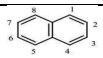
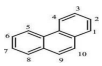
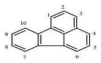
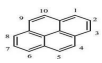
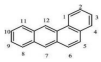
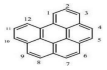
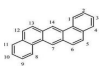
assessments. While extractable PAH concentrations may decrease, a considerable fraction may persist as NERs, whose bioavailability and ecological risk vary (Alexander, 2000; Kästner et al., 2014). This has prompted interest in incorporating NER quantification and sequestration endpoints into monitored natural attenuation (MNA) strategies. In summary, the environmental fate of phenanthrene involves a dynamic interplay of mineralisation, biomass assimilation, and NER formation. Understanding the long-term stability and ecological relevance of these residues, especially under changing environmental conditions, remains a research priority.

2.1.3 Impact of PAH properties on PAH bioaccessibility and biodegradation

The molecular weight, structure, ring number, and hydrophobicity of PAHs amongst other factors control their behaviour in soils (Riding et al., 2013). An increase in molecular weight, ring number, and complexity of structure, increases hydrophobicity and reduces mobility and biodegradation (Stroud et al., 2007; Papadopoulos et al., 2007). Therefore, complex PAHs and HMW PAHs are less susceptible to biodegradation than their simple structure and low molecular weight (LMW) counterparts. This is due to larger molecular size, low diffusion, and increased dispersion forces binding the molecules. This is consistent with the findings of Guo et al. (2020) that complex PAHs with high ring number were toxic to microbes, and showed reduced bioavailability, and biodegradation. Hydrophobicity contributes to the accumulation and persistence of PAHs in soils as an increase in hydrophobicity enhances accumulation and persistence (Baldantoni et al., 2017). It favours PAH affinity to soil organic matrix thereby increasing persistence. The catabolism of hexadecane (straight chain) and PAHs (naphthalene, phenanthrene, pyrene, and benzo[a]pyrene) as demonstrated in studies, showed that molecular structure and ring number

affects biodegradation (Stroud et al., 2007; Yuan et al., 2009). Hexadecane had the shortest lag phase (though it has a higher octanol-water coefficient; K_{ow}) while the lag phase for the PAHs increased with increase in ring number (Stroud et al., 2007). Oleszczuk and Baran (2003) demonstrated that PAHs sorption and degradation in soil is dependent on PAH properties ($logK_{ow}$ and water partition coefficient; $logK_{oc}$), soil physico-chemical properties, and soil microbial activity. Table 1 shows some PAHs and the properties influencing their hydrophobicity. PAH's bioaccessibility is inversely related to the ring number and K_{ow} (Wu et al., 2013), hence confirming hydrophobicity to affect desorption. As the ring number increases, so does the octanol-water partition coefficient (K_{ow}), leading to greater hydrophobicity. Higher hydrophobicity enhances a compound's bioaccumulation potential while reducing its desorption and likelihood of biodegradation.

Table 1: Physicochemical properties of 16 PAHs in the USEPA list of priority pollutants (Oleszczuk and Baran, 2003; Papadopoulos et al., 2007; Patel et al., 2020)

PAHs	Molecular structure	RN	MW (g/mol)	MP (^{0}C)	BP (^{0}C)	VP (mmHg)	AS (mg/L)	$t_{1/2}$ (d)	LogK_{ow}	LogK_{oc}
Naphthalene		2	128	80.26	218	0.0087	31	5.66	3.37	3.30
Acenaphthylene		2	152.2	92	265	0.0029	16.1	30.7	4.07	1.40
Acenaphthene		2	154	95	96	4.5×10^{-3}	3.8	18.77	3.98	3.85
Fluorene		2	166	118	215	3.2×10^{-3}	1.9	15.14	4.21	4.14
Phenanthrene		3	178	100	340	6.8×10^{-4}	1.1	14.97	4.46	4.36
Anthracene		3	178	218	341	1.8×10^{-6}	0.045	123	4.50	4.42
Fluoranthene		3	202	110.8	375	5.0×10^{-6}	0.26	191.4	4.90	6.38
Pyrene		4	202	156	404	2.5×10^{-6}	0.132	283.4	4.88	4.80
Benzo(a)anthracene		4	228	158	438	2.5×10^{-6}	0.011	343.8	5.63	7.30
Chrysene		4	228	253	448	6.4×10^{-9}	0.0015	343.8	5.63	3.66
Benzo(b)fluoranthene		4	252	168.3	480	5.0×10^{-7}	0.0015	284.7	6.04	5.74
Benzo(k)fluoranthene		4	252	215.7	480	9.6×10^{-11}	0.0008	284.7	6.12	5.74
Benzo(a)pyrene		5	252	179	495	5.6×10^{-9}	0.0038	421.6	6.06	8.30
Benzo(g,h,i)perylene		5	276	273	550	1.0×10^{-10}	0.00026	517.1	6.78	6.01
Indeno(1,2,3-cd)pyrene		5	276	163.6	530	10^{-10}-10^{-16}	0.0062	349.2	6.58	6.14
Dibenzo(a,h)anthracene		5	278	262	524	1.0×10^{-10}	0.0005	511.4	6.86	5.97

RN = Ring number; AS = Aqueous solubility; MP = Melting point; BP = Boiling point; $t_{1/2}$ = Half-life; MW = Molecular weight; VP = Vapour pressure; LogK_{ow} = Octanol water coefficient; LogK_{oc} = water partition coefficient.

2.1.4 Impact of PAH concentration and co-occurrence of contaminants on PAH bioaccessibility and biodegradation

Aging and contaminant concentration affects PAHs bioaccessibility in soil (Chung and Alexander, 1999; Tang et al., 1998). Low contaminant concentration will reduce bioaccessibility with time through sequestration, which is same in higher contaminant concentration, but the increase in concentration compensates for the sequestered fraction and increases percentage bioavailability. High PAHs concentration and presence of multiple PAHs can negatively affect PAHs microbial catabolism (Couling et al., 2010; Macleod and Semple, 2006). High concentration of PAHs can be toxic to catabolic microbes while presence of other contaminants can enhance or inhibit the degradation of others (Couling et al., 2010; White et al., 1999a). Degradation of ^{14}C-naphthalene was inhibited by other PAHs (^{14}C-pyrene and ^{14}C-phenanthrene) whereas present of ^{14}C-naphthalene enhanced the degradation of other contaminants (Couling et al., 2010). Introduction of anthracene and pyrene in contaminated aged soil enhanced the removal of aged phenanthrene (White et al., 1999a; White et al., 1999b). This could be through sorption displacement of aged/sorbed PAHs by fresh PAH (Wang et al., 2005). The simultaneous existence of two PAHs can stimulate their biodegradation as phenanthrene and pyrene degraded faster together than singly (Yuan et al., 2009). The presence of phenanthrene was observed to enhance the biodegradation of pyrene in unamended soil through co-metabolism and this effect increased with biochar amendment (Li et al., 2021).

Although high pollutant level affects microbial activity, low initial concentration of PAH may result in low removal rate as low removal rate of naphthalene was observed due to a lower initial

concentration (Guo et al., 2020). This may imply that very high PAH concentration can disrupt microbial activity, but a given level of concentration is needed to achieve good bioavailability and to stimulate the microbes to mineralise the contaminant. Therefore, for effective mineralisation, the contaminant concentration may be required to be high enough to stimulate microbial degradation but low enough to circumvent toxicity. The presence of metals (Cu, Al, Zn at 50 mg/kg and 500 mg/kg) increased the extractability of ^{14}C-phenanthrene in soil, and affected the efficacy of HP-β-CD in predicting mineralisation (Obuekwe and Semple, 2013). This implies that potential toxic elements (PTEs) can increase the mobility of PAHs in soil which can lead to overestimation of biodegradation using NEETs. On the contrary, a review by Saeedi et al. (2020) indicated that PTEs enhance PAHs sorption and repress desorption and extractability. Hence, further studies are necessary to understand the impact of PTEs on PAHs bioavailability and biodegradation in soil.

2.2 Compost and spent mushroom compost (SMC) as an amendment for PAH-contaminated soil.

2.2.1 Compost as an amendment to PAH – contaminated soil.

Compost (rich in organic matter and nutrients) is a product of controlled aerobic decomposition of organic material (Bernal et al., 2017; Ejileugha, 2022). The use of compost in contaminated soil is effective for soil bioremediation through stimulating microbial activities and enhancing the breakdown of organic contaminants and mitigation of toxicity (Gandolfi et al., 2010). Compost application improved soil structure and nutrients, which resulted in improved microbial community, reduced ecotoxicity, and enhanced biodegradation of a 4 – ring PAH in soil (Gandolfi

et al., 2010). The amended soil showed 10.4% higher PAHs reduction than the control. High soil-to-compost ratio with high pollutant level may limit microbial activity and nutrient hence low rate of PAHs removal (Guo et al., 2020). However, a very high compost level can cause a shift in microbial nutrient choice. Compost from varying feedstocks reduced PAH concentration in soils and mitigated PAH transfer to vegetables (Attanayake et al., 2015). The reduction in PAH concentration was by biodegradation which resulted in safer vegetables with lower toxicity risk. Similarly, compost improved the biodegradation of PAHs in soil with a higher level of degradation on LMW – PAHs compared to HMW – PAHs (Baldantoni et al., 2017). The difference in degradation level can be attributed to the constraints of ring number, structure, and molecular weight of PAHs. The amended soil had 8.0% higher reduction in PAH concentration than the control. The ecotoxicity and bioaccessibility of PAHs in soil were reduced by compost application (Bari et al., 2010). The reduction improved as soil – PAH contact time (aging) increased, and the reduction in ecotoxicity can be explained by the reduction in bioaccessibility. This shows the impact of aging on PAH bioaccessibility and ecotoxicity in soil. At higher PAH level (150 mg/kg), there was no significant difference in bioavailability reduction under lower (10 tha^{-1}) and higher (30 tha^{-1}) compost application, which indicates that high compost amount may not be necessary in heavily contaminated soil. However, the higher compost amount significantly reduced PAH bioavailability than the lower amount after 20 d. Additionally, the impact of compost amendment on PAH – contaminated soil may be influenced by compost stability as a stable compost was 52% better than a less stable compost in improving PAH biodegradation in soil (Sayara et al., 2010). However, this was soil composting remediation and may not represent the impact of compost application in PAH contaminated soil.

Irrespective of soil type, compost amendment resulted in >90% PAH removal in spiked soil through improving desorption and biodegradation (Wu et al., 2013). The compost type and applied amount had minimal influence on PAH removal but the impact of their interaction with soil type and contact time, on PAH removal was significant. Therefore, it is necessary to understand the independent impact of contact time and interaction with soil type or amendment, in the bioremediation of PAH contaminated soil. Degradation of 3-ring PAH was >94% in spiked soil but 6-ring PAH was 45% in spiked soil, 17% in coal tar, and 8% in coal ash (Wu et al., 2013). This indicates that findings using artificially spiked soils in laboratory experiments may not represent the outcomes in field aged soil. Compost can sorb PAHs in soil reducing the bioavailability, but degradation is also enhanced due to microbes in compost promoting PAH degradation (Puglisi et al., 2007). There was no significance difference in bioavailability reduction when two compost amounts (0.38% and 1.15%) were compared, therefore, high compost amount may not be necessary in reducing PAH bioavailability or enhancing biodegradation in soil. After a 240 d incubation, the bioavailable fraction of phenanthrene was 30% in 0.38% amended soil, 29% in 1.15% amended soil, and 45% in the control. The aged fraction was 62% in 0.38% amended soil, 63% in 1.15% amended soil, and 37% in the control. This shows that compost application increases aging and reduces bioavailability but there may be no long-term benefit of higher compost amount. The addition of 10% compost resulted in a 10 – fold increase in PAH sorption and a 2 – fold increase in PAH biodegradation (Sigmund et al., 2018). Overall, compost can sorb PAHs, but the impact of sorption is offset by humic acid – like substances in compost which enhance desorption and stimulate microbial degradation of PAHs (Puglisi et al., 2007; Wu et al., 2013). Additionally, 10% compost application was better than 25% and 50% for PAH

dissipation in vegetated soil (Feng et al., 2014). However, this was to an extent, attributable to the PAHs in the sludge compost which increased the PAH concentration in the soil at higher compost to soil ratio.

2.2.2 SMC as an amendment to PAH – contaminated soil

The management of SMC is a challenge and there is growing suggestions for re-use as soil amendment, animal feed, biofertilizer, and biocontrol agent (Leong et al., 2022). SMC as a cheaper compost alternative, possesses similar properties as compost (Umor et al., 2021), therefore it may have a similar impact on PAH – contaminated soil. SMC contains relevant properties (nitrogen, organic matter, carbonates) and can be applied to soil to mitigate soil degradation and improve soil quality (Becher et al., 2021). The use of SMC at 0.5 – 8% application was demonstrated to improve the nitrogen and organic carbon content of degraded soil making it a suitable amendment for the restoration of degraded soils (Gümüş & Şeker, 2016). Using SMC at 29 t/ha improved micronutrients (Fe, Zn, and Cu) and the percentage of sucrose in sugar beet when compared to chemical fertilizer and sheep manure (Ahmadpoor Dehkordi et al., 2019). A recent study demonstrated that SMC can improve soil nutrients (N, organic carbon, P), enzyme activities, increase microbial diversity, and cause a shift to beneficial microbial groups in soil (Huang et al., 2023). Though at a very high application amount (40% and 30%), SMC improved soil pH (from 4.3 to 6.8), CEC, and soil nutrients (Asemoloye et al., 2020). The amendment amount is too high and may be difficult to manage in real-life situations, but the study did demonstrate the potential of SMC as a soil amendment.

Several studies have demonstrated that SMC is a suitable amendment for improving crop growth and yield (e.g., Jonathan et al., 2011; Vahid Afagh et al., 2019; Ma et al., 2021; Zubkova & Vinogradov, 2023; Idowu et al., 2023). Nevertheless, there are limited studies on the impact of SMC on the biodegradation and dissipation of PAHs in soils. An earlier study demonstrated that SMC contains degradative enzymes, macronutrients, and microbial population which enhances total petroleum hydrocarbon (TPH) degradation in soil (Chiu et al., 2009). Similar studies demonstrated that SMC could stimulate soil indigenous bacteria and can also enhance the removal of PAHs in soil (García-Delgado, D'Annibale, et al., 2015; García-Delgado, Yunta, et al., 2015). As a cheaper compost alternative, SMC was effective in the dissipation of LMW PAHs in soil (Gasecka et al., 2012). Fresh SMC is more effective than air-dried SMC for dissipation of PAHs in soil because active enzymes and nutrients in fresh SMC are high and abundant thereby promoting microbial activities and degradation of PAHs (Zhou et al., 2020). Extracts (containing laccase) from SMC degraded PAHs which further supports the impact of fresh SMC on PAH degradation (Xuanzhen et al., 2010). An earlier study demonstrated the effectiveness of SMC and SMC – associated enzymes as a stimulant for the degradation of PAHs (Lau et al., 2003). This study by Lau et al. (2003) conducted at a temperature of 80°C led to a complete degradation of all PAHs (naphthalene, benzo[a]pyrene, phenanthrene, and benzo[g,h,i]perylene). This study showed the impact of temperature and SMC – associated enzymes on PAH degradation, but such temperature condition will be challenging and expensive to maintain under real-world conditions.

2.3 Biochar as an amendment to PAH – contaminated soil

Biochar is a carbon-rich material produced through biomass pyrolysis (Ejileugha, 2022). Biochar is a soil conditioner as it improves the physico-chemical properties of soil and has a beneficial interaction with soil microbes (Piscitelli et al., 2019). Biochar improves soil physico-chemical properties, crop yield, soil microbial activities, and prevents nutrient leaching (Ahmad et al., 2014; Kaur and Sharma, 2019; Kaur and Sharma, 2020; Zhang et al., 2020; Kaur and Sharma, 2021). This is possible due to the high organic carbon content of biochar, its hydrophobic nature, and its high surface area. The pH (neutral to alkaline) of biochar is favourable for biodegradation and can bring a liming effect in acidic soils (Ahmad et al., 2014; Liu et al., 2018). Biochar has a beneficial impact on soil properties and this can be maintained for a long period after first time application (Giagnoni et al., 2019). Biochar's impact on soil microbes influences the bacterial community at the genus level while the fungal community is affected at genus, phylum, and class levels (Zhang et al., 2020). This shows that biochar influences soil microbial community structure and may have a greater impact on soil fungal community. This is important as the fungal community in a recent study, significantly correlated with PAH degradation in soil (Wu et al., 2023). Zhang et al. (2020) reported that biochar had limited or negative impact on fungal richness but may enhance fungal evenness in PAH contaminated soil. In contrast, they may enhance bacterial richness but reduce bacterial evenness (Zhang et al., 2020).

Biochar application improved PAH degradation through stimulating the growth/activity of PAH degraders by increasing PAH – degradation gene copies and altering the microbial community structure in soil (Liu et al., 2015; Song et al., 2017). Biochar reduced the concentration of heavy

PAHs in contaminated soil with a far greater effect than compost (Beesley et al., 2010). Biochar increased soil available nutrients, increased PAH adsorption, reduced bioconcentration factor, and improved plant height and biomass (Kaur & Sharma, 2020). The application of biochar to crude oil contaminated soil improved soil enzymatic activities, microbial diversity, microbial respiration, and showed a degradation efficiency that was 34% better than the control (Saeed et al., 2021). Biochar applied at 2 – 10% improved soil properties, microbial activity, and the biodegradation of benzo(a)pyrene by 11.9 – 63.1% compared to the control (Guo et al., 2021). Similarly, Zhang et al. (2020) reported that biochar from different feedstocks, enhanced the biodegradation of PAHs of varying ring numbers by 3.0 – 60.3%. A meta-analysis by Li et al. (2023) showed that biochar application reduced total PAH concentration by 15.4%, the C-free PAHs by 55.6%, and the bioaccessible PAHs by 46.5%. A long-term field and laboratory experiment showed biochar to significantly reduce C-free PAHs, hence suggesting biochar as a safe soil amendment for reducing the bioaccessibility of PAHs in soil (Oleszczuk et al., 2016; Zielinska & Oleszczuk, 2016b).

Immobilizing microbes or enzymes on biochar enhances the efficacy of biochar for PAH removal (Omoni et al., 2020b; Imam et al., 2021). Biochar improved organic content, available P and K in the soil, and synergistically accelerated PAH biodegradation in association with immobilized PAH-degrading microbes (Guo et al., 2022). Biochar also improved soil health and synergistically elevated PAH removal together with bound PAH degrading bacterium (Song et al., 2021). Biochar functioned in synergy with inoculated PAHs-degrading microbes to enhance PAHs mineralisation (Li et al., 2021). Immobilized enzyme (laccase) on biochar, greatly enhanced the mineralisation

of anthracene (Imam et al., 2021). In immobilizing microbes on biochar for PAH removal in soil, biochar binds the PAHs and the attached microbes accelerate the PAH degradation (Xiong et al., 2017).

2.3.1 Impact of soil and PAH properties on PAH bioaccessibility and biodegradation in biochar amended soil

Biochar can immobilise microbes and contaminants in biochar micro and nanopores, and create effective contact time between microbes and contaminants leading to biodegradation (Jaafar et al., 2015). The molecular size and structure of the PAHs also affect sorption to biochar. Biochar reduced the dissipation of LMW and MMW PAHs but increased that of HMW PAHs (Zhang et al., 2021). The LMW and MMW could absorb tightly to biochar micropores thereby reducing their bioavailability. Despite the high hydrophobicity and $LogK_{ow}$ of HMW PAHs which aid their binding affinity to biochar, their dissipation increased as they bound mostly on the biochar surface which made them more accessible to microbial degradation. Therefore, the use of biochar and immobilized PAH-degrading microbes may be effective for the biodegradation of HMW PAHs and reduce biodegradation constraints caused by complex structure and ring number. A similar study assumed the effect of biochar on PAHs to be molecular weight dependent because phenanthrene removal was impeded while benzo[a]pyrene removal improved (Cao et al., 2016). Biochar reduced the bioaccessibility, bioaccumulation, and phytotoxicity of PAHs in soil, and improved soil health and fertility (Oleszczuk et al., 2017). The 5 – 6 ring PAHs were more reduced than the 2 – ring PAH. This was attributed to the high affinity of HMW PAHs to carbonaceous materials and the possible leaching of LMW PAHs from the biochar. Similar finding was reported by Zhang

et al. (2020) with higher degradation rates for 4 – 6 ring PAHs than 2 – 3 ring PAHs. These findings demonstrate that biochar enhances the biodegradation and sequestration of PAHs with a possible greater impact on HMW PAHs.

The SOM is an important factor in biochar soil amendment (Kong et al., 2021). In soil with low SOM, biochar acts mainly by adsorption of PAHs but in soil with high SOM biochar improves biodegradation (by improving soil properties and microbial activity) in addition to sorption (Kong et al., 2021). Repeated biochar application, aging, and high clay content suppress the effect of biochar on enhancing PAH sorption (Kumari et al., 2014). The enhanced effect of biochar positively correlated with soil organic carbon (SOC) and negatively correlated with soil clay content (Kumari et al., 2014). Dissolved organic matter (DOM) in soils can affect biochar sorption efficiency by coating biochar surface and reducing contaminant accessibility to biochar sorption sites and pores (Jaafar et al., 2015; Marchal et al., 2013).

2.3.2 Impact of biochar pyrolysis condition on PAH bioaccessibility and biodegradation in biochar amended soil

The property of a biochar depends on the feedstock and pyrolysis conditions such as temperature, retention time, and heating rate (Semple et al., 2013, Ejileugha, 2022). Biochar can sorb PAHs but biochar pyrolyzed at high temperature (HT) shows a better sorption efficiency than low temperature (LT) biochar (Chen and Chen, 2009; Chen et al., 2012; Ogbonnaya et al., 2016; Janus et al., 2020; Ding et al., 2021a; Liu et al., 2021). Biochar produced at HT (900 – 1000℃ for 1 h) showed better sorption efficiency than the one from LT (400 – 500℃ for 16 – 18 h)

(Ogbonnaya et al., 2016). This is due to the high surface area and high porosity of the HT – biochar. Biochar (wheat straw) produced at LT (100 – 400℃) decreased phenanthrene degradation while those produced at HT (500 – 700℃) increased phenanthrene degradation (Ding et al., 2021a). This is due to a greater improved effect of the HT – biochar DOC on the soil DOC which improves PAH – degraders in soil. Phenanthrene desorption and extractible fraction decreased with increasing pyrolysis temperature which can be attributed to increased surface area and porosity in the HT – biochar (Ding et al., 2021a). The LT – biochar may contain some bound contaminants which are toxic to microbes as HT reduces certain contaminants in biochar during production (Freddo et al., 2012; Tomczyk et al., 2020; Godlewska et al., 2021; Mosko et al., 2021). The toxicity of sewage sludge-derived biochar reduced with an increase in pyrolysis temperature (Tomczyk et al., 2020). Those produced at 600℃ and 700℃ were less toxic to organisms and improved soil properties compared to 500℃.

Different plant feedstocks were pyrolyzed for biochar production at 250℃, 400℃, and 600℃ for 4 h to examine the effect of biochar pyrolysis temperature on its impact on soil N, P, and K (Zhang et al., 2017). The biochar affected soil N, P, and K which was dependent on biochar pyrolysis temperature. The soil available N decreased while the P and K increased with an increase in the biochar pyrolysis temperature. The reduction in available N could be linked to increase in microbial biomass due to biochar application which led to higher N utilization. It could also be that the N was strongly sorbed to biochar reducing the available fraction. Carbon and ash content of biochar increase with increasing pyrolysis temperature and the ash content of biochar may affect PAH dissipation in soil (Chen et al., 2019a). Sorption capacity of biochar varies based on

feedstock as corn straw was influenced by surface functional group while rice husk was influenced by surface area, surface functional groups, and ash content (Chen et al., 2019a). Biochar ash content may affect its effect in the soil as there was a negative correlation between residual PAHs (3 – 6 rings) and biochar ash content in soils amended with biochar (Zhang et al., 2020). Chicken manure biochar effectively sorbed naphthalene and the sorption effect was better using biochar produced at 700℃ when compared to 300℃ and 500℃ (Liu et al., 2021). This is attributed to the high pore volume and increased surface area of the HT – pyrolyzed biochar. Biochar produced at HT and LT reduced PAH accumulation in tubers, roots, shoots, and vegetables (Khan et al., 2013; Ni et al., 2017; Zhu et al., 2018). Through sorption, biochar reduced PAH bioavailability and plant accumulation (Zhu et al., 2018). The HT - pyrolyzed biochar had better immobilizing efficiency than those produced at low temperatures. Biochar produced at 700℃ mitigated PAH accumulation mainly through immobilization while that produced at 300℃ reduced PAH accumulation mainly by stimulating biodegradation (Ni et al., 2017; Ni et al., 2018). Similarly, biochar pyrolyzed at 600℃ was better in immobilizing PAHs while biochar at 300℃ was better in improving soil microbial diversity (Song et al., 2017). In contrast, biochar produced at 100 – 400℃ showed poor mineralisation due to low PAH-degraders and low bioavailability while biochar produced at 500 – 700℃ showed improved PAH mineralisation through improved sorption, PAH degraders, and dissolved organic carbon (Ding et al., 2021b). Therefore, the impact of LT-biochar on PAH mineralisation requires further assessment. Despite the higher sorption capacity of HT-biochar, PAH mineralisation can still be higher due to improved soil nutrients, biochar facilitated transfer of water and nutrients into biochar pores, and sorbed PAHs could still

be available to soil microbes especially microbes attached to biochar surface and pores (Zhang et al., 2020).

Biochar from sawdust and wheat straw accelerated PAH biodegradation through improving soil microbial activity and soil properties (Kong et al., 2018). The impact of biochar feedstock was insignificant, but pyrolysis temperature had a significant effect as biochar produced at 500℃ initiated better biostimulation than those produced at 300℃. Wheat straw-derived biochar produced at 400℃ and 700℃ reduced phenanthrene rapidly desorbing concentration by 44.8% and 92.5% respectively (Ding et al., 2021a). Mineralisation was higher in soil amended with biochar pyrolyzed at 700℃ than in 400℃, because rapidly desorbing and slowly desorbing phenanthrene was degraded in 700℃ biochar amended soil, while only the rapidly desorbing phenanthrene was degraded in 400℃ biochar amended soil. This indicates that HT-biochar can improve the degradation of reversibly biochar bound PAHs due to improved soil properties aiding the interaction of soil microbes with bound PAHs, while LT-biochar could only improve the degradation of bioavailable PAH fractions. Phenanthrene and microbial biomass were concentrated on the biochar separated from the soil, hence showing that sorbed PAH can be degraded by attached microbial community. Overall, biochar pyrolyzed at ≥500℃ can be regarded as HT – biochar while those pyrolyzed at ≤500℃ can be regarded as LT – biochar according to information in the literature. While it is generally accepted that HT-biochar has high sorption efficiency, the impact of HT-biochar and LT-biochar on the mineralisation of PAHs of different molecular size require further research.

2.3.3 Impact of biochar amount and particle size on PAH bioaccessibility and biodegradation

The influence of biochar on PAHs in soil is mainly through sorption and immobilization, and indirectly through improving soil properties and microbial activities. Biochar can sorb PAHs; this sorption improves with an increase in biochar application amount, though at high application rates (≥10%), the sorption effect can be sabotaged by biochar-induced toxicity (Bielska et al., 2018). The level of extraction and mineralisation of PAHs in soil decreased with an increase in contact time and biochar application amount which can be related to contaminant sorption and reduced bioaccessibility (Rhodes et al., 2008; Rhodes et al., 2012; Oyelami et al., 2014; Ogbonnaya et al., 2016). The sorption effect of biochar produced at 300℃ improves above 0.5% application while that for biochar produced between 400 – 700℃ improves above 0.1% application (Chen & Yuan, 2011). A biochar amount >1% will affect PAHs extraction and mineralisation in soils by reducing bioaccessible and bioavailable fractions (Rhodes et al., 2008; Bielska et al., 2018). Though high biochar amounts lead to reduced biodegradation, it is not a serious concern as non-bioaccessible contaminants cannot initiate toxicity. However, it has been argued that low bioavailability does not imply no toxicity (Andersson et al., 2009), and bound PAHs could become remobilised and available overtime due to environmental changes and soil disturbance. The use of <1% biochar in soil amendment is effective and can prevent certain toxic effects of biochar on organisms caused by high pH, salinity, and bound contaminants in biochar (Godlewska et al., 2021). A 0.01 – 0.2% biochar was more effective than 0.5 – 1.0% biochar amendment in both enhanced and non-enhanced microcosms during ^{14}C – phenanthrene mineralisation (Omoni et al., 2020b). Therefore, high biochar may not be necessary for enhancing PAHs mineralisation in soils. Low biochar amount below 1%, will enhance PAHs biodegradation

and circumvent biochar – related soil toxicity (Rhodes et al., 2008; Godlewska et al., 2021). Biochar effect on reducing mineralisation and extractible concentration of ^{14}C – naphthalene increased with rise in application amount and biochar produced at HT had greater effect (Ogbonnaya et al., 2016). In addition, black carbon amount above 0.5% affects the effectiveness of HP-β-CD extraction for predicting possible mineralisation leading to underprediction (Rhodes et al., 2008; Rhodes et al., 2012). Therefore, the effectiveness of NEETs for predicting PAHs bioaccessiility and related risks may be jeopardized under high biochar application leading to poor risk assessment or estimation of sequestration endpoint.

Biochar particle size may be a factor in PAHs sorption due to particle number and surface area. Biochar particle size has effect on the sorption efficiency of biochar as biochar with finer particles had significantly greater sorption efficiency (Kang et al., 2018). Fine particle biochar reduced leaching and mobility of PAHs and the sorption effect was better for HMW PAHs than LMW PAHs (Zand & Grathwohl, 2016). Similarly, granular and pulverized biochar could bind PAHs and reduce their mobility and leaching in soil but pulverised biochar have a better effect (Zand & Grathwohl, 2016). Fine biochar particle (≤0.5mm) was better than other particle sizes (0.5 – 1.0 mm and 1.0 – 2.0 mm) in improving soil P, pH, and microbial diversity and this was aided by incubation temperature of 25℃ as opposed to 15℃ (Sarfraz et al., 2020). Greater mineralisation and extractability were reported in 3 – 7 mm particles biochar compared to ≤2 mm particles biochar (Ogbonnaya et al., 2014a; Ogbonnaya et al., 2014b). In contrast, Kang et al. (2019) reported greater phenanthrene desorption in fine particles biochar compared to coarse particles. This discrepancy in findings required further assessment.

2.4 Biochar and compost (SMC) mixture/blends as an amendment to PAH – contaminated soil

Studies have demonstrated the beneficial effects of biochar and compost amendment on soil health and fertility (e.g., Gandolfi et al., 2010; Ahmad et al., 2014; Zhang et al., 2020; Kaur and Sharma, 2021). Biochar and compost can improve soil bacterial and fungal diversity and soil nutrient content hence enlarging the soil microbial network and nutrient (Yan et al., 2021; Yan et al., 2022). Since both amendments can improve soil properties, their combination may give a more beneficial impact than their single use. Mixing biochar with compost mitigates the low nutrient challenges in biochar and also improves and extends the agronomic benefits of compost (Liao et al., 2021). SMC in combination with other ameliorants was promising in the biodegradation of PAHs in soil (Russo et al., 2012; Liu et al., 2019; Omoni et al., 2020a). It promoted the biodegradation of phenanthrene and PAHs containing 5 – 6 rings. Though there was no significant difference in the removal of TPH in amended soils, SMC – biochar amended soils had a significant impact on the biodegradation of HMW – PAHs, microbial abundance, and bioavailable PAH concentrations (Atai et al., 2023a). The effect on microbial abundance was high on fungi, possibly due to the fungi remnants in SMC. In similar study by Atai et al. (2023b), SMC, biochar, and SMC – biochar improved TPH biodegradation in saline and non-saline soil, and soil amended with a combination of biochar and SMC showed substantial impacts in non-saline soil. Biochar and biochar – compost suppressed plant parasitic nematodes in soil and this effect was enhanced under biochar-compost (Cao et al., 2018). Biochar – compost outperformed biochar and had a lasting effect on soil quality which was similar to that with compost (D'Hose et al.,

2020). Biochar – compost mix in soil, enhanced the efficient use of nutrients (N and P) in soil than biochar or compost making it more stable and efficient for long – term release of nutrients (Liao et al., 2021).

Biochar – compost combination significantly improved soil nutrient and soil quality with a better effect on plants when compared to biochar or compost (Ghosh et al., 2015). Biochar – compost mix was better than biochar in improving soil properties (Al-Omran et al., 2019; Al-Omran et al., 2021). Biochar – compost blend had better effect on available nutrients and carbon sequestration compared to compost or biochar, and plant yield is close to that of mineral fertiliser though cautious consideration of feedstock is important to avoid soil contamination (Oldfield et al., 2018). Application of compost – biochar led to a 100 – fold increase in PAHs sorption and a 10 – fold decrease in biodegradation when compared to compost addition at 10 – fold increased sorption and 2 – fold increased biodegradation (Sigmund et al., 2018). There was still PAHs biodegradation despite the high sorption efficiency of the biochar. Biochar – compost combination was more effective than the use of biochar or compost, reducing the toxicity and concentration of pyrene in contaminated soil (Bielska et al., 2017). Biochar and compost was screened for the removal of 2,2',4,4'-tetrabrominated diphenyl ether (BDE-47) in contaminated soil (Xiang et al., 2019). Biochar (applied singly) reduced BDE-47 bioavailability but was not effective in BDE-47 biodegradation while compost enhanced the biodegradation of BDE-47. A combination of both enhanced biodegradation, reduced plant BDE-47 uptake, and reduced soil extractible fraction (bioavailability) of BDE-47. This implies that biochar – compost combination

could mitigate toxicity and enhance bioremediation of contaminated soils better than a single use of biochar or compost.

However, the conclusion from a short term field experiment stated that the application of biochar – compost mixture did not improve crop yield and soil properties as expected and recommended long term study of the effects and best possible application/combination techniques (Doan et al., 2021). A combination of biochar and compost can decrease availability of nutrients when compared to compost only treatments (Doan et al., 2021). This could be due to the impact of biochar on available nutrient through sorption. Similarly, compost only application had better total N and available P than biochar and their mixture, while biochar had higher available P than biochar – compost mixture (Hannet et al., 2021). Therefore, determining the appropriate blending ratio is crucial for optimizing the effectiveness of the biochar–compost blend, ensuring it aligns with the intended application or desired outcomes. A case by case consideration is needful in the use of biochar and biochar – compost as soil amendment as the behaviour of the contaminants and soil properties may affect the impact of the amendment (Prodana et al., 2019).

2.5 Concerns on compost and biochar application in soil

2.5.1 Concerns on the use of compost as amendment to PAH – contaminated soil

It is crucial to understand the source of compost feedstock and level of contaminants as a contaminated feedstock is likely to yield a contaminated compost. Compost from several feedstocks were reported to contain LMW PAHs, HMW PAHs, polychlorinated biphenyls (PCBs),

and PTEs (Barcauskaite et al., 2020). The PAHs contamination was caused by feedstocks as among sewage sludge, mixed municipal waste, and green waste composts, the most contaminated was mixed municipal waste compost and the least contaminated was green waste compost. Similar study had earlier demonstrated that municipal waste compost had higher PAHs than green waste compost (Sadej & Namiotko, 2010). Sewage sludge compost can still contain high levels of PAHs (Feng et la., 2014). Compost can leach PTEs in soil and the leachability of the PTEs depends on the compost feedstock (Paradelo et al., 2017). The more contaminated the compost feedstock the higher the possibility of PTEs leaching. Different manures for land application were found to contain antibiotics, antibiotic resistant genes (ARGs), and PTEs (Xue et al., 2021). ARGs were more persistent than antibiotics, Cu and Zn had higher concentration than other PTEs. Certain organic pollutants sequester in sewage sludge compost and the concentration and abundance of ARGs have been reported to increase after composting (Lu et al., 2021). However, the use of additives such as biochar, can reduce the level of contaminants and bioavailable concentrations in compost (Ejileugha, 2022; Ejileugha et al., 2024).

2.5.2 Concerns on the use of biochar as amendment to PAH – contaminated soil

Biochar may contain PAHs, and the enhanced adsorptive property of biochar may aid PAHs persistence in soil. The application of biochar to soil may lead to initial increase in PAHs level which decreases with contact time (Kusmierz et al., 2016; Rombolà et al., 2019). The feedstock, pyrolysis temperature, contaminants, pH, and electrical conductivity are major contributors of biochar ecotoxicity (Godlewska et al., 2021). The bioavailable and total concentrations of PAHs in biochar can differ based on production conditions, as biochar produced under slow pyrolysis

generally have lower PAH concentrations (Hale et al., 2012). Soil that received biochar for 3 years had higher PAHs concentration than control and biochar addition did not reduce leaching at all times (Quilliam et al., 2013). Rather, biochar addition reduced PAH catabolism due to increased sorption and reduced bioavailability.

The degree and behaviour of biochar for contaminant sorption depend on the biochar properties and current state of weathering (Semple et al., 2013). Aging reduces the bioavailability and toxicity of biochar – bound PAHs and biochar produced at HT possess better sorption efficiency (Zielinska & Oleszczuk, 2016a; Oleszczuk & Koltowski, 2018; Zhu et al., 2018; Liu & Fan, 2022). Aging also reduced the sorption efficiency of biochar through the formation of oxygen containing polar moieties on biochar surface (Huang et al., 2020). Aging biochar through physical and chemical processes reduces the distribution of PAHs especially LMW PAHs, and biochar surface area and pore volume significantly affect PAHs distribution in biochar (Liu & Fan, 2022). Aging biochar under the sun and rain prior to agricultural application will help reduce PAHs contamination from biochar application in soil (Khalid & Klarup, 2015). Reduction in the biochar application amount can help mitigate the negative impact of contaminated biochar in soil (Kong et al., 2019; Godlewska et al., 2021). Biochar applied to soil undergoes aging in soil which leads to reduction in sorption efficiency with time. This reduced sorption efficiency may equal unamended soil within 2.5 years soil – biochar contact time (Ren et al., 2018b).

Biochar pyrolyzed at HT reduced microbial population and microbial enzyme in aged PAHs contaminated soil which correlated with the high aromatic C content in the biochar (Zhang et al.,

2018). Though it reduced to baseline after 105 days, application of biochar to soil elevated the soil – PAHs concentration and the PAHs could migrate few centimetres down the soil horizon (Kusmierz et al., 2016). A study identified volatile organic compounds (VOCs) content in biochar as a concern and recommended their inclusion in assessing biochar quality (Buss et al., 2015). Despite the report on the removal of organic pollutants in biochar at high pyrolysis temperature, a review reported that biochar produced at 750℃ can have high PAHs content (Buss et al., 2016). A similar article demonstrated that sewage sludge biochar produced at 700℃ contain more PAHs leachate than those produced at around 300℃ (Chen et al., 2019b). Feedstock and pyrolysis temperature influences PAHs in biochar and high pyrolysis temperature (500 – 700℃) can lead to the formation of toxic PAHs derivatives (Krzyszczak et al., 2021). Pyrolysis of sewage sludge increased the level of trace elements in biochar compared to the biosolid (Zielinska & Oleszczuk, 2015). Aqueous extract from a biochar amended soil contained PTEs and 16 USEPA priority PAHs at concentration within EU regulation for surface water (Bastos et al., 2014). This represents a risk of biochar application in soil to aquatic lives. Sewage sludge derived biochar had negative effect on wheat growth which correlated with the PTEs content of the biochar (Kong et al., 2019). Root exudates encourage PAHs release from biochar in soil and this increase transport to vegetables posing potential human cancer risks (Wang et al., 2018). These findings have shown that while biochar can improve soil properties and remediation, it should be cautiously applied to prevent soil contamination. Biochar should be screened for possible contaminants before soil application, and biochar feedstock should be carefully selected to reduce the concentration of contaminants in biochar. This is important to avoid toxicity swapping.

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Chapter 3 Effect of phenanthrene soil contact time and amendment amount on microbial mineralisation of freshly added ^{14}C–phenanthrene in soil slurry systems

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3.1 Abstract

Biochar and spent mushroom compost (SMC) can be used as amendments to reduce the adverse impacts of PAHs in soil. However, the effectiveness of the amendments is influenced by contact time and amendment amount. This study investigated the impact of increasing amount of biochar and SMC (0%, 0.1%, 0.5%, 1.0%, and 10.0%) on ^{14}C-phenanthrene mineralisation in soil at increasing soil-PAH contact time (1, 25, 50, 75, and 100 d). The results showed that both amendments enhanced ^{14}C-phenanthrene mineralisation in soil, although this effect diminished over time. The impact of increasing contact time and amendment amount on reducing mineralisation was higher in biochar amended soils. All amended soils showed shorter lag phases, faster rates, and higher extents of ^{14}C-phenanthrene mineralisation than control except biochar at 10%. There was no significant ($p > 0.05$) difference in lag phases, rates, and extents of mineralisation between SMC and biochar amendment at 0.1% and 0.5%, but SMC amendment showed shorter lag phases, faster rates, and higher extents of mineralisation than biochar amendment at 1.0% and 10.0%. The influence of contact time and interaction with amendment amount on the mineralisation kinetics were notably greater than that of the amendment amount itself. These findings emphasize the importance of accounting for both the independent and combined effects of contact time and amount when assessing the long – term effectiveness of amendments in PAH-contaminated soils.

Keywords: aging, biochar, biodegradation, lag phase, polycyclic aromatic hydrocarbon, spent mushroom compost

3.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of hydrophobic organic contaminants (HOCs) that are released into the environment mainly through the incomplete combustion of fossil fuels (Lawal, 2017). They are of concern because of their putative teratogenic, genotoxic, and carcinogenic effects. Due to the hydrophobic properties of PAHs and their affinity to soil organic matter, and as such, soil acts as a major sink for PAHs in the environment (Okere and Semple, 2012; Patel et al., 2020). The interactions between PAHs and soil and its components can affect their bioaccessibility through aging, sorption, and sequestration (Riding et al., 2013; Semple et al., 2013; Umeh et al., 2017). The indigenous microbes in the soil can catabolise PAHs, but an increase in soil-PAHs contact time will decrease mineralisation due to reduced bioaccessibility, as a result of sorption and sequestration (Reid et al., 2001; Macleod and Semple, 2000; Macleod and Semple, 2002; Macleod and Semple, 2003). However, microbial degradation may be a limiting factor in soil containing recalcitrant higher molecular weight PAHs, due to their hydrophobicity, structural complexity, and molecular size (Huesemann et al., 2002; Huesemann et al., 2003; Huesemann et al., 2004).

Biochar and compost are soil conditioners used to improve soil health and fertility (Kästner and Miltner, 2016; Baldantoni et al., 2017; Xiang et al., 2019; Saeed et al., 2021; Kaur & Sharma, 2021). They are also utilized for the amendment/restoration of degraded soils (Alkharabsheh et al., 2023), and soil containing HOCs and/or potential toxic elements (Gonzaga et al., 2020; Medynska-Juraszek et al., 2020; Patel et al., 2022). Biochar acts mainly by sorption of HOCs and improving soil microbial community structure, by promoting the proliferation of beneficial

microbial groups (Guo et al., 2017; Hameed et al., 2020), while compost acts mainly by improving soil nutrients and introducing beneficial hydrocarbonoclastic microorganisms (Puglisi et al., 2007; Kästner and Miltner, 2016). The use of organic amendments to stimulate microbial degradation of PAHs in soil is environmentally friendly, and it has been demonstrated to be beneficial for the remediation of soils contaminated by PAHs (Rhodes et al., 2008; Gandolfi et al., 2010; Ogbonnaya et al., 2016; Baldantoni et al., 2017; Omoni et al., 2020a; Omoni et al., 2020b; Guo et al., 2021; Guo et al., 2022).

An estimated 3 million tonnes of spent mushroom compost (SMC) is generated as waste by commercial mushroom industries in Europe (Horizon 2020, 2019), and its management and disposal are a concern. SMC can be used as an amendment for improving soil properties and restoring degraded soil (Gümüş & Şeker, 2017; Rajavat et al., 2022). The amendment of soil with SMC for agricultural purposes produced improved agronomic benefits in plants (Afagh et al., 2019; Chaudhary et al., 2022). SMC contains nutrients though not at the same level as compost as some of its nutrients have been utilised by the fungi (Umor et al., 2021). It contains important nutrients (nitrogen, phosphorus, organic matter, and carbonates) which can improve soil health and fertility (Becher et al., 2021; Huang et al., 2023). Additionally, SMC contains enzymes, nutrients, and microbes which can enhance the degradation of PAHs in soil (Chiu et al., 2009; García-Delgado et al., 2015).

The bioavailability of PAHs in soil decreases with an increase in soil-PAH contact time due to the aging effect (Riding et al., 2013; Semple et al., 2013; Duan et al., 2015). The decrease in bioavailability, therefore, reduces the possibility of significant levels of biodegradation occurring,

even where there are suitable microbial degraders present in the soil (Semple et al., 2006; Allan et al., 2007; Oyelami et al., 2013). Therefore, greater attention is needed to understand the extent to which soil-PAH contact time reduces biodegradation, particularly in comparison to the impact of organic amendments on stimulating PAH biodegradation in soil, under varying amendment amount. The optimisation of organic amendment application for managing PAH-contaminated soil is crucial due to the interplay between amendment amount and soil-PAH contact time. Excessive amendment amounts can be costly, labour-intensive, and impractical for large-scale applications. Conversely, insufficient amendment levels may fail to enhance biodegradation effectively. Striking the right balance is essential to ensure cost-effectiveness, minimize resource wastage, and avoid detrimental alterations to soil properties, enabling efficient remediation strategies. Therefore, this study was carried out to investigate (a) the impact of soil-phenanthrene contact duration on phenanthrene mineralisation in soil, (b) the effect of amount and type of amendment on phenanthrene mineralisation in soil, (c) the interaction of contact time and amendment amount on phenanthrene mineralisation in soil. This is relevant because these factors influence phenanthrene bioavailability and biodegradation which are important for the management and risk assessment of phenanthrene-contaminated soil.

3.3 Material and methods

3.3.1 Collection of samples

The soil samples were collected at a depth of 5 – 20 cm from Cockerham Green Energy at Hillam Farm, Cockerham, Lancaster, United Kingdom. The soil was air-dried, homogenized by sieving through 2 mm mesh (to remove stones, debris, plant roots, and large particles), and stored at 4

°C in the dark until needed (Macleod and Semple, 2006; Couling et al., 2010; Omoni et al., 2020b). The SMC was collected from Drinkwater Mushrooms Limited, Lancaster, United Kingdom. It was stored at 4 °C in the dark until when needed. Biochar was purchased from SoilFixers, Royal Wootton Bassett, United Kingdom. The properties of the biochar as given by the manufacturer are shown in Table 1, and the properties of the soil and SMC are shown in Table 2.

Table 1: Biochar properties and pyrolysis conditions as provided by the supplier (SoilFixers, UK).

Parameter	Value
Feedstock	Hardwood logs
Pyrolysis equipment	Retort Kiln
Pyrolysis temperature	700 – 900 °C
Pyrolysis duration	5 – 12 h
Particle size	0 – 8 mm
pH	8.8 – 10
Volatiles	9 – 15%
Ash max.	3 – 6%
Moisture max.	8 – 12%
C Fixed	>76%

Table 2: Properties of the soil and SMC used for this study. Values are in mean ± SD

Parameters	Soil	SMC
Sand (%)	71.4	-
Silt (%)	26.2	-
Clay (%)	2.4	-
Soil texture	Sandy loam	-
pH	7.3±0.0	7.4±0.1
Electrical conductivity	382±4.4 (μScm^{-1})	6.8±0.1 ($mScm^{-1}$)
Organic matter (%)	6.5±0.3	62.5±4.2
C:N	10.4±0.1	13.3±0.5
Total carbon (mg/kg)	102.4±4.4	566.4±35.7
Total organic carbon (mg/kg)	98.9±3.6	523.3±32.6
Inorganic carbon (mg/kg)	3.6±1.0	43.0±2.9
Ammonium nitrogen (mg/kg)	0.4±0.0	12.8±1.1
Nitrate nitrogen (mg/kg)	3.6±0.2	0.2±0.0

3.3.2 Soil spiking and organic amendment levels

Soil (100 g dry weight; n = 3) was spiked in triplicate with ^{12}C–phenanthrene at a concentration of 100 mg/kg (dry weight) at field moisture content (30%). Approximately one-quarter of the soil was initially spiked using acetone as the carrier solvent at a solvent-to-soil ratio of 1:20 (v/w) and left to vent in a fume hood for 2 h to allow solvent volatilisation. The remaining three-quarters

of the soil was then added in three portions and thoroughly mixed to ensure homogeneity (Reid et al., 2001; Doick et al., 2003; Semple et al., 2006). The biochar and SMC amendments were then added to the soil at 10%, 1%, 0.5%, 0.1%, and 0% (dry wt) respectively and mixed thoroughly and subsequently incubated in the dark at 21 ± 2 °C for 100 d. The soils were sampled at 1 d, 25 d, 50 d, 75 d, and 100 d respectively, to determine ^{14}C-phenanthrene catabolism (Macleod & Semple, 2006; Couling et al., 2010, Omoni et al., 2020b). In this study, only the ^{12}C-phenanthrene was aged in soil and contact time as used in this study refers to ^{12}C-phenanthrene aging and not ^{14}C-phenanthrene.

3.3.3 Measurement of the mineralisation of ^{14}C–phenanthrene in soil

The measurement of ^{14}C–phenanthrene in soil was carried out in respirometers (Reid et al., 2001; Doick et al., 2003; Macleod & Semple, 2006; Allan et al., 2007; Couling et al., 2010; Omoni et al., 2020a). At each sample time point, soil (10 ± 0.2 g) from each soil treatment condition was weighed into a clean Schott bottle, and 30 ml of sterile minimal basal salt (MBS) was added to form a slurry. The contents of the MBS have been listed by Ogbonnaya et al. (2014). The soil was then spiked with 9-^{14}C–phenanthrene (0.045 kBq/g) and a 7 ml glass vial containing 1 ml 1M NaOH (for trapping $^{14}CO_2$) was immediately attached to the bottle cap using the crocodile clip. The respirometer was tightly closed and subsequently incubated at 21 ± 2 °C on a rotary shaker (at 100 RPM) for 14 d. Each respirometer was sampled at 3 h intervals for the first 9 h and then sampled every 24 h for 14 d. At each sampling, the 7 ml vial was removed and replaced with a fresh one. Then, 5 ml of Goldstar scintillating fluid was added to the sampled vial and the vial was

stored in the dark (to prevent the effect of chemiluminescence) for 24 hours before counting. The stored vials were analysed using a Liquid Scintillation Counter (LSC; Canberra Packard Tri-Carb2250CA). The effect of the amendments on the catabolism of ^{14}C–phenanthrene was assessed by measuring the lag phases (time taken to achieve 5% mineralisation), the fastest rates of mineralisation (highest $^{14}CO_2$ evolved in an hour), and cumulative extents of mineralisation after 14 days of incubation.

3.3.4 Enumeration of phenanthrene degrading microbes

The phenanthrene degrading microbes were enumerated using freshly prepared sterile MBS medium solidified with 15 g/l agar (Omoni et al., 2020a, b). Two separate 500 ml Erlenmeyer flasks were used to prepare MBS media for enumerating phenanthrene degrading bacteria and fungi, respectively. The media were autoclaved and enriched with 50 mg/l ^{12}C–phenanthrene. Penicillin-Streptomycin-Glutamine (5 µl/ml) and Amphotericin-B (5 µl/ml) were added to specific ^{12}C-phenanthrene enriched MBS media to inhibit bacteria and fungi, respectively. The media were poured into sterile Petri dishes and allowed to solidify. A 100 µL of a serially diluted sample (10^{-5}) of the soil was used to inoculate the respective plates (by spread plate technique) and incubated at 25 ± 2 °C for 2 – 7 days. Control agar plates (n = 3) with no sample inoculation were incubated simultaneously with the inoculated plates to ensure the observed growth is from the inoculated samples and not due to contamination. Colonies were counted and recorded as colony-forming units per gram (CFU/g).

3.3.5 Statistical analysis

Data was analysed using multivariate analysis of variance (MANOVA), univariate ANOVAs, Tukey's HSD post hoc, Pearson correlation, Hotelling's T^2 test, and independent sample t-test. The mineralisation parameters were calculated using MS Excel and data analysis was done using SPSS (IBM SPSS 27.0). Data plots were done using SigmaPlot.

3.4 Results

3.4.1 Impact of SMC on ^{14}C-phenanthrene mineralisation in soil

The changes in lag phases, fastest rates, extents of ^{14}C-phenanthrene mineralisation, and phenanthrene degrading microbial numbers over time in the amended soils were monitored (Figure 1; Table 3). The contact time (^{12}C-phenanthrene aging) and amendment amount significantly ($p < 0.05$) influenced the changes observed in lag phases, fastest rates, and extents of mineralisation. Results showed that the contact time, amendment amount, and their interaction had significant Wilk's Lambda ($p < 0.01$) and partial Eta squared (η_p^2) values of 0.825, 0.391, and 0.669 respectively. Furthermore, ANOVA on lag phases, rates, and extents of mineralisation revealed significant effects ($p < 0.05$) of contact time on lag phases, rates, and extents of mineralisation. The amendment amount had a significant ($p < 0.05$) impact on the extents of mineralisation but a non-significant ($p > 0.05$) effect on lag phases and rates. In addition, the interaction between contact time and amendment amount showed a significant effect ($p < 0.05$) on lag phases, rates, and extents of mineralisation.

Table 3: Respirometry data for SMC amended soils showing lag phases, fastest rates, extents of mineralisation, and microbial numbers for phenanthrene (Phe) degraders. Values are in mean ± SD (n = 3). Values in columns followed by different letters are statistically different (Tukey's HSD; n = 3; $p < 0.05$); also indicated are shorter lag phases (+), faster rates (*), higher extents of mineralisation (-), and higher microbial numbers (#) than the controls that are not statistically significant ($p > 0.05$)

Contact time (d)	SMC amount (%)	Lag phase (h)	Fastest rate (%$^{14}CO_2$/h)	Extent of mineralisation (%)	Phe Degraders (Log_{10}CFU/g)	
					Bacteria	Fungi
1	0.0	40.6±0.4^{a}	1.1±0.2^{a}	44.1±0.4^{a}	8.4±0.2^{a}	8.6±0.1^{a}
	0.1	38.3±3.3^{a+}	1.0±0.3^{a}	49.3±0.5^{b}	8.5±0.2$^{a\#}$	8.5±0.3^{a}
	0.5	38.8±0.4^{a+}	1.2±0.2^{a*}	46.1±1.1^{a-}	8.3±0.2^{a}	8.5±0.2^{a}
	1.0	38.0±2.5^{a+}	1.2±0.2^{a*}	49.5±1.3^{b}	8.4±0.4^{a}	8.6±0.3^{a}
	10.0	41.2±3.4^{a}	1.3±0.0^{a*}	45.6±1.5^{a-}	8.7±0.3$^{a\#}$	8.8±0.1$^{a\#}$
25	0.0	56.0±11.5^{a}	0.6±0.1^{a}	35.3±1.3^{a}	8.5±0.3^{a}	8.5±0.3^{a}
	0.1	40.2±1.5^{b}	0.5±0.0^{a}	45.8±0.6^{b}	8.5±0.1^{a}	8.3±0.1^{a}
	0.5	38.5±0.8^{b}	0.4±0.1^{a}	46.7±0.9^{b}	8.7±0.2$^{a\#}$	7.9±0.5^{a}
	1.0	40.2±3.6^{b}	0.5±0.1^{a}	49.4±1.1^{c}	8.5±0.4^{a}	8.5±0.2^{a}
	10.0	47.4±3.2^{c}	0.5±0.1^{a}	38.9±0.5^{a-}	8.5±0.4^{a}	8.5±0.1^{a}
50	0.0	42.0±1.1^{a}	0.7±0.2^{a}	41.3±0.7^{a}	9.1±0.0^{a}	8.8±0.1^{a}
	0.1	43.2±0.9^{a}	0.5±0.1^{b}	41.4±0.4^{a-}	8.9±0.1^{a}	8.8±0.1^{a}
	0.5	47.6±2.4^{a}	0.5±0.1^{b}	36.3±0.7^{c}	8.8±0.1^{a}	8.8±0.2^{a}
	1.0	32.5±1.7^{b}	0.8±0.1^{a*}	50.3±0.8^{b}	9.0±0.1^{a}	8.8±0.1^{a}
	10.0	35.4±0.8^{b}	0.4±0.2^{b}	48.5±0.8^{b}	8.9±0.1^{a}	8.8±0.0^{a}
75	0.0	50.3±2.9^{a}	0.4±0.0^{a}	35.5±1.4^{a}	7.5±0.2^{a}	7.8±0.2^{a}
	0.1	45.5±3.2^{a+}	0.7±0.1^{b}	40.4±2.3^{b}	7.9±0.3$^{a\#}$	7.9±0.2$^{a\#}$
	0.5	38.8±0.7^{b}	0.7±0.2^{b}	46.1±0.4^{c}	8.1±0.2$^{a\#}$	8.0±0.3$^{a\#}$
	1.0	48.6±2.2^{a+}	0.4±0.2^{a}	37.7±0.8^{a-}	8.1±0.2$^{a\#}$	7.8±0.1^{a}
	10.0	48.7±2.2^{a+}	0.5±0.0^{a*}	36.1±1.8^{a-}	8.0±0.0$^{a\#}$	7.3±0.2^{a}
100	0.0	50.6±1.9^{a}	0.9±0.1^{a}	34.3±1.1^{a}	8.8±0.1^{a}	7.3±0.3^{a}
	0.1	63.7±5.8^{b}	0.6±0.1^{b}	27.6±1.8^{b}	8.8±0.1^{a}	7.5±0.2$^{a\#}$
	0.5	61.2±7.2^{b}	0.5±0.2^{b}	26.1±0.5^{b}	8.8±0.1^{a}	7.6±0.1$^{a\#}$
	1.0	58.6±7.5^{b}	0.6±0.1^{b}	30.8±3.7^{b}	8.8±0.2^{a}	7.4±0.2$^{a\#}$
	10.0	60.7±5.5^{b}	0.6±0.3^{b}	30.0±3.0^{b}	8.8±0.1^{a}	7.1±0.2^{a}

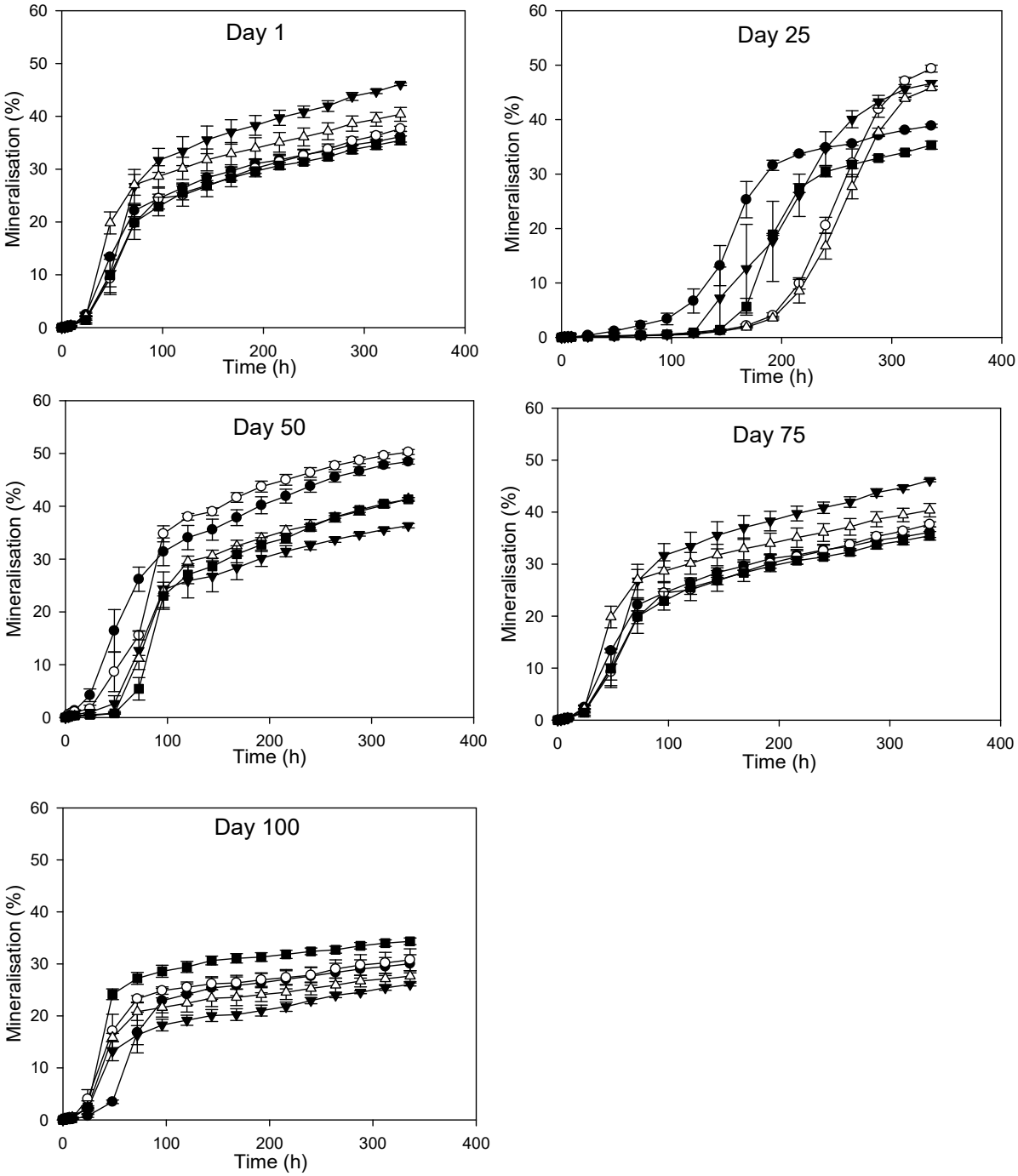

Figure 1: Extents of mineralisation in SMC amended soils at 1 d, 25 d, 50 d, 75 d, and 100 d. Amendment amount is represented as ●(10%), ○(1%), ▲(0.5%), △(0.1%), and ■(0%; control) respectively. Data points are plotted as mean (n = 3) with error bars showing the standard deviation (SD).

3.4.1.1 Impact of SMC amendment on lag phases

The lag phases in the SMC amended soils were monitored at 1 – 100 d (Table 3). The effect of contact time on lag phases was significant ($p < 0.01$) indicating that contact time influenced the changes in lag phases observed in the SMC soil treatments (Table 3). The lag phases ranged from approximately 38 – 41 h at 1 d and 51 – 64 h at 100 d. The effect of SMC amount on lag phases was not significant ($p > 0.05$) revealing that, if the impact of contact time was ignored, the amendment amount is highly unlikely to cause the changes observed in lag phases (Table 3). For instance, the lag phases at 1 d, with minimal impact from contact time, ranged from 38 – 41 h, while it was 33 – 48 h at 50 d, and 51 – 64 h at 100 d (Table 3). Significantly different ($p < 0.05$) lag phases were observed in the amended soils from 25 – 75 d. All amended soils had shorter lag phases than the control at 25 d. However, 1.0% amendment had shorter lag phases than the control from 1 – 75 d. The effect of the interaction of contact time and SMC amount on lag phases was significant ($p < 0.05$) revealing that the effect of contact time on lag phases differed across treatment levels. There was no significant difference ($p > 0.05$) in lag phases in the amended soils and control at 1 d , but the control had significantly ($p < 0.05$) shorter lag phases than the amended soils at 140 d (Table 3).

3.4.1.2 Rates of mineralisation in SMC amended soils.

The rates of mineralisation were monitored from 1 – 100 d (Table 3). The contact time significantly influenced the rates ($p < 0.05$) observed in the SMC soil treatments. The rates at 1 d were significantly faster ($p < 0.05$) than at 25 – 100 d (Table 3). All soil treatment conditions had

significantly ($p < 0.05$) faster rates at 1 d compared to 25 – 100 d. The difference between the rates at 25 – 100 d was not statistically significant ($p > 0.05$). The effect of SMC amount on rates was not significant ($p > 0.05$) revealing that, if the impact of contact time was kept constant, it was highly unlikely for amendment amount to cause the changes observed in rates. There was no significant difference ($p > 0.05$) in rates across amended soils at 1 d, 25 d, and 140 d. Furthermore, the interaction of contact time and SMC amount on rates was significant ($p < 0.05$) revealing that the effect of contact time on rates differed across treatment levels. There was no significant difference ($p > 0.05$) in rates across all soil treatment conditions at 1 – 25 d. There was significant difference ($p < 0.05$) in rates from 50 – 100 d, with the control showing a significantly ($p < 0.05$) higher faster rates than all amended soils at 100 d.

3.4.1.3 Extents of mineralisation in SMC amended soils

The SMC amended soils were monitored at 1 – 100 d for changes in the extents of mineralisation (Table 3; Figure 1). Noticeably, the extents of mineralisation reduced over time. The effect of contact time on extents of mineralisation was significant ($p < 0.01$) which indicated that contact time substantially influenced the changes in the extents of mineralisation in SMC soil treatments (Table 3, Figure 1). The extents of mineralisation at 1 d were significantly higher ($p < 0.05$) than at 25 – 100 d (Table 3, Figure 1). In addition, the SMC amount significantly ($p < 0.05$) impacted the changes observed in extents of mineralisation. Overall, amended soils showed higher extents of mineralisation than the control from 1 – 75 d, and the 1.0% SMC amendment showed a higher extents of mineralisation than other levels of treatment (Table 3, Figure 1). The extents of mineralisation in 0.1% and 0.5% SMC amounts were higher than 10.0% at 1 d, 25 d, and 75 d,

while the extents of mineralisation in 10% were higher at 50 d and 100 d. The effect of the interaction of contact time and SMC amount on extents of mineralisation was significant ($p < 0.05$) showing that the effect of contact time on extents of mineralisation varied across amendment amount. At 1 d, 1.0% amendment showed higher ($p < 0.05$) extents of mineralisation than other treatment levels except for 0.1% ($p > 0.05$). At 100 d, the control showed a higher ($p < 0.05$) extents of mineralisation than all amended soils revealing the impact of contact time on the extents of mineralisation. Overall, at 100 d, the control showed shorter lag phases, faster rates, and higher extents of mineralisation thereby showing the relationship between the extents of mineralisation, lag phases, and rates (Table 3; Figure 1). This relationship is further demonstrated by the strong correlation as lag phases correlated with the extents of mineralisation in 0.1% ($r = -0.95$, $p < 0.001$), 0.5% ($r = -0.95$, $p < 0.001$), 1.0% ($r = -0.72$, $p < 0.01$), and 10.0% ($r = -0.99$, $p < 0.01$). The fastest rates did not correlate with the extents of mineralisation in SMC amended soils. Generally, 1.0% amendment showed shorter lag phases, faster rates, and higher extents of ^{14}C-phenanthrene mineralisation.

3.4.1.4 Phenanthrene degrading microbial numbers in SMC amended soils

The phenanthrene degrading microbial numbers were monitored at 1 – 100 d to examine their relationship with the mineralisation parameters (Table 3). There was no significant difference ($p > 0.05$) in the phenanthrene degrading microbial numbers in all soil treatment conditions from 1 – 100 d. The fastest rates did not correlate with the phenanthrene degrading microbial numbers. The bacteria numbers did not correlate with the extents of mineralisation but correlated with the lag phases ($r = -0.56$, $p < 0.05$) and fastest rates ($r = 0.62$, $p < 0.05$) in soil amended at 0.5%. Lag phases correlated with fungal numbers in soils amended at 0.1% ($r = -0.71$,

$p < 0.01$), 1.0% ($r = -0.95$, $p < 0.001$), and 10.0% ($r = -0.71$, $p < 0.01$). Also, the extents of mineralisation correlated with the fungal numbers in soils amended at 0.1% ($r = 0.67$, $p < 0.01$), 1.0% ($r = 0.95$, $p < 0.001$), and 10.0% ($r = 0.80$, $p < 0.001$).

3.4.2 Impact of biochar amendment on ^{14}C-phenanthrene mineralisation in soil

The temporal changes in lag phases, fastest rates, extents of ^{14}C-phenanthrene mineralisation, and ^{14}C-phenanthrene degrading microbial numbers were monitored (Table 4; Figure 2). The results showed that the contact time (^{12}C-phenanthrene aging) and amendment amount significantly ($p < 0.05$) affected lag phases, fastest rates, and extents of mineralisation. The contact time, amendment amount, and their interaction had a significant ($p < 0.01$) Wilk's lambda and partial Eta squared (η_p^2) value of 0.891, 0.626, and 0.694 respectively. Furthermore, ANOVA on lag phases, rates, and extents of mineralisation revealed significant effects ($p < 0.05$) of contact time and amendment amounts on lag phases, rates, and extents of mineralisation. The interaction between contact time and amendment amount showed a significant effect ($p < 0.05$) on lag phases, rates, and extents of mineralisation.

Table 4: Respirometry data for biochar amended soils showing lag phase, fastest rate, extent of mineralisation, and microbial numbers for phenanthrene (Phe) degraders. Values are in mean ± SD (n = 3). Values in columns followed by different letters are statistically different (Tukey's HSD; n = 3; $p < 0.05$); also indicated are shorter lag phases (+), faster rates (*), higher extents of mineralisation (-), and higher microbial numbers (#) than the controls that are not statistically significant ($p > 0.05$)

Contact time (d)	Biochar amount (%)	Lag phase (h)	Fastest rate (%$^{14}CO_2$/h)	Extent of mineralisation (%)	Phe Degraders (Log_{10}CFU/g)	
					Bacteria	Fungi
1	0.0	40.6±0.4^{a}	1.1±0.2^{a}	44.1±0.4^{a}	8.4±0.2^{a}	8.6±0.1^{a}
	0.1	37.8±3.4^{a+}	1.2±0.0^{a*}	50.6±3.0^{b}	8.7±0.1$^{a\#}$	8.7±0.2$^{a\#}$
	0.5	35.9±0.8^{a+}	1.2±0.2^{a*}	50.3±0.6^{b}	8.6±0.1$^{a\#}$	8.5±0.1^{a}
	1.0	40.4±3.8^{a+}	1.0±0.2^{a}	47.9±1.0^{b}	8.7±0.3$^{a\#}$	8.7±0.2$^{a\#}$
	10.0	47.2±4.0^{b}	0.7±0.2^{b}	38.6±1.6^{c}	8.8±0.2$^{a\#}$	8.8±0.2$^{a\#}$
25	0.0	56.0±11.5^{a}	0.6±0.1^{a}	35.3±1.3^{a}	8.5±0.3^{a}	8.5±0.3^{a}
	0.1	48.1±2.7^{b}	0.4±0.1^{a}	40.7±0.9^{b}	8.6±0.5$^{a\#}$	8.6±0.3$^{a\#}$
	0.5	41.8±2.1^{b}	0.4±0.3^{a}	43.8±0.6^{c}	8.6±0.0$^{a\#}$	8.5±0.4^{a}
	1.0	44.1±1.3^{b}	0.3±0.1^{a}	40.4±1.1^{b}	8.6±0.3$^{a\#}$	8.6±0.2$^{a\#}$
	10.0	45.8±0.8^{b}	0.4±0.3^{a}	38.6±0.7^{b}	8.7±0.2$^{a\#}$	8.6±0.2$^{a\#}$
50	0.0	42.1±1.1^{a}	0.7±0.2^{a}	41.3±0.7^{a}	9.1±0.0^{a}	8.8±0.1^{a}
	0.1	41.5±0.5^{a+}	0.4±0.2^{b}	41.2±0.4^{a}	9.0±0.1^{a}	8.8±0.1^{a}
	0.5	40.9±0.4^{a+}	0.6±0.1^{a}	42.3±0.1^{a-}	9.0±0.1^{a}	8.8±0.0^{a}
	1.0	42.6±12.0^{a+}	0.2±0.0^{b}	35.6±1.0^{b}	8.9±0.1^{a}	8.7±0.2^{a}
	10.0	48.3±0.8^{b}	0.3±0.1^{b}	35.9±1.0^{b}	9.0±0.1^{a}	8.7±0.2^{a}
75	0.0	50.3±2.9^{a}	0.4±0.0^{a}	35.5±1.4^{a}	7.5±0.2^{a}	7.8±0.2^{a}
	0.1	37.1±0.6^{c}	1.0±0.1^{b}	48.5±1.3^{b}	7.7±0.2$^{a\#}$	7.8±0.2^{a}
	0.5	42.1±1.1^{b}	0.6±0.1^{a*}	42.6±0.8^{c}	7.9±0.2$^{a\#}$	7.8±0.3^{a}
	1.0	44.0±1.2^{b}	0.8±0.0^{b}	40.4±1.3^{c}	8.0±0.2$^{a\#}$	8.0±0.1$^{a\#}$
	10.0	50.5±0.3^{a}	0.6±0.1^{a*}	34.6±0.6^{a}	7.8±0.4$^{a\#}$	7.7±0.1^{a}
100	0.0	50.6±1.9^{a}	0.9±0.1^{a}	34.3±1.1^{a}	8.8±0.1^{a}	7.3±0.3^{a}
	0.1	65.9±2.1^{b}	0.5±0.1^{b}	27.6±0.9^{b}	8.8±0.1^{a}	7.4±0.6$^{a\#}$
	0.5	66.5±3.1^{b}	0.3±0.1^{b}	26.7±0.6^{b}	8.9±0.0$^{a\#}$	7.5±0.1$^{a\#}$
	1.0	61.9±9.8^{b}	0.4±0.1^{b}	27.1±1.1^{b}	8.8±0.0^{a}	7.6±0.1$^{a\#}$
	10.0	91.6±7.2^{c}	0.2±0.1^{b}	18.6±1.7^{c}	8.9±0.1$^{a\#}$	7.6±0.5$^{a\#}$

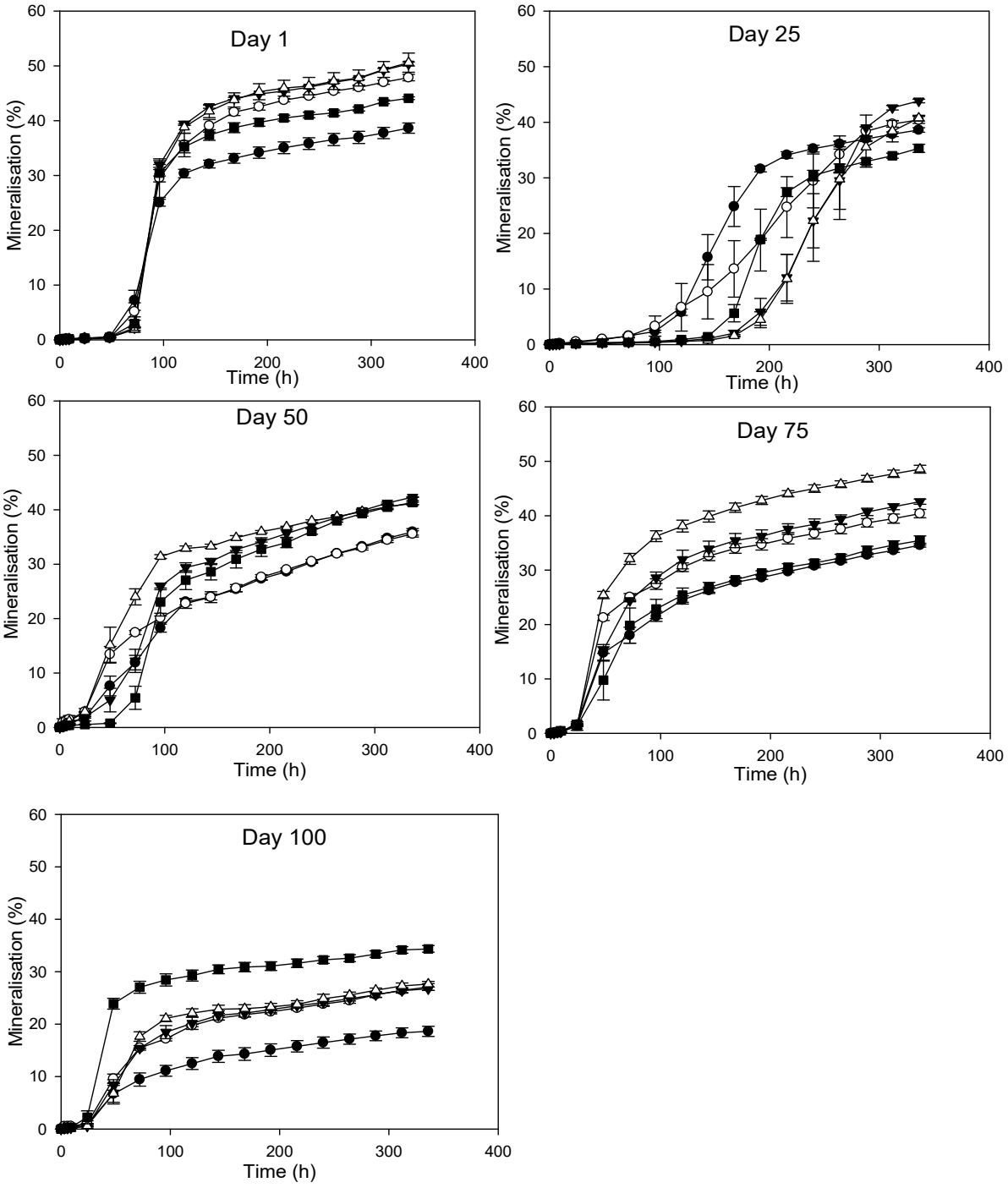

Figure 2: Extents of mineralisation in biochar amended soils on 1d, 25d, 50d, 75d, and 100d. Amendment amount is represented as ●(10%), ○(1%), ▲(0.5%), △(0.1%), and ■(0%; control) respectively. Data points are plotted as mean (n = 3) with error bars showing the standard deviation (SD).

3.4.2.1 Effect of biochar amendment on lag phases

The lag phase in biochar amended soils were monitored from 1 – 100 d (Table 4). The changes in lag phases were significantly ($p < 0.01$) influenced by contact time. The lag phases at 100 d were significantly ($p < 0.05$) longer than 1 – 75 d (Table 4). This was similar to the results observed under SMC amendments. The effect of biochar amount on lag phases was significant ($p < 0.05$) which revealed that biochar amount affected lag phases. The lag phases at 10.0% biochar amendment were longer than in other soil treatment conditions (Table 4). Additionally, the effect of the interaction of contact time and biochar amounts on lag phases was significant ($p < 0.05$) which showed that the effect of contact time on lag phases varied with the amount of amendment. However, this did not follow a specific pattern. The lag phases in 10% biochar amendment were significantly ($p < 0.05$) longer than other treatment conditions at 1 d, 50 d, and 140 d (Table 4). All amended soils showed longer ($p < 0.05$) lag phases than the control at 100 d, while 10.0% biochar amendment showed longer lag phases ($p < 0.05$) than other levels of treatments (Table 4). This was similar but longer than the lag phases observed at 100 d in SMC amended soils.

3.4.2.2 Effect of biochar amendment on rates of mineralisation

The rates of ^{14}C-phenanthrene mineralisation were monitored in biochar amended soils at 1 – 100 d (Table 4). The effect of contact time on rates was significant ($p < 0.05$) indicating that contact time influenced the changes in rates observed in the biochar amended soils. The rates at 1 d were significantly faster ($p < 0.05$) than at 25 – 100 d, and this is similar to the result observed in SMC amendment (Table 3). Furthermore, the effect of biochar amount on rates was significant ($p < 0.05$) revealing that biochar amount influenced the changes observed in rates. Overall, the

rates at 0.1% were faster than in other amended soils (Table 4). The interaction of contact time and biochar amendments had a significant ($p < 0.05$) impact on the rates of mineralisation. The rates at 10.0% biochar amendment were slower ($p < 0.05$) than the rates in other soil treatment conditions at 1 d (Table 4). There was no significant difference ($p > 0.05$) in the rates across amended soils and control at 25 d. At 100 d, the rates in control were faster ($p < 0.05$) than in all amended soils (Table 4).

3.4.2.3 Effect of biochar amendment on the extents of mineralisation

Noticeably, the extents of mineralisation decreased over time (1 – 100 d) in biochar amended soils (Table 4; Figure 2). Contact time significantly ($p < 0.05$) influenced the changes in the extents of mineralisation observed in the biochar soil treatments (Figure 2, Table 4). Significantly ($p < 0.05$) lower extents of mineralisation were observed at 100 d, and significantly ($p < 0.05$) higher extents of mineralisation were observed at 1 d. Similar trends at 1 d and 100 d was observed in SMC amended soils. Biochar amount significantly ($p < 0.05$) affected the extents of mineralisation. All amended soils and control showed significantly ($p < 0.05$) higher extents of mineralisation than 10.0% (Table 4, Figure 2). The extents of mineralisation at 0.1% biochar amendment were higher than at 0.5% but the difference was not significant ($p > 0.05$; Figure 2). However, both showed significantly ($p < 0.05$) higher extents of mineralisation than the control. Furthermore, the extents of mineralisation at 1.0% were higher than control but the difference was not statistically significant ($p > 0.05$). The interaction effect of contact time and biochar amendments on extents of mineralisation was significant ($p < 0.05$) showing that the effect of contact time on extents of mineralisation was different across amendment amounts. At 1 d, all

treatment levels (except 10.0%) showed higher ($p < 0.05$) extents of mineralisation than the control (Table 4, Figure 1). The extents of mineralisation at 10.0% were significantly lower ($p < 0.05$) than in other amended soils at 1 d and 75 d. The control showed higher extents of mineralisation than all amended soils, while 10.0% showed lower extents of mineralisation than other treatment levels at 100 d ($p < 0.05$). This could be linked to the shorter lag phases and longer lag phases showed by control and 10.0% respectively at 100 d (Table 4, Figure 2). The 10.0% biochar showed the longest lag phases, slowest rates, and the lowest extents of ^{14}C-phenanthrene mineralisation in this study. This shows the negative impact of high biochar amount on the biodegradation of phenanthrene in soil. This inhibitory effect of 10.0% biochar is higher than that of 10.0% SMC. Lag phases correlated with the extents of mineralisation at 0.1% ($r = -0.95$, $p < 0.001$), 0.5% ($r = -0.97$, $p < 0.001$), 1.0% ($r = -0.94$, $p < 0.01$), and 10.0% ($r = 0.96$, $p < 0.01$). The fastest rates correlated with the extents of mineralisation at 0.1% ($r = 0.72$, $p < 0.01$), 0.5% ($r = 0.67$, $p < 0.01$), and 1.0% ($r = 0.67$, $p < 0.01$).

3.4.2.4 Phenanthrene degrading microbial numbers in biochar amended soils

The numbers of phenanthrene-degrading microbes in the biochar amended soils were monitored for 100 d (Table 4). There was no significant difference ($p > 0.05$) in the phenanthrene degrading microbial numbers in all the soil treatment conditions from 1 – 100 d. The bacteria numbers did not correlate with any of the mineralisation parameters. Lag phases correlated with fungi numbers in soils amended at 0.1% ($r = -0.56$, $p < 0.05$), 0.5% ($r = -0.69$, $p < 0.01$), 1.0% ($r = -0.61$, $p < 0.05$), and 10.0% ($r = -0.69$, $p < 0.01$). The rates did not correlate with phenanthrene degrading fungal numbers. The extents of mineralisation correlated with the fungi numbers in soils

amended at 0.1% ($r = 0.52$, $p < 0.05$), 0.5% ($r = 0.66$, $p < 0.01$), 1.0% ($r = 0.65$, $p < 0.01$), and 10.0% ($r = 0.72$, $p < 0.01$). This indicates that the fungal population in this study likely contributed more to changes in ^{14}C-phenanthrene mineralisation than the bacterial population.

3.4.3 Comparing the impacts of biochar and SMC amendment on ^{14}C-phenanthrene mineralisation in soil

Despite observed higher extents of ^{14}C-phenanthrene mineralisation in SMC amendment compared to biochar (at 1.0% and 10.0%), Hotelling's T^2 test showed that there was no significant difference ($p > 0.05$) between the impacts of SMC and biochar amendments on a combination of lag phases, fastest rates, and extents of ^{14}C-phenanthrene mineralisation, likely due to the high correlation between two of the dependent variables ($r = 0.59 – 0.99$). Follow-up independent t-tests revealed a significant difference at higher amendment amount (1.0% and 10%). The lag phases at 0.1% SMC amendment were not significantly ($p = 0.993$) longer than at 0.1% biochar. The rates at 0.1% SMC were not significantly ($p = 0.950$) slower than 0.1% biochar. The extents of mineralisation at 0.1% SMC amendment were not significantly ($p = 0.780$) lower than at 0.1% biochar. The lag phases at 0.5% SMC amendment were not significantly ($p = 0.907$) shorter than at 0.5% biochar. The rates at 0.5% SMC were not significantly ($p = 0.932$) faster than at 0.5% biochar. The extents of mineralisation at 0.5% SMC were not significantly ($p = 0.761$) lower than at 0.5% biochar. Additionally, the lag phases at 1.0% SMC amendment were not significantly ($p = 0.420$) shorter than at 1.0% biochar. The rates at 1.0% SMC were significantly ($p = 0.019$) faster than at 1.0% biochar. However, the extents of mineralisation at 1.0% SMC were significantly ($p = 0.047$) higher than at 1.0% biochar. Furthermore, the lag phases at 10.0% SMC amendment were

significantly ($p = 0.017$) shorter than at 10.0% biochar. The rates at 10% SMC were not significantly ($p = 0.09$) faster than at 10.0% biochar. The extents of mineralisation at 10.0% SMC were significantly ($p = 0.023$) higher than at 10.0% biochar. The differences in the impact of SMC and biochar amount became substantial on lag phases, rates, and extents of mineralisation from 1.0% amendment. Additionally, the impact of biochar and SMC at 0.1% and 0.5% on the rates, lag phases, and extents of mineralisation of ^{14}C-phenanthrene were similar.

In addition, calculated omega squared values (ω^2) for biochar amendment showed the main effect sizes for contact time, amendment amount, and their interactions on lag phases, rates, and extents of mineralisation. The ω^2 value for lag phases was 0.58 for contact time, 0.10 for amendment amount, and 0.18 for their interaction. It was 0.56 for contact time, 0.10 for amendment amount, and 0.19 for interaction for rates; and 0.70, 0.17, and 0.14 for contact time, amendment amount, and interaction respectively for extents of mineralisation. This showed that contact time and interaction had more effect than biochar amount, and this is similar to the η_p^2 observed in this study. Furthermore, calculated omega squared values (ω^2) for SMC amendment showed the main effect sizes for contact time, amendment amount, and their interactions on lag phases, rates, and extents of mineralisation. The ω^2 value for lag phases was 0.65 for contact time, 0.002 for amendment amount, and 0.18 for the interaction. It was 0.62 for contact time, 0.008 for amendment amount, and 0.09 for interaction on rates; and 0.66, 0.06, and 0.24 for contact time, amendment amount, and interaction respectively for extents of mineralisation. This shows that contact time and interaction had more effect than SMC amount which is similar to the initial observed η_p^2. Additionally, the effect size of SMC amount on lag phases and rates

was low which explains why the impact of SMC amount on lag phases and rates was not significant ($p > 0.05$).

3.5 Discussion

3.5.1 Effect of contact time and increasing SMC amount on ^{14}C–phenanthrene mineralisation

This study has shown that SMC application to soil at ≤10.0% could be beneficial for enhancing phenanthrene mineralisation by shortening the lag phases, quickening the rates, and enhancing the extents of mineralisation. A similar study demonstrated that spent brewery grain and SMC can improve the rates and extents of ^{14}C-phenanthrene microbial degradation (Omoni et al., 2020b). This present study revealed that the effect size of contact time (^{12}C-phenanthrene aging) and interaction effects between contact time and amendment amount were substantially larger than that of the amendment amount alone. This indicates that temporal dynamics and the combined influence of variables play a more critical role in phenanthrene mineralisation than the amendment amount itself. This aligns with previous studies, which indicated that contact time and soil type have a more significant impact on PAH bioavailability than compost type and amount (Wu et al., 2013; Wu et al., 2014). However, the interaction between compost type and amount with contact time and soil type was found to be significant (Wu et al., 2014). The lag phases and extents of mineralisation were shortest and highest respectively at 1 d, and longest and lowest at 100 d, while the rates didn't follow a defined pattern. Earlier studies have demonstrated that PAH mineralisation reduce with increase in soil-PAH contact time (Macleod and Semple, 2000; Macleod and Semple, 2003; Duan et al., 2015). Microbial numbers were high

in all soil treatments throughout the study. Therefore, variation in microbial numbers was not responsible for the difference in lag phases, rates, and extents of mineralisation in the soil treatments. The observed changes were attributed to changes in soil properties and microbial communities due to soil-phenanthrene-amendment interactions. This is consistent with past studies which have demonstrated that, aging of PAHs in soil affected the bioavailability and possible mineralisation by suitable microbes (Semple et al., 2007; Bari et al., 2010; Riding et al., 2013; Umeh et al., 2017), and amendment amount influenced phenanthrene catabolism in soil (Bari et al., 2010; Omoni et al., 2020b).

Generally, shorter lag phases, faster rates, and higher extents of ^{14}C-phenanthrene mineralisation were observed at 1.0% amendment compared to other amended soils. Additionally, on average, similar extents of mineralisation were observed in 0.1%, 0.5%, and 10.0% SMC amendment indicating that a higher amount of amendment does not necessarily translate to higher extents of ^{14}C-phenanthrene mineralisation. Wu et al. (2014) found no significant difference in loss of bioavailable PAHs when comparing two compost amounts. Puglisi et al. (2007) found no difference in recovered biovailable fractions of phenanthrene in soils amended with 0.38% and 1.15% of compost after 240 d. The bioaccessibility of pyrene at 20 d and 240 d in soils amended with compost at 10 t/ha and 30 t/ha ranged from 64 – 33% and 41.4 – 44.3% respectively (Bari et al., 2010) indicating that lower amendment amounts may provide more long-term benefits. Furthermore, soil amended with 0.37% of compost had higher phenanthrene biodegradation than soil amended with 0.83% of compost (Scelza et al., 2007). Additionally, SMC:soil ratio of 1:5

and 1:10 had shorter lag phases and higher extents of mineralisation than 1:2, 1:1, and 2:1 (Omoni et al., 2020b).

The phenanthrene degrading fungal numbers had a positive relationship with the extents of mineralisation, while the lag phases showed a negative relationship with the extents of mineralisation. This means that as phenanthrene degrading fungal numbers increased, phenanthrene mineralisation increased; and as the lag phases decreased, extents of mineralisation increased. There was no correlation between phenanthrene degrading bacteria numbers and extents of mineralisation. Therefore, it is likely that the fungi in this study contributed more to ^{14}C-phenanthrene mineralisation than the bacteria. Studies have demonstrated synergistic activity between the microbes in SMC and the soil indigenous microbial community (Adam et al., 2015; Liu et al., 2019). SMC promoted soil microbial activity (Zeng et al., 2023; Vafa et al., 2023; Atai et al., 2023), and enhanced the biodegradation of petroleum hydrocarbons (Mohammadi-Sichani et al., 2019; Vafa et al., 2023; Atai et al., 2023). Compost can transform and improve soil microbial community (Kastner et al., 1999; Hickman and Reid, 2008a, 2008b; Wu et al., 2020), and the microbes in compost contribute to PAH degradation in soil (Bastida et al., 2016; Baldantoni et al., 2017; Bellino et al., 2019).

3.5.2 Effect of contact time and increasing biochar amount on ^{14}C-phenanthrene mineralisation

The findings from this study showed that biochar amendments at ≤1.0% can improve the catabolism of phenanthrene in soil. It also showed that contact time (^{12}C-phenathrene aging), amendment amount, and their interaction influenced the mineralisation of phenanthrene in soil. Similar to the effect sizes in SMC amended soils, the effect sizes for contact time and interaction were higher than the biochar amount. These findings underscore the importance of interpreting effect sizes in the context of remediation outcomes. While the amendment amount had a smaller effect size, its interaction with contact time played a pivotal role in shaping the mineralisation trajectory. This suggests that time – dependent sequestration processes dominate over direct microbial degradation, particularly in the long term. Longer lag phases and lower extents of mineralisation were observed at 100d which is similar with SMC amended soils, and consistent with past studies that aging reduces mineralisation of PAHs in soil over time (e.g. Macleod and Semple, 2000; Macleod and Semple, 2003; Zhang et al., 2016; Song et al., 2017; Ren et al., 2018; Ukalska-Jaruga et al., 2020). Zhang et al. (2010) and Zhang et al. (2013) also reported that the interaction of contact time and biochar influences PAH sorption in soil.

The higher interaction effect implies that increasing the amount may not counteract the time – dependent decline in mineralisation. Instead, it highlights a complex situation, where higher amendment amounts accelerate contaminant sequestration but limit microbial degradation over extended periods. The impact of a high biochar amendment was obvious as 0.1% generally had

shorter lag phases and higher extents of mineralisation than other amendment levels, and all amended soils (except 10%) showed shorter lag phases and higher extents of mineralisation than the control from 1 – 75 d. However, the extents of mineralisation at 1.0% amendment were not higher than the control at 50 d. A similar finding was reported by Ogbonnaya et al. (2014) with no significant difference between control and 1.0% biochar amendment. Biochar amendment at 0.1%, 0.2%, and 0.01% had shorter lag phases, faster rates, and higher extents of mineralisation than 0.5% and 1.0% (Omoni et al., 2020a). Furthermore, 0.01% biochar amendment had higher rates and extents of ^{14}C-phenanthrene than 0.1% and 1.0% in Kettering loam soil (Ogbonnaya et al., 2014). A <1.0% amount of black carbon has been recommended for reduced impact of sorption on PAH bioavailability and mineralisation (Rhodes et al., 2008), and for preventing biochar-induced toxicity by biochar-bound contaminants (Godlewska et al., 2021). At ≥1% biochar amendment, the sorption effect increases and interferes with the stimulating effect, hence causing a reduction in phenanthrene mineralisation as seen in this study (Rhodes et al., 2008; Ogbonnaya et al., 2014).

The observed decline in microbial mineralisation of freshly added ^{14}C-phenanthrene, as indicated by reduced $^{14}CO_2$ evolution in the respirometric assays at 100 d and at 10.0% biochar, is attributable to the aging of ^{12}C-phenanthrene in soil due to soil-phenanthrene-amendment interaction. Aging could lead to a significant reduction in the bioavailability of phenanthrene, primarily due to time-dependent sorption and sequestration processes within the soil and biochar (Semple et al., 2013; Riding et al., 2013; Umeh et al., 2017). Over time, ^{12}C-phenanthrene could become increasingly associated with soil organic matter, mineral surfaces, and biochar, or

diffuse into soil and biochar pores making it physically inaccessible to soil microbes. These processes limit the fraction of phenanthrene available for microbial degradation. Therefore, as bioavailable phenanthrene declines, microbial populations may experience reduced substrate exposure, leading to the downregulation of key catabolic enzymes involved in phenanthrene degradation. Additionally, microbial communities may shift in composition, which may favour non-phenanthrene degrading community. However, the phenanthrene degrading microbial number in this study was high from 1 – 100 d which indicates that any possible shift still favoured phenanthrene degrading microbes and there was probably no downregulation of degradation pathways. Furthermore, ^{14}C-phenanthrene may rapidly sorb to the same soil domains already altered by the aging of ^{12}C-phenanthrene, resulting in non-equilibrium partitioning and a reduction in the freely available aqueous-phase concentration. In addition, sorption displacement of aged or sorbed PAH by fresh PAH has been reported by Wang et al. (2005). This would further limit microbial access to the freshly spiked ^{14}C-phenathrene and slow its biodegradation. Overall, the reduction in ^{14}C-phenanthrene mineralisation observed with increasing ^{12}C-phenanthrene aging in this study, is more attributable to a combination of physicochemical constraints on phenanthrene availability and not necessarily microbial adaptation to long-term substrate limitation. Semple et al. (2006) and Allan et al. (2007) had erlier reported that biodegradation of most PAHs is limited by bioavailability and not microbial catabolic ability.

The phenanthrene degrading fungal numbers had a positive relationship with the extents of mineralisation in all biochar treatments. Omoni et al. (2020a) also reported a positive correlation between phenanthrene degrading fungal numbers and extents of mineralisation in soils

amended with biochar at 0.01% and 1.0%. The lag phases had a strong negative relationship with the extents of mineralisation in SMC and biochar treatments. Therefore, amendments that could shorten the lag phases may increase the extents of mineralisation. For instance, lower biochar amendments (0.1%, 0.2%, 0.01%) led to shorter lag phases, faster rates, and higher extents of mineralisation in soil (Omoni et al., 2020a). The phenanthrene degrading bacteria numbers showed no statistically significant relationship with the extents of mineralisation. Okere et al. (2017) reported no correlation between ^{14}C-phenanthrene degraders and extents of mineralisation. However, phenanthrene degrading bacteria numbers were observed to negatively correlate with rates at 0.1%, 0.5%, and 1.0% biochar amendments, and extents of mineralisation at 0.1% and 0.2% biochar amendments (Omoni et al., 2020a). Earlier studies inferred that bacteria may be responsible for pyrene mineralisation in soil (Macleod and Semple, 2002; Macleod and Semple, 2003). Biochar can improve phenanthrene degradation due to its positive impact on the abundance of PAH degraders and soil properties (Ding et al., 2021). The application of biochar to soil improves soil physicochemical properties, thereby enhancing soil microbial activity and subsequent contaminant biodegradation (Song et al., 2017; Zhang et al., 2020; Saeed et al., 2021; Kaur & Sharma, 2021). Biochar application improved PAHs biodegradation through stimulating the growth/activity of PAHs degraders by increasing microbial enzyme activity, PAHs-degradation gene copies, and altering the microbial community structure in soil (Liu et al., 2015; Song et al., 2017; Guo et al., 2021).

In this current study, to assess the effect of aging on phenanthrene mineralisation, fresh ^{14}C-phenanthrene was spiked into soils pre-contaminated with ^{12}C-phenanthrene, at various contact times. While the ^{14}C-phenanthrene is freshly introduced and chemically available at the point of

addition, its subsequent availability to microbial degradation is influenced by the altered sorption characteristics and soil conditions resulting from the aging of the ^{12}C-phenanthrene. Aging can lead to reduction in bioaccessibility and shifts in microbial community composition due to soil-phenanthrene-amendment interactions, all of which would influence the mineralisation of the freshly added ^{14}C-phenanthrene. Therefore, $^{14}CO_2$ evolution from ^{14}C-phenanthrene serves as a proxy for the bioavailability of ^{12}C-phenanthrene under aged conditions, rather than a direct measure of aged contaminant mineralisation. Also, the mineralisation assays were conducted in soil slurries under shaking conditions to enhance contact between microbes and the freshly added ^{14}C-phenanthrene. While this approach improves microbial access and reflects the biodegradation potential of the system, it may reduce the influence of aging on bioavailability. Thus, $^{14}CO_2$ evolution in the slurry setup primarily reflects microbial mineralisation capacity under optimal conditions, while still serving as a relative indicator of changes in phenanthrene bioavailability due to aging and amendment effects.

3.6 Conclusions

This study revealed that contact time and its interaction with amendment amount accounted for a larger proportion of the variance in phenanthrene mineralisation, compared to the effect of amendment amount alone. This indicates that temporal dynamics and synergistic processes between amount and contact time were the primary drivers of the observed reduction in mineralisation. The declining trend may result from time – dependent sequestration of phenanthrene by the amendment, limiting their bioavailability for microbial degradation. This explains why lower mineralisation was observed in biochar amended soil at higher amounts (1%

and 10%) compared to compost due to higher sorption capacity. Furthermore, the significant interaction effect suggests that the relationship between amount and contact time is non-linear, with higher amounts potentially amplifying sequestration processes at the expense of microbial activity. These findings highlight the need to consider both the individual and combined effects of contact time and amendment amount when evaluating the long - term efficacy of amendments in PAH contaminated soils. Future studies incorporating co-aging of both isotopes and comparing static and slurry mineralisation in parallel would provide a more comprehensive understanding of how contaminant aging and amendment interact to influence phenanthrene fate under realistic environmental conditions.

3.7 CRediT author statement

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3.8 Declaration of conflict of interest

The authors declare no conflicting financial, professional, or personal interests.

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Chapter 4 The impact of organic amendment blends on phenanthrene-contaminated soil.

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4.1 Abstract

Biochar and spent mushroom compost (SMC) can be used as amendments for reducing the adverse impacts of polycyclic aromatic hydrocarbons (PAHs) in soil. A blend of both amendments may be more beneficial than using either biochar or SMC. Therefore, this study investigated the impact of SMC:biochar blends on soil spiked with phenanthrene. SMC – biochar blends were prepared at a ratio (SMC:biochar) of 10:1, 5:1, 2:1, and 1:1 (w/w; dry wt). Phenanthrene-spiked soils were amended with the SMC:biochar blends, SMC, and biochar at 0.5% (w/w; dry wt). The amended and unamended soils were incubated in the dark and monitored at 1 d, 25 d, 50 d, 75 d, and 140 d for ^{14}C–phenanthrene mineralisation and changes in soil properties. The results showed that soil amended with SMC:biochar blend of 5:1 had shorter lag phases, faster rates, and higher extents of ^{14}C-phenanthrene mineralisation than the control. Higher extents of mineralisation were observed in soil amended with 5:1 amendment blend compared to other amended soils. All amendment blends showed higher extents of mineralisation than SMC amended soil, biochar amended soil, and the control. The soils' available phosphorus and ammonium nitrogen showed stronger relationships with the ^{14}C-phenanthrene mineralisation parameters than other monitored soil properties. This study has demonstrated that SMC:biochar blend is a more beneficial amendment for phenanthrene mineralisation in soil than biochar or SMC. This study provides novel insights into the synergistic effects of SMC:biochar blends on PAH mineralisation, which is crucial for advancing sustainable and effective strategies for mitigating the adverse effects of PAHs in soil.

Keywords: Available P, ammonium nitrogen, biochar, spent mushroom compost, polycyclic aromatic hydrocarbons, biodegradation, soil.

4.2 Introduction

Soil is important as it supports the survival and growth of populations of terrestrial plants and animals, including humans (Lal, 2008). Soil that has been contaminated by organic chemicals adversely impacts soil functions. The contamination of soil is mainly through anthropogenic activities (FAO and UNEP, 2021), with polycyclic aromatic hydrocarbons (PAHs) being primarily introduced into soil through human activities (Okere and Semple, 2012; Singh et al., 2016; Lawal, 2017). PAHs are organic chemicals with two or more benzene rings (Lawal, 2017; Patel et al., 2020) and are of concern due to their genotoxic, carcinogenic, mutagenic, and teratogenic effects (Semple et al., 2003; Lawal, 2017). Due to the hydrophobic nature of PAHs, they can sorb to soil organic matter and persist in soil (Semple et al., 2003). Their molecular weight, ring number, and structural complexity contribute to their hydrophobicity, thereby influencing their environmental persistence (Okere and Semple, 2012; Lawal, 2017; Patel et al., 2020). Their presence in the soil is of ecological and public health concern due to their toxicity and potential accumulation in soil biota (Zungum & Imam, 2021).

Chemical and physical remediation techniques may be used to remove PAHs from soil; however, they may not be environmentally friendly, cost-effective, or sustainable compared to biological methods (Patel et al., 2020). Using organic amendments is cost – effective, environmentally friendly, sustainable, and effective for the remediation of PAH – contaminated soil (Gandolfi et al., 2010; Baldantoni et al., 2017; Patel et al., 2020; Guo et al., 2021; Guo et al., 2022). Studies have demonstrated that compost and biochar are effective as amendments for the

biodegradation of PAHs in soils (e.g., Wu et al., 2013; Ogbonnaya et al., 2016; Sayara & Sanchez, 2020; Valizadeh et al., 2022; Li et al., 2023; Atai et al., 2023; Li et al., 2024). The use of compost as a soil amendment provides a double advantage of biostimulation and bioaugmentation due to the high nutrient content and rich microbial community in compost (Sayara & Sanchez, 2020). Compost is rich in nutrients and microbes which aid in its effect on improving PAH biodegradation, as nutrients and microbes are prerequisites for contaminant biodegradation (Ren et al., 2018; Sigmund et al., 2018).

Spent mushroom compost (SMC) may not contain the same nutrient levels as compost, because the fungus has utilized some of its nutrients, but the nutrient content of SMC is similar to compost and suitable for soil amendment (Umor et al., 2021). As a cheaper compost alternative, the use of SMC is cost-effective and sustainable. It will reduce the waste management burden on mushroom farmers, and improve PAH remediation in soil because it contains nutrients, enzymes, and microorganisms that possess PAH degrading potential (Chiu et al., 2009; Xuanzhen et al., 2010; Gasecka et al., 2012; Zhou et al., 2020; Huang et al., 2023). Studies have demonstrated that SMC can improve soil nutrients, crop growth, enzyme activities, and crop yield; increase microbial diversity, and cause a shift to beneficial microbial groups in soil (Idowu et al., 2023; Huang et al., 2023; Zubkova & Vinogradov, 2023). The improvement of soil nutrients, enzymes, and microbial activities as reported in these studies can be beneficial for improving the biodegradation of PAHs in soil.

Biochar has been demonstrated as a quality amendment for improving soil health and fertility (Giagnoni et al., 2019; Piscitelli et al., 2019; Guo et al., 2021; Kaur & Sharma, 2021), and compost enhances soil properties and microbial activities (Kästner and Miltner, 2016; Bernal et al., 2017; Ejileugha, 2022). Improving soil health and fertility improves soil microbial community structures and activities which could promote the biodegradation of organic contaminants (Zhu et al., 2017). Studies have shown that the combined application of biochar and compost is more effective in enhancing soil properties and microbial activities (Frimpong et al., 2021; Bong et al., 2021; Bello et al., 2023). Blending biochar with compost may mitigate the low nutrient challenges in biochar and also improve and extend the agronomic benefits of compost (Liao et al., 2021). Application of biochar or compost enhances the biodegradation of PAHs in soil (Wu et al., 2013; Zhang et al., 2020; Li et al., 2023). Therefore, the blending of both amendments is promising for the biodegradation of PAHs in soil. The combined application of biochar and compost can immobilise PAHs in soil while facilitating the microbial degradation of remaining accessible fractions (Sigmund et al., 2018). In this study, the impact of SMC:biochar blends on the mineralisation of ^{14}C-phenanthrene in soil was investigated to examine if amendment blends will be more effective for shortening the lag phases, expediting rates, and increasing the extents of ^{14}C-phenanthrene mineralisation in soil when compared to a single use of SMC or biochar. To the best of our knowledge, this is the first study to investigate the impact of SMC:biochar blends on the kinetics of phenanthrene biodegradation in soil.

4.3 Materials and methods

4.3.1 Sample collection.

The soil sample was collected from cattle grazing grassland at Hillam Farms Cockerham, Lancaster, United Kingdom. The soil was collected at a 5 – 25 cm depth, air dried and sieved through a 2 mm mesh to remove large stones, debris, and plant materials. The sieved soil was stored at 4 ℃ in the dark. The biochar was bought from a commercial biochar-producing company (SoilFixers), Royal Wootton Bassett, United Kingdom. The SMC was obtained from Drinkwater Mushrooms, Lancaster, United Kingdom. The properties of the biochar are shown in Table 1, and the properties of the soil and SMC are shown in Table 2.

Table 1: Biochar properties and pyrolysis conditions as provided by the supplier (SoilFixers, UK)

Parameter	Value
Feedstock	Hardwood logs
Pyrolysis equipment	Retort Kiln
Pyrolysis temperature	700 – 900 °C
Pyrolysis duration	5 – 12 h
Particle size	0 – 8 mm
pH	8.8 – 10
Volatiles	9 – 15%
Ash max.	3 – 6%
Moisture max.	8 – 12%
C Fixed	>76%

Table 2: Properties of the soil and SMC samples. Values are in mean ± SD (n = 3) except for soil texture, sand, clay, and silt.

Parameters	Soil	SMC
Sand (%)	71.4	-
Clay (%)	2.4	-
Silt (%)	26.2	-
Soil texture	Sandy loam	-
pH	7.2±0.0	7.4±0.1
Electrical conductivity	382±4.2 (μScm^{-1})	6.8±0.1 ($mScm^{-1}$)
Organic matter (%)	6.5±0.2	62.5±4.2
C:N	10.4±0.1	13.3±0.5
Total carbon (ppm)	102.4±4.4	566.4±35.7
Total organic carbon (ppm)	98.8±3.6	523.3±32.6
Inorganic carbon (ppm)	3.6±1.0	43.0±2.9
Ammonium nitrogen (ppm)	0.4±0.0	12.8±1.0
Nitrate (ppm)	3.6±0.2	0.2±0.0

4.3.2 Soil spiking and monitoring

Soil (200 g dry weight; n = 3) was spiked with ^{12}C–phenanthrene at a concentration of 100 mg/kg (dry weight) at a field moisture content of 30%. Approximately ¼ of the soil was first spiked using acetone as the carrier solvent at a 1:20 (v/w) solvent-to-soil ratio. This portion was left to vent in a fume hood for 2 h to allow complete volatilisation of the solvent. The remaining unspiked soil

was then added in three roughly equal portions, with thorough mixing after each addition to ensure homogeneity (Reid et al., 2001; Doick et al., 2003; Semple et al., 2006; Allan et al., 2007). Afterwards, the spiked soil was amended with SMC:biochar blend (10:1, 5:1, 2:1, and 1:1), biochar only, and SMC only at 0.5% (dry weight), respectively. Spiked soil without any amendment was prepared as the control. The soils were stored in an amber bottle and incubated at 21 ± 2 °C in the dark for 140 d and sampled at 1 d, 25 d, 50 d, 75 d, and 140 d for monitoring ^{14}C – phenanthrene mineralisation and soil properties.

4.3.3 Respirometry for ^{14}C – phenanthrene mineralisation

Soil (10 ± 0.2 g) was weighed from respective incubated soils into a 250 ml modified Schott bottles with a Teflon lined screw cap (Reid et al., 2001; Doick et al., 2003; Semple et al., 2006; Allan et al., 2007). The modification is the fitting of a crocodile clip to the cap for attaching vials for trapping evolved $^{14}CO_2$ (Reid et al., 2001). Sterile MBS (30 ml) was added to the modified Schott bottles to form a soil slurry, and the bottles (except ^{12}C – blank) were spiked with 9-^{14}C – phenanthrene (22.7 Bq/g). The bottles were immediately closed tightly with the cap which was fitted with a 7 ml vial containing 1 ml of 1M NaOH for trapping evolved $^{14}CO_2$. The bottles were placed on an orbital shaker at 100 RPM and incubated at 21 ± 2 °C. The vials were sampled at 3 h intervals for the first 9 h, and then every 24 h for 14 d. At each sampling time point, the 7 ml vial was removed and replaced with a fresh vial. Then, 5 ml Ultima Goldstar scintillation fluid was added to the sampled vial. The sampled vial was stored for 24 h in the dark before quantifying using a liquid scintillation counter (LSC) (Canberra Packard Tri-Carb2250CA).

4.3.4 Soil properties

The pH and electrical conductivity (EC) were determined in a soil – water ratio of 1:5 (w/v) using a pH meter (METTLER TOLEDO, SevenCompact™ pH/Ion S220) and EC meter (METTLER TOLEDO, FiveEasy™ FE30) respectively. The available phosphorus (OLSEN P) was measured by extracting 2 g of soil in 40 ml of 0.5 M Na_2CO_3 and analysing the filtrate using AA3 Seal Autoanalyzer. The ammonium nitrogen (NH_4^+-N) was determined by extracting 5 g soil with 25 ml of 2 M KCl and analysed with AA3 Seal autoanalyzer. For total organic carbon (TOC), 5 g soil was extracted with 0.5 M K_2SO_4 (25 ml) and analysed using Shimadzu TOC-L with TN. A 20 – 24 mg of ball-milled dry soil was analysed for C:N using Elementar CN analyser (Vario EL).

4.3.5 Data analysis

The lag phases were calculated as the time taken to mineralise 5% of the spiked ^{14}C-phenanthrene, the fastest rates as the highest ^{14}C-phenanthrene mineralised per hour, and the extents of mineralisation as the cumulative ^{14}C-phenanthrene mineralised in 14 d. Data were analysed using multivariate analysis of variance (MANOVA), univariate ANOVAs, LSD post hoc test, and Pearson's correlation. Data were blank corrected and mineralisation parameters calculated using MS Excel. Data plots were done using SigmaPlot 10, and data analysis was done using IBM SPSS (version 28).

4.4 Results

4.4.1 Impact of organic amendments on ^{14}C – phenanthrene mineralisation in the soils

The mineralisation of ^{14}C – phenanthrene in the soils was monitored for 140 d (Figure 1; Table 3). Figure 1 shows the changes in the extents of mineralisation from 1 – 140 d in the soils amended with SMC-biochar blends while Table 3 shows the changes in the lag phases, fastest rates, and the extents of mineralisation in the soils from 1 – 140 d. The MANOVA conducted showed that there was a significant multivariate effect of ^{12}C-phenanthrene aging (Wilk's Lambda = 0.013, $p < 0.001$) and SMC:biochar blends (Wilk's Lambda = 0.295, $p < 0.001$) on lag phases, fastest rates, and extents of mineralisation. Follow-up univariate analyses revealed a significant effect of the amendments on lag phases ($p < 0.05$), fastest rates ($p < 0.001$), and extents of mineralisation ($p < 0.05$).

The lag phases in biochar amended soil were shorter than the control from 1 – 75 d (Table 3). There was no significant difference ($p > 0.05$) in lag phases in all soil treatment conditions on 1 d and 75 d. The lag phases were shorter on 1 d and longer on 140 d in all soil treatment conditions (Table 3). The difference in lag phases did not follow a defined pattern. All amended soils had lower lag phases than the control on 50 d except SMC amended soil (Table 3; $p < 0.05$), and all amended soils had lower lag phases than the control on 140 d except biochar amended soil (Table 3; $p < 0.05$). The fastest rates for biochar-amended soil were faster than the control from 1 – 50 d (Table 3). There was no significant difference ($p > 0.05$) in fastest rates in all soil treatment conditions on 75 d and 140 d (Table 3). The changes in fastest rates did not follow a given pattern, however, the fastest rates in 10:1 and 5:1 amended soil were faster than in SMC amended soil

and in soil amended with 2:1 and 1:1 amendment blend (Table 3). The fastest rates in SMC-amended soil were slower than in all soil treatment conditions (Table 3). The fastest rates at 140 d were slower compared to the fastest rates at 1 – 75 d (Table 3). Overall (1 – 140 d), biochar amended soil showed faster rates than other soil treatment conditions. There was no significant difference ($p > 0.05$) in the extents of mineralisation in all soil treatment conditions on 1 d, 50 d, and 75 d (Table 3 and Figure 1). Higher extents of mineralisation were observed on 1 d compared to 25 – 140 d (Table 3 and Figure 1). All amended soils showed higher extents of mineralisation than the control on 50 d except SMC amended soil. Similarly, all amended soils showed higher extents of mineralisation than the control on 75 d. On 140 d, the extents of mineralisation in the amended soils were higher than the control except in biochar-amended soil (Table 3 and Figure 1). Overall (1 – 140 d), higher extents of mineralisation were observed in soils treated with amendment blends compared to biochar and SMC only treatments (Table 3 and Figure 1). Similarly, other SMC:biochar blends showed higher extents of mineralisation (1 – 140 d) compared to 1:1 (Table 3 and Figure 1).

Table 3: Mineralisation of ^{14}C-phenanthrene in the soil treatments. Values are in mean ± SD (n = 3). Values in columns followed by different letters are statistically different (LSD; n = 3; $p < 0.05$); also indicated are shorter lag phases (*), faster rates (+), higher extents of mineralisation (#) than the controls that are not statistically significant ($p > 0.05$)

Contact time (d)	Amended soil	Lag phase (h)	Fastest rate (%$^{14}CO_2$/h)	Extent of mineralisation (%)
1	Control	35.7 ± 5.9^{a}	0.7 ± 0.3^{a}	57.5 ± 5.4^{a}
	10:1	36.1 ± 4.0^{a}	0.9 ± 0.1^{a+}	57.6 ± 1.2^{a}
	5:1	41.0 ± 5.3^{a}	0.8 ± 0.1^{a+}	49.8 ± 4.4^{a}
	2:1	36.9 ± 1.0^{a}	0.6 ± 0.4^{a}	55.3 ± 4.0^{a}
	1:1	38.5 ± 5.8^{a}	0.7 ± 0.3^{a}	53.2 ± 3.0^{a}
	Biochar	34.0 ± 1.2^{a*}	1.0 ± 0.2^{b}	53.6 ± 1.5^{a}
	SMC	36.4 ± 2.7^{a}	0.6 ± 0.3^{a}	53.4 ± 1.0^{a}
25	Control	38.5 ± 0.9^{a}	0.7 ± 0.1^{a}	34.1 ± 2.9^{a}
	10:1	58.4 ± 4.2^{b}	0.4 ± 0.1^{a}	29.3 ± 1.2^{a}
	5:1	42.7 ± 5.3^{a}	0.6 ± 0.1^{a}	36.7 ± 2.8$^{a\#}$
	2:1	54.2 ± 3.4^{b}	0.4 ± 0.1^{a}	30.0 ± 1.3^{a}
	1:1	63.9 ± 2.4^{b}	0.3 ± 0.1^{a}	26.4 ± 1.3^{c}
	Biochar	24.6 ± 2.0^{c}	1.5 ± 0.2^{b}	39.0 ± 1.0^{b}
	SMC	45.0 ± 4.9^{a}	0.5 ± 0.2^{a}	30.7 ± 4.4^{a}
50	Control	50.50± 4.2^{b}	0.6 ± 0.2^{a}	27.2 ± 2.1^{a}
	10:1	43.6 ± 3.6^{a}	1.0 ± 0.2^{b}	32.3 ± 2.8$^{a\#}$
	5:1	47.1 ± 4.8^{a}	1.0 ± 0.2^{b}	32.6 ± 4.2$^{a\#}$
	2:1	45.7 ± 4.4^{a}	0.8 ± 0.2^{a+}	31.8 ± 3.9$^{a\#}$
	1:1	40.0 ± 1.1^{c}	1.0 ± 0.2^{b}	32.6 ± 1.0$^{a\#}$
	Biochar	43.9 ± 3.1^{a}	0.8 ± 0.2^{a+}	28.5 ± 1.1$^{a\#}$
	SMC	57.9 ± 6.0^{d}	0.7 ± 0.1^{a+}	25.9 ± 3.2^{a}
75	Control	39.2 ± 1.9^{a}	0.8 ± 0.2^{a}	44.1 ± 1.4^{a}
	10:1	38.5 ± 2.9^{a*}	0.7 ± 0.2^{a}	46.7 ± 3.0$^{a\#}$
	5:1	36.5 ± 2.4^{a*}	0.9 ± 0.2^{a+}	48.2 ± 3.3$^{a\#}$
	2:1	35.5 ± 1.5^{a*}	0.9 ± 0.1^{a+}	49.7 ± 2.2$^{a\#}$
	1:1	35.3 ± 5.5^{a*}	0.7 ± 0.2^{a}	46.3 ± 5.0$^{a\#}$
	Biochar	38.7 ± 0.7^{a*}	0.8 ± 0.1^{a}	44.3 ± 0.9$^{a\#}$
	SMC	38.5 ± 2.3^{a*}	0.7 ± 0.1^{a}	45.9 ± 3.4$^{a\#}$
140	Control	57.9 ± 3.2^{a}	0.5 ± 0.1^{a}	30.1 ± 2.2^{b}
	10:1	48.5 ± 5.2^{b}	0.5 ± 0.2^{a}	37.3 ± 5.7^{a}
	5:1	45.3 ± 2.7^{b}	0.5 ± 0.1^{a}	38.5 ± 1.8^{a}
	2:1	47.2 ± 1.7^{b}	0.5 ± 0.2^{a}	36.8 ± 1.1^{a}
	1:1	46.0 ± 2.2^{b}	0.5 ± 0.1^{a}	37.1 ± 2.5^{a}
	Biochar	69.9 ± 2.3^{c}	0.5 ± 0.1^{a}	26.9 ± 0.5^{b}
	SMC	49.4 ± 6.2^{b}	0.5 ± 0.2^{a}	35.5 ± 4.2^{a}

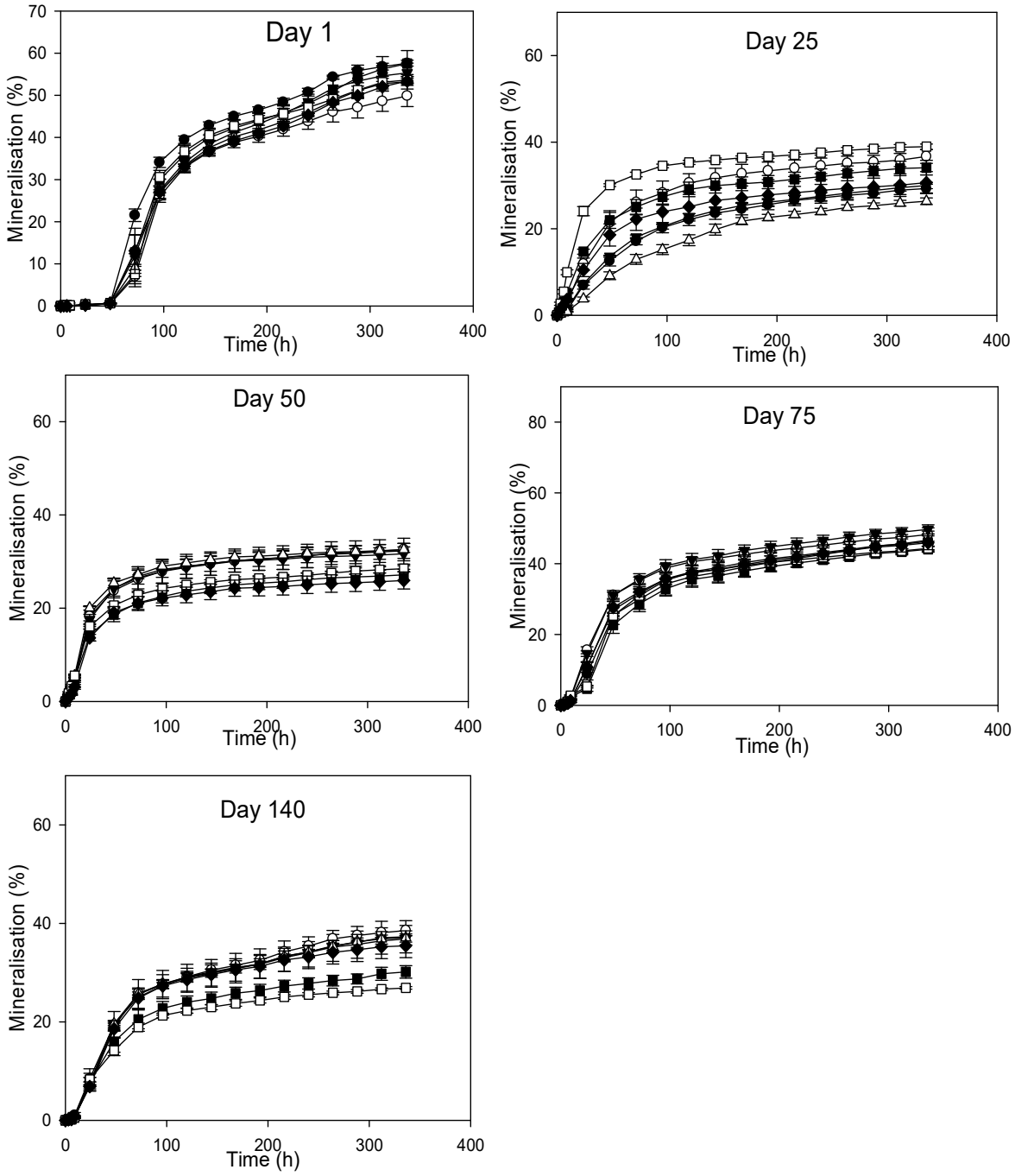

Figure 1: Extents of mineralisation in SMC:biochar blend, SMC-, and biochar-amended soils at 1 d, 25 d, 50 d, 75 d, and 140 d. Soil treatments are represented with ●(10:1), ○(5:1), ▼(2:1), △(1:1), □(biochar only), ■(control), and ◆(SMC only) respectively. Mean values are plotted with error bars showing the SD (n = 3).

4.4.2 Relationship between soil properties and ^{14}C-phenanthrene mineralisation

The pH, EC, OLSEN P, TOC, C:N ratio, and NH_4^+-N in the soil treatment conditions were monitored from 1 – 140 d (Figure 2). The soil properties were monitored to observe if there was a relationship between the soil properties and the lag phases, fastest rates, and extents of mineralisation. Examination of the data revealed that there were correlations between the soil properties and the mineralisation parameters. The lag phases correlated with the OLSEN P in 5:1 ($r = -0.634$, $p < 0.05$) and SMC ($r = -0.837$, $p < 0.01$) amended soils. The fastest rates correlated with OLSEN P ($r = 0.663$, $r = p < 0.05$) in biochar amended soils, and correlated with TOC in biochar amended soil ($r = 0.661$, $p < 0.05$) and SMC amended soil ($r = -0.787$, $p < 0.01$). The extents of mineralisation correlated with OLSEN P in 10:1 ($r = 0.786$, $p < 0.01$), 5:1 ($r = 0.764$, $p < 0.01$), 2:1 ($r = 0.775$, $p < 0.01$), 1:1 ($r = 0.668$, $p < 0.05$), and SMC ($r = 0.821$, $p < 0.01$) amended soils. The ammonium nitrogen correlated with extents of mineralisation in 10:1 ($r = 0.636$, $p < 0.05$), 5:1 ($r = 0.646$, $p < 0.05$), 1:1 ($r = 0.672$, $p < 0.05$), and SMC ($r = 0.652$, $p < 0.05$) amended soils. The extents of mineralisation in biochar amended soil correlated with the EC ($r = 0.644$, $p < 0.05$) and TOC ($r = 0.702$, $p < 0.05$). In the control soil, the lag phases correlated with the EC ($r = -0.723$, $p < 0.01$) and the extents of mineralisation also correlated with the EC ($r = 0.656$, $p < 0.05$).

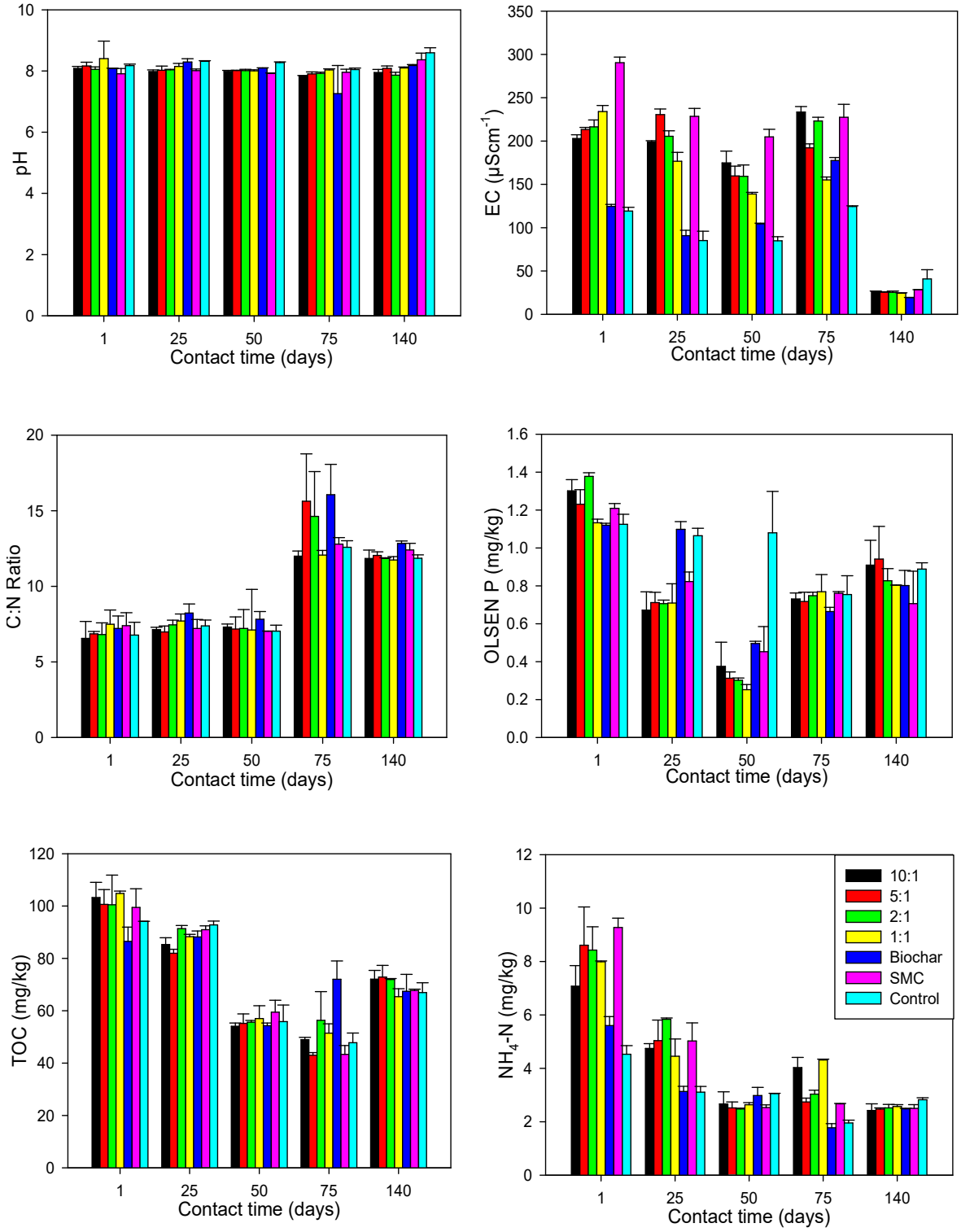

Figure 2: Temporal changes in the monitored soil properties (pH, EC, OLSEN P, TOC, C:N ratio, and NH_4^+-N) in the soil treatments from 1 – 140 d. The soil treatment conditions are represented with different coloured bars as shown. Mean values were plotted with error bars showing the SD (n = 3).

4.5 Discussion

4.5.1 Impact of amendments and contact time on ^{14}C – phenanthrene mineralisation in the soils.

Biochar and SMC have been demonstrated as amendments for improving soil properties (Guo et al., 2021; Kaur & Sharma, 2021; Idowu et al., 2023; Huang et al., 2023), and a mixture of biochar and compost has been shown to have greater impacts on improving agronomic benefits (Abideen et al., 2020; Al-Omran et al., 2021). The findings from this present study demonstrated that SMC:biochar blends could shorten lag phases, expedite mineralisation rates, and increase the extents of mineralisation. However, this was influenced by the blending ratio of the amendments. Generally, soils amended with amendment blends showed similar lag phases, but 5:1 and 10:1 (SMC:biochar) showed faster rates than 2:1 and 1:1, and higher extents of mineralisation were observed in other SMC:biochar blends when compared to 1:1. Amendments with shorter lag phases and faster rates have shown higher extents of mineralisation in past studies (Ogbonnaya et al., 2014a, Omoni et al., 2020a, Omoni et al., 2020b). In contrast, biochar-amended soil generally showed shorter lag phases and faster rates than all soil treatment conditions but lower extents of mineralisation. The faster rates and shorter lag phases were attributed to improved soil microbial activities by biochar (Liu et al., 2015; Song et al., 2017; Li et al., 2023), while the lower cumulative extents of mineralisation were attributed to reduced bioavailability of phenanthrene over time due to biochar sorption properties (Ogbonnaya et al., 2014b, Ogbonnaya et al., 2016, Sigmund et al., 2018), which reduced overall mineralisation in biochar amended soil when compared to SMC:biochar blends.

The SMC:biochar blends (10:1, 5:1, 2:1, and 1:1) generally showed higher extents of mineralisation than SMC and biochar amendments alone, especially from 50 – 140 d. This shows that SMC:biochar blends are better alternatives to biochar or SMC for improving long-term phenanthrene biodegradation in soil. This has been confirmed by Bao et al (2020) who reported that a combined application of biochar and mushroom residue significantly improved the removal of PAHs in soil when compared to the control and biochar only treatment. The blending of compost and biochar mitigates the low nutrient challenges in biochar and also improves the agronomic benefits of compost (Liao et al., 2021). The SMC:biochar blends (10:1, 5:1, and 2:1) generally showed higher extents of mineralisation than the 1:1 blend. This was attributed to increased sorption due to a higher biochar content in the 1:1 blend compared to other blend ratios. This is consistent with the results of Sigmund et al. (2018) who reported that the addition of biochar to compost-amended soil increased sorption by 100 – fold and reduced the degradation rate of PAHs. This indicates that in blending SMC and biochar for phenanthrene mineralisation in soil, an optimal blending ratio is important to prevent the impact of biochar phenanthrene sorption on phenanthrene mineralisation. The SMC:biochar blend of 5:1 was better in this present study over time for increasing the extents of mineralisation.

The extents of mineralisation at 1 d were higher than 25 – 140 d, and the lag phases were shorter than 25 – 140 d. This is due to higher phenanthrene bioavailability at 1 d and increased phenanthrene sequestration over time, reducing bioavailability and mineralisation. This is consistent with past studies which have demonstrated that an increase in soil – PAH contact time reduces the extractability and biodegradation of PAH in soil due to reduced bioavailability (e.g., Alexander & Kelsey, 1997; White et al., 1997; Macleod and Semple, 2000; Macleod and Semple,

2003; Bielská et al., 2013; Duan et al., 2015). In this study, fresh ^{14}C-phenanthrene was added to soils that had previously been aged with ^{12}C-phenanthrene to assess changes microbial mineralisation of phenanthrene over time. While this approach enables tracking of phenanthrene mineralisation through $^{14}CO_2$ emission, it does not fully capture the fate of the aged ^{12}C-phenanthrene, which may have undergone sorption, sequestration, or microbial adaptation processes not mirrored by the freshly added ^{14}C-phenanthrene. Additionally, while ^{14}C-phenanthrene was freshly spiked into aged soils for respirometry, its mineralisation reflects not only microbial activity but also changes in phenanthrene bioavailability resulting from prior contact time of ^{12}C-phenanthrene with the soil and amendment. The aging of ^{12}C-phenanthrene likely altered sorption site availability and microbial responsiveness, indirectly affecting the degradation of the freshly added ^{14}C-phenanthrene. Therefore, the results represent relative shifts in bioavailability and microbial degradative capacity (or microbial community), modelled by the freshly spiked ^{14}C-phenanthrene, rather than direct mineralisation of aged ^{12}C-phenanthrene. The aging of ^{12}C-phenanthrene in soil alters the physicochemical and microbiological conditions that influence the mineralisation kinetics of subsequently added ^{14}C-phenanthrene. Aging promotes the sequestration of phenanthrene into soil organic matter and mineral matrices, through processes such as slow diffusion into micropores, adsorption to hydrophobic domains, and potential incorporation into non-extractable residues (Semple et al., 2013; Riding et al., 2013; Umeh et al., 2017). These aging processes alter the distribution and bioaccessibility of phenanthrene in the soil, reducing the readily available phenanthrene pool that typically sustains microbial degradative activity. As a result, microbial communities may undergo functional shifts or downregulate phenanthrene-degrading pathways due to prolonged substrate limitation. When ^{14}C-phenanthrene is freshly introduced, these existing effects can lead

to reduced enzymatic induction and delayed metabolic response to the freshly added compound, manifesting as longer lag phases and slower mineralisation rates as seen on 140 d in this study. Therefore, even though ^{14}C-phenanthrene is freshly spiked, its mineralisation is modulated by the existing phenanthrene exposure and aging-induced changes in both the soil and indigenous microbes, highlighting the importance of considering contaminant aging when assessing biodegradation potential and predicting the environmental fate of PAHs in contaminated soils.

The extents of mineralisation observed at 75 d and 140 d were higher than those at 25 d and 50 d. A similar increase in ^{14}C-phenanthrene mineralisation over time was reported by Yu et al. (2016) which was attributed to sorptive attenuation. Apparently, the sorption capacity of the organic amendment reduced over time due to the blocking of sorption sites by competing soil organic matter thereby preventing/reducing the sorption of the spiked organic contaminant (Jonker et al., 2004; Rhodes et al., 2008; Yu et al., 2016). Additionally, microbial community structure and abundance may increase and stabilise over time in soil amended with SMC or biochar (Song et al., 2017; Huang et al., 2023), which could improve microbial degradation of phenanthrene.

4.5.2 Relationship between soil properties and ^{14}C-phenanthrene mineralisation in the soils

Organic amendments can be applied to soil to improve soil properties and enhance soil health and fertility. There were changes in the pH and C:N ratio in the soil treatments over time but these soil properties did not correlate with lag phases, fastest rates, and extents of mineralisation. The pH in this study from 1 – 140 d ranged from 7.26 – 8.60 which is within the

suitable pH range for biodegradation reported in the literature (e.g., Wu et al., 2013; Abdulsalam et al., 2018; Sangodoyin and Igbode, 2019; Chikere et al., 2020; Tran et al., 2021). Yu et al. (2016) demonstrated that ^{14}C-phenanthrene solvent extractability was higher at pH 7 and 8, due a higher concentration of phenanthrene in the aqueous phase caused by increased dissolved organic matter. The C:N ratio in this study from 1 – 140 d ranged from 6.55 – 16.06 which is within the range of effective C:N ratio reported in past studies (e.g., Amellal et al., 2001; Teng et al., 2010; Han et al., 2017). However, in this study, the pH and C:N did not correlate with lag phases, rates, and extents of mineralisation. Therefore, any difference observed in the lag phases, rates, and extents of mineralisation in the soil treatments was highly unlikely to be due to the difference in pH and C:N. Although correlations between phenanthrene mineralisation and soil properties were observed, it is important to interpret these within the context of the slurry-based assay. The use of MBS and continuous agitation may have diminished the influence of natural pH gradients, nutrient heterogeneity, and organic matter-related sorption. These changes could obscure the natural influence of soil properties such as pH, TOC, Olsen P, C:N ratio, ammonium nitrogen, and EC on degradation. As such, the relationships observed likely reflect the soils' underlying microbial capacity for degradation under nutrient-rich, well-oxygenated conditions, rather than true in-situ limitations. While this reduces environmental realism, it enhances comparability across treatments and allows for clearer interpretation of microbial responses.

In this study, EC, TOC, NH_4^+-N, and OLSEN P correlated positively with the extents of mineralisation, and the OLSEN P and NH_4^+-N correlated negatively with the lag phases. In this study, the EC correlated positively with extents of mineralisation in biochar-amended soil, and positively with the extents of mineralisation in the control. The EC also showed a negative

correlation with lag phases in control and biochar-amended soil. Studies have shown that EC improves PAH biodegradation and influences the changes in microbial community structure and microbial abundance (Meng et al., 2019; Rahman et al., 2021; Wang et al., 2023). In contrast, studies have also demonstrated a negative relationship between EC and PAH biodegradation (Betancur-Galvis et al., 2006; Yaun et al., 2023). In this study, the TOC correlated positively with extents of mineralisation in biochar-amended soil. The TOC also showed a negative correlation with lag phases in biochar-amended soil. This is consistent with Yaun et al. (2023) who stated that the removal of total petroleum hydrocarbon showed a strong positive significant correlation with TOC. However, the TOC in this study correlated negatively with the rates of mineralisation in SMC-amended soil. This could indicate that the impact of TOC is influenced by amendment properties. Earlier studies stated that TOC significantly correlated positively with PAH retention in soil, as PAHs may be sequestered by soil organic matter and become less available for biodegradation (Tang et al., 2005; Li et al., 2008; Nam et al., 2008). Similarly, TOC reportedly correlated positively with lag phases in UK soil (Okere et al., 2017). The EC and TOC did not correlate with the mineralisation parameters in soils amended with amendment blends further indicating that amendment type may influence the impact of soil properties in PAH mineralisation. The contradicting reports on EC and TOC imply that further research is needed to understand these parameters' implications on phenanthrene mineralisation under different amendment conditions.

In this present study, ammonium nitrogen correlated positively with the extents of mineralisation in all soil treatments except 2:1 and biochar-amended soils, and the available P correlated positively with extents of mineralisation in all soil treatments except in biochar-amended soils.

Therefore, both soil properties are highly likely to have had a greater influence on the observed ^{14}C-phenanthrene mineralisation than other monitored soil properties in this study. A recent study demonstrated that available phosphorus improved the biodegradation of 3 – 4 ring PAHs (Kończak et al., 2023). Nitrogen and phosphorus have been demonstrated in earlier studies to influence microbial communities and improve PAH removal in soil (Betancur-Galvis et al., 2006, Zhang et al., 2010). The increase in the C:N ratio over time in this present study is an indication of N utilisation in the soils. The application of ammonium nitrogen has been demonstrated to increase PAH removal (Wang et al., 2022). Similarly, available P favoured microbial growth and influenced microbial community composition (Zhang et al., 2010; Wu et al., 2021; Martinez-Toledo et al., 2022).

4.6 Conclusion

This study has demonstrated that SMC:biochar blends can shorten lag phases, expedite rates, and increased the extents of phenanthrene mineralisation in soil. However, the effectiveness of these blends is influenced by the blending ratio, as higher biochar content may enhance sorption, potentially limiting biodegradation. These findings provide valuable insights into the effectiveness of SMC:biochar blends as soil amendments and highlight the importance of optimising the blending ratio. The optimal ratio should be selected based on the remediation objective – higher compost content to enhance biodegradation or higher biochar content to promote sorption while allowing for the gradual degradation of the remaining bioavailable contaminants. However, further research is needed to assess the impact of different application amounts on PAH mineralisation kinetics. This study used freshly added ^{14}C-phenanthrene to

probe the bioavailability of aged ^{12}C-phenanthrene, though this may not fully represent the fate of the aged compound. Simultaneous aging of both isotopes would offer a more accurate representation of aging effects on bioavailability and mineralisation. Additionally, using MBS in slurry assays may alter native soil chemistry and microbial responses; replacing it with distilled water in future studies would help preserve native soil conditions and improve the ecological relevance of observed correlations.

4.7 Declaration of interest

The authors declare no conflicting financial, professional, or personal interests.

4.8 CRediT author statement

Chisom Ejileugha: Conceptualisation, Methodology, Investigation, Data interpretation, Visualisation, Formal analysis, Writing - Original draft, Writing – Review & Editing **Kirk T.**

Semple: Conceptualisation, Methodology, Validation, Resources, Supervision, Writing – Review & Editing.

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Chapter 5 Influence of biochar particle size on ^{14}C-phenanthrene extractability and mineralisation in soil

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5.1 Abstract

Biochar can influence the bioavailability and biodegradation of polycyclic aromatic hydrocarbons (PAHs) in soil, but the role of biochar particle size in this process has not been fully explored. In this study, soil was spiked with ^{14}C-phenanthrene and subsequently amended with biochar of <0.6 mm and 2 – 4 mm particle size at 0.0%, 0.1%, 1.0%, and 10.0%, respectively. The amended soils were aged for 60 d and ^{14}C-phenanthrene extractability and mineralisation were monitored at 1 d, 15 d, 30 d, 45 d, and 60 d. The total residual ^{14}C-activity and extractability reduced over time with increasing biochar amounts irrespective of biochar particle size. Similarly, longer lag phases, slower rates, and lower extents of mineralisation were observed over time with increasing biochar amounts. Solvent extractability and reduction in residual activity were higher in <0.6 mm amended soils which were attributed to a higher surface area and shorter diffusion length. Hydroxypropyl-β-cyclodextrin (HP-β-CD) extracted ^{14}C-phenanthrene correlated with the extents of mineralisation with stronger agreement in <0.6 mm amended soils (R^2 = 0.77 – 0.86) than in 2 - 4 mm amended soils (R^2 = 0.57 – 0.82). The weakest correlation was observed at 10.0% of 2 - 4 mm. This study demonstrated HP-β-CD's potential for predicting phenanthrene microbial availability in biochar-amended soils and highlighted the influence of biochar particle size on phenanthrene bioavailability and mineralisation. These findings will support informed decisions in risk assessment and the likelihood of successful bioremediation of PAH-contaminated soil.

Keywords: bioavailability, biochar, cyclodextrin, mineralisation, organic amendment, polycyclic aromatic hydrocarbon.

5.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic chemicals with two or more benzene rings (Lawal, 2017). They can be produced from the incomplete combustion of fossil fuels or organic matter (Quilliam et al., 2013; Lawal, 2017). They are considered to be persistent organic pollutants (POPs) as they display are persistent, toxic and bioaccumulatory properties and pose risks to human and environmental health (Wu et al., 2019; Patel et al., 2020). PAHs are hydrophobic and can sorb to soil, making soil a sink for PAHs in the environment (Okere and Semple, 2012). PAHs may also have negative impacts on soil health and function; however, organic amendments can be used to reduce these impacts of PAHs by reducing PAH mobility and bioavailability (Puglisi et al., 2007; Sigmund et al., 2018; Kaur and Sharma, 2020). PAHs have different molecular weights, ring numbers, and structural complexity which influence their hydrophobicity, bioavailability, biodegradation, and environmental persistence (Riding et al., 2013). As a result, PAHs with high molecular weight, ring number, and complex structure are more recalcitrant to biodegradation and persist more in the environment (Stroud et al., 2007; Papadopoulos, Reid et al., 2007; Baldantoni et al., 2017). Soil-indigenous microbes can biodegrade PAHs, but susceptibility to biodegradation is influenced by their bioavailability (Reid et al., 2001). Thus, the biodegradation of most PAHs in soil is not limited by microbial population or catabolic ability but by their desorption and bioavailability (Allan et al., 2007; Semple et al., 2006).

Recently, environmental remediation strategies have moved towards more sustainable approaches, where possible (Patel et al., 2020). The use of biochar as an amendment for PAH-

contaminated soil, is cost-effective, environmentally friendly, and sustainable (Zahed et al., 2021; Guo et al., 2021; Guo et al., 2022). Biochar has been demonstrated to improve soil physicochemical properties and soil microbial activities thereby improving PAH biodegradation (Kong et al., 2021). Biochar can also mitigate the impact of PAHs in the soil through sorption by transferring PAHs in the soil to biochar thereby reducing the leaching and bioavailability of PAHs in soil (Chen & Yuan, 2011; Ogbonnaya et al., 2014; Jimenez et al., 2018). Reduction in bioavailability reduces potential exposure thereby mitigating possible toxicity, and the bioavailable concentration of a PAH is of interest in risk assessment as it controls potential biodegradation and toxicity (Riding et al., 2013; Semple et al., 2013; Umeh et al., 2017). Non-exhaustive extraction techniques (NEETs) have been demonstrated as effective for predicting the concentration of PAHs available for microbial degradation (e.g. Alexander & Kelsey, 1997; Reid et al., 2000; Rhodes et al., 2008a; Rhodes et al., 2008b; Semple et al., 2013; Cachada et al., 2014; Oyelami et al., 2014; Ogbonnaya et al., 2016; Cao et al., 2022). In this study, hydroxypropyl-β-cyclodextrin (HP-β-CD) solvent extraction (a NEET) was used to predict ^{14}C-phenanthrene microbial degradation because it has been demonstrated as an efficient, reliable, and time-effective technique for predicting PAH microbial degradation in soil (e.g., Patterson et al., 2004; Swindell & Reid, 2006; Semple et al., 2006; Papadopoulos et al., 2007; Bernhardt et al., 2013; Ogbonnaya et al., 2014; Adedigba et al., 2018; Leech et al., 2020; Vázquez-Cuevas et al., 2021; Posada-Baquero et al., 2022; Jin et al., 2023).

Biochar improves the sorption of PAHs in soil which is dependent on the properties of biochar, soil, PAHs, and soil-biochar contact duration (Zhang et al., 2010). Studies have demonstrated that

HP-β-CD extraction and microbial degradation in soil amended with black carbon reduces with an increase in contact time and amendment amount (Rhodes et al., 2008a; Rhodes et al., 2010; Rhodes et al., 2012; Ogbonnaya et al., 2014a; Ogbonnaya et al., 2014b, Oyelami et al., 2014; Oyelami et al., 2015; Ogbonnaya et al., 2016). Additionally, desorption could predict PAH mineralisation in black carbon amended soils (Oyelami et al., 2014; Ogbonnaya et al., 2014a; Ogbonnaya et al., 2016; Yu et al., 2016), but desorption could not predict mineralisation at higher black carbon amounts (Rhodes et al., 2008a; Rhodes et al., 2012). This raises concern about the suitability of HP-β-CD extraction for predicting the mineralisation of PAHs in soils amended with black carbon. In addition, phenanthrene biodegradation exceeded solvent extraction in past studies (Rhodes et al., 2008a; Rhodes et al., 2012; Ogbonnaya et al., 2016) though phenanthrene desorption has been reported to be higher than mineralised (Oyelami et al., 2014; Kang et al., 2019). There is notable information in the literature regarding the sorption and desorption behaviour of biochar and a disparity in the impact of biochar particle size on PAH mineralisation. Fine particle biochar has been shown to demonstrate higher sorption capacities and rates compared to coarse particle biochar (Kang et al., 2018; Kang et al., 2019; Jin et al., 2022; He et al., 2022). However, it has also exhibited higher desorption for phenanthrene (Kang et al., 2019), ammonium nitrogen (He et al., 2022), and phosphorus (Sarfraz et al., 2020). Additionally, while powdered biochar (<250 μm) showed greater phenanthrene mineralisation than raw biochar (<2–4 mm) (Kang et al., 2019), contradictory findings indicate that coarse biochar (3–7 mm) resulted in higher phenanthrene mineralisation than fine biochar (≤2 mm) (Ogbonnaya et al., 2014a; Ogbonnaya et al., 2014b). These conflicting results warrant further investigation.

Although studies have demonstrated that fine particle biochar can improve microbial activity and abundance in soil (Chen et al., 2017; Sarfraz et al., 2020; Zhao et al., 2020; Özenç et al., 2023), and smaller particle-sized biochar can enhance soil pH and water holding capacity, influencing the mobility and availability of PAHs (Chen et al., 2017; Liao & Thomas, 2019; Sarfraz et al., 2020), there remains insufficient information on how biochar particle size affects PAH bioavailability and mineralisation. While it is widely accepted that fine biochar particles have higher sorption capacities (Kang et al., 2018; Kang et al., 2019; Jin et al., 2022; He et al., 2022), most studies focus on the impact of biochar particle size on soil physicochemical properties (e.g., Singh et al., 2010; Yao et al., 2012; Jin et al., 2016; Pratiwi et al., 2016; Xu et al., 2016; Esmaeelnejad et al., 2017; Lim et al., 2017; Lim & Spokas, 2018; Billah et al., 2019; Duarte et al., 2019; Alghamdi et al., 2020; Edeh et al., 2021; Özenç et al., 2023). Therefore, this study investigated the effect of biochar particle size on ^{14}C-phenanthrene bioavailability and mineralisation in soil, which are crucial factors in the risk assessment and remediation of contaminated soils.

5.3 Materials and methods

5.3.1 Sample collection.

The soil sample was collected from Hillam Farm green energy site in Cockerham Lancaster, United Kingdom. The soil was air-dried, sieved through a 2 mm mesh to remove unwanted materials (stones, debris, etc.), and was stored at 4 °C. Biochar was purchased from a commercial source (SoilFixers), Royal Wootton Bassett, United Kingdom. The properties of the biochar and soil are shown in Table 1 and Table 2, respectively.

Table 1: Biochar properties and pyrolysis conditions as provided by the supplier (SoilFixers, UK)

Parameter	**Value**
Feedstock	Hardwood logs
Pyrolysis equipment	Retort Kiln
Pyrolysis temperature	700 – 900 °C
Pyrolysis duration	5 – 12 h
Particle size	0 – 8 mm
pH	8.8 – 10
Volatiles	9 – 15%
Ash max.	3 – 6%
Moisture max.	8 – 12%
C Fixed	>76%

Table 2: Properties of the soil sample used in this study. Values are in mean ± SD except the values for sand, silt, and clay

Parameters	Value
Sand (%)	71.4
Silt (%)	26.2
Clay (%)	2.4
Soil texture	Sandy loam
pH	7.3±0.0
Electrical conductivity (µS cm^{-1})	382±4.4
Organic matter (%)	6.5±0.3
C:N	10.4±0.1
Total carbon (mg/kg)	102.4±4.4
Total organic carbon (mg/kg)	98.9±3.6
Inorganic carbon (mg/kg)	3.6±1.0
Ammonium nitrogen (mg/kg)	0.4±0.0
Nitrate nitrogen (mg/kg)	3.6±0.2

5.3.2 Soil spiking and amendment

Soil (2.1 kg; dry weight) was rehydrated to field moisture content (30%) and was spiked with ^{12}C and 9-^{14}C– phenanthrene (100 mg/kg and 3,800 DPM/g; dry weight) (Reid et al., 2000; Doick et al., 2003; Stokes et al., 2005). Soil (approx. ¼) was initially spiked with ^{12}C and 9-^{14}C–phenanthrene using acetone as the carrier solvent at a 1:20 (v/w) solvent-to-soil ratio. This portion was left in a fume hood for 3 h to allow complete volatilisation of the solvent. The remaining soil was subsequently added in three roughly equal portions, with thorough mixing after each addition to ensure uniform distribution of the contaminant and overall homogeneity. The spiked soil was mixed thoroughly in a glass bowl using a stainless-steel spoon (Doick et al., 2003). Soil (300 g; dry weight) was weighed into a clean glass bowl, rehydrated, and spiked with ^{12}C-phenanthrene as ^{12}C blank. Soil (300 g) for ^{14}C-blank was weighed into a clean glass bowl. The remaining soils were amended with biochar (<0.6 mm and 2 – 4 mm particle size) at 0.1 %, 1.0%, and 10%, respectively. The soils (amended and blanks) were weighed (100 g; n = 3) into amber bottles and incubated in the dark at 21 ± 2 ℃ for 60 d. The incubated soils were sampled at 1 d, 15 d, 30 d, 45 d, and 60 d to measure the total residual ^{14}C-activity, solvent extractability, and mineralisation.

5.3.3 Determination of total ^{14}C – activity in the soil

Soil (1.0 g; wet weight) was weighed into a cellulose combustion cone and Combustaid (200 µl) was added to the soil (Reid et al., 2000; Doick et al., 2003; Stokes et al., 2004; Doick et al., 2005).

The soil was combusted for 3 mins using a sample oxidiser (Packard 307). The released $^{14}CO_2$ was trapped with 10 ml of CarbonTrap and was delivered into a 20 ml vial with 10 ml of CarbonCount as a scintillation fluid. The efficiency (>96%) of the sample oxidiser to trap the evolved $^{14}CO_2$ was determined before combustion. The 20 ml vial was stored in the dark for 24 h before quantifying for 10 min with a liquid scintillation counter (LSC; Canberra Packard Tri-Carb 2250CA).

5.3.4 Extractability of ^{14}C – phenanthrene in the soil

3.4.2.5 Dichloromethane (DCM) extraction of ^{14}C – phenanthrene

Soil (1.5 g) was weighed into a 50 ml Teflon-lined centrifuge tube. Anhydrous sodium sulphate (1.5 g) was added to the soil and 20 ml of DCM was added to the tube (Reid et al., 2000; Doick et al., 2003; Papadopoulos, Paton et al., 2007; Rhodes et al., 2008b). All tubes were tightly closed and placed in an orbital shaker at 100 RPM for 24 h. Afterwards, the tubes were centrifuged at 4000 RPM for 1 h. The supernatant (5 ml) was pipetted into a 20 ml vial, and 14 ml of Ultima Goldstar scintillation fluid was added to the vial. The vial was stored in the dark for 24 h before quantifying for 10 min using an LSC. The remaining supernatant was safely and carefully discarded.

3.4.2.6 Hydroxypropyl-β-cyclodextrin (HP-β-CD) extraction of ^{14}C – phenanthrene

Soil (1.5 g) was weighed into a 50 ml Teflon-lined centrifuge tube, and 25 ml of 50 mM HP-β-CD solution was added to the tube (Reid et al., 2000; Stokes et al., 2005; Doick et al., 2006; Papadopoulos, Reid et al., 2007). All tubes were sealed and placed on an orbital shaker at 100

RPM for 24 h. Afterwards, the tubes were centrifuged at 4000 RPM for 1 h. The supernatant (5 ml) was transferred into a 20 ml vial and 14 ml of Ultima Goldstar scintillation fluid was added to the vial. The vial was stored in the dark for 24 h before quantifying for 10 mins using an LSC. The remaining supernatant was properly discarded.

5.3.5 Respirometric monitoring of ^{14}C – phenanthrene mineralisation in soil

Soil (10 ± 0.2 g) was weighed into a 250 ml modified Schott bottle and 30 ml of sterile MBS (1:3 soil – liquid ratio) was added to the soil to form a slurry (Reid et al., 2001; Doick et al., 2003; Allan et al., 2007). A 7 ml vial containing 1 ml of 1 M NaOH was attached to the bottle to trap evolved $^{14}CO_2$. The bottles were tightly closed, placed on an orbital shaker (100 RPM) and monitored at 21 ± 2 ℃. The bottles were sampled at 3 h intervals for an initial 9 h, then daily for 14 d. At each sampling, the 7 ml vial was removed and replaced with a fresh vial. Then, 5 ml Ultima Goldstar scintillation fluid was added to the sampled vial. The sampled vial was stored for 24 h in the dark before quantifying using LSC.

5.3.6 Statistical analysis

The data was processed using MS Excel and analysed using IBM SPSS 28. Data plots were done using SigmaPlot. Data were analysed using a one-way analysis of variance (ANOVA), Tukey's HSD post hoc, independent sample t-test, and simple linear regression.

5.4 Results

5.4.1 Temporal changes in the total residual ^{14}C-phenanthrene activity in the amended soils

The total residual ^{14}C-phenanthrene activity in the amended soils was monitored at 1 – 60 d (Figure 1). Noticeably, the residual ^{14}C-activity in the amended soils reduced as the soil contact duration increased in <0.6 mm and 2 – 4 mm amendments (Figure 1). The decrease followed a similar trend irrespective of amendment particle size. However, the residual activity was lower in <0.6 mm amended soils. The residual ^{14}C-activity was significantly higher at 1 d and significantly lower at 60 d (all $p < 0.05$). In soils amended with <0.6 mm, the residual ^{14}C-activity at 0.1% was lower than in 1.0% ($p > 0.05$) and 10.0% ($p < 0.05$) at 60 d (Figure 1). The residual ^{14}C-activity in <0.6 mm amended soils was higher than in the control (0.1% and 1.0%, $p > 0.05$; 10.0%, $p < 0.05$). The 10.0% amendment showed higher ($p < 0.05$) residual activity at 60 d compared to other amended soils irrespective of biochar particle size. In soils amended with 2 – 4 mm, the residual ^{14}C-activity at 1.0% amendment was lower than in 0.1% ($p > 0.05$) and 10.0% ($p < 0.05$) at the end of incubation (Figure 1). All soils amended with 2 – 4 mm biochar also had a higher residual ^{14}C-activity than control ($p < 0.05$). Noticeably, at 15 d, 10.0% of 2 – 4 mm had only lost 1.77% of the initial spiked activity. Overall, at 60 d, <0.6 mm amended soil lost 61.64 – 75.91% of the initial spiked activity, while 2 – 4 mm amended soils lost 58.38 – 61.29% of the initial spiked activity.

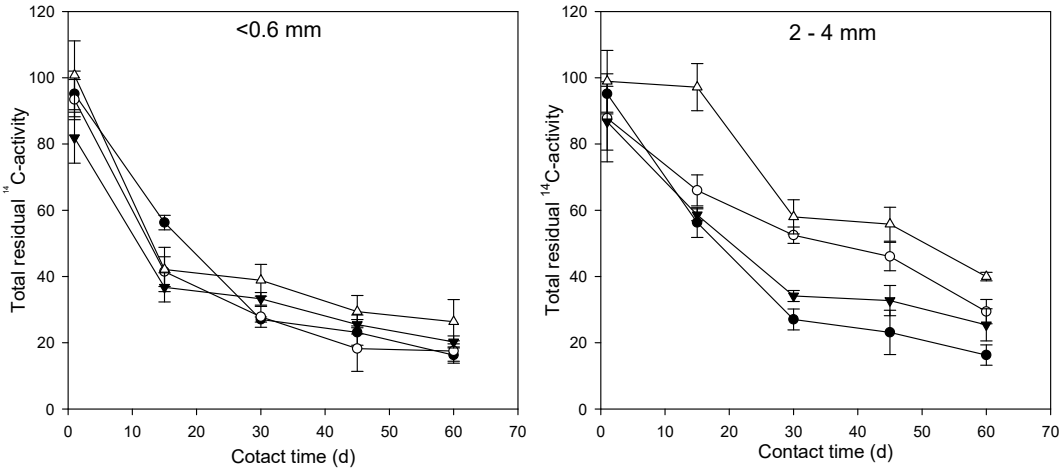

Figure 1: Temporal changes in total residual ^{14}C-phenanthrene activity in <0.6 mm and 2 – 4 mm amended soils at 1 – 60 d. The soils are represented as ●(Control), ○(0.1%), ▼(1%), and △(10%). Data points show mean values (n = 3) with error bars showing the standard error of mean (SEM).

5.4.2 Temporal changes in the extractability of ^{14}C-phenanthrene in amended soils

The extraction of ^{14}C-phenanthrene using DCM and HP-β-CD in the amended soils was measured over the 60 d incubation (Figure 2 and Figure 3). This was expressed as a percentage of the residual ^{14}C-activity at each contact time. Noticeably, DCM and HP-β-CD extractability of ^{14}C-phenanthrene decreased over time in soils amended with <0.6 mm and 2 – 4 mm (Figure 2; Figure 3). The DCM-extractable ^{14}C-phenanthrene was substantially higher than the HP-β-CD-extractable ^{14}C-phenanthrene in the amended soils regardless of particle size. The DCM-extractable ^{14}C-phenanthrene at 1 d was higher than that at 15 – 60 d, and that at 60 d was significantly lower than that at 1 – 45 d ($p < 0.05$). At the end of incubation, in soils amended with <0.6 mm amendment, the DCM-extractable ^{14}C-phenanthrene at 10.0% amendment was lower

than in other amended soil ($p < 0.05$), and the DCM-extractable ^{14}C-activity at 1.0% amendment was lower ($p > 0.05$) than at 0.1% (Figure 2). Also, in soils amended with 2 – 4 mm, the DCM-extractable ^{14}C-phenanthrene at 10.0% was lower than other amended soils ($p < 0.05$), and the DCM extractable ^{14}C-activity at 1.0% was lower ($p > 0.05$) than at 0.1%. All biochar amended soils had lower DCM-extractable ^{14}C-activity than the control regardless of the particle size (Figure 2). This was significant ($p < 0.05$) for all 2 – 4 mm amended soils and significant ($p < 0.05$) for <0.6 mm amended soils only at 1.0% and 10.0% (Figure 2). Furthermore, at 60 d in soils amended with <0.6 mm, HP-β-CD-extractable ^{14}C-phenanthrene at 10.0% was lower than at 1.0% ($p > 0.05$) and 0.1% ($p < 0.05$), and that at 1.0% amendment was lower ($p > 0.05$) than at 0.1% (Figure 3). In soils amended with 2 – 4 mm, the HP-β-CD-extractable ^{14}C-phenanthrene at 10.0% amendment was lower ($p < 0.05$) than at 1% and 0.1%, while there was significant ($p > 0.05$) difference between 1.0% and 0.1% (Figure 3). Generally, the reduction in solvent extractability increased with an increase in biochar amount in <0.6 mm and 2 – 4 mm amended soils. Additionally, <0.6 mm amended soils had higher extractability than 2 – 4 mm amended soils ($p > 0.05$).

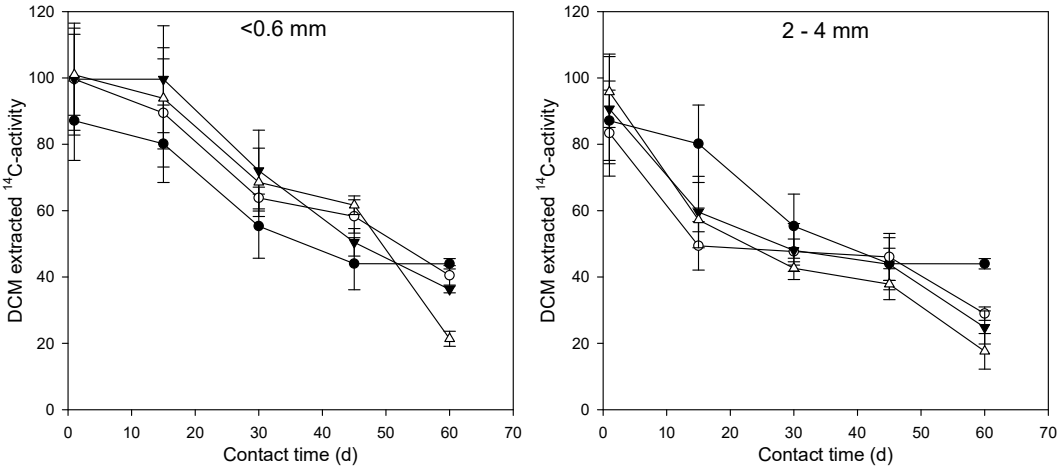

Figure 2: DCM extractable ^{14}C-phenanthrene in the <0.6 mm and 2 – 4 mm amended soils. The soils are represented as ●(Control), ○(0.1%), ▼(1%), and △(10%). Data points show mean (n = 3) values with error bars showing the standard error of mean (SEM).

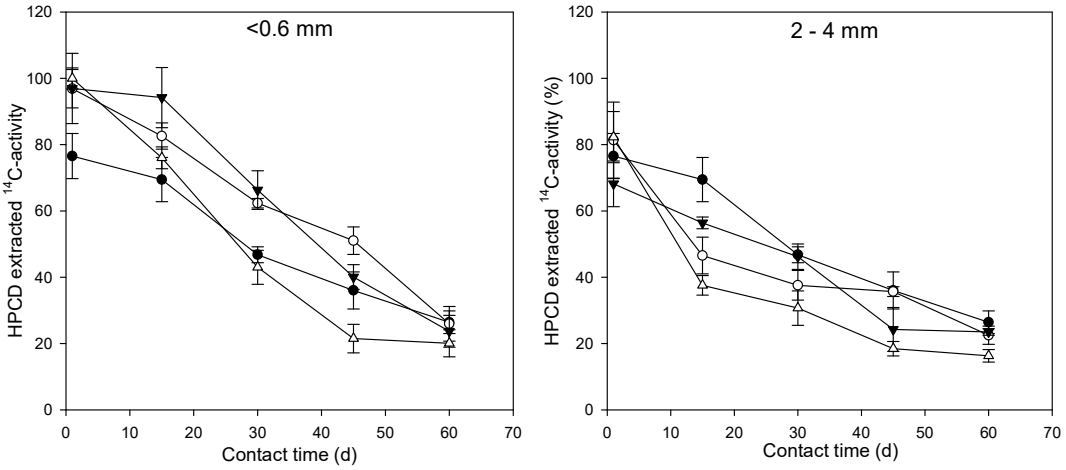

Figure 3: HP-β-CD-extractable ^{14}C-phenanthrene in the <0.6 mm and 2 – 4 mm amended soils. The soils are represented as ●(Control), ○(0.1%), ▼(1%), and △(10%). Data points show mean (n = 3) values with error bars showing the standard error of mean (SEM).

5.4.3 Temporal changes in ^{14}C-phenanthrene catabolism in amended soils

The lag phases, rates, and extents of ^{14}C-phenanthrene mineralisation were monitored in the soil treatments from 1 – 60 d (Table 3; Figure 4; Figure 5). The mineralisation kinetics were calculated from the residual activity at each contact time. Noticeably, the lag phases were longer, and the extents of mineralisation were lower over time in <0.6 mm and 2 – 4 mm amended soils (Table 3, Figure 4, and Figure 5). In <0.6 mm amended soils, there were no significant differences between the lag phases at 1 d and 30 d ($p > 0.05$). The lag phases at other contact times were longer than at 15 d (Table 3; $p < 0.001$), with those at 60 d significantly longer than at 1–45 d ($p < 0.05$), and at 45 d longer than at 1–30 d ($p < 0.05$). The fastest rates at 15 d were more rapid than the rates at 1 d, 30 d, 45 d, and 60 d (Table 3; $p < 0.001$). The rates at 1 d were faster than at 30 - 60 d ($p < 0.01$), while rates at 30 d were faster than at 45 d ($p < 0.01$), with no significant difference between 45 and 60 d ($p > 0.05$). There was no significant difference in the extents of mineralisation at 1 d and 15 d ($p > 0.05$), but both contact times showed significantly higher extents of mineralisation than 30 – 60 d (Table 3; Figure 4; $p < 0.001$). The extents of mineralisation at 30 d were significantly higher than at 45 – 60 d ($p < 0.01$), but there was no significant difference between the extents of mineralisation at 45 d and 60 d (Figure 4; $p > 0.05$).

Table 3: Mineralisation kinetics for the soil treatments. Values are in mean ± SEM (n = 3). Values in columns followed by different letters are statistically different (Tukey's HSD; n = 3; $p < 0.05$); also indicated are shorter lag phases (*), faster rates (+), higher extents of mineralisation (-) than the controls that are not statistically significant ($p > 0.05$).

Contact time (d)	Particle size (mm)	Amendment amount (%)	Lag phase (h)	Fastest rate (%$^{14}CO_2$/h)	Extent of mineralisation (%)
1		0.0	37.9±1.6^{a}	0.6 ± 0.0^{a}	47.5 ± 2.3^{a}
	<0.6	0.1	35.8±5.7^{a*}	0.8 ± 0.3^{a+}	53.6 ± 4.5^{b}
		1.0	46.8±9.7^{b}	0.5 ± 0.3^{b}	41.3 ± 5.4^{a}
		10.0	67.6±9.6^{c}	0.3 ± 0.1^{b}	26.8 ± 3.7^{c}
	2 – 4	0.1	27.3±2.2^{d}	0.9 ± 0.0^{c}	69.5 ± 5.8^{d}
		1.0	29.1±4.0^{d}	1.1 ± 0.1^{c}	65.4 ± 5.6^{d}
		10.0	39.7±3.0^{a}	0.9 ± 0.2^{c}	45.5 ± 3.8^{a}
15		0.0	27.4±6.4^{a}	0.8 ± 0.2^{a}	40.5 ± 5.2^{a}
	<0.6	0.1	16.4±4.3^{b}	1.1 ± 0.1^{b}	52.6 ± 1.0^{b}
		1.0	12.8±3.3^{b}	1.3 ± 0.1^{b}	46.1 ± 3.6^{a-}
		10.0	13.4±4.4^{b}	1.1 ± 0.2^{b}	56.3 ± 3.4^{b}
	2 – 4	0.1	30.3±5.7^{a}	0.7 ± 0.1^{a}	38.9 ± 4.0^{a}
		1.0	23.5±2.7^{a*}	0.9 ± 0.1^{a+}	42.0 ± 3.6^{a-}
		10.0	100.7±15.0^{c}	0.3 ± 0.0^{c}	15.4 ± 2.2^{c}
30		0.0	75.3±8.5^{a}	0.4 ± 0.1^{a}	18.8 ± 1.9^{a}
	<0.6	0.1	53.3±4.9^{b}	0.4 ± 0.0^{a}	26.2 ± 2.4^{b}
		1.0	61.6±2.3^{b}	0.3 ± 0.1^{a}	24.2 ± 1.2^{b}
		10.0	82.6±29.7^{a}	0.3 ± 0.1^{a}	23.1 ± 4.3^{b}
	2 – 4	0.1	131.4±24.8^{c}	0.3 ± 0.1^{a}	13.1 ± 2.9^{c}
		1.0	202.0±49.1^{d}	0.2 ± 0.0^{a}	9.5 ± 2.7^{c}
		10.0	144.3±1.1^{c}	0.2 ± 0.0^{a}	10.8 ± 0.2^{c}
45		0.0	115.2±20.6^{a}	0.2 ± 0.0^{a}	15.8 ± 3.5^{a}
	<0.6	0.1	163.7±26.8^{b}	0.1 ± 0.0^{a}	11.1 ± 1.7^{a}
		1.0	124.0±12.2^{a}	0.1 ± 0.0^{a}	14.1 ± 1.0^{a}
		10.0	155.8±33.5^{b}	0.1 ± 0.0^{a}	11.8 ± 2.1^{a}
	2 – 4	0.1	165.2±39.0^{b}	0.2 ± 0.0^{a}	11.8 ± 3.3^{a}
		1.0	124.4±26.5^{a}	0.1 ± 0.0^{a}	15.3 ± 3.4^{a}
		10.0	142.6±21.1^{c}	0.2 ± 0.0^{a}	12.5 ± 2.0^{a}
60		0.0	98.7 ± 18.0^{a}	0.4 ± 0.1^{b}	16.9 ± 3.0^{a}
	<0.6	0.1	192.6±40.6^{b}	0.1 ± 0.0^{a}	9.5 ± 1.6^{b}
		1.0	183.5 ± 6.1^{b}	0.1 ± 0.0^{a}	9.0 ± 0.4^{b}
		10.0	184.3±13.6^{b}	0.2 ± 0.0^{a}	9.0 ± 0.7^{b}
	2 – 4	0.1	202.9±8.2^{c}	0.1 ± 0.0^{a}	8.3 ± 0.3^{b}
		1.0	140.7±37.8^{d}	0.2 ± 0.0^{a}	13.1 ± 2.8^{b}
		10.0	195.4±47.1^{b}	0.2 ± 0.0^{a}	10.0 ± 2.7^{b}

In <0.6 mm amended soils, significant difference ($p < 0.05$) was observed in lag phases in the amended soils on 1 d, 30d, and 45 d, while there was in significant difference ($p > 0.05$) in lag phases at 15 d and 60 d. Higher lag phases were observed in soil amended at 0.1% on 15 d, 45 d, and 60 d compared to other amended soils while this was observed on 1 d and 30 d at 10.0% amendment. The control had lower lag phases than amended soils at 45 d and 60 d. The lag phases in the amended soils were not significantly different at 60 d ($p > 0.05$), but all amended soils showed longer lag phases than the control ($p < 0.001$). There was no significant difference in the fastest rates in amended soils from 15 – 60 d. Significantly ($p < 0.05$) higher rates were observed in 0.1% at 1 d compared to other amended soils. The control showed faster rates than all amended soils from 30 – 60 d. Significant difference ($p < 0.05$) was observed in the extents of mineralisation in the amended soils from 1 – 15 d, with no significant difference ($p > 0.05$) observed from 30 – 60 d. The soil amended at 10.0% showed significantly ($p < 0.05$) lower extents of mineralisation at 1 d and had overall lower extents of mineralisation compared to other amendment levels. The amended soils showed significantly ($p < 0.05$) higher extents of mineralisation than the control from 15 – 30 d. The extents of mineralisation in the control at 60 d was higher than in all amended soils ($p < 0.05$).

In 2 – 4 mm amended soils, there was no significant difference in lag phases at 1 d and 15 d ($p > 0.05$) but the lag phases at 15 d were significantly shorter than 30 – 60 d ($p < 0.001$). There were no significant differences in lag phases at 30 – 60 d ($p > 0.05$). In 2 – 4 mm amended soils, there were no significant differences in the rates at 1 d and 15 d ($p > 0.05$), but both contact times showed faster rates than 30 – 60 d (Table 3; $p < 0.001$). The rates at 30 d were faster than at 45 d ($p < 0.05$), and there was no significant difference in rates at 45 d and 60 d ($p > 0.05$). In 2 – 4

mm amended soils, the extents of mineralisation at 1 d were higher ($p < 0.001$) than at 15 d, and the extents of mineralisation at 15 d were higher ($p < 0.001$) than at 30 – 60 d (Table 3, Figure 5). There was no significant difference in the extents of mineralisation at 30 – 60 d ($p > 0.05$).

In 2 – 4 mm amended soils, significant ($p < 0.05$) difference in lag phases were observed in the amended soils from 1 – 60 d. The control showed shorter lag phases than all amended soils from 30 – 60 d. The soil amended at 10.0% showed higher lag phases than the control from 1 – 60 d. At 15 d, the lag phases at 10.0% amendment were longer than in other soil treatment conditions (Table 3; $p < 0.05$). At 30 d, the lag phases at 1.0% amendment were longer than in other soil treatment conditions ($p < 0.05$). The control had significantly ($p < 0.050$ shorter lag phases at 60 d compared to amended soils. There was no significant ($p > 0.05$) difference in fastest rates in the amended soils on 1 d and from 30 – 60 d. Significantly ($p < 0.05$) slower rates were observed in 10.0% at 15 d compared to other amended soils. The control showed higher rates than the amended soils from 30 – 60 d. There was a significant difference ($p < 0.05$) in the extents of mineralisation in the amended soils from 1 – 15 d, but the difference in the extents of mineralisation from 30 – 60 d was no significant ($p > 0.05$). At 1 d, the extents of mineralisation at 1.0% and 0.1% amendment were significantly higher (Figure 5; $p < 0.001$) than at 10.0% amendment and in the control. Also, at 15 d, the extents of mineralisation at 10.0% amendment were significantly lower than in other soil treatment conditions ($p < 0.001$). The soil amended at 10.0% showed significantly ($p < 0.05$) lower extents of mineralisation than other amendment levels from 1 - 15 d and had an overall lower extent of mineralisation compared to other amendment levels. The difference in the extents of mineralisation observed at 0.1% and 1.0% amendment from 1 – 60 d was not statistically significant ($p > 0.05$).

At 1 d, 2 – 4 amended soils (0.1% and 1.0%) showed non-significant shorter lag phases ($p > 0.05$), but significantly faster rates and higher extents of mineralisation ($p < 0.05$) compared to ≤0.6 mm (0.1% and 1.0%) amended soils (Table 3, Figure 4, Figure 5). Soil amended with 2 – 4 mm at 10.0% showed statistically significant ($p < 0.05$) shorter lag phases, faster rates, and greater extents of mineralisation at 1 d compared to soil amended with <0.6 mm at 10.0%. At 15 d, <0.6 mm amended soils showed significantly ($p < 0.05$) shorter lag phases and faster rates than 2 – 4 mm amended soils. The extents of mineralisation in soil amended at 1.0% with <0.6 mm was not significantly ($p > 0.05$) higher than in soil amended at 1.0% with 2 – 4 mm at 15 d. However, the extents of mineralisation at 15 d in <0.6 mm (0.1% and 10%) amended soil were higher ($p < 0.05$) than in 2 – 4 amended soils (0.1% and 10%). At 30 d, <0.6 mm amended soils showed significantly shorter lag phases, faster rates, and higher extents of mineralisation compared to 2 – 4 mm treatments (Table 3; $p < 0.05$). The extents of mineralisation significantly reduced at 30 d in soils amended with 2 – 4 mm (Table 3). Noticeably, at 15 d, there was an obvious significant ($p < 0.05$) reduction in the extents of mineralisation, with slower rates, and longer lag phases at 10.0% amendment for 2 – 4 mm (Table 3). There was no significant ($p > 0.05$) difference in the extents of mineralisation at 45 – 60 d in <0.6 mm and 2 – 4 mm amended soils (Table 3). The control showed shorter lag phases, faster rates, and higher extents of mineralisation than amended soils ($p < 0.05$) at 60 d, showing the impact of contact time on mineralisation kinetics.

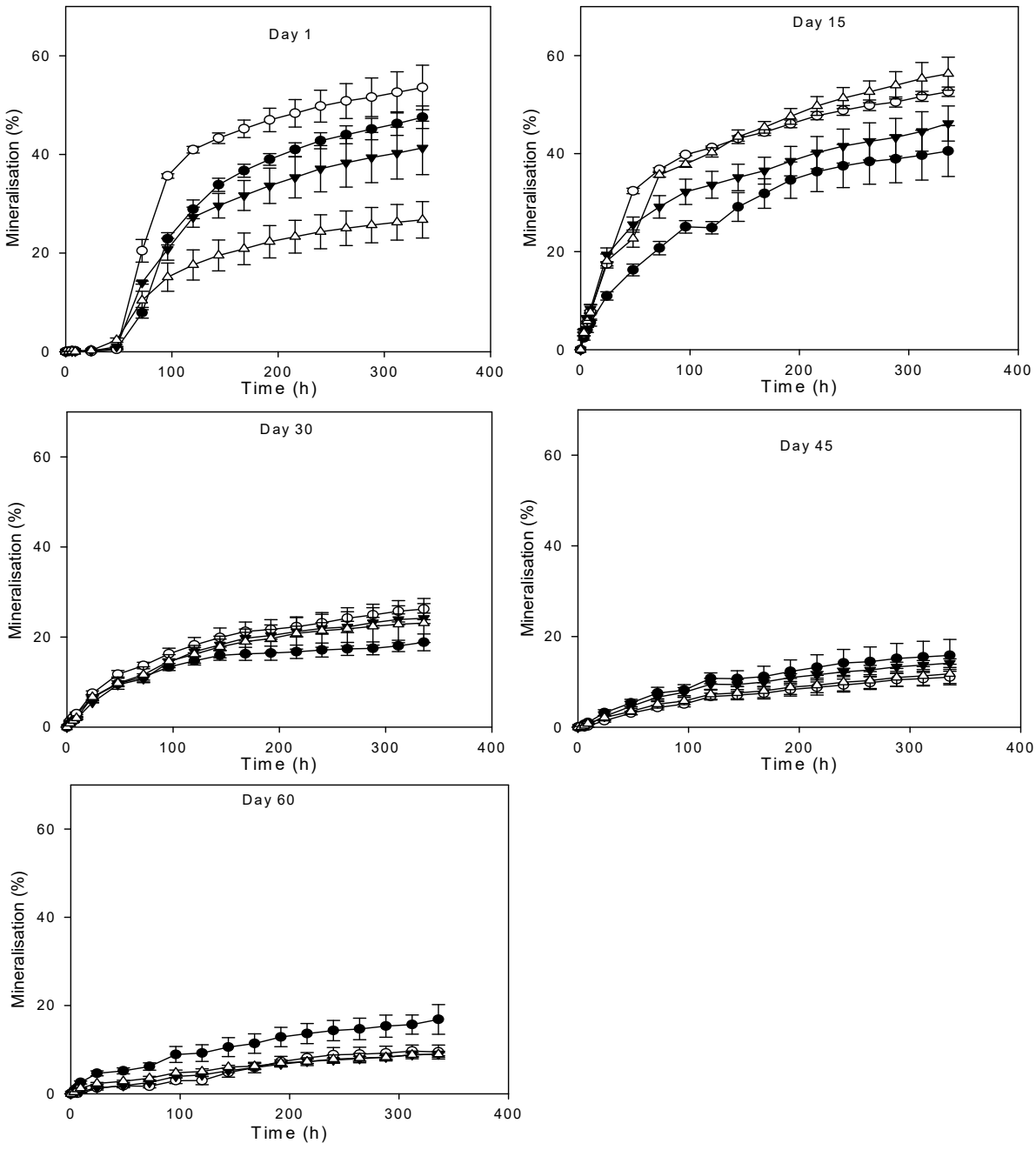

Figure 4: Extents of mineralisation in soils amended with <0.6 mm biochar. The soils are represented as ●(Control), ○(0.1%), ▼(1%), and △(10%). Data points show mean values (n = 3) with error bars showing the standard error of mean (SEM).

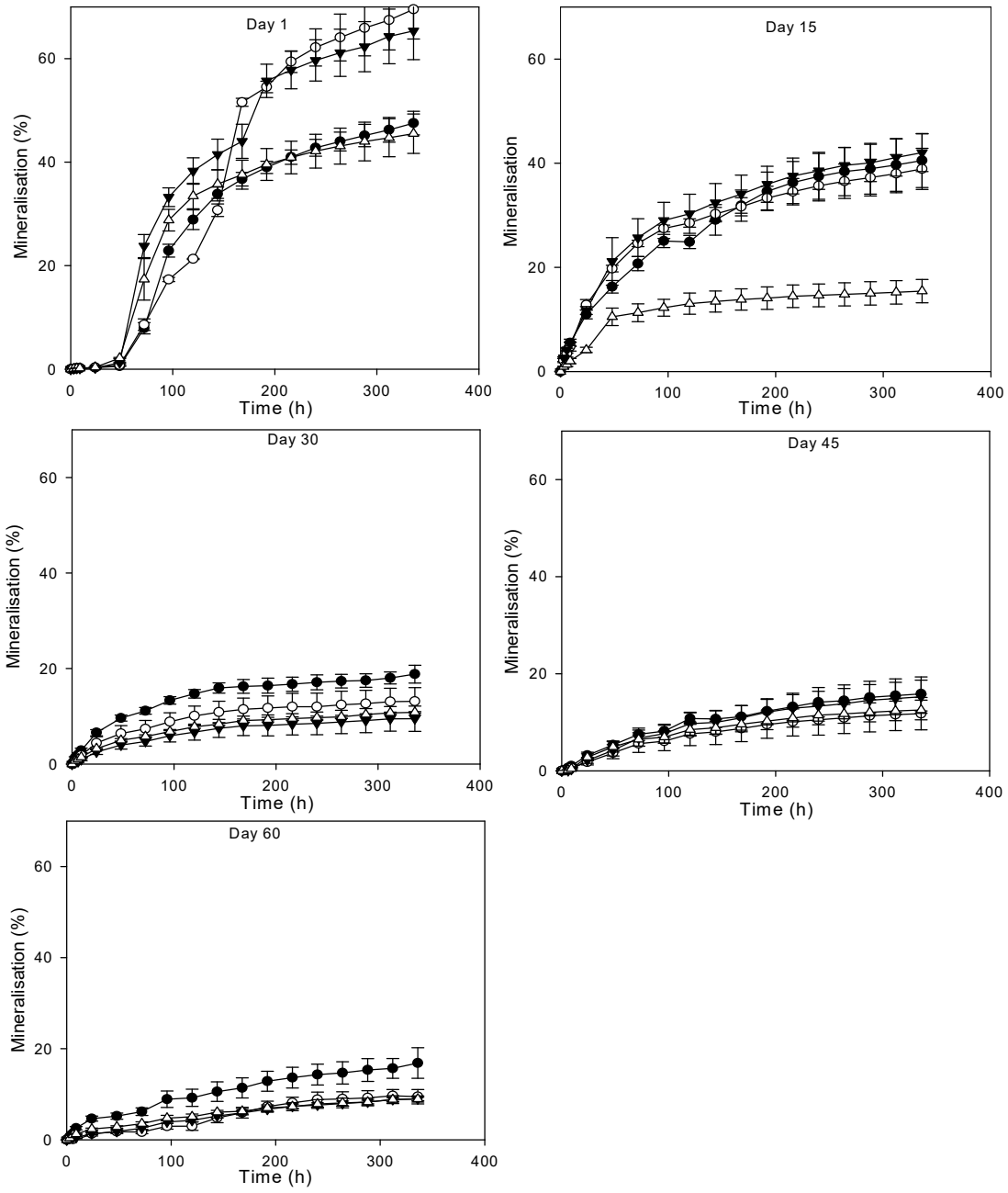

Figure 5: Extents of mineralisation in soils amended with 2 – 4 mm biochar. The soils are represented as ●(Control), ○(0.1%), ▼(1%), and △(10%). Data points show mean values (n = 3) with error bars showing the standard error of mean (SEM).

5.4.4 Relationship between HP-β-CD extracted ^{14}C-phenanthrene and extents of mineralisation

The linear relationship between the HP-β-CD extractable ^{14}C-phenanthrene and the extents of mineralisation was carried out to check the suitability of solvent extraction for predicting ^{14}C-phenanthrene microbial degradation in the biochar soils. The HP-β-CD extracted ^{14}C-phenanthrene correlated with the extents of mineralisation in the amended soil but this was influenced by the level of amendment and particle size. In <0.6 mm amended soils, at 0.1% amendment, HP-β-CD extracted ^{14}C-phenanthrene (R^2 = 0.86, slope = 0.99, intercept = -16.37). At 1.0% amendment, HP-β-CD extracted ^{14}C-activity (R^2 = 0.77, slope = 0.85, intercept = -1.22) also correlated with the extents of mineralisation, and at 10.0% amendment, HP-β-CD extracted ^{14}C-activity (R^2 = 0.82, slope = 0.65, intercept = 0.85) correlated with the extents of mineralisation. Similarly, in 2 – 4 mm amended soils, at 0.1% amendment, the HP-β-CD extracted ^{14}C-phenanthrene also showed a relationship with the extents of mineralisation (R^2 = 0.82, slope = 0.68, intercept = -20.06). Also, at 1.0% amendment, HP-β-CD (R^2 = 0.73, slope = 0.46, intercept = -7.01) correlated with the extents of mineralisation. Furthermore, at 10.0% amendment, HP-β-CD extracted ^{14}C-activity (R^2 = 0.57, slope = 0.54, intercept = -3.26) correlated with the extents of mineralisation.

5.5 Discussion

5.5.1 Changes in the total residual ^{14}C-phenanthrene in the amended soils

The initial recovery of spiked ^{14}C-phenanthrene ranged from 86.69% to 100.75%, meeting the acceptable variability threshold (<20%) required to validate spiking procedures (Doick et al., 2003). The reduction in recovered ^{14}C-phenanthrene activity was attributed to volatilisation, adhesion to the blending vessel, and soil heterogeneity, which can create subsystems with varying contaminant concentrations (Doick et al., 2003). Additional factors such as sampling variation and potential microbial degradation within 1 d may have also contributed to reduced ^{14}C-activity. Indigenous soil microbes, capable of degrading PAHs, may play a significant role in this reduction, which is consistent with previous findings (Macleod and Semple, 2000; Doick et al., 2005 Couling et al., 2010; Okere et al., 2017). Comparisons between sterile and non-sterile soils further highlight the influence of soil microbiota on residual ^{14}C-activity (Macleod and Semple, 2000; Oyelami et al., 2014). Residual ^{14}C-phenanthrene activity decreased over time in both amended soils, with greater reductions observed in soils amended with <0.6 mm. This trend aligns with findings by Ogbonnaya et al. (2014a) using wood biochar in sandy soils. At a 10.0% biochar amendment level, residual ^{14}C-activity was higher regardless of particle size compared to 0.1% and 1.0%, indicating reduced ^{14}C-phenanthrene losses. The higher sorption capacity at 10.0% amendment minimized volatilisation and biodegradation (Rhodes et al., 2008a; Ogbonnaya et al., 2016; Bielska et al., 2018).

5.5.2 Changes in DCM and HP-β-CD extractability of ^{14}C-phenanthrene in the soils

The solvent extractability of ^{14}C-phenanthrene declined over time, with greater reductions observed at higher amendment levels regardless of biochar particle size. Extractability decreased progressively, with the reduction at 60 d being more significant than at earlier time points (1 - 45 d). This aligns with studies showing that prolonged soil-PAH contact time enhances aging, leading to reduced PAH extractability in soil (Alexander & Kelsey, 1997; White et al., 1997; Macleod and Semple, 2000; Doick et al., 2005; Bielská et al., 2013). The reduction in solvent extractability of ^{14}C-phenanthrene was greater in the 10.0% amendment than in the 1.0% and 0.1% amendments. This supports previous findings that extractability decreases as black carbon content increases (Rhodes et al., 2008a; Ogbonnaya et al., 2014a; Yu et al., 2016; Ogbonnaya et al., 2016). Biochar addition resulted in reduced HP-β-CD extractability, with more significant effects at 5% and 10% amendments compared to 1% in soils amended with ≤2 mm and 3 - 7 mm biochar (Ogbonnaya et al., 2014a). The DCM extraction yielded higher ^{14}C-phenanthrene activity than HP-β-CD in all amended soils, which is consistent with the fact that HP-β-CD is a milder extraction solvent (Reid et al., 2000; Semple et al., 2007). As noted by Doick et al. (2003), neither contaminant concentration nor spiking procedure significantly affects DCM or HP-β-CD extractability. Therefore, the observed differences in ^{14}C-activity were attributed to aging, soil heterogeneity, and biochar particle size and amount.

Biochar's sorption capacity depends on pore size, particle size, and surface area (Zhang et al., 2010; He et al., 2022). Fine particle biochar has a higher surface area and sorption capacity than

coarse biochar (He et al., 2022). Smaller particles improve phenanthrene sorption rate and capacity (Kang et al., 2018). In this study, soils amended with fine particle biochar (<0.6 mm) showed higher solvent extractability and reduced residual ^{14}C-activity. This may be due to the larger surface area and shorter diffusion lengths of smaller biochar particles, which provide more improved sorption but enhance microbial degradation through faster desorption. Smaller particles facilitate faster sorption and desorption, improving phenanthrene availability for microbial activity. Recent studies support these findings (Kang et al., 2018; Kang et al., 2019; Sarfraz et al., 2020; Jin et al., 2022; He et al., 2022). Smaller biochar particles, with higher surface area and micropores, improve contaminant sorption and microbial degradation by providing faster access to contaminants and enhancing desorption (Kang et al., 2018; Sarfraz et al., 2020). Fine particle biochar shows better sorption capacity than coarse biochar, making it more effective in contaminant removal (He et al., 2022; Jin et al., 2022). However, for reducing contaminant bioaccessibility in soil, coarse biochar may offer better long-term sorption stability.

5.5.3 Changes in ^{14}C-phenanthrene mineralisation in the amended soils

Soil-PAH contact time affects PAH bioavailability and catabolism (Macleod and Semple, 2000; Reid et al., 2001; Wu et al., 2013; Wu et al., 2014; Omoni et al., 2020). In this study, ^{14}C-phenanthrene catabolism decreased with an increase in contact time. This is consistent with the findings in earlier studies (Rhodes et al., 2008a; Ogbonnaya et al., 2014a; Ogbonnaya et al., 2014b; Ogbonnaya et al., 2016; Omoni et al., 2020). Similarly, the amount of biochar added to the soils also influenced ^{14}C-phenanthrene mineralisation. Higher amounts of biochar (e.g.

10.0%) led to reduced mineralisation irrespective of biochar particle size. This is consistent with earlier findings where increase in amounts of black carbon caused a reduction in extents of mineralisation (Rhodes et al., 2008a; Ogbonnaya et al., 2014a; Oyelami et al., 2015, Ogbonnaya et al., 2016; Bielska et al., 2018). This is likely due to PAH sorption and decreased bioavailability (Ogbonnaya et al., 2016). In this study, higher extractability and catabolism was observed in <0.6 mm amended soils compared to 2 – 4 mm. This is consistent with the reports of Kang et al. (2019) which reported higher phenanthrene biodegradation with powdered (<250 µm) biochar compared to raw biochar (<2 mm). Significantly ($p < 0.05$) greater phenanthrene catabolism was observed at 0.1% in <0.6 mm amended soil, and a non-significantly ($p > 0.05$) greater mineralisation at 0.1% and 1% in 2 – 4 mm amended soil compared to the control. Ogbonnaya et al. (2014b) reported non-significant lower extents of mineralisation at 1.0% amendment compared to the control in ≤2 mm and 3 – 7 mm amended soils. Also, in soils amended at 1.0% using black carbon, the control showed better rates and extents of mineralisation than amended soils (Yu et al., 2016). Therefore, <1% biochar amendment should be used if the objective is to enhance contaminant biodegradation.

5.5.4 Relationship between HP-β-CD extractability and microbial degradation

According to Semple et al. (2007), the bioaccessible concentration provides a more realistic description of the microbial degradation endpoint for an organic contaminant in soil. Over two decades, non-exhaustive extraction using HP-β-CD has been demonstrated as a robust and reliable predictor of PAH microbial availability in soil (Reid et al., 2000; Stokes et al., 2005; Doick

et al., 2006; Papadopoulos et al., 2007; Rhodes et al., 2008b; Ogbonnaya et al., 2014; Adedigba et al., 2018; Vázquez-Cuevas et al., 2021; Posada-Baquero et al., 2022; Jin et al., 2023). HP-β-CD extraction has been demonstrated to be suitable for predicting microbial degradation of PAHs in soil amended with black carbon (Rhodes et al., 2008a; Ogbonnaya et al., 2014a; Oyelami et al., 2014; Ogbonnaya et al., 2016; Yu et al., 2016). HP-β-CD extraction predicts the extents of mineralisation better than DCM extraction as DCM extraction overpredicts microbial degradation (Reid et al., 2000; Doick et al., 2003; Papadopoulos, Paton et al., 2007; Adedigba et al., 2018). In this study, the HP-β-CD extracted ^{14}C-phenanthrene showed a strong association with the extents of ^{14}C-phenanthrene mineralisation, which is consistent with earlier reports (Wu et al., 2013; Adedigba et al., 2018; Vázquez-Cuevas et al., 2021; Posada-Baquero et al., 2022). However, the correlation was lower in 2 – 4 mm amended soils (R^2 = 0.82 – 0.57) than in <0.6 mm amended soils (R^2 = 0.86 – 0.77), especially at 10.0% of 2 – 4 mm amendment. Linear regression revealed a strong relationship between HP-β-CD extracted and total mineralisation at 0.1% amendment (R^2 = 0.67, slope = 0.95), but the R^2 and slope for 0.5 – 5% activated carbon amendment ranged from 0.51 – 0.13 and 2.19 – 12.73 respectively, indicating that HP-β-CD underpredicted total mineralisation at higher amendment (Rhodes et al., 2008a). Similar findings with a weaker correlation after 0.1% amendment were reported by Rhodes et al. (2012). Ogbonnaya et al. (2014a) demonstrated that HP-β-CD extraction was in good agreement with the extents of mineralisation in ≤2 mm and 3 – 7 mm biochar amended soils, but the correlation was better in ≤2 mm biochar compared to 3 – 7 mm biochar. Therefore, the amount and particle size of biochar influence the efficiency of HP-β-CD extraction for prediction microbial degradation of phenanthrene.

5.6 Conclusions

The higher extractability and mineralisation observed in soils amended with <0.6 mm biochar particles are attributed to shorter diffusion lengths, which facilitate easier desorption and greater bioavailability for microbial degradation. Conversely, larger biochar particles (2–4 mm) exhibit longer diffusion lengths, reducing desorption and bioavailability. While biochar with a larger surface area enhances contaminant binding, it may also promote faster desorption, whereas biochar with a smaller surface area slows desorption due to extended diffusion pathways. This study has also demonstrated that HP-β-CD extraction can effectively predict microbial degradation of ^{14}C-phenanthrene in biochar-amended soils. However, this prediction is influenced by biochar amount and particle size. Stronger correlations were observed in <0.6 mm amended soils compared to 2–4 mm soils, particularly at 10% amendment, and at lower amendment levels (0.1% and 1.0%) across both particle sizes. Additionally, soils amended with 10.0% biochar, especially in the 2–4 mm, exhibited longer lag phases, slower mineralisation rates, and lower extents of mineralisation. These findings are important as they highlight the critical role of biochar particle size and amount in determining PAH desorption dynamics, bioavailability, and microbial degradation in contaminated soils which are critical for mitigating the adverse impacts of PAHs in soil.

5.7 Declaration of interest

The authors declare no conflicting financial, professional, or personal interests.

5.8 CRediT author statement

Chisom Ejileugha: Conceptualisation, Methodology, Investigation, Data interpretation, Visualisation, Formal analysis, Writing - Original draft, Writing – Review & Editing **Kirk T. Semple:** Conceptualisation, Methodology, Validation, Resources, Supervision, Writing – Review & Editing.

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Chapter 6 Impact of amendment particle size on the mineralisation of ^{14}C-phenanthrene in soil.

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6.1 Abstract

Spent mushroom compost (SMC) has similar properties as compost and therefore can be used as an amendment for polycyclic aromatic hydrocarbon (PAH) contaminated soil. However, a difference in particle size may influence SMC's impact on PAH mineralisation in soil. In this study, SMC of different particle sizes (<1 mm, 1 – 4 mm, 4 – 11 mm, and <15 mm) was applied at 1.0% to phenanthrene spiked soil. The spiked soil and control were monitored for 140 d and sampled at 1 d, 25 d, 50 d, 75 d, and 140 d to monitor ^{14}C – phenanthrene mineralisation and change in soil properties. Results showed that soil amended with <15 mm and 1 – 4 mm showed shorter lag phases than the control. All amended soils had significantly ($p < 0.05$) faster mineralisation rates than the control. Higher extents of mineralisation were observed in <15 mm, 4 – 11 mm, and 1 – 4 mm compared to the control. Shorter lag phases and faster rates were observed in 1 – 4 mm amended soils while higher extents of mineralisation were observed in <15 mm amended soil compared to other soil treatment conditions. The total organic carbon (TOC) showed a strong positive correlation with the extents of mineralisation in all amended soils. Therefore, the TOC was highly likely the more impactful soil property that influenced ^{14}C-phenanthrene mineralisation in the amended soils. The findings of this study underscore the importance of tailoring SMC particle size to enhance its efficacy in remediating PAH-contaminated soils. However, more studies are needed to understand the interaction of amount and particle size of SMC on its impacts on phenanthrene mineralisation in soil.

Keywords: particle size, soil, polycyclic aromatic hydrocarbons, spent mushroom compost, biodegradation, total organic carbon

6.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds with two or more benzene rings in their structure (Lawal, 2017; Wu et al., 2019). They are broadly recognized as persistent organic pollutants (POPs) because of their environmental persistence, strong affinity for organic matter, hydrophobic nature, potential to bioaccumulate, and harmful impacts on both ecosystems and human health (Okere and Semple, 2012, Lawal, 2017, Patel et al., 2020). Their toxic, teratogenic, mutagenic, and carcinogenic characteristics make them a significant public health concern. PAHs are ubiquitous in soil because of their high affinity with soil organic matter caused by their predominantly non-polar and hydrophobic nature (Okere and Semple, 2012). As a result, numerous physical, chemical and biological methodologies have been developed to mitigate the adverse impacts of PAHs in soil; however, some of these techniques may not achieve desirable sustainable solutions (Alvarez et al., 2011; Ning et al., 2017). This has provoked the need to investigate alternative sustainable solutions that are effective in the remediation of PAH-contaminated soil, as well as improving the health and fertility of soil. Organic amendments offer a sustainable and environmentally friendly approach to remediating PAH-contaminated soils. Compost offers an efficient, cost-effective alternative to conventional methods by supporting microbial degradation of PAHs, improving soil health, and mitigating secondary pollution (Bernal et al., 2017; Kästner and Miltner, 2016; Baldantoni et al., 2017).

Studies have shown that the use of compost in soil improves soil physicochemical and microbiological properties which enhances PAH biodegradation (e.g., Marc et al., 2005; Anastasi et al., 2008; Anastasi et al., 2009; Kästner & Miltner, 2016; Ren et al., 2018; Wu et

al., 2020; Feizi et al., 2020). Due to the challenges of disposal and management of spent mushroom compost (SMC), SMC is suggested for re-use for mushroom cultivation, as a soil amendment for improving soil health and fertility, as an animal feed, as a biofertilizer, and as a biocontrol agent (Leong et al., 2022; Gupta et al., 2024). SMC is a cheaper alternative to compost and its use as an amendment is cost-effective, sustainable, and reduces the burden of SMC management on mushroom farmers (Becher et al., 2021; Umor et al., 2021; Chaudhary et al., 2022; Rajavat et al., 2022). SMC contains relevant nutrients (nitrogen, organic matter, carbonates) and can be applied to soil to mitigate soil degradation and improve soil quality (Becher et al., 2021). A recent study demonstrated that SMC can improve soil nutrients (nitrogen, organic carbon, phosphorus), enzyme activities, increase microbial diversity, and cause a shift to beneficial microbial groups in soil (Huang et al., 2023). These improvements in soil health and functions by SMC can improve soil microbial activities and possibly PAH microbial degradation. An earlier study demonstrated that SMC contains degradative enzymes, macronutrients, and microbial population which could enhance the degradation of soil organic contaminants (Chiu et al., 2009). Similarly, SMC could stimulate soil indigenous bacteria which could promote the biodegradation of organic contaminants in soil (García-Delgado, D'Annibale, et al., 2015; García-Delgado, Yunta, et al., 2015).

SMC contains valuable nutrients and shares similar properties as compost (Becher et al., 2021; Umor et al., 2021), therefore its application to soil could have a similar effect as compost. Several studies have demonstrated that compost and SMC amendment improve soil microbial activity and enhance organic contaminant biodegradation and dissipation in soil (e.g., Lau et al., 2003; Hesnawi & McCartney, 2006; Yuan et al., 2009; Gandolfi et al., 2010; Wu et al., 2013; Liu et al., 2019; Omoni et al., 2020). However, the influence of compost/SMC particle size on

organic contaminant biodegradation has been neglected. SMC of fine particle size have a larger surface area, providing more space for the interaction of the SMC with soil and PAHs (Yuan et al., 2009). The larger surface area could improve the effectiveness of the SMC in stimulating microbial catabolism of the PAHs by improving soil properties. Similarly, smaller particles can decompose easily contributing to soil nutrients and organic matter (Lata Verma & Marschner, 2013; Haynes et al., 2015), which can influence soil microbial activities and PAH behaviour. Also, fine particles will ensure easy application and uniform distribution providing a more homogenous effect of the amendment in soil. Therefore, this study investigated the impact of SMC particle size on phenanthrene mineralisation in soil. This is important to understand how the particle size of SMC influences its effectiveness in enhancing phenanthrene biodegradation, which will be beneficial for optimising SMC application to improve soil remediation, pollution control, and sustainable waste management practices.

6.3 Materials and Methods

6.3.1 Sample collection and microscopy.

The soil sample was collected from Hillam Farm, Cockerham, Lancaster, United Kingdom. The site is a grassland used as a grazing field for cattle. The soil was collected at a depth of 4 – 25 cm, air – dried and sieved through a 2 mm mesh before storing at 4 ℃ in the dark. The SMC was obtained from Drinkwater Mushroom Limited, Lancaster, United Kingdom, and stored at 4 ℃. The SMC surface morphology was observed using a field emission scanning electron microscope (FE – SEM; JEOL JSM – 7800F; JEOL (UK) Ltd Welwyn Garden City, UK) equipped with a secondary electron detector, and operated at a voltage of 5.0 kV with working distance (WD) as given on the image. The SMC was mounted on aluminium pin stubs using double –

sided conductive carbon mounts (G3348N, Agar Scientific, Rotherham) and excess samples not stuck on the sticky dots were removed. The mounted samples were coated with gold to a thickness of ca. 5 nm using Quorum Technologies, Q150RES, sputter coater. The FE – SEM images of the SMC surface morphology are shown in Figure 1. The properties of the soil and SMC samples are shown in Table 1. For this study, the SMC was separated into different particle size fractions using a sieve with mesh sizes of 1 mm, 4 mm, 11 mm, and 15 mm to give particle size fractions of <1 mm, 1 – 4 mm, 4 – 11 mm, and <15 mm. The same SMC was sieved through 1 mm, 4 mm, and 11 mm to get the first 3 particle size fractions, and then another sample from the same SMC was sieved through 15 mm mesh to get the <15 mm particle size fraction.

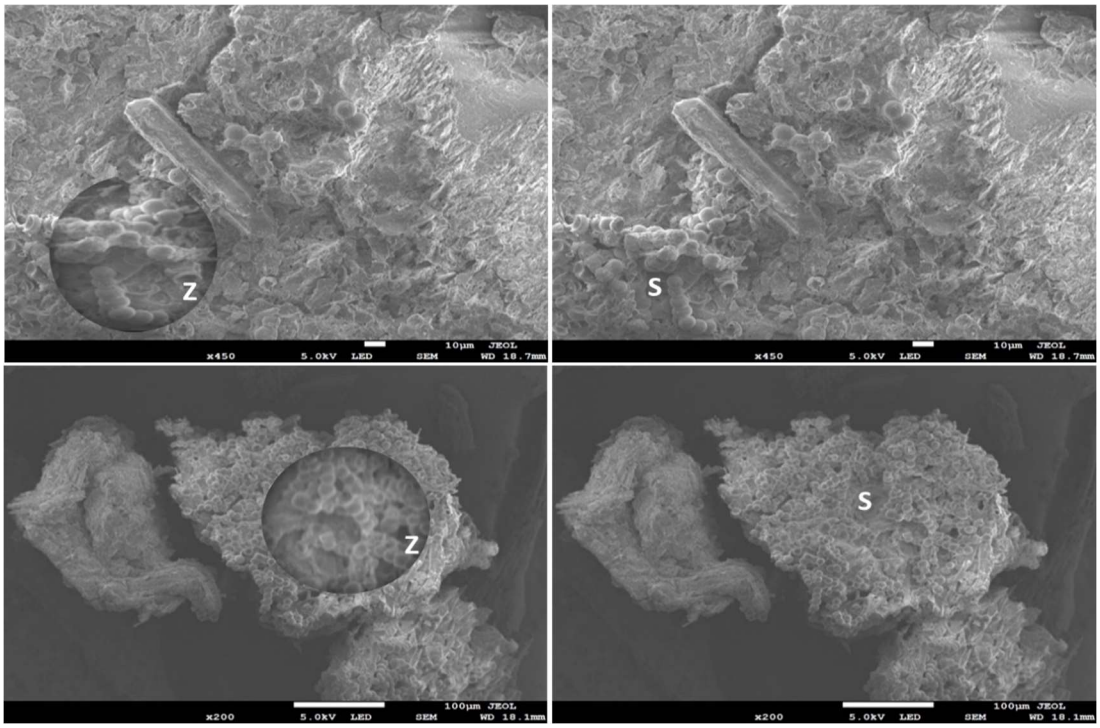

Figure *1*: Field emission SEM images of the SMC used in this study. Microbes can be seen attached to the surface of the SMC in all the images. Images are marked with Z (showing a magnified image of the microbes) and S (showing the microbes on the original image). These images show that SMC contain microbes which could enhance PAH degradation in amended soils.

Table 1: Properties of the soil and spent mushroom compost used in this study. Values are in mean ± SD (n = 3) except the values for sand, clay, and silt.

Parameters	Soil	SMC
Sand (%)	71.4	-
Clay (%)	2.4	-
Silt (%)	26.2	-
Soil texture	Sandy loam	-
pH	7.2±0.0	7.4±0.1
Electrical conductivity	382±4.2 (μScm^{-1})	6.8±0.1 ($mScm^{-1}$)
Organic matter (%)	6.5±0.2	62.5±4.2
C:N	10.4±0.1	13.3±0.5
Total carbon (ppm)	102.4±4.4	566.4±35.7
Total organic carbon (ppm)	98.8±3.6	523.3±32.6
Inorganic carbon (ppm)	3.6±1.0	43.0±2.9
Ammonium nitrogen (ppm)	0.4±0.0	12.8±1.0
Nitrate (ppm)	3.6±0.2	0.2±0.0

6.3.2 Soil spiking and monitoring

Soil (200 g dry weight; n = 3) was spiked with ^{12}C–phenanthrene (100 mg/kg; dry weight) and maintained at field moisture content (30%). To achieve this, approximately one-quarter of the soil was first spiked using acetone as a carrier solvent at a solvent-to-soil ratio of 1:20 (v/w). The spiked portion was left to vent in a fume hood for 2 h to allow complete volatilisation of

the solvent. The remaining soil was then added in three increments, with thorough mixing after each addition to ensure even distribution of the contaminant and soil homogeneity (Reid et al., 2001; Doick et al., 2003; Semple et al., 2006; Allan et al., 2007). The different SMC particle sizes (<1 mm, 1 – 4 mm, 4 – 11 mm, and <15 mm) were added to respective soils at an amendment amount of 1.0 % (w/w; dry wt). The soil was thoroughly mixed, and the amended soils were incubated (at 21 ± 2 ℃) in the dark for 140 d using amber glass bottles, and sampled at 1 d, 25 d, 50 d, 75 d, and 140 d. All soil amendments were prepared in triplicate and were sampled to measure the mineralisation of ^{14}C-phenanthrene respirometrically.

6.3.3 Respirometric assay of the soils

Soil (10 ± 0.2 g) was weighed into a 250 ml modified Schott bottle with a Teflon-lined screw cap fitted with a crocodile clip (Reid et al., 2001; Doick et al., 2003; Semple et al., 2006; Allan et al., 2007). Sterile MBS (30 ml) was added to the bottle and 9-^{14}C-phenanthrene (0.022 kBq/g) was spiked into each bottle. The bottle was immediately closed with the cap which had been attached with a 7 ml vial containing 1 ml of 1M NaOH to trap evolved $^{14}CO_2$. The bottle was incubated on a rotary shaker (100 RPM) at 21 ± 2 ℃. The 7 ml vial was changed every 3 h for the first 9 h and then every 24 h for 14 d. At each sampling, 5 ml of Goldstar scintillating fluid was added to each sampled 7 ml vial and the vial was kept in the dark for 24 h before counting using a Liquid scintillating counter (Canberra Packard Tri-Carb2250CA). The data from the counts was used to determine the impacts of the biochar on phenanthrene mineralisation by calculating the lag phase (time took to mineralise 5% of spiked ^{14}C-

phenanthrene), fastest rate (highest mineralisation in an hour), and extent of mineralisation (percentage of total mineralised ^{14}C-phenanthrene).

6.3.4 Soil properties

The electrical conductivity (EC) and pH were measured in a soil – water (1:5; w/v) by using EC meter (Mettler Toledo, FiveEasy™ FE30) and a pH meter (Mettler Toledo, SevenCompact™ pH/Ion S220). A 2 g soil sample was extracted into 40 ml of 0.5M Na_2CO_3 and analysed using AA3 Seal Autoanalyzer for OLSEN P. A soil sample (5 g) was dissolved into 25 ml of 2 M KCl and analysed for ammonium nitrogen and nitrate (NH_4^+-N) using AA3 Seal autoanalyzer. A 5 g soil sample was extracted in 25 ml 0.5 M K_2SO_4 for TOC and analysed with Shimadzu TOC-L. For C:N, 20 – 24 mg of ball-milled soil was analysed using Elementar CN analyser (Vario EL).

6.3.5 Statistical analysis

Data were blank corrected, and the lag phases, fastest rates, and extents of mineralisation were calculated using MS Excel. Data were analysed using multivariate analysis of variance (MANOVA), univariate ANOVAs, Tukey's HSD post hoc test, and Pearson correlation. Data were plotted using SigmaPlot and statistical analysis was done using IBM SPSS (version 28).

6.4 Results

6.4.1 Mineralisation of ^{14}C – phenanthrene in the soils

The changes in ^{14}C-phenanthrene mineralisation in the amended soils and the control were monitored for 140 d (Table 2, Figure 2). The ^{14}C-phenanthrene was monitored by measuring the lag phases, the fastest rates, extents of mineralisation. The MANOVA results indicated a significant multivariate effect of both ^{12}C-phenanthrene aging (Wilk's Lambda = 0.007, $p < 0.001$) and amendment particle size (Wilk's Lambda = 0.335, $p < 0.001$) on lag phases, fastest rates, and extents of mineralisation. Subsequent univariate analyses showed that amendment particle size fractions had a significant influence on lag phases ($p < 0.01$) and fastest rates ($p < 0.001$), but no significant effect on the extent of mineralisation ($p = 0.252$).

Higher significant lag phases were observed at 140 d compared to other contact times (Table 2). There was no significant difference ($p > 0.05$) in the lag phases in the soil treatments on 1 d and 75 d (Table 2). Soil amended with <15 mm showed lower lag phases than the control from 1 – 75 d. Similarly, soil amended with <15 mm showed lower lag phases than other amended soils from 1 – 75 d (Table 2). The 1 – 4 mm amended soil showed lower lag phases compared to the control. Lower lag phases were also observed in 1 – 4 mm amended soils compared to <1 mm and 4 – 11 mm (Table 2). Faster rates were observed in all amended soils compared to the control Table 2). Higher mineralisation rates were observed in 1 – 4 mm amended soil compared to other amended soils (Table 2). Noticeably, the extents of mineralisation after 1 d were higher than at 25 – 140 d (Table 2 and Figure 2). There was no significant difference ($p > 0.05$) observed in extents of mineralisation in all soil treatment

conditions from 1 – 50 d (Table 2 and Figure 2). Significant difference ($p < 0.05$) in extents of mineralisation was observed at 75 – 140 d (Figure 2, Table 2). The extents of mineralisation in <15 mm amended soil were higher than in other amended soils from 1 – 75 d. higher extents of mineralisation were observed in 4 – 11 mm amended soil from 50 – 140 d compared to 1 – 25 d (Table 2; Figure 2). The changes in extents of mineralisation did not follow a given pattern by overall, soil amended with <15 mm showed higher extents of mineralisation than other amended treatments.

Table 2: Mineralisation of ^{14}C – phenanthrene in SMC amended soils showing the lag phases, fastest rates, and extents of mineralisation. Values are in mean ± SD (n = 3). Values in columns followed by different letters are statistically different (Tukey's HSD; n = 3; $p < 0.05$); also indicated are shorter lag phases (*), faster rates (+), higher extents of mineralisation (#) than the controls that are not statistically significant ($p > 0.05$)

Soil-PAH contact time (d)	Particle size (mm)	Lag phase (h)	Fastest rate (%$^{14}CO_2$/h)	Extent of mineralisation (%)
1	Control	35.7 ± 5.9^{a}	0.7 ± 0.3^{a}	57.5 ± 5.4^{a}
	< 1	38.0 ± 1.5^{a}	1.0 ± 0.2^{a+}	57.2 ± 2.3^{a}
	1 – 4	36.0 ± 2.1^{a}	1.1 ± 0.2^{b}	56.4 ± 2.7^{a}
	4 – 11	37.2 ± 5.5^{a}	0.9 ± 0.3^{a+}	55.6 ± 6.3^{a}
	< 15	34.6 ± 9.0^{a*}	0.8 ± 0.4^{a+}	57.5 ± 9.5^{a}
25	Control	38.5 ± 0.9^{a}	0.7 ± 0.1^{a}	34.1 ± 2.9^{a}
	< 1	30.2 ± 2.5^{b}	1.1 ± 0.1^{b}	32.0 ± 1.1^{a}
	1 – 4	25.1 ± 5.8^{b}	1.1 ± 0.1^{b}	31.2 ± 2.0^{a}
	4 – 11	50.1 ± 2.5^{c}	0.6 ± 0.2^{a}	28.9 ± 0.6^{a}
	< 15	24.5 ± 4.8^{b}	1.1 ± 0.3^{b}	33.2 ± 1.8^{a}
50	Control	50.5 ± 4.2^{a}	0.6 ± 0.2^{a}	27.2 ± 2.1^{a}
	< 1	57.8 ± 9.3^{a}	0.7 ± 0.2^{a+}	22.4 ± 3.3^{a}
	1 – 4	44.6 ± 10.0^{b}	0.9 ± 0.2^{b}	24.7 ± 1.4^{a}
	4 – 11	52.5 ± 5.7^{a}	0.7 ± 0.2^{a+}	25.0 ± 3.5^{a}
	< 15	44.3 ± 8.6^{b}	0.7 ± 0.2^{a+}	25.0 ± 0.7^{a}
75	Control	39.2 ± 1.9^{a}	0.8 ± 0.2^{a}	44.1 ± 1.4^{a}
	< 1	42.3 ± 3.1^{a}	0.8 ± 0.2^{a}	41.7 ± 3.6^{a}
	1 – 4	37.6 ± 0.8^{a*}	0.9 ± 0.1^{a+}	47.2 ± 1.5^{b}
	4 – 11	36.2 ± 1.8^{a*}	1.0 ± 0.1^{a+}	49.2 ± 2.2^{b}
	< 15	34.6 ± 3.0^{a*}	1.1 ± 0.1^{a+}	51.0 ± 3.7^{b}
140	Control	57.9 ± 3.2^{a}	0.5 ± 0.1^{a}	30.1 ± 2.2^{a}
	< 1	58.6 ± 5.6^{a*}	0.7 ± 0.1^{a+}	30.5 ± 2.3^{a}
	1 – 4	51.7 ± 5.3^{a*}	0.9 ± 0.3^{b}	34.0 ± 3.4$^{a\#}$
	4 – 11	46.5 ± 5.2^{b}	1.0 ± 0.3^{b}	37.9 ± 4.6^{b}
	< 15	59.1 ± 6.4^{a}	0.7 ± 0.2^{a+}	30.5 ± 4.0$^{a\#}$

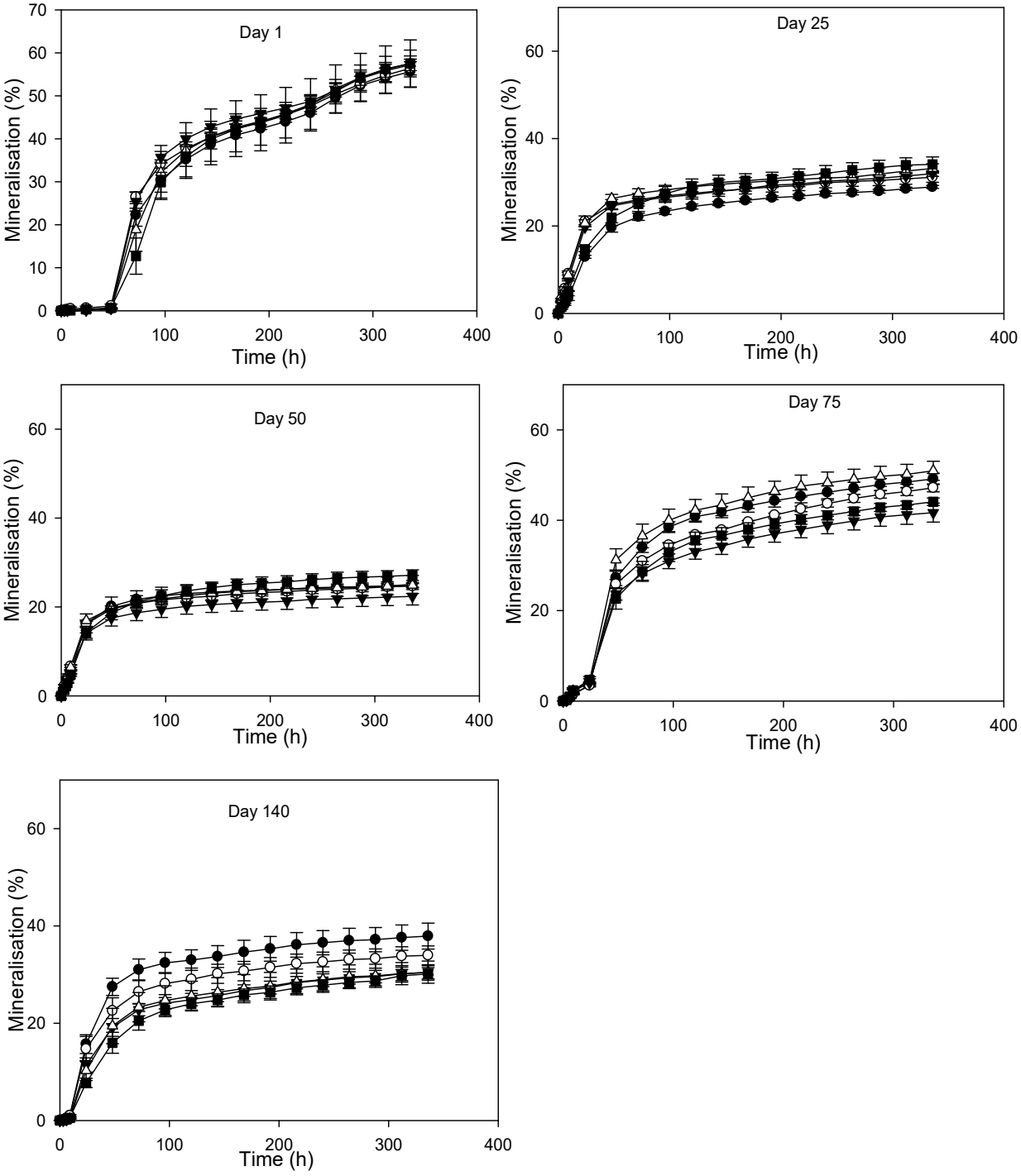

Figure 2: Extents of mineralisation in SMC amended soils on 1 - 140d. Amendment particle size fractions are represented with ●(4 – 11 mm), ○(1 – 4 mm), ▼(<1 mm), △(<15 mm), and ■(0%; control) respectively. The mean values are plotted with error bars showing the SD (n = 3).

6.4.2 Correlation of monitored soil properties with ^{14}C-phenanthrene mineralisation kinetics in the soils.

The changes in the selected soil properties were monitored (1 – 140 d) during the study to investigate the association with lag phases, fastest rates, and extents of mineralisation of ^{14}C-phenanthrene in the soils (Figure 3). In <15 mm amended soils, the lag phases correlated negatively with the EC ($r = -0.657$, $p < 0.05$), and the rates in <1 mm amended soils correlated positively with the OLSEN P ($r = 0.702$, $p < 0.05$). There was no correlation between lag phases or rates with the monitored soil properties in other amended soils. The TOC correlated positively with the extents of mineralisation in 4 – 11 mm ($r = 0.738$, $p < 0.01$), 1 – 4 mm ($r = 0.716$, $p < 0.05$), <1 mm ($r = 0.752$, $p < 0.01$), and <15 mm ($r = 0.763$, $p < 0.01$). The NH_4^+-N correlated ($r = 0.633$, $p < 0.05$) with the extents of mineralisation in <1 mm amended soils, and the OLSEN P correlated with the extents of mineralisation in <1 mm amended soils ($r = 0.649$, $p < 0.05$) and <15 mm amended soils ($r = 0.653$, $p < 0.05$). The EC did not correlate with the extents of mineralisation in the amended soils. However, the EC in the control showed a relationship with lag phases ($r = -0.723$, $p < 0.01$) and extents of mineralisation ($r = 0.656$, $p < 0.05$). The pH did not correlate with the extents of mineralisation in all soil treatment conditions.

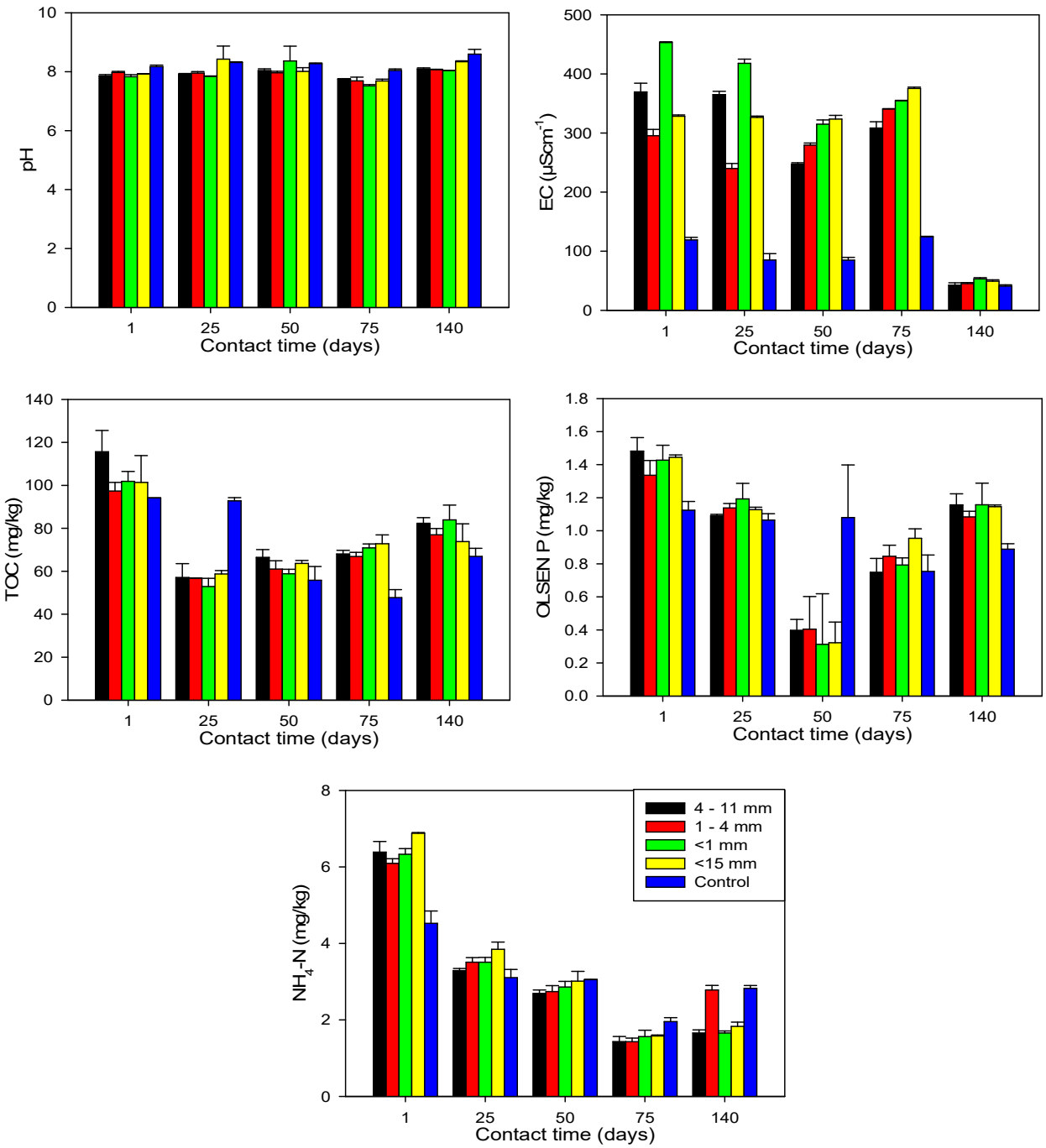

Figure 3: Monitored soil properties (pH, OLSEN P, TOC, EC, and NH_4^+-N) from 1 – 140 d in soils amended with SMC of different particle sizes (<1 mm, 1 – 4 mm, 4 – 11 mm, <15 mm, control). Mean values are plotted with error bar showing the SD (n = 3). Each different coloured bar represents a different soil treatment condition.

6.5 Discussion

6.5.1 Effect of SMC amendment on ^{14}C – phenanthrene mineralisation in soil.

The levels of mineralisation after 1 d incubation were higher and the lag phases after 140 d were longer than at other contact times, which in past studies, has been attributed to reduced bioavailability over time due to aging (Macleod & Semple, 2000, 2003; Reid et al., 2000). Faster rates were observed in all amended soils compared to the control, and greater extents of phenanthrene mineralisation and shorter lag phases were observed in some of the amended soils compared to the control, which indicates that SMC can stimulate PAH mineralisation in soil (Xuanzhen et al., 2010; Gąsecka et al., 2012; García-Delgado, D'Annibale, et al., 2015; Zhou et al., 2020). An earlier study noted that compost particle size influenced the rates of PAH degradation with smaller particles showing higher degradation rates but when the compost was applied to soil, the particle size of compost had no significant effect on the rates of PAH degradation (Yuan et al., 2009). However, in this present study, significant differences were observed in the lag phases, fastest rates, and extents of mineralisation at some contact times in the soil treatments.

Soil amended with <1 mm generally showed substantially longer lag phases and lower extents of mineralisation compared to other amended soils. This could be due to the rapid release of nutrients, which caused intense microbial competition and delayed microbial adaptation to phenanthrene. In other words, this provided an easy carbon source thereby limiting the microbial use of phenanthrene as a carbon source. Though organic amendments enhance PAH mineralisation, high amendment amount may inhibit microbial degradation because

when the amendment carbon source is preferred over the target contaminant, microbial degradation of the target contaminant may be impeded (Swindoll et al., 1988; Rittmann, 1992; Yuan et al., 2009). Despite increased microbial population, hydrocarbon removal decreased after organic manure addition because the microbes chose the easily degradable nutrient to the complex hydrocarbon which is less biodegradable (Schaefer and Juliane, 2007). Additionally, the small particle size of <1 mm may have increased the sorption of phenanthrene, reducing its bioavailability and preventing sustained microbial degradation, ultimately leading to lower overall mineralisation. Compost application to soil increased PAH sorption and reduced overall PAH removal by 26 – 89% during the first 3 months of incubation compared to the control (Wu et al., 2013). However, this could have less impact due to the humic acid - like substances, nutrients, and microbes in compost enhancing desorption and stimulating PAH mineralisation (Puglisi et al., 2007; Bari et al., 2010; Wu et al., 2013; Lin et al., 2016; Sigmund et al., 2018).

The 1 – 4 mm amended soils had shorter lag phases than the control and faster rates compared to other soil treatment conditions. The shorter lag phases and faster rates were attributed to the high surface area that favoured microbial access and attachment, leading to a quicker release of nutrients, which promoted rapid microbial degradative activity. This is consistent with Yuan et al. (2009) and Delhoménie et al. (2002) who stated that smaller compost particles possess greater specific surface, creating favourable conditions for microbial colonisation and subsequently boosting microbial degradation activity. Although not statistically significant ($p > 0.05$), the extent of mineralisation in soils amended with 4 - 11 mm particle fraction was higher compared to those amended with <1 mm and 1 - 4 mm

particles from 50 – 140 d. This is consistent with the findings of Lata Verma & Marschner (2013) who reported that coarse compost (>5 mm) increased microbial activity in the soil more than other particle fractions (<3 mm and 3 – 5 mm) but didn't increase the microbial biomass. The greatest soil respiration was observed in soil amended with >5 mm compost fraction and the greatest microbial biomass C and P was observed in <3 mm (Lata Verma & Marschner, 2013). The high microbial biomass observed in fine particles where attributed to high surface area and increased accessibility to the microbes (Yuan et al., 2009; Lata Verma & Marschner, 2013). However, the higher accessibility and decomposability did not result in a higher cumulative respiration which was attributed to preferential C utilisation for growth rather than respiration (Lata Verma & Marschner, 2013). Additionally, the higher extents of mineralisation observed in 4 – 11 mm amended soils (50 – 140 d) compared to <1 mm and 1 – 4 mm could also be due to a slow decomposition of the SMC, leading to a slow release of nutrients thereby providing sustained nutrients for ^{14}C-phenanthrene mineralisation, which promoted continuous microbial activity over time. This is consistent with Haynes et al. (2015), who reported that larger compost particles decompose more slowly than smaller ones due to their higher C/N ratio and greater lignified woody content.

The soils amended with <15 mm had shorter lag phases and higher extents of mineralisation than other amended soils. This showed that the SMC in its original form, comprising of different particle sizes, could be a better amendment for improving the extent of ^{14}C-phenanthrene mineralisation in soil than the <1 mm, 1 – 4 mm, and 4 – 11 mm range of particle sizes. Studies have demonstrated that SMC amendment can enhance the microbial degradation of PAHs in soil (Omoni et al., 2020; Zhou et al., 2020; Atai et al., 2023; Ge et al.,

2023). The <15 mm particle fraction likely offered an optimal balance between nutrient release, microbial habitat stability, and phenanthrene bioavailability. The larger particle size range provided a more diverse and sustained nutrient release, promoting longer-term microbial activity and less competition among microbes. Additionally, <15 mm may have enhanced the desorption of phenanthrene, making it more available for microbial degradation while maintaining a favourable environment for microbial colonisation and growth, ultimately leading to higher overall mineralisation. These are consistent with the findings of Wu et al. (2013) and Sigmund et al. (2018), who reported that compost can enhance PAH desorption and microbial degradation in soil.

6.5.2 Relationship of soil properties with ^{14}C-phenantherene mineralisation in the amended soils

A recent study pointed out that soil chemical and physical properties are the main factors influencing PAH residuals in soil (Chen et al., 2023). The TOC, available P, EC, and NH_4^+-N in this study showed an association with lag phases, rates, and extents of mineralisation, while the pH showed no correlation with the monitored mineralisation parameters. However, studies have reported that a decrease in pH supports the formation of easily degradable hydrocarbon fractions, and high pH reduces PAH degradation (Betancur-Galvis et al., 2006; Rahman et al., 2021; Yaun et al., 2023). In contrast, high pH improved the desorption of phenanthrene as a result of improved solubility in aqueous phase (Yu et al., 2016; Yu et al., 2018). In this study, the pH in all the soil treatments from 1 – 140 d was approximately 7 – 8 which is within the reported suitable pH range for hydrocarbon desorption and

biodegradation (e.g., Wu et al., 2013; Yu et al., 2016; Abdulsalam et al., 2018; Sangodoyin and Igbode, 2019; Chikere et al., 2020; Tran et al., 2021).

In this present study, the TOC correlated positively and strongly with extents of mineralisation in all amended soils. This is consistent with Guo et al. (2020) who reported that biodegradation of PAHs correlated positively with TOC, and Yuan et al. (2023) who demonstrated that total petroleum hydrocarbon removal positively correlated strongly with TOC. Past studies have shown that the interaction of PAHs with compost's dissolved organic matter (or dissolved organic carbon) increases PAH mobility and enhances bioavailability, desorption, and biodegradation in soils (e.g., Janzen et al., 1996; Cheng et al., 2008; Kobayashi et al., 2008; Kobayashi et al., 2009; Yu et al., 2011; Ghanem et al., 2013; Yu et al., 2014). The NH_4^+-N correlated positively with extents of mineralisation in <1 mm, and the available P correlated positively with extents of mineralisation in <1 mm and <15 mm amended soils. Recent studies demonstrated that available P affects microbial community composition; it favoured microbial growth which could improve the biodegradation of organic contaminants (Wu et al., 2021; Martinez-Toledo et al., 2022). Additionally, the use of N and P was reported to significantly increase PAH removal (Betancur-Galvis et al., 2006; Yang et al., 2018). According to Wang et al. (2022), the application of nitrate and ammonium nitrogen resulted in improved PAH removal.

The EC in <15 mm amended soil and the control correlated negatively with the lag phases and correlated positively with extents of mineralisation in the control. This is corroborated by studies where an increase in EC has been reported to improve the degradability and

extractability of hydrocarbons (Meng et al., 2019; Rahman et al., 2021). The results from this study have shown that the soil properties that influence the mineralisation of phenanthrene in soil are affected by the soil treatment conditions. The TOC showed a correlation in all amended soils, but EC, available P, and NH_4^+-N correlated with mineralisation parameters under different treatment conditions. The TOC, available P, and NH_4^+-N correlated with extents of mineralisation only in <1 mm treatment. Therefore, further studies are needed to investigate the conditions at which specific soil parameter influence PAH mineralisation. This will inform the choice of parameters to monitor or optimise based on soil conditions, to prevent the waste of resources and optimise PAH mineralisation in soil.

Although correlations between phenanthrene mineralisation and certain soil properties were identified, these findings should be interpreted with caution given the slurry-based assay conditions. The use of MBS and constant agitation with a rotary shaker likely minimised the influence of natural soil factors such as pH variation, nutrient distribution, and organic matter-driven sorption. Moreover, employing a soil slurry system with MBS likely boosted microbial activity and reduced mass transfer limitations, thereby highlighting microbial potential under favourable conditions. As a result, the roles of pH, TOC, Olsen P, NH_4^+-N, and EC in phenanthrene biodegradation may have been obscured. Thus, the observed associations more accurately reflect the soils' intrinsic microbial capacity to degrade phenanthrene in nutrient-rich, aerated environments, rather than their performance under actual field conditions. Although this setup compromises environmental realism, it improves consistency across treatments and allows for clearer interpretation of microbial responses.

6.6 Conclusions

The SMC comprising of different particle sizes showed a better improved mineralisation kinetics than the particle size fractions which indicates that raw SMC could be a better amendment for improving phenanthrene biodegradation than particle size fractions. The TOC showed a strong positive correlation with extents of mineralisation in all amended soils which suggests that TOC was highly likely the monitored soil property with a higher influence on the observed phenanthrene mineralisation. The mineralisation kinetics varied across particle sizes showing that SMC particle size distribution affects its impact on phenanthrene mineralisation kinetics in soil. This is significant for optimising the effectiveness of SMC as soil amendment and in reducing the adverse impacts of PAHs in soils.

This study assessed phenanthrene degradation using freshly spiked ^{14}C-phenanthrene to infer the mineralisation of aged ^{12}C-phenanthrene, though this may not fully reflect the behaviour of the aged contaminant due to potential differences in sequestration and microbial interactions. However, it showed the influence of soil-phenanthrene-amendment interaction on microbial degradation over time. The slurry-based assay with MBS enhanced microbial activity and standardised conditions but reduced environmental realism by minimising the effects of native soil properties. Consequently, the findings reflect microbial degradation potential under optimal conditions rather than actual field condition. Future studies should consider co-aging of both isotopes and conducting respirometry with distilled water to better simulate field conditions.

6.7 Declaration of interest

The authors declare no conflicting financial, professional, or personal interestss.

6.8 CRediT author statement.

Chisom Ejileugha: Conceptualisation, Methodology, Investigation, Data interpretation, Visualisation, Formal analysis, Writing - Original draft, Writing – Review & Editing **Kirk T. Semple:** Conceptualisation, Methodology, Validation, Resources, Supervision, Writing – Review & Editing.

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Chapter 7 Effect of dimethyl suphoxide (DMSO) on ^{14}C-phenanthrene extractability and biodegradation in soil

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7.1 Abstract

The mineralisation of polycyclic aromatic hydrocarbons (PAHs) in soil is limited by their bioavailability. Therefore, improving PAH mineralisation in soil could be achieved through improving bioavailability. Dimethyl sulphoxide (DMSO) could solubilise PAHs and increase their availability and delivery to suitable microbial degraders in soil. In this study, soil was spiked with $^{12/14}$C-phenanthrene and treated with DMSO at 0.0%, 0.01%, 0.1%, and 1.0% respectively. The treated soils were aged for 60 d and sampled at 1 d, 15 d, 30 d, 45 d, and 60 d to monitor residual activity, solvent extractability, and mineralisation. Results showed that residual activity, solvent extractability, and mineralisation reduced over time. The residual activity in soil treated with DMSO at 0.01% and 0.1% was significantly ($p < 0.05$) lower than in 1.0%. Solvent extractability was higher ($p < 0.05$) in 1.0% compared to 0.1% and 0.01%. However, extents of mineralisation were higher ($p < 0.05$) in 0.1% compared to other treated soils. Hydroxypropyl-β-cyclodextrin (HP-β-CD) extraction overpredicted extents of mineralisation which was attributed to enhanced solubilisation of phenanthrene by DMSO. However, the HP-β-CD extraction correlated strongly (R^2 = 0.60 – 0.80) with the extents of mineralisation in the soil treatments. This study has shown that DMSO at appropriate amounts can enhance ^{14}C-phenanthrene bioavailability and mineralisation in soil. This finding represents a novel approach with implications for advancing the remediation of PAH-contaminated soil.

Keywords: bioavailability, cyclodextrin, dichloromethane, dimethyl sulphoxide, microbial degradation, polycyclic aromatic hydrocarbons, soil

7.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that contain two or more fused benzene rings in their structure (Lawal, 2017; Patel et al., 2020). They are toxic and are produced mainly through human activities. They are mainly found in soil due to their affinity with soil organic matter (Okere & Semple, 2012). The ring number, molecular weight, and structural complexity of PAHs are among the properties that influence their behaviour in soil (Patel et al., 2020). PAHs with high ring number and molecular weight (or complex structure), are less bioavailable, persist more in soil, and are more resistant to microbial degradation. PAHs can also bind and partition in soil and soil organic matter thereby increasing their accumulation and persistence (Scelza et al., 2010; Yang et al., 2011; Baldantoni et al., 2017; Umeh et al., 2017). This effect increases with soil contact duration, reducing bioaccessibility and biodegradation (Macleod & Semple, 2000).

One school of thought in soil remediation is to remove as much contaminant as possible. To achieve this through biodegradation, soil contaminants must be accessible for microbial degradation. Since the mineralisation of most PAHs in soil is not limited by microbial catabolic activity but by desorption and bioavailability (Allan et al., 2007; Semple et al., 2006), improving PAH bioavailability in soil could enhance mineralisation and mitigate possible adverse impacts. Conditions that improve the solubility of PAHs in soil, such as surfactant application, have been demonstrated to enhance their microbial degradation (Rathankumar et al., 2022; Li et al., 2023; Liu et al., 2023; Zhang et al., 2024). Additionally, soil treatment with organic solvents, such as ethanol, acetone, and acetonitrile, has been reported to enhance PAH microbial degradation (White & Alexander, 1996; Bonten et al., 1999; Lee et al., 2001).

The use of non-exhaustive solvent extraction, such as hydroxypropyl-β-cyclodextrin (HP-β-CD) extraction, can predict PAH bioaccesibility in contaminated soils (Tang & Alexander, 1999; Reid et al., 2000). Studies have shown that HP-β-CD extraction can adequately predict PAH microbial availability in laboratory spiked soils and field contaminated soils (e.g., Reid et al., 2000; Patterson et al., 2004; Doick et al., 2005; Stokes et al., 2005; Allan et al., 2006; Semple et al., 2006; Doick et al., 2006; Papadopoulos et al., 2007; Rhodes et al., 2008; Duan et al., 2015). Therefore, HP-β-CD was used in this study to predict the microbial availability of ^{14}C-phenanthrene in spiked soils. Phenanthrene is a model PAH and was chosen as a PAH of choice because it has been extensively used in several PAH extractability and mineralisation studies (Reid et al., 2000; Doick et al., 2003; Semple et al., 2006; Rhodes et al., 2008; Oyelami et al., 2014; Vazquez-Cuevas et al., 2020).

Dimethyl sulphoxide (DMSO) is a solvent that is widely used to solubilise polar and non-polar compounds (Swanson, 1985; Galvao et al., 2014). It is used as an extraction solvent for soil (Hayes et al., 1975; Castle et al., 2011; Song et al., 2023), and as a delivery solvent in ecotoxicity and pharmaceutical studies (Andrade-Vieira et al., 2022; Maher et al., 2022; Pashynska et al., 2022; Ramkar et al., 2023; Sun et al., 2023). However, DSMO has not been explored in the biodegradation of PAHs in soil. DMSO's solvent and penetrability properties could be utilized in soil remediation to improve the bioavailability and delivery of PAHs to soil microbes. DMSO can improve the desorption (Yang et al., 2015) and microbial degradation (Nguyen et al., 2022) of PAHs and PAH-degrading bacteria can use DMSO as a carbon and energy source (Balachandran et al., 2012; Nguyen et al., 2022). This implies that DMSO may not inhibit PAH degradation but rather promote PAH degradation through co-metabolism and

improved bioavailability. Therefore, this study investigated the impact of safe amounts of DMSO on the bioavailability and mineralisation of ^{14}C-phenanthrene in soil. This represents a novel approach with substantial implications for advancing PAH remediation in soil.

7.3 Materials and methods

7.3.1 Sample collection.

Soil was collected at a depth of 5 – 20 cm from Hillam Farm, Cockerham, Lancaster, United Kingdom. The soil was air-dried and sieved through a 2 mm mesh to remove debris, stones, and plant roots. The sieved soil was stored at 4 °C in the dark until when needed. The properties of the soil are shown in Table 1.

Table 1: Properties of the soil sample used in this study. Values are in mean ± SD except soil texture, clay, sand, and silt.

Parameters	Value
Sand (%)	71.4
Clay (%)	2.4
Silt (%)	26.2
Soil texture	Sandy loam
pH	7.3±0.0
Electrical conductivity (µScm^{-1})	382.0±4.4
Organic matter (%)	6.5±0.3
C:N	10.4±0.1
Total carbon (mg/kg)	102.4±4.4
Total organic carbon (mg/kg)	98.9±3.6
Inorganic carbon (mg/kg)	3.6±1.0
Ammonium nitrogen (mg/kg)	0.4±0.0
Nitrate nitrogen (mg/kg)	3.6±0.2

7.3.2 Soil spiking and treatment

Soil (1.2 kg; dry weight) was rehydrated to field moisture condition (30%) and was spiked with ^{12}C and 9-^{14}C–phenanthrene (100 mg/kg and 3,800 DPM/g; dry weight) (Reid et al., 2000; Doick et al., 2003; Stokes et al., 2005). Soil (ca. ¼) was first spiked using acetone as a carrier solvent at a solvent-to-soil ratio of 1:20 (v/w). The spiked portion was left to vent in a fume hood for 3 h to volatilise carrier solvent. The remaining soil was then added in three roughly equal parts, with thorough mixing after each addition to ensure even distribution of the contaminant and soil homogeneity. The spiked soil was homogenised in a glass bowl using a stainless-steel metal spoon (Doick et al., 2003). Soil (300 g; dry weight) was weighed into a separate clean glass bowl as ^{14}C blank. Soil (300 g; dry weight) was rehydrated and spiked with ^{12}C-phenanthrene as experimental blank and weighed into a separate clean glass bowl. The remaining $^{12/14}C$-spiked soil was divided into 3 approximately equal portions and were treated with DMSO at 0.01%, 0.1 %, and 1.0% respectively. The amount of DMSO added was determined from reported safe amounts (Sciuchetti & Mingis, 1965; Erdman & Hsieh, 1969; Kumar et al., 1976; Galvao et al., 2014; Petruccelli et al., 2020; Andrade-Vieira et al., 2022). Afterwards, the soils were weighed (100 g; n = 3) into amber bottles and incubated in the dark at 21 ± 2 ℃ for 60 d. The soils were sampled at 1 d, 15 d, 30 d, 45 d, and 60 d to monitor the ^{14}C – phenanthrene total residual activity, solvent extractability, and mineralisation.

7.3.3 Determination of total ^{14}C – activity in the soil

Soil (ca. 1.0 g) was weighed into a cellulose combustion cone and 200 µl of Combustaid was added to the soil (Reid et al., 2000; Doick et al., 2003; Stokes et al., 2004; Doick et al., 2005).

The soil was combusted for 3 min using a sample oxidiser (Packard 307). The released $^{14}CO_2$ was trapped with 10 ml of CarbonTrap and was delivered into a 20 ml vial with 10 ml of CarbonCount as a scintillation fluid. The efficiency (>96%) of the sample oxidiser to trap the evolved $^{14}CO_2$ was determined before soil combustion. The 20 ml vial was stored in the dark for 24 h before being quantified for 10 min with a liquid scintillation counter (LSC; Canberra Packard Tri-Carb 2250CA) using a relevant protocol.

7.3.4 Solvent extractability of the ^{14}C – phenanthrene in the soil

3.4.2.7 Dichloromethane (DCM) extractability of the ^{14}C – phenanthrene

Soil was extracted as demonstrated in past studies (Macleod & Semple, 2000; Macleod & Semple, 2003; Papadopoulos et al., 2007; Rhodes et al., 2008). Soil (ca. 1.5 g) was weighed into a 50 ml Teflon-lined centrifuge tube. Anhydrous sodium sulphate (1.5 g) was added to the soil and 20 ml of DCM was added to the tube. All tubes were tightly closed and placed in an orbital shaker at 100 RPM (21 ± 2 ℃) for 24 h. Afterwards, the tubes were centrifuged at 4000 RPM for 1 h. The supernatant (5 ml) was pipetted into a 20 ml vial, and 14 ml of Ultima Goldstar scintillation fluid was added to the vial. The vial was stored in the dark for 24 h before quantifying for 10 min using an LSC. The remaining supernatant was properly discarded.

7.3.4.2 Hydroxypropyl-β-cyclodextrin (HP-β-CD) extractability of the ^{14}C – phenanthrene

This was performed as reported in past studies (Reid et al., 2000; Stokes et al., 2005; Doick et al., 2006). Soil (ca. 1.5 g) was weighed into a 50 ml Teflon-lined centrifuge tube, and 25 ml of 50 mM HP-β-CD solution was added to the tube. All tubes were tightly closed and placed on an orbital shaker at 100 RPM (21 ± 2 ℃) for 24 h. Afterwards, the tubes were centrifuged at

4000 RPM for 1 h. The supernatant (5 ml) was transferred into a 20 ml vial and 14 ml of Ultima Goldstar scintillation fluid was added to the vial. The vial was stored in the dark for 24 h before quantifying for 10 min using an LSC. The remaining supernatant was safely discarded.

7.3.5 Respirometric monitoring of ^{14}C – phenanthrene mineralisation in the soils.

Soil (10 ± 0.2 g) was weighed into 250 ml modified Schott bottles and 30 ml of sterile MBS (1:3 soil – liquid ratio) was added to the soil to form a slurry (Reid et al., 2001; Macleod & Semple, 2002; Doick et al., 2003; Macleod & Semple, 2006; Allan et al., 2007). A 7 ml vial containing 1 ml of 1 M NaOH was attached to the bottle to trap evolved $^{14}CO_2$. The bottles were tightly closed and incubated (at 21 ± 2 °C) on an orbital shaker at 100 RPM, and sampled at 3 h intervals for an initial 9 h, then daily for 14 d. At each sampling, the 7 ml vial was replaced with a fresh vial, and 5 ml Ultima Goldstar scintillation fluid was added to the sampled vial. The vial was stored for 24 h in the dark before quantifying using LSC (Canberra Packard Tri-Carb 2250CA).

7.3.6 Statistical analysis

The residual activity, solvent extractability, and mineralisation parameters were calculated using MS Excel following blank corrections. The data analysis was done using IBM SPSS 28 and figures were produced using SigmaPlot. Data were analysed using one – way analysis of variance (ANOVA), Tukey's HSD post hoc, and simple linear regression.

7.4 Results

7.4.1 Total residual ^{14}C-activity in the treated soils over time

The changes in the total residual ^{14}C-activity in the treated soils were monitored at 1 – 60 d by sample oxidation (Figure 1). The total residual activity decreased with an increase in soil-^{14}C-phenanthrene contact time. The total residual ^{14}C-phenanthrene in the soils ranged from 94.55 – 99.18% at 1 d to 16.28 – 32.22% at 60 d. As expected, the total residual activity at 1 d was significantly higher ($p < 0.01$) than at 15 – 60 d. A reduction in total residual ^{14}C-activity of 37.98 – 51.03% was observed at 15 d in the treated soils compared to at 1 d. The reduction was 60.61 – 67.52% at 30 d and 62.82 – 71.44% at 45 d compared to at 1 d. There was no statistical difference ($p > 0.05$) in residual ^{14}C-activity for soil treated at 0.01% and 0.1% at 60 d. However, the residual activity at 60 d in 0.01% and 0.1% was significantly ($p < 0.05$) lower than 1.0%. At 60 d, the reduction in total residual ^{14}C-activity in the treated soils ranged from 66.94 – 78.85%. The reduction in total residual ^{14}C-activity after 60 d was in the order control > 0.1% > 0.01% > 1.0% (Figure 1).

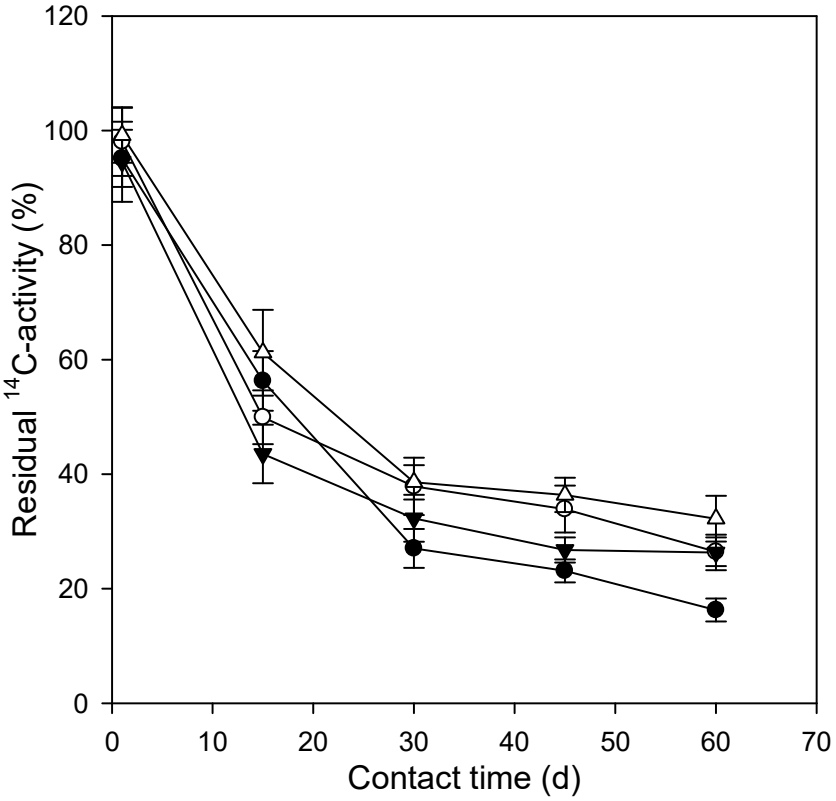

Figure 1: Total residual 14C-phenanthrene activity in DMSO-treated soils. Soil treatments are represented as ●(Control), ○(0.01%), ▼(0.1%), and △(1.0%). Data points show mean (n = 3) values with error bars showing the SEM.

7.4.2 DCM and HP-β-CD extractability of ^{14}C-phenanthrene in the treated soils

The extractable ^{14}C-phenanthrene activity in the treated soils was monitored at 1 – 60 d using DCM and HP-β-CD (Figure 2). This was expressed as a percentage of the residual ^{14}C-activity at each contact time. The solvent extractability decreased over time in all soil treatment conditions (Figure 2) with lower extractability observed at each sampling time compared to previous sampling time. Noticeably, the DCM extracted ^{14}C-phenanthrene at 1 d was

significantly ($p < 0.05$) higher than at 15 – 60 d. The DCM extractability ranged from 85.53 – 100.96% at 1 d to 31.21 – 46.99% at 60 d. At 60 d, the DCM extracted ^{14}C-activity was higher at 1.0% treatment and lower at 0.01% (Figure 2; $p < 0.05$). There was a 43.1 – 59.8% reduction in DCM extracted ^{14}C-activity at 60 d compared to 1 d. Noticeably, the HP-β-CD extracted ^{14}C-activity was lower than the DCM extracted activity in all treatment conditions. The HP-β-CD extractability reduced over time, and the extracted ^{14}C-activity at 1 d was significantly higher ($p < 0.01$) than at 15 – 60 d (Figure 2). The HP-β-CD extracted ^{14}C-activity ranged from 65.64 – 94.23% at 1 d to 18.09 – 26.43% at 60 d (Figure 2). The reduction in HP-β-CD extracted ^{14}C-activity after 60 d was significantly ($p < 0.05$) higher at 0.1% treatment than in other soil treatment conditions (Figure 2). There was no significant difference ($p > 0.05$) in HP-β-CD extracted ^{14}C-activity in 0.01% and 1.0% after 60 d.

The relationship between HP-β-CD extractability and extents of mineralisation was examined to understand how well it predicted mineralisation in DMSO treated soils. The HP-β-CD extraction gave an approximately 1:1 ratio with the extents of mineralisation in some of the soil treatments but overpredicted the extents of mineralisation at most contact times in the soil treatments (Table 2). However, linear regression analysis revealed strong correlations between the HP-β-CD extraction and extents of mineralisation. At 0.01% DMSO treatment, the HP-β-CD extracted activity correlated with extents of mineralisation ($R^2 = 0.60$, slope = 0.44, intercept = 0.36). At 0.1% DMSO treatment, the HP-β-CD extracted ^{14}C-activity showed a stronger correlation with the extents of mineralisation ($R^2 = 0.75$, slope = 1.43, intercept = -24.86). The correlation between HP-β-CD extracted ^{14}C-activity at 1.0% DMSO treatment ($R^2 = 0.80$, slope = 0.95, intercept = -17.18) was also strong.

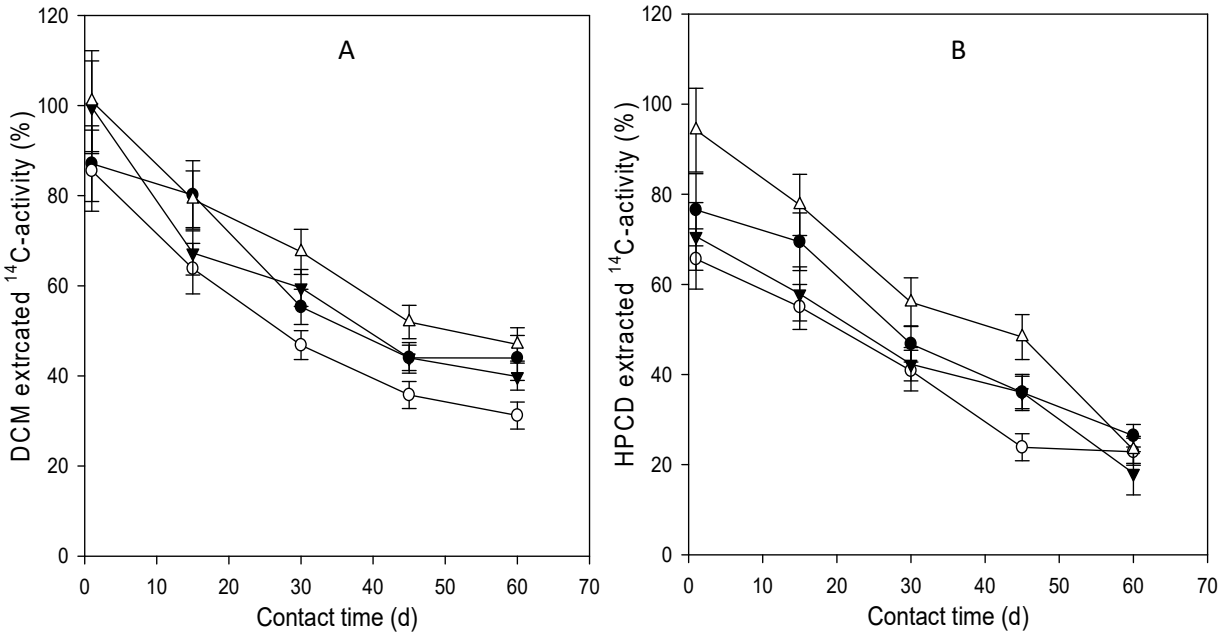

Figure 2: DCM (A) and HP-β-CD (B) extractability of ^{14}C – phenanthrene in DMSO treated soils. Soil treatments are represented as ●(Control), ○(0.01%), ▼(0.1%), and △(1.0%). Data points show mean (n = 3) values with error bars showing the SEM.

7.4.3 Mineralisation of ^{14}C-phenanthrene in the soil treatment conditions

The ^{14}C-phenanthrene mineralisation in all the soil treatment conditions was monitored over time at 1 – 60 d using respirometric assay (Figure 3, Table 2). The mineralisation kinetics were calculated from the residual activity at each sampling time. Longer lag phases, slower rates, and lower extents of mineralisation were observed over time (Table 2; Figure 3). Longer lag phases were observed at 60 d, significantly longer than at 1 – 45 d (Table 2; $p < 0.001$). There was no significant difference in lag phases at 1 – 30 d ($p > 0.05$) but the lag phases at 1 – 15 d were shorter than at 45 – 60 d ($p < 0.001$). There was no significant difference in lag phases at 30 – 45 d ($p > 0.05$) but the lag phases at 30 d were shorter than at 60 d ($p < 0.001$). At 1 d,

significantly ($p < 0.05$) shorter and longer lag phases were observed in 0.1% treated soil and 0.01% treated soil respectively compared to other treatment conditions. Significantly ($p < 0.001$) longer lag phases were observed in 1.0% treatment at 30 d and 60 d compared to other treatments (Table 2). The control showed shorter ($p < 0.05$) lag phases at 45 d and 60 d compared to the treated soils. Longer lag phases were observed in 0.01% at 1d and 45 d which were significantly ($p < 0.05$) longer than in 0.1% treated soil (Table 2).

The fastest rates in the soil treatments did not follow a defined pattern. The fastest rates at 15 d were faster than at 1 – 60 d ($p < 0.001$) and the rates at 1 d were faster than at 30 – 60 d ($p < 0.001$). At 1 d, the rates at 0.1% treatment were faster than in other treatment conditions ($p < 0.001$). The control showed faster rates at 30 d and 60 d compared to the treated soils. As expected, the extents of mineralisation at 1 d were higher than 15 – 60 d ($p < 0.01$; Table 2, Figure 3). The extents of mineralisation decreased with an increase in contact time. Overall, the extents of mineralisation at 0.1% treatment were higher than in all soil treatment conditions. However, this was expected at 1.0% treatment with higher HP-β-CD extractability. At 0.1% treatment, the average extent of mineralisation was approximately 8.0% short of 100% at 1 d (Table 2; Figure 3). The extents of mineralisation in soil treated with DMSO at 0.1% were significantly higher than in other soil treatment conditions at 1 d and 15 d ($p < 0.05$). The mean extent of mineralisation in 1.0% treatment was approximately 10% at 30 d and 4% at 60 d, which was significantly lower than in other treatment conditions. The control showed higher extents of mineralisation than 1.0% treatment from 15 d (Table 2, Figure 3). Significantly ($p < 0.05$) higher extents of mineralisation were observed in the control

at 60 d compared to treated soils. Overall, higher extents of mineralisation were observed in soil treated at 0.1% while lower extents of mineralisation were observed at 1.0%.

Table 2: The ^{14}C-phenanthrene mineralisation kinetics in the DMSO soil treatment conditions over time. Values are in mean ± SEM (n = 3). Values in columns followed by different letters are statistically different (Tukey's HSD; n = 3; $p < 0.05$); also indicated are shorter lag phases (*) and higher extents of mineralisation (+) than the controls that are not statistically significant ($p > 0.05$). Also shown in the table is the ratio of HP-β-CD extracted ^{14}C-phenanthrene to the extents of mineralisation.

Contact time (d)	DMSO treatment (%)	Lag phase (h)	Fastest rate (%$^{14}CO_2$/h)	Extent of mineralisation (%)	HP-β-CD extracted: Mineralised
1	0.0	37.9±1.6^{a}	0.6±0.0^{a}	47.5±2.3^{a}	1.6
	0.01	67.1±3.6^{b}	0.3±0.0^{c}	45.5±1.4^{a}	1.4
	0.1	21.1±0.5^{c}	0.9±0.0^{b}	92.2±3.0^{b}	0.8
	1.0	35.1±1.4^{a*}	0.5±0.1^{a}	51.8±2.5^{a+}	1.8
15	0.0	27.4±6.4^{a}	0.8±0.2^{a}	40.5±5.2^{a}	1.7
	0.01	17.3±8.4^{b}	1.2±0.2^{b}	44.0±6.3^{a+}	1.2
	0.1	23.1±1.4^{b}	0.8±0.0^{a}	69.4±4.8^{b}	0.8
	1.0	19.7±2.5^{b}	1.1±0.1^{b}	37.0±4.0^{a}	2.0
30	0.0	75.3±8.5^{a}	0.4±0.1^{a}	18.8±1.9^{a}	2.4
	0.01	52.7±7.6^{b}	0.3±0.0^{a}	29.1±4.0^{b}	1.4
	0.1	71.7±9.9^{a*}	0.3±0.0^{a}	21.6±3.4^{a+}	1.9
	1.0	161.7±11.0^{c}	0.2±0.0^{a}	10.4±1.0^{c}	5.3
45	0.0	115.2±20.6^{a}	0.2±0.0^{a}	15.8±3.5$^{a\#}$	2.2
	0.01	174.0±21.6^{b}	0.1±0.0^{a}	10.0±1.2^{a}	2.3
	0.1	139.2±16.4^{c}	0.2±0.0^{a}	12.4±1.4^{a}	2.9
	1.0	167.3±18.9^{b}	0.1±0.0^{a}	10.7±1.3^{a}	4.5
60	0.0	98.7 ± 18.0^{a}	0.4±0.1^{b}	16.9±3.0^{a}	1.5
	0.01	221.0 ±41.7^{b}	0.2±0.0^{a}	8.1±1.7^{b}	2.8
	0.1	222.3±57.3^{b}	0.1±0.0^{a}	9.0±3.0^{b}	2.0
	1.0	444.3±124.7^{c}	0.1±0.0^{a}	4.3±1.0^{c}	5.3

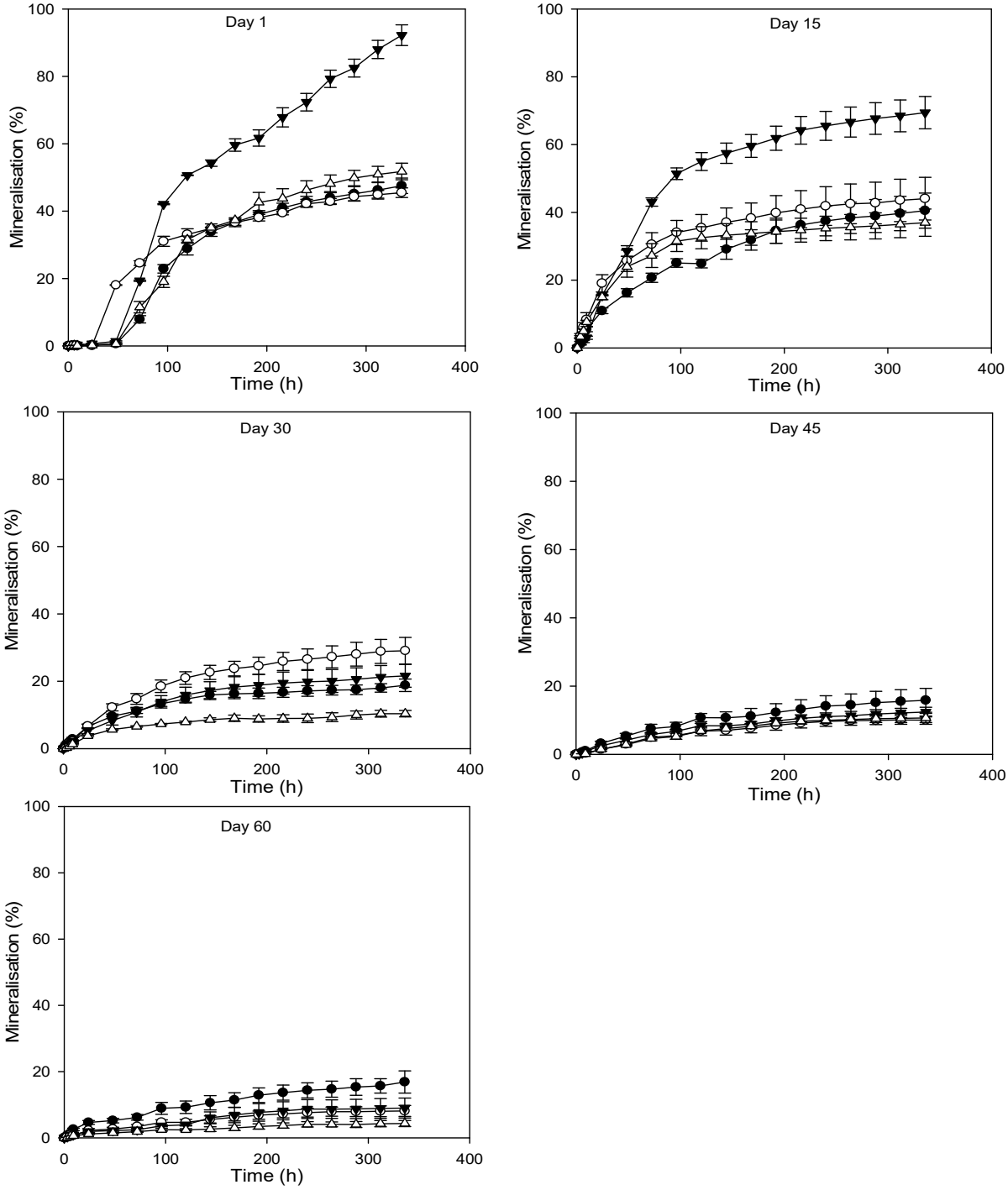

Figure 3: Changes in extents of ^{14}C-phenanthrene mineralisation in the DMSO soil treatment conditions. The soil treatments are represented as ●(Control), ○(0.01%), ▼(0.1%), and △(1%). Data points represent mean values with error bars showing the SEM (n = 3).

7.5 Discussions

7.5.1 Residual activity and solvent extractability in the treated soils

The total residual ^{14}C-phenanthrene activity and solvent extractability reduced over time, and similar findings have been reported in earlier studies (e.g., Chung & Alexander, 1999; Macleod & Semple, 2000, Doick et al., 2003; Macleaod & Semple, 2003; Reid et al., 2004; Doick et al., 2005). After 60 d, among the treated soils, soil treated at 1.0% showed the lowest reduction in total residual activity. This implies a lower loss in spiked activity in soils treated at 1.0%. Microbial degradation has been associated with loss in spiked PAHs (Macleod & Semple, 2000; Doick et al., 2005; Couling et al., 2010; Oyelami et al., 2014), therefore 1.0% treatment may have inhibited microbial degradation. This could be possible as ≥1.0% DMSO has been reported to inhibit microbial proliferation (Galvao et al., 2014; Petruccelli et al., 2020). The difference in residual activity after 60 d in 0.01% and 0.1% was statistically insignificant ($p > 0.05$). Therefore, 0.01% and 0.1% treatment improved the microbial degradation of ^{14}C-phenanthrene than 1.0% due to potentially improved bioavailability and low microbial inhibition. However, due to the simpler structure, higher water solubility, and higher bioavailability of DMSO compared to phenanthrene, soil microbes may have chosen DMSO over phenanthrene at 1.0% treatment.

The ^{14}C-phenanthrene solvent extractability was higher at 1.0% treatment. DMSO is a solvent that can solubilise polar and non-polar compounds (Hayes et al., 1975; Swanson, 1985; Castle et al., 2011; Galvao et al., 2014; Song et al., 2023), therefore it was expected for the 1.0% treatment to show higher extractability. In this study, DCM extracted ^{14}C-phenanthrene was higher than HP-β-CD extracted ^{14}C-phenanthrene which is consistent with past studies (Reid

et al., 2000; Doick et al., 2003; Adedigba et al., 2018). However, in most soil treatments and at most contact times, HP-β-CD extraction overpredicted microbial degradation in this study, which was attributed to increased solubilisation of phenanthrene by DMSO. According to Reid et al. (2004) in a study using sandy loam soil (treated with HP-β-CD), HP-β-CD extraction overestimated mineralisation by 13%. This shows that HP-β-CD extraction can overestimate mineralisation especially when the soil is treated with a solubility/bioavailability enhancer. In addition, Yu et al. (2016) reported higher HP-β-CD extraction (3 – 15 times) to mineralised in phosphate buffer solution (pH 7), which was attributed to a higher dissolution of soil organic matter producing more dissolved organic matter, which subsequently led to a higher solubility of phenanthrene. There was an approximately 1:1 relationship between HP-β-CD extraction and mineralisation in some of the treatments in this study between 1 – 30 d, which is consistent with past studies where the ratio of HP-β-CD extracted and mineralised have ranged between 0.5 – 1.42 for a 2 – 3 ring PAHs (e.g., Doick & Semple, 2003; Doick et al., 2003; Doick et al., 2005; Doick et al., 2006; Semple et al., 2006; Papadopoulos et al., 2007; Rhodes et al., 2010). In this study, the ratio ranged from 0.8 – 5.3 with higher overestimation observed over time (30 – 60 d) and in soil treated with DMSO at 1.0% (1.8 – 5.3). However, linear regression analysis showed a strong correlation (R^2 = 0.60 – 0.80) between HP-β-CD extracted and mineralised, which indicates that HP-β-CD extraction can predict microbial mineralisation of phenanthrene as demonstrated in past studies (e.g., Reid et al., 2000; Doick et al., 2003; Semple et al., 2006; Rhodes et al., 2008). Rhodes et al. (2008) pointed out that despite overestimating by over 20%, HP-β-CD extraction accurately predicted phenanthrene mineralisation with R^2 of 0.95 – 0.99 and a gradient of 0.90 – 1.01.

7.5.2 Influence of DMSO treatment on the mineralisation of ^{14}C-phenanthrene in the soils

Several studies have demonstrated the positive effect of surfactants in improving the microbial degradation of soil organic contaminants (e.g., Posada-Baquero et al., 2019; Perini et al, 2020; Guo & Wen, 2021; Rathankumar et al., 2022; Li et al., 2023; Liu et al., 2023; Zhang et al., 2024). Soil treatment with HP-β-CD has been demonstrated to improve the microbial degradation of PAHs in soil (Reid et al., 2004; Wang et al., 2005; Allan et al., 2007). In this study, soil treatment with DMSO at 0.1% led to a higher ($p < 0.05$) mineralisation of ^{14}C-phenanthrene in soil than other treatment conditions. This was postulated to be through DMSO improving the solubility and bioavailability of ^{14}C-phenanthrene in the treated soil. In a study by Ngugen et al. (2022) microbial cultures grown using DMSO as the only energy and carbon source showed the highest rates of 2'3'7'8-tetrachlorodibenzo-p-dioxin degradation. This indicates that DMSO can improve the microbial degradation of organic contaminants. Similarly, a combination of DMSO and Triton X-100 improved the solubilisation of 4,4'-dibromodiphenyl ether than a single use of Triton X-100 at 92.9% and 67.3% respectively (Yang et al., 2015).

The amount of DMSO added to the soils also affected the level of mineralisation. Soil treated with 0.1% DMSO had significantly ($p < 0.05$) higher extents of ^{14}C-phenanthrene mineralisation than other soil treatments despite higher extractability at 1.0%. The lower ^{14}C-phenanthrene mineralisation at 0.01% and 1.0% treatment may be caused by bioavailability and toxicity constraints. The DMSO treatment at 0.01% may not have an effective impact on improving bioavailability when compared to 0.1%, while it may have negatively influenced the soil microbial community at 1.0%. Studies have indicated that DMSO at ≥1% may elicit

antimicrobial and phytotoxic effects (e.g., Kumar et al., 1976; Galvao et al., 2014; Petruccelli et al., 2020). DSMO is used for the treatment of interstitial cystitis due to its inhibitory effect on urease (Deutch, 2020), and DMSO is referred to as a strong antibacterial agent (Bharathiraja et al., 2020). Additionally, higher extractability does not necessarily result in higher mineralisation. Citric acid and malic acid enhanced the desorption and extractability of phenanthrene in soil but there was no evidence to suggest they enhanced the level of mineralisation (Gao et al., 2012; Vazquez-Cuevas et al., 2020).

Furthermore, while DMSO was used in this study with the intention of stimulating phenanthrene degradation, its metabolic pathway is distinct from that of phenanthrene and does not directly induce PAH-degrading enzymes. DMSO may serve as a carbon, sulphur, or electron acceptor for certain microbes (Mohebali et al., 2008; Rosenbaum et al., 2022; Dixon et al., 2023; Carrión et al., 2023), potentially altering microbial community composition and diverting metabolic activity away from PAH degradation. Moreover, if there is preferential utilisation of DMSO by some microbes due to its simpler structure and availability compared to phenanthrene, this could suppress the expression of phenanthrene-catabolising enzymes. These could explain the high residual activity and low mineralisation at 1.0% DSMO application in this study. Therefore, the observed enhancement in degradation at 0.1% application may reflect indirect shifts in microbial dynamics rather than a direct stimulatory effect on phenanthrene-degrading pathways. In addition, DMSO may enhance the bioavailability of phenanthrene and other PAHs by improving solubility, particularly in aged soils where contaminants are tightly sorbed to organic matter. Due to its high solvent property, DMSO can desorb or mobilise PAHs, potentially increasing their accessibility to

microbial degraders. Furthermore, while DMSO is not inherently benign, it shares functional similarities with other solvents such as ethanol, acetone, surfactants, and dispersants that have been used in environmental applications to enhance pollutant desorption and bioavailability. In aged PAH-contaminated soils, the controlled use of such agents may offer a practical strategy to overcome bioavailability limitations, provided their application is site-specific and coupled with appropriate risk mitigation.

7.5.3 Cost and environmental impact considerations

Solvent treatment to enhance PAH mineralisation in soil is a decades-old technique. For instance, an acetone and ethanol pre-treated soil showed a faster rate of phenanthrene, chrysene, and benzo(a)pyrene degradation (Lee et al., 2001). The impact of the solvent pre-treatment on the difference in degradation between pre-treated soils and control increased with an increase in PAH ring number, showing that desorption and bioavailability limited mineralisation. In addition, methanol improved the mineralisation of desorption-resistant phenanthrene (White & Alexander, 1996). Similarly, the addition of acetonitrile increased the bioavailability and degradation of chrysene, and the growth of chrysene degrading bacterial strain (Caldini et al., 1995). Soaking in an acetone-water mixture increased PAH degradation by more than 2 – folds (Bonten et al., 1999). Bis(ethylhexyl) sebacate improved the rate and level of degradation of LMW PAHs (phenanthrene, pyrene, anthracene, naphthalene) in a two-phase partitioning bioreactor (MacLeod & Daugulis, 2003).

Among these solvents, ethanol and acetone are considered more environmentally benign, particularly due to their lower toxicity. DMSO also exhibits relatively low environmental impact when applied at low concentrations (Galvao et al., 2014; Petruccelli et al., 2020; Andrade-Vieira et al., 2022), making it a viable alternative in certain remediation contexts. The selection of organic solvents such as DMSO, ethanol, and acetone to enhance the bioavailability of phenanthrene in contaminated soils necessitates a careful balance between solvent efficacy, environmental persistence, and microbial compatibility. DMSO exhibits lower volatility and longer half-life in soil (1–7+ days) (Han et al., 2017)**,** providing sustained desorption of aged and strongly sorbed PAHs. While ethanol and acetone degrade more rapidly (0.1–3 and 2–7 days, respectively) (Staples, 2000; Zhang et al., 2006; De Bruyn et al., 2020) and are less persistent, they pose reduced risk of long-term microbial toxicity and are thus more compatible with sensitive microbial communities. However, the half-life and degradation of these solvents is influenced by physicochemical conditions such as pH, aeration, and temperature which could reduce or increase the half-lives (Hwang et al., 2007). In terms of microbial toxicity, ethanol is generally well-tolerated at low concentrations, whereas acetone can exhibit moderate toxicity, particularly at higher amounts (Cápiro et al., 2008; Zheng et al., 2010). DMSO, despite its excellent PAH solubilisation properties and deeper soil penetration, may suppress microbial activity if applied excessively like observed at 1.0% application in this study. Its longer residence time, however, allows for gradual PAH desorption, making it particularly suited to aged or recalcitrant soils where bioavailability limits microbial degradation. Therefore, the longer half-life and lower volatility of DMSO confer advantages for mobilising tightly bound phenanthrene, enhancing the potential for microbial mineralisation in weathered soils. However, its application should be coupled with microbial bioaugmentation or biostimulation strategies to offset potential inhibitory effects.

The efficacy of phenanthrene bioremediation in soil systems is influenced by the physicochemical characteristics of the soil and the extent of contaminant aging, which typically reduces PAH bioavailability. The use of chemical agents such as organic solvents and surfactants has shown promise in improving desorption and subsequent microbial mineralisation (Lee et al., 2001; Rathankumar et al., 2022; Li et al., 2023; Liu et al., 2023; Zhang et al., 2024). However, their selection must consider not only their desorptive capacity but also their environmental safety and economic viability. DMSO demonstrates greater potential for enhancing bioavailability in aged soils due to its low volatility and high affinity for hydrophobic compounds. Its relatively long residence time in soil may facilitate sustained desorption of sorbed phenanthrene, allowing indigenous or introduced microbial communities more time to degrade the compound. However, the cost of DMSO (£60 – 80/L) is higher than that of ethanol or acetone (£5–10/L), which are more volatile and less persistent but more accessible and economically feasible for large-scale applications. Ethanol offers a favourable combination of low toxicity, biodegradability, and compatibility with microbial processes. The application of synthetic surfactants such as Tween 80 or Triton X-100 (£50 – 109/L) has also been widely documented (Rathankumar et al., 2022; Li et al., 2023; Liu et al., 2023; Zhang et al., 2024), particularly for their ability to reduce interfacial tension and mobilise hydrophobic contaminants. Nevertheless, their potential ecotoxicity and limited biodegradability in certain soil environments remain concerns, especially at higher concentrations or with repeated application. In contrast, biosurfactants, such as rhamnolipids, have garnered attention for their high environmental compatibility, low toxicity, and excellent biodegradability (Posada-Baquero et al., 2019; Guo & Wen, 2021). Despite their advantages, biosurfactants remain cost-prohibitive in purified forms (£1000–

1500/g), which limits their scalability. From a practical standpoint, solvent and surfactant selection should align with the contaminant aging profile, remediation scale, and project budget. DMSO may be more appropriate for targeted treatment of highly weathered contamination, whereas ethanol or acetone are more practical for large-area applications. Biosurfactants offer an attractive, sustainable option where environmental regulations and long-term site recovery are prioritized, provided production costs can be mitigated.

7.6 Conclusion

The findings from this study demonstrate that DMSO can enhance the mineralisation of phenanthrene in soil, with the effect potentially influenced by the amount applied. The higher residual activity, higher extractability, but lower mineralisation at 1.0% indicate some level of microbial inhibition. The HP-β-CD extraction overpredicted the extents of mineralisation which was attributed to enhanced solubility of phenanthrene by DMSO because extractability increased with DMSO amount. Therefore, DMSO application should be done cautiously to avoid secondary contamination or solubilisation-driven toxicity. A high DMSO amount may be toxic and there may be a toxicity concern if solubilisation exceeds mineralisation. This will increase the concentration of bioavailable PAH in the soil thereby increasing the likelihood of PAH-related toxicity. A similar concern was expressed by Andersson et al. (2009). This study pioneered the use of DMSO to enhance PAH bioavailability and mineralisation in soil, offering an innovative solution to PAH soil contamination and advancing PAH remediation strategies. DMSO may offer benefits in aged PAH-contaminated soils to overcome the constraints of bioavailability and improve microbial degradation of aged contaminants. The findings from this current study suggest that solvent-facilitated mobilisation, though not universally

suitable, merits further exploration as a targeted tool to enhance biodegradation of aged organic contaminants in soil.

7.7 Declaration of interest

The authors declare no conflicting financial, professional, or personal interests.

7.8 CRediT author statement

Chisom Ejileugha: Conceptualisation, Methodology, Investigation, Data interpretation, Visualisation, Formal analysis, Writing - Original draft, Writing – Review & Editing **Kirk T. Semple:** Conceptualisation, Methodology, Validation, Resources, Supervision, Writing – Review & Editing.

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7.10 Data availability

All relevant data were included in this article

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Chapter 8 Discussion, conclusions, and future work

8.1 Discussion

This thesis has provided evidence-based insights that biochar and SMC can enhance the biodegradation of PAHs in soil, which is consistent with past studies (e.g. Omoni et al., 2020a; Omoni et al., 2020b; Kaur & Sharma, 2021; Patel et al., 2022; Atai et al., 2023; Ge et al., 2023). The mineralisation and extractability of phenanthrene reduced over time in all the studies in this thesis. Contact time has been consistently shown to impact PAH bioaccessibility and possible mineralisation due to aging (e.g., Alexander & Kelsey, 1997; White et al., 1997; Macleod and Semple, 2000; Reid et al., 2000; Macleod and Semple, 2003; Stokes et al., 2005; Doick et al., 2006). High amendment amount does not necessarily result in improved PAH biodegradation as revealed by the results in Chapter 3. In Chapter 3, an increase in amendment amounts reduced mineralisation in biochar-amended soils, and lower amendment amounts (0.1 – 1%) improved mineralisation than 10% in SMC-amended soils. Similar findings have been reported in past studies (e.g., Puglisi et al. 2007; Scelza et al., 2007; Rhodes et al., 2008; Omoni et al., 2020a; Omoni et al., 2020b).

The results in Chapter 3 indicate that the effect of amendment amounts on the lag phases, maximum mineralisation rates, and overall mineralisation extents was dependent on the type of amendment used. The mineralisation of ^{14}C-phenanthrene decreased over time, with biochar amendments showing a greater reduction than SMC, and is likely due to differences in their sorption capacities, carbon content, and nutrient availability. This is consistent with the reports of Sigmund et al. (2018) that compost caused 10 – fold increase in PAH sorption

but the addition of biochar caused a 100 – fold increase in the sorption of PAHs. As reported in Chapter 3, there were negligible differences in the level of mineralisation for 0.1% and 0.5% SMC and biochar-amended soils. There were significant ($p < 0.05$) differences at 1% and 10% with SMC having a greater impact on improving phenanthrene mineralisation than biochar. Contact time and its interaction with amendment amount had a greater impact on the mineralisation kinetics compared to amounts, which underscores the importance of assessing the independent and combined impacts of contact time and amendment amount. Earlier studies demonstrated that contact time has a greater impact on PAH biodegradation than amendment type or amount (Wu et al., 2013; Wu et al., 2014). Amendment conditions that shorten mineralisation lag phases may enhance overall mineralisation in soil, as treatments with shorter lag phases generally showed higher mineralisation levels. This aligns with the findings of Omoni et al. (2020a).

Amendment blends showed higher cumulative extents of mineralisation than biochar and SMC alone which indicates that there was a synergistic benefit of biochar – SMC blends. This is consistent with the findings of Bao et al. (2020), who combined biochar and SMC, showing that there was greater PAH removal than in biochar alone in soil. The combined amendment significantly improved the biodegradation of LMW and HMW PAHs while biochar improved only the biodegradation of HMW PAHs (Bao et al., 2020). This was through the sorption of LMW in biochar micropores reducing microbial accessibility, and binding of HMW on the biochar surface which aided faster microbial access and biodegradation (Cao et al., 2016; Zhang et al., 2021). The combined application of biochar and compost can immobilise PAHs in the soil while facilitating the microbial degradation of remaining accessible fractions

(Sigmund et al., 2018). Blending biochar with compost can address the nutrient limitations of biochar while enhancing and prolonging the agronomic benefits of compost (Liao et al., 2021). Fine particles SMC (<1 mm and 1 – 4 mm) showed lower cumulative extents of mineralisation than 4 – 11 mm and <15 mm, due to easily accessible nutrients and fast decomposition which could cause a shift in microbial preference (Schaefer & Juliane, 2007; Yuan et al., 2009), leading to a lower cumulative extent of mineralisation. The wide particle range (<15 mm) provided a nutrient balance while the 4 – 11 mm contained slow degrading lignified wood (Haynes et al., 2015) leading to sustained slow nutrient release and higher cumulative extents of mineralisation. Available phosphorus, ammonium nitrogen, and TOC were strongly correlated with the mineralisation kinetics in the soil treatments which indicates their strong influence on the observed mineralisation kinetics. This is in line with past studies that have demonstrated that ammonium nitrogen (Wang et al., 2022), available phosphorus (Kończak et al., 2023), and TOC (Guo et al., 2020; Yaun et al., 2023) improve the biodegradation of PAHs.

Smaller biochar particles (<0.6 mm) enhanced ^{14}C-phenanthrene extractability, reduced ^{14}C-residual activity, and promoted mineralisation compared to 2 – 4 mm. This could be due to their shorter diffusion lengths and larger surface areas, which facilitated easier desorption, increased bioavailability, and microbial degradation (Kang et al., 2018; Kang et al., 2019; Sarfraz et al., 2020; Jin et al., 2022; He et al., 2022). In contrast, larger biochar particles (2–4 mm) had longer diffusion paths, slowing desorption and decreasing bioavailability. While biochar with a larger surface area enhances contaminant sorption, it may also promote faster desorption; whereas biochar with a smaller surface area slows desorption due to the lesser

surface – solvent contact and extended diffusion pathways. Furthermore, biochar amount and particle size substantially impacted mineralisation rates, with stronger correlations between HP-β-CD extractability and mineralisation of ^{14}C-phenanthrene observed at lower biochar amounts and in the smaller particle size. This is consistent with the reports of Rhodes et al. (2008) and Rhodes et al. (2012) who reported a poor correlation between HP-β-CD extraction and mineralisation of ^{14}C-phenanthrene in soil amended with black carbon above 0.1%. It is also in line with the reports of Ogbonnaya et al. (2014) who demonstrated that there was a better agreement with mineralisation in soil amended with smaller particles of biochar than in the larger particles.

Solvent treatment has been demonstrated in earlier studies to enhance PAH biodegradation (e.g., White & Alexander, 1996; Lee et al., 2001; MacLeod & Daugulis, 2003). Surface active agents are used to solubilise hydrophobic organic contaminants, like PAHs, to increase possible microbial degradation (Li et al., 2023; Liu et al., 2023; Zhang et al., 2024). Dimethyl sulphoxide (DMSO) as a solubilising agent enhanced phenanthrene bioavailability and mineralisation as reported in Chapter 7. DMSO at 0.1% improved mineralisation by shortening lag phases, accelerating rates, and increasing extents of mineralisation. However, higher amounts of DMSO (1.0%) increased phenanthrene extractability without enhancing mineralisation. This was attributed to a possible inhibitory effect of DMSO on soil microbes at 1.0%. However, PAH solubilisation could outpace microbial degradation, increasing the bioavailability of PAHs and potentially causing toxicity (Anderson et al., 2009). This situation could make treated soil more toxic than untreated soil.

8.2 Conclusions

The findings reveal that the ^{14}C-phenanthrene mineralisation kinetics in soil were influenced not only by the amounts of the organic amendments but also by a significant interaction with soil-PAH contact time. These findings concur with the initial hypothesis that the amount of amendments, contact time, and the interaction of both factors influence phenanthrene mineralisation kinetics. This interaction was found to have a more substantial influence on mineralisation kinetics than the amendment amount itself, with the potential for mineralisation decreasing over time. However, the amount of the amendment also influenced the mineralisation kinetics with a reduction in mineralisation observed at higher amounts, especially in the biochar-amended soils. This highlights a complex and time-dependent relationship, where increasing amounts of amendment may not necessarily counteract the decline in mineralisation over time. This is novel as it has demonstrated that the interaction between the amount of the amendment and contact time is pivotal in shaping the mineralisation trajectory, revealing that time-dependent sequestration processes are more influential than direct microbial degradation, particularly over extended periods. The sorption capacity of the amendment played a key role, as larger amounts of amendment, especially in biochar-amended soils, led to a more significant reduction in mineralisation over time compared to SMC-amended soils. This provides new insights into how the interaction of amendment amount and contact time should be considered when designing soil treatment strategies, with the implication that simply increasing the amounts of amendment may not be the most effective approach for promoting long-term biodegradation. Amendment amount >1.0% may not offer further benefits for improving mineralisation kinetics than ≤1.0% in biochar or SMC amended soil. However, further research is needed to determine whether

the greater reduction in mineralisation at biochar amounts ≥1.0% is solely due to sorption, or if it is also associated with changes in microbial community structure as influenced by soil-phenanthrene-amendment interactions.

The results in Chapter 4 support the hypothesis that amendment blends can improve ^{14}C-phenanthrene mineralisation kinetics in soil compared to individual amendments. Blending biochar and compost in specific ratios proved to be a more effective strategy for enhancing mineralisation, highlighting the importance of optimising the blending ratio to achieve the desired outcome. While higher biochar content could favour sorption and reduced biodegradation, higher compost content could promote biodegradation over sorption. This underscores the need for a balanced approach that utilises biochar's sorption benefits while ensuring adequate biodegradation of the bioaccessible fraction of ^{14}C-phenanthrene. This has provided novel insights that amendment blends, when appropriately balanced, offer a more efficient alternative to single amendments for improving mineralisation kinetics, providing a more effective strategy for amending phenanthrene contaminated soil. Further studies on SMC-biochar blends exploring the impacts of the different blends on phenanthrene sorption, incorporation in microbial biomass, changes in microbial communities, and formation of bound residues (parent compound or metabolites) could accurately predict the long-term fate of phenanthrene under SMC-biochar blend and other organic amendment applications.

The findings in Chapters 5 and 6 support the hypothesis that amendment particle size influences ^{14}C-phenanthrene extractability and mineralisation kinetics in soil. Specifically, smaller particle biochar exhibited lower ^{14}C-residual activity, higher extractability, and greater

mineralisation compared to larger particle size fraction. While smaller particle biochar offers high sorption capacity, larger particles may be more effective for long-term sorption stability, particularly when the objective is to reduce bioaccessibility and prevent re-mobilisation. Interestingly, SMC in its original form outperformed its particle size fractions, suggesting that the original form provides better mineralisation benefits. In contrast, smaller particle compost showed lower cumulative extents of mineralisation, but this effect could be mitigated by reducing the application amount to provide just enough nutrients to sustain microbial activity without causing a shift in microbial preferences. These findings highlight the importance of particle size in amendment efficacy, demonstrating that small and large particles may have distinct roles depending on the amendment objective, particularly in balancing bioaccessibility, sorption, and biodegradation. The sorption – desorption dynamics in biochar particle size was attributed to surface area and difference in diffusion pathway. However, more studies are needed to fully understand how other factors like pore structure, pore size and volume, ash content, and surface properties influence the sorption stability of biochar of different particle sizes. Raw SMC outperformed particle fractions at 1.0% application. However, studies monitoring the mineralisation kinetics at different application amounts below and above 1.0% could provide a more holistic understanding of the impact of SMC particle sizes, and studies monitoring the impact of organic matter sorption could provide more evidence as smaller particle could decompose faster contributing to higher organic matter driven bound residues.

The findings from DMSO study confirm that treatment of ^{14}C-phenanthrene-spiked soil with DMSO enhances extractability and improves mineralisation kinetics, supporting the

hypothesis that DMSO positively impacts these processes. Specifically, 0.1% DMSO was found to improve mineralisation kinetics more effectively than other treatment amounts. Despite concerns about the potential toxicity of higher DMSO concentrations, the results suggest that DMSO has significant potential for enhancing PAH mineralisation, which could be beneficial in enhancing the biodegradation of PAHs in situations where bioavailability is a limitation such as in HMW PAH-contaminated soil and long-term aged soils. This demonstrates the promising application of DMSO in soil remediation, as it facilitated both extractability and microbial degradation of phenanthrene, offering a potential solution for more effective remediation of aged contaminants in soil. Future studies using aged field contaminated soil consisting of several PAHs of different molecular weight and structure could provide evidence of how well DMSO improves the bioavailability and mineralisation of aged PAHs in soil.

These findings have provided substantial information that is useful for exposure mitigation, risk assessment, and possible remediation of PAH-contaminated soil. This has important implications in agriculture, environmental science, and land management. This could contribute to good environmental and sustainable soil management practices, and impact policy decisions and existing practices aimed towards managing contaminated land. The use of biochar and SMC as soil amendments align with the principles of circular economy and sustainable agricultural practice and waste management, through recycling and re-using waste materials for environmental gain. SMC is a cheaper alternative to compost and its use as an amendment is cost-effective, environmentally friendly, sustainable, and will reduce the burden of SMC management on mushroom farmers.

8.3 Limitation and recommendations for future studies

A key limitation of this study was the lack of funding for molecular analysis to monitor changes in microbial diversity and abundance in the amended soils, particularly in the DMSO treatment. Such analysis would have provided valuable insights into microbial community dynamics across different amendment amounts and contact times. Additionally, it would have identified the microbial species involved in phenanthrene mineralisation and any community shifts induced by the contaminant or amendments. This data would have significantly enhanced the understanding of the DMSO experiment. Therefore, future studies incorporating molecular analysis would provide a more comprehensive understanding of microbial responses to treatments/amendments and their role in PAH biodegradation. The aging of only ^{12}C-phenanthrene in the experiments in chapters 3, 4, and 6, and spiking of the ^{14}C-phenanthrene during respirometry may not show the actual effect of contact time and amendment on phenanthrene mineralisation over time. Therefore, future studies should consider aging both isotopes together to closely simulate the fate of the phenanthrene over time.

With the promising effect of SMC-biochar blends on PAH biodegradation in soil, co-composted biochar could provide a better alternative due to the improved nutrient content of co-composted biochar compared to compost, biochar, or SMC (Akdeniz, 2019; Ejileugha, 2022). Additionally, co-composted biochar will reduce the risk of introducing biochar- and compost-related contaminants in soil (Ejileugha, 2022; Ejileugha et al., 2024). The amount used in this thesis for biochar-SMC blend and SMC particle size experiments was 0.5% and

1.0% respectively. Therefore, investigating the impacts of biochar-SMC blends and SMC particle size fractions at different amounts on PAH bioavailability and mineralisation in soil could provide more useful insights. A given blend or particle size may be better at a lower or higher amount and the single amount used in this thesis cannot provide such information.

Biochar properties are influenced by both feedstock selection and pyrolysis conditions (Semple et al., 2013; Ejileugha, 2022; Ghorbani et al., 2024). However, further research is needed to understand how feedstock composition affects the relationship between biochar particle size and the bioavailability and biodegradation of PAHs in soil. Investigating biochar produced from different feedstocks, under consistent pyrolysis conditions, could help determine whether the impact of particle size on contaminant sorption and desorption is influenced by feedstock type and other biochar properties, such as functional groups and pore structure. Additionally, exploring how these factors affect the sorption, desorption, and mineralisation of PAHs with varying molecular weights and structures would provide valuable insights for mitigating adverse impacts of PAHs in soil. Biochar can be modified using metal nanoparticles (Lalhriatpuia & Tiwari, 2024; Ahmad et al., 2024) or polymers (Babalar et al., 2024; Saleh et al., 2024; Yang et al., 2024) to improve the properties of the biochar for pollutant removal. Optimising biochar sorption capacity and stability, and engineering biochar to respond to varying environmental conditions, could provide a significant breakthrough in contaminant removal using biochar.

8.4 References

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Appendix

The influence of contact time on biodegradation and extractability of 9 –^{14}C–phenanthrene in digestate and wood – ash amended soils.

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Abstract

The environmental persistence and ecotoxicity potential of polycyclic aromatic hydrocarbons (PAHs) has stimulated significant interest in the bioaccessibility and biodegradation of PAHs in soils. Contact time, organic amendment, and microbial inoculation can influence PAH bioaccessibility and/or biodegradation in soil. This study investigated the influence of contact time on chemical extractability and biodegradation of 9-^{14}C-phenanthrene in soils amended with anaerobic digestate (AD), wood–ash (WA), and AD + WA. It also investigated the impact of bacteria inoculation on PAH degradation in soil during 90 days of soil incubation, with soil samples analysed at 0, 15, 30, 60, and 90 d soil-PAH contact time. Significantly low amounts of 9-^{14}C-phenanthrene activity were recovered in most of the soils, particularly with the AD + WA, and the amounts recovered reduced with increasing soil-PAH contact time. Dichloromethane (DCM) -extractable and hydroxypropyl-β-cyclodextrin (HP-β-CD) -extractable 9-^{14}C-phenanthrene fractions also reduced with increasing soil-PAH contact time. The DCM-extractable fractions reduced from 88.9% - 79.1% to 41.9% - 35.4%, and HP-β-CD extractable fraction reduced from 83.5% - 63.7% to 5.6% - 1.8% from time zero to 90 d, respectively. The extents of mineralisation in inoculated soil and uninoculated soils were similar, but the former was relatively higher, especially with longer soil-PAH contact times. A nearly 1:1 relationship existed between the HP-β-CD extractable and mineralized 9-^{14}C-phenanthrene fractions in indigenous and inoculum-assisted mineralisation. These results show that ageing is a significant factor in PAH bioaccessibility and mineralisation in soil, and biostimulation of the indigenous microbial community can enhance PAH biodegradation in soil. Therefore, bioaugmentation may not be necessary where suitable indigenous microbes are present in the soil.

Keywords: Bioaccessibility, Dichloromethane extraction, Hydroxypropyl-β-cyclodextrin extraction, Mineralisation, Phenanthrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic contaminants, which can be introduced into the environment through natural activities like thermal geologic production, and anthropogenic sources that involve industrial activities (Okere & Semple, 2012; Oyelami et al., 2013; Lang et al., 2016; Patel et al., 2020). Due to their hydrophobic properties, PAHs can be sequestered in soils through sorption onto soil organic matter and/or clay fractions, resulting in desorption-resistant fractions, that can persist in soils (Reid et al., 2000; Papadopoulos et al., 2007; Marini & Frapiccin, 2013). This enhances the resistance of PAHs to biological, physical, and chemical degradation (Couling et al., 2010). This persistent property of PAHs in soil increases the possible exposure to their putative toxic effects (Das and Chandran, 2011). Despite this, the intrinsic catabolic potential of soil microflora has been shown to be a significant route for the removal of PAHs from soils (Leys et al., 2005; Tyagi et al., 2011; Premnath et al., 2021). However, the increased interaction of PAHs with the soil particles over time has been known to reduce the microbial accessibility of PAHs through the sequestration of these aromatic chemicals (Doick & Semple, 2003; Doick et al., 2003; Semple et al., 2003; Doick et al., 2005a).

Microbial degradation of PAHs has been known to be efficient, environmentally sustainable, and less expensive (Alvarez et al., 2011; Maletić et al., 2013; Naseri et al., 2014; Tiwary et al., 2015; Ning et al., 2017). This makes bioremediation a suitable technology for the long-term restoration of soil contaminated with PAHs and their derivatives. Studies have demonstrated that indigenous microbes in soil and wetland can degrade PAHs (Mills et al., 2003; Picariello et al., 2020; Roszak et al, 2021). The increase in the soil carbon as a result of the entry of

organic contaminants can affect the stoichiometric composition of the soil nutrients (Rojas-Avelizapa et al., 2000), and consequently reduce microbial growth and degradation activity. In nutrient-limited environments, biostimulation approach has been used to enhance the biodegradation of organic contaminants (Lang et al., 2016). Where biodegradation needs to be improved, the indigenous microbial populations have been supported with pre-adapted microbial inoculants, that possess the relevant hydrocarbon degradation potential (Lang et al., 2016). In past studies, strategies involving the addition of nutrients and pre-adapted catabolic inoculants with required catabolic capability have been explored to enhance microbial degradation of PAHs in soil (Tyagi et al., 2011; Haleyur et al., 2019; Brzeszcz et al., 2020). However, there is a paucity of information on the impact of organic amendments, especially studies focusing on the impact of anaerobic digestate (AD) or wood ash (WA) singly or as mixtures on indigenous and inoculum-assisted biodegradation of PAHs in soil.

In this present study, two nutrient-rich bioenergy residues, AD and WA (fly ash fraction) were added to soil to enhance microbial growth and degradation of PAH using phenanthrene as a model PAH. Both AD and WA are known to contain considerable amounts of major elements (e.g., Ca, K, and Na) (García-Sánchez et al., 2015). Also, AD is known to have a large amount of processed organic carbon and N contents (Insam et al., 2015; Tiwary et al., 2015), while N is mostly absent in WA (Perucci et al., 2006). The positive effects of AD and WA on soil properties have been documented in the literature (Sharma & Kalra, 2006; Insam et al., 2009; García-Sánchez et al., 2015; Ibeto et al., 2020, 2023). Due to the complementary nutrients of AD and WA (Bougnom et al., 2012), their use as mixtures can be a valuable soil amendment for enhancing the biodegradation of PAHs. Therefore, this study examined the effect of

contact time on solvent extractability and evaluated the indigenous and inoculum-assisted mineralization of 9–^{14}C-phenanthrene in soils amended with AD and/or WA.

2. Materials and Methods

2.1 Materials

Non-labelled phenanthrene (>96 %) and 9-^{14}C-phenanthrene (55.7 mCi mmol^{-1}; >99 % purity) were supplied by Sigma-Aldrich, Pool United Kingdom (UK). Sodium hydroxide (NaOH), acetone (>99.8 %, HPLC grade), Gold Star multipurpose liquid scintillation cocktail, and Combustaid® were obtained from Meridian, UK. Nutrient agar was supplied by Sigma Life Science; Ringer's pellets and plate count agar (PCA) powder were supplied by Oxoid. AD (pasteurized) was obtained from an anaerobic digestion plant in the UK, and it consists of anaerobically digested household food wastes. WA (fly ash) was generated in the UK, with timber and bark as feedstocks.

2.2 Soil sampling and characteristics

The soil used in this study was collected (5 - 20 cm depth) from Myerscough, UK. The soil is known to be a Dystric Cambisol soil with a clayey-loam texture, high organic matter content, and no record of previous exposure to PAHs. Other physicochemical characteristics of the soil are presented in Table 1. The soil was passed through a ≤2 mm sieve to remove debris and stones; its moisture content (30%) was determined by oven drying at 105°C for 24 h. The soil's carbon-to-nitrogen ratio was 6.7±0.3 (Couling et al., 2010).

2.3 Soil spiking and amendments

Soil (2.396 kg w/w; moisture content = 30%) was spiked with ^{12}C-phenanthrene (100 mg kg^{-1}), using acetone as the carrier solvent. The mixtures were carefully homogenized and left in the fume cupboard for 2 h to allow the acetone to volatilize (Macleod & Semple, 2000). Afterwards, the soil was amended in triplicate as follows: (1) soil + $^{12/14}$C-phenanthrene + AD; (2) soil + $^{12/14}$C-phenanthrene + WA, and (3) soil + $^{12/14}$C-phenanthrene + AD + WA. Control soils were similarly spiked with $^{12/14}$C-phenanthrene without the addition of AD and/or WA and separately with ^{12}C-phenanthrene without the addition of AD and/or WA, which provided background levels of radioactivity. The concentrations of AD (17.31 g) and WA (0.94 g) added to the soils were derived from the N:P (3:1) reference doses of agricultural practice recommendations for soil amendment (AHDB, 2017) and wheat plantation (DEFRA, 2017). All the AD and WA amended and unamended soils remained in the fume cupboard for 24 h before spiking with 9-^{14}C-phenanthrene (35 kBq kg^{-1}) alongside its non-radiolabelled analogue (10 mg kg^{-1}), using acetone as the delivery solvent. The spiked soils were left in the fume cupboard for 24 h, before ageing for 90 d at 20 ± 2 ^{o}C in separate pre-cleaned amber glass jars with loose Teflon–lined screw caps, to allow ambient oxygen exchange.

Table 1: Physicochemical characteristics of Myerscough soil, AD, and WA; measurements were in dry weight (d/w), except those indicated wet weight (w/w); n = 3 ± SEM (standard error of mean). <BDL = Below detectable limit.

Parameters		Values		
		Soil	Anaerobic digestate	Wood-ash
pH (in dH_2O) (w/w)		6.5 ± 0.1	9.0 ± 0.0	12.7 ± 0.0
Electrical conductivity (w/w)		35.7 ± 3.0	9.6 ± 0.2	50.3 ± 0.5
Organic matter (LOI) (%)		5.7 ± 2.0	66.0 ± 2.0	1.0 ± 0.0
Elemental analysis (mg kg^{-1}):				
	Total carbon	19.0 ± 1.0	349.0 ± 1.0	13.0 ± 0.1
	Total nitrogen	2.8 ± 0.1	43.5 ± 0.5	0.7 ± 0.1
	Total phosphorus	1.1 ± 0.08	12.9 ± 0.5	25.2 ± 0.0
Water Soluble PO_4-P * (g kg^{-1})		0.0736±5.9	0.316 ± 0.0	0.027 ± 0.0
Ammonium nitrogen (NH_4^+-N) (g kg^{-1})		0.0003±0.2	4.281 ± 0.1	<BDL
Nitrate nitrogen (NO_3^--N) (g kg^{-1})		0.0014±0.3	0.001 ± 0.0	0.032 ± 0.0
*Soil particle analysis (%):				
	Clay	19.5 ± 0.7		
	Silt	20.0 ± 0.9		
	Sand (total)	60.4 ± 1.2		
	Coarse sand	0.12 ± 0.0		
	Medium sand	6.9 ± 0.1		
	Fine sand	53.3 ± 0.6		

2.4 Measurement of total ^{4}C-activity from 9-^{14}C-phenanthrene amended soils.

The total activity applied as 9-^{14}C-phenanthrene to the AD and/or WA amended and unamended ^{12}C-phenanthrene spiked soils was determined by combustion of soil samples using a Sample Oxidizer (Packard 307) with increasing soil-PAH contact times (0, 15, 30, 60, and 90 d). This allowed the total ^{14}C-activity to be measured and loss to be quantified over the 90 d incubation. Each of the soil samples was weighed (ca. 1 g) into separate cellulose combustion cones and combusted for 5 minutes with the aid of Combustaid® (200 µl). Evolved $^{14}CO_2$ was trapped with 10 ml of Carbosorb-E®, and 10 ml Permafluor-E® was added as a scintillation cocktail. The trapping efficiency of the sample oxidizer (>96 %) was determined before the soil combustion. The trapped ^{14}C-activity was quantified with a liquid scintillation

counter (Canberra Packard Tri-Carb 2250CA) by using standard calibration and quench correction techniques and protocols (Reid et al., 2004).

2.5 The extractability of 9-^{14}C-phenanthrene in soils

2.5.1 Dichloromethane (DCM) extraction of 9-^{14}C-phenanthrene from soil incubations

The total extractable 9-^{14}C-phenanthrene in the AD and/or WA amended and unamended soils was carried out through an exhaustive extraction method using DCM as an extraction solvent (Semple et al., 2003; Allan et al., 2007). The soils were extracted at increasing soil-PAH contact times (0, 15, 30, 60, and 90 d). At each sampling, 2.5 g (n = 3) of amended and unamended soil was weighed into separate 50 ml Teflon centrifuge tubes, and DCM (30 ml) and anhydrous Na_2SO_4 granules (2.5 g) were added. The tubes were tightly closed with screw caps and agitated end-to-end at 150 rpm on an orbital shaker (SANYO Gallenkamp) for 22 h at 21 °C. The resultant extracts were centrifuged at 4000 rpm for 30 minutes, and the supernatants were separately decanted into similar pre-cleaned Teflon centrifuge tubes. The soil pellets were re-washed with fresh 30 ml DCM solution as described above. A 3 ml of the supernatant was pipetted into clean 20 ml economy vials, followed by the addition of 17 ml of Ultima Gold scintillation fluid to each of the vials. The vials were subsequently analyzed by liquid scintillation counting using relevant protocols. The remaining supernatant was discarded, and the resultant pellets were dried in the fume hood and oven. The pellets were separately weighed (ca. 1 g) into cellulose combustion cones and combusted in a sample oxidizer, to determine the non-extractable 9-^{14}C-phenanthrene fraction in each of the soil samples.

2.5.2 Hydroxypropyl-β-cyclodextrin (HP-β-CD) extraction of 9-^{14}C-phenanthrene from soil incubations

Non-exhaustive chemical extraction of 9-^{14}C-phenanthrene residues from the soil samples was carried out with HP-β-CD, to mimic bioavailability and estimate its extent in the soils, at increasing soil-PAH contact times (0, 15, 30, 60, and 90 d) (Reid et al., 2000; Doick et al., 2003). A 1.25 g (w/w; n = 3) of soil was weighed into separate 50 ml Teflon centrifuge tubes. 25 ml of 50 mM HP-β-CD solution was added to each of the tubes and properly closed with screw-caps followed by end-to-end agitation at 150 rpm on an orbital shaker (SANYO Gallenkamp) for 22 h at 21°C (Semple et al., 2006). The mixtures were centrifuged for 30 minutes at 3600 rpm (using Beckman JA 21/2 Centrifuge), and the supernatants were separately decanted into similar pre-cleaned and labeled Teflon centrifuge tubes. The soil pellets were re-suspended in fresh 50 mM HP-β-CD solution (25 ml) and the above process was repeated. 6 ml of the supernatants were separately sampled into clean 20 ml economy vials followed by the addition of 14 ml of Ultima Gold scintillation fluid. The mixtures were quantified by liquid scintillation counting, and the amounts of solvent nonextractable 9-^{14}C-phenanthrene fractions in the soil pellets were determined by sample oxidation.

2.6 Mineralisation of 9-^{14}C-phenanthrene in soil incubations

The mineralisation of 9-^{14}C-phenanthrene to $^{14}CO_2$ was carried out at increasing soil-PAH contact times (0, 15, 30, 60, and 90 d) using [a] ^{14}C-respirometric system. The mineralisation was performed in modified 250 ml Schott bottles with Teflon-lined screw-caps incorporated with metal clips (Couling et al., 2010). Soil (13.7 g w/w; n = 3) from each of the soil treatments

was weighed into a respirometer, and sterile mineral basal salts (MBS) medium was added to form a slurry, to ensure an even $^{12/14}$C-PAH distribution (Doick & Semple, 2003). Each inoculated respirometer contains 25 ml of sterile MBS and 5 ml of a bacterial inoculum of 9-^{14}C-phenanthrene degrading *Pseudomonas* sp. (~10^7 bacteria kg^{-1}soil) in MBS medium. Each uninoculated respirometer contains 30 ml of MBS medium. A 7 ml scintillation vial containing 2 ml of 1 M NaOH was suspended in each respirometer using a metal clip from the bottle cap, to trap any catabolically evolved $^{14}CO_2$ during the mineralisation of the 9-^{14}C-phenanthrene. The MBS medium contains 0.3 gl^{-1} NaCl, 0.6 gl^{-1} $(NH_4)_2SO_4$, 0.6 gl^{-1} KNO_3, 0.25 gl^{-1} KH_2PO_4, 0.75 gl^{-1} K_2HPO_4, 0.15 gl^{-1} $MgSO_4.7H_2O$, and the following micronutrients: $LiCl(LiBO_2)$ (20 mgl^{-1}), $CaSO_4.5H_2O$ (80 mgl^{-1}), $ZnSO_4.7H_2O$ (100 mgl^{-1}), $Al(SO_4)_3.16H_2O$ (100 mgl^{-1}), $NiCl.6H_2O(CoNO_3)$ (100 mgl^{-1}), $CoSO_4.7H_2O(CoNO_3)$ (100 mgl^{-1}), KBr (30 mgl^{-1}), KI (30 mgl^{-1}), $MnCl_{2.}2H_2O$ (600 mgl^{-1}), $SnCl_2.2H_2O$ (40 mgl^{-1}) and $FeSO_4.7H_2O$ (300 mgl^{-1}).

The respirometers were incubated at 20 ± 2 ^oC for 14 d on a flat-bed orbital shaker (SANYO Gallenkamp) at 100 rpm to ensure adequate mixing of the slurries. The NaOH-$^{14}CO_2$ traps were removed and instantly replaced with fresh ones at 2, 4, 8, 12, and 24 h respectively, and subsequently every 24 h for 14 d. The collected NaOH-$^{14}CO_2$ traps were instantly mixed with 5 ml of Ultima Gold scintillation fluid and tightly screw-capped. The vials were wiped clean with acetone-moistened tissues to remove any residual ^{14}C-activity on the surface and allowed to rest in the dark for 12 h (to prime the mixtures and normalize the chemiluminescence effect). The ^{14}C-activity in the traps was quantified by liquid scintillation counting.

2.7 Microbial inoculation of soil incubations

The density of the catabolically active phenanthrene degrading *Pseudomonas* sp. used in this study was ~10^7 CFUs kg^{-1} soil. The population density of the inoculant agreed with past studies where catabolically active bacteria of relatively high inoculum densities ranging between ~10^6 to 10^8 cells kg^{-1} soil were employed for the biodegradation of PAHs (Doick et al., 2005a). The inoculum was obtained from a pure strain of *Pseudomonas* sp. previously cultured and subsequently re-cultured in fresh MBS medium with ^{12}C-phenanthrene, as the sole carbon source. This concurs with past studies where the successful adaptation of microorganisms to mineralize PAHs was linked to their prior exposures to PAHs, which resulted in faster degradation of freshly added PAHs (Macleod & Semple 2006; Rhodes et al., 2008; Couling et al., 2010). The inoculant was harvested after 4 days (late exponential phase) of incubation by centrifugation (4000 rpm) at 15 ^{o}C for 30 minutes. The cell pellets were recovered and re-washed in a fresh MBS medium to ensure the total removal of any residual phenanthrene (Reid et al., 2004). Pseudomonads were used because it is one of the bacterial strains identified to possess the metabolic routes required for the degradation of recalcitrant compounds (Hwang & Cutright, 2002; Tyagi et al., 2011).

2.8 Statistical analysis

Following blank correction, the data were plotted with SigmaPlot 10.0, and statistical analysis was done with IBM SPSS 27. The differences in the mineralisation of the 9-^{14}C-phenanthrene in the AD and/or WA amended and unamended soils were evaluated using one-way analysis of variance (ANOVA), at a 95 % confidence level ($P < 0.05$) to determine the least significant

difference. The comparison of the means within and across the soils was analyzed using Turkey and LSD's post-hoc tests. Pearson correlation coefficient (r) was performed to describe the relationship between HP-β-CD extractable 9-^{14}C-phenanthrene and mineralized 9-^{14}C-phenanthrene in inoculum-assisted mineralisation and uninoculated mineralisation.

3. Results

3.1 Recovery of total residual 9-^{14}C-phenanthrene in the amended soils.

The 9-^{14}C-phenanthrene associated residues (%) as obtained in the amended and unamended soils with increasing contact times are shown in Figure 1. The loss of the ^{14}C activity from the soils increased with increasing soil contact time. Generally, there were greater rates of losses in the AD, WA and AD + WA amended soils as compared to the unamended soil incubations. The losses were greater in the AD + WA amendment as compared to the other soil conditions after time zero. The amounts of 9-^{14}C-phenanthrene residues recovered from the soils amended with AD + WA were significantly lower ($P < 0.05$) than the control soils incubations after time zero, and WA after 30 d. Greater amounts of 9-^{14}C-phenanthrene residues were lost in the presence of WA (30 d and 60 d) and AD (30 d, 60 d, and 90 d) compared to the controls, but these differences were not statistically significant ($p > 0.05$).

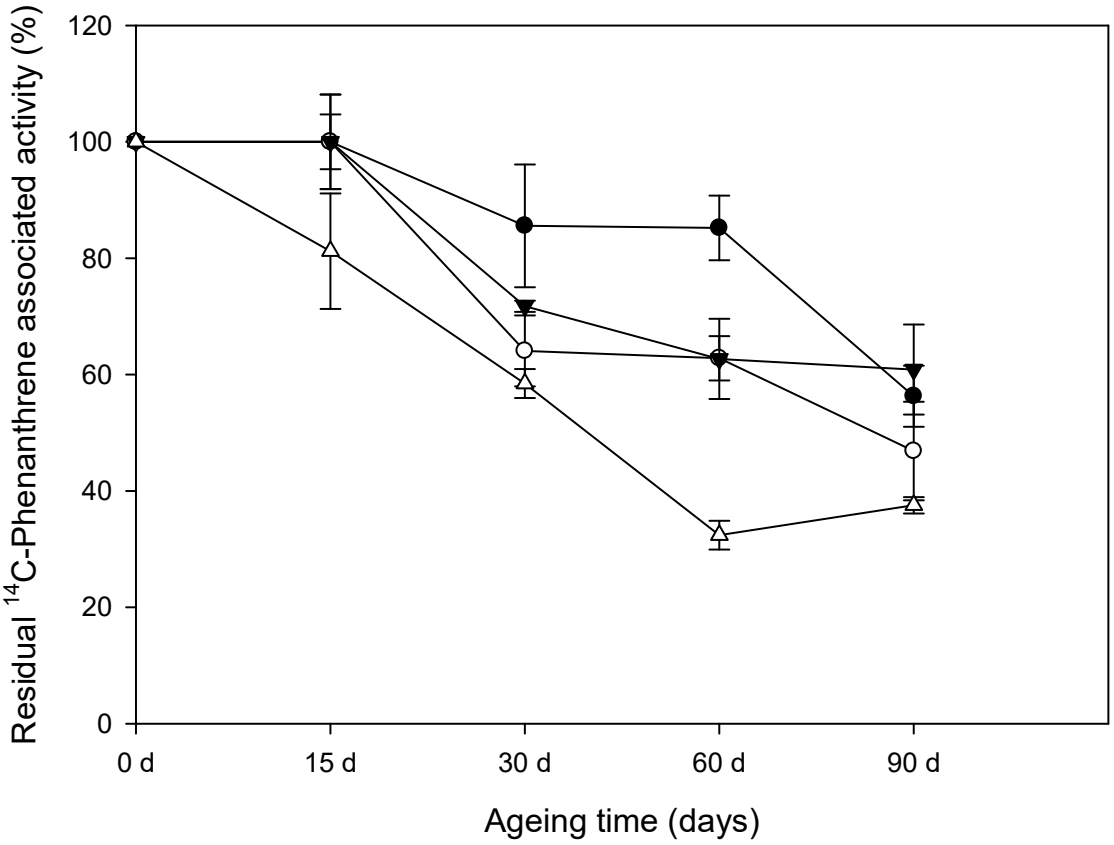

Figure 1: The 9-^{14}C-phenanthrene's loss curves in amended and unamended soils: AD (○), WA (▼), AD + WA (△), and unamended soils (●) over 90 days; values (n = 3) represent the mean ± SEM.

3.2 Chemical extraction of ^{14}C-phenanthrene in the soils

The total amounts of DCM extractable 9-^{14}C-phenanthrene residues (%) in the spiked soils at various contact times are shown in Table 2. DCM-extractable 9-^{14}C-phenanthrene fractions were higher across time zero to 90 d in the unamended control soils compared to the amended soils. However, in AD amended soils, DCM-extractable 9-^{14}C-phenanthrene residues slightly became increased than the controls at 30 d and 90 d. At most sampling times, the DCM-extractable 9-^{14}C-phenanthrene residues from the amended soils were not statistically

different (P > 0.05) from the controls. The amounts of HP-β-CD-extractable 9-^{14}C-phenanthrene fractions (%; Table 2) in the spiked amended soils were significantly lower (P < 0.05) than the unamended controls across time zero to 90 d. The AD amended soil showed a higher HP-β-CD-extractable amount on 30 d and 90 d compared to the control.

Table 2: DCM and HP-β-CD extractable 9-^{14}C-phenanthrene residues in amended and unamended soils; AD and/or WA. Values in columns followed by different letters are statistically different (Turkey, LSD; p < 0.05); values (n = 3) represent the mean ± SEM.

Soil-PAH contact times (d)	Soil amendments	DCM-extractable ^{14}C-phenanthrene activity (%)	HP-β-CD-extractable ^{14}C-phenthrene activity (%)
0	Control	88.9 ± 1.5 a	83.5 ± 0.0 a
	Soil + AD	79.1 ± 1.5 a	71.2 ± 6.2 b
	Soil + WA	85.6 ± 7.0 a	63.7 ± 1.3 b
	Soil + AD + WA	82.6 ± 4.0 a	74.2 ± 2.8 b
15	Control	69.3 ± 12.7 a	66.5 ± 5.6 a
	Soil + AD	68.4 ± 1.1 a	62.1 ± 1.5 a
	Soil + WA	61.7 ± 18.1 a	59.2 ± 0.4 a
	Soil + AD + WA	65.0 ± 5.8 a	54.7 ± 6.9 b
30	Control	43.7 ± 5.4 a	37.9 ± 1.9 a
	Soil + AD	44.5 ± 1.5 a	40.5 ± 10.2 a
	Soil + WA	28.5 ± 5.6 b	18.2 ± 11.1 b
	Soil + AD + WA	31.7 ± 4.7 a	11.2 ± 16.1 b
60	Control	37.4 ± 3.1 a	8.4 ± 4.5 a
	Soil + AD	22.1 ± 4.8 b	5.4 ± 2.7 b
	Soil + WA	23.8 ± 4.3 b	1.3 ± 1.3 b
	Soil + AD + WA	25.6 ± 4.3 a	4.6 ± 1.1 b
90	Control	40.2 ± 1.8 a	5.2 ± 0.4 a
	Soil + AD	41.9 ± 6.4 a	5.6 ± 5.7 a
	Soil + WA	36.0 ± 5.1 a	5.5 ± 4.5 a
	Soil + AD + WA	35.4 ± 5.6 a	1.8 ± 3.9 b

3.3 Mineralisation of ^{14}C-phenanthrene in inoculated soils

The inoculum-assisted mineralisation of the 9-^{14}C-phenanthrene in the amended and unamended soils is shown in Figure 2 and Table 3. The lag phases for amended soils were significantly shorter ($P < 0.05$) than the controls at time zero. Shorter lag phases were also observed with the additions of AD at time zero, as well as AD + WA at 60 d and 90 d, but they were not statistically different ($P > 0.05$) from the controls. The rates of mineralisation were significantly higher ($P < 0.05$) than the controls under the additions of WA and AD + WA at time zero. Also, the rates of mineralisation were high with the additions of AD at time zero, as well as AD + WA at 30 d, but these were not statistically different ($P > 0.05$) from the controls. The extents of mineralisation decreased over time in all the soil treatment conditions (Figure 2 and Table 3). Higher extents of mineralisation were observed in the control at time zero, 15 d, and 30 d. The extents of mineralisation in AD + WA were higher at 60 d but were lower at time zero, 15 d, and 30 d when compared to other amended soils.

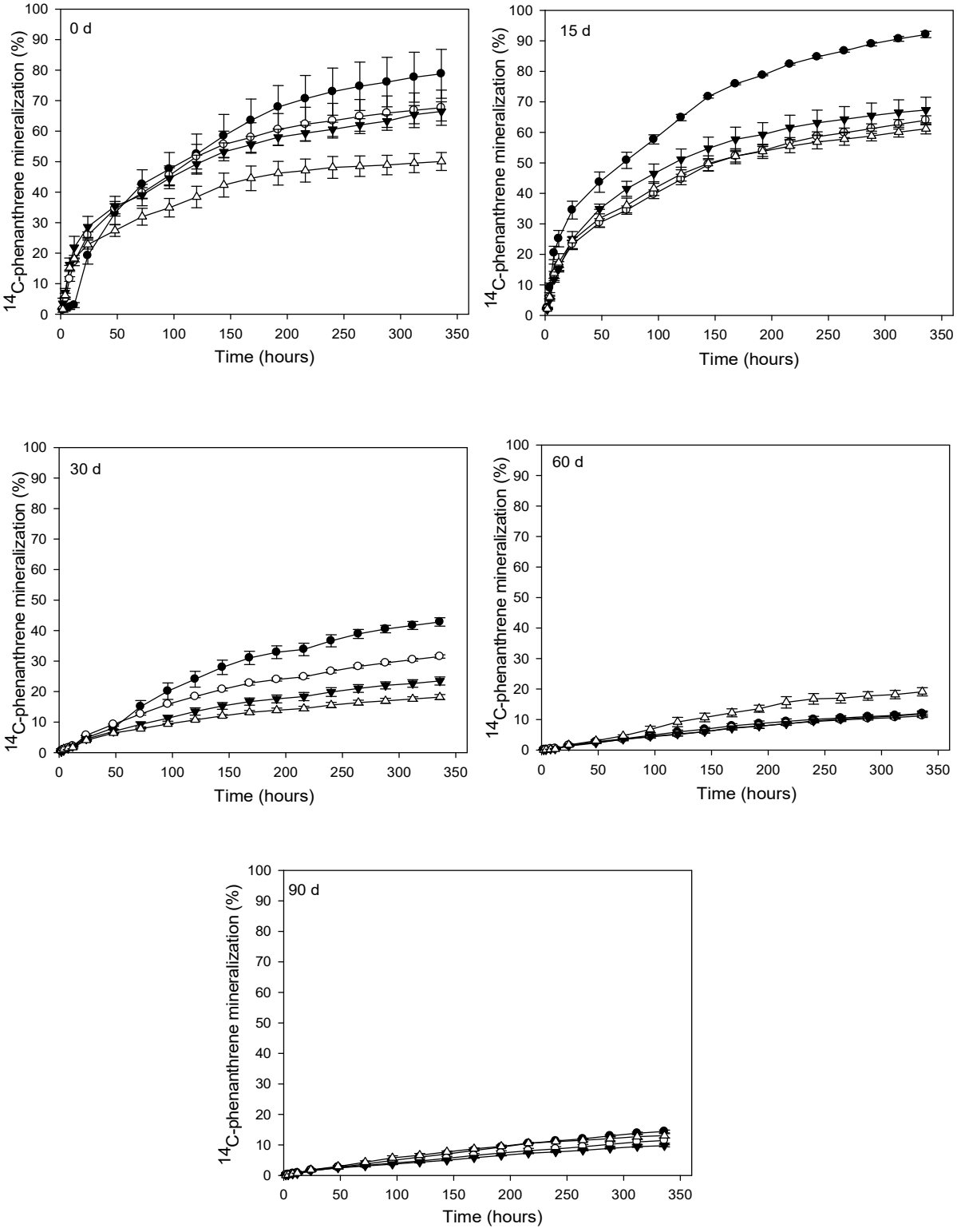

Figure 2: Mineralisation of 9-^{14}C-phenanthrene (%) in inoculated amended and unamended soils: AD (○), WA (▼), AD + WA (△), and unamended soils (●) over 90 days, values (n = 3) represent the mean ± SEM.

Table 3: Mineralisation of 9-^{14}C-phenanthrene in inoculated and unamended soils. Values in columns followed by different letters are statistically different (Turkey, LSD; n = 3; $p < 0.05$); also indicated are shorter lag phases (+) and higher maximum rate (*) than the controls that are not statistically significant ($p > 0.05$); values (n = 3) represent the mean ± SEM.

Soil-PAH contact times (d)	Soil amendments	Lag phase (h)	Maximum rates (% h^{-1})	Cumulative extents (%)
0	Control	13.6 ± 0.7 a	1.4 ± 0.2 a	78.8 ± 8.0 a
	Soil + AD	9.7 ± 2.8 a+	1.8 ± 0.2 a*	67.7 ± 5.8 a
	Soil + WA	3.9 ± 1.4 b	2.7 ± 0.5 b	66.5 ± 3.1 a
	Soil + AD + WA	3.6 ± 0.3 b	2.5 ± 0.0 b	50.1 ± 2.9 b
15	Control	2.8 ± 0.2 a	3.4 ± 0.7 a	92.1 ± 1.0 a
	Soil + AD	3.5 ± 0.2 a	1.9 ± 0.2 b	64.0 ± 1.5 a
	Soil + WA	4.0 ± 0.2 b	1.7 ± 0.1 b	67.3 ± 4.2 a
	Soil + AD + WA	3.6 ± 0.3 b	2.2 ± 0.4 a	61.1 ± 1.6 a
30	Control	25.7 ± 3.4 a	0.3 ± 0.0 a	42.8 ± 1.4 a
	Soil + AD	21.7 ± 0.2 a+	0.3 ± 0.0 a	31.5 ± 0.5 a
	Soil + WA	29.5 ± 5.1 a	0.2 ± 0.0 a	23.5 ± 1.3 a
	Soil + AD + WA	32.8 ± 3.5 a	0.4 ± 0.2 a*	18.2 ± 0.8 a
60	Control	102.9 ± 12.3 a	0.1 ± 0.0 a	12.0 ± 0.9 a
	Soil + AD	114.7 ± 13.2 a	0.1 ± 0.0 a	11.2 ± 0.5 a
	Soil + WA	113.2 ± 15.0 a	0.1 ± 0.0 a	11.8 ± 0.6 a
	Soil + AD + WA	78.1 ± 5.2 a+	0.1 ± 0.0 a	19.1 ± 1.3 b
90	Control	83.8 ± 2.0 a	0.2 ± 0.0 a	15.3 ± 0.5 a
	Soil + AD	102.3 ± 15.2 a	0.1 ± 0.0 a	11.9 ± 0.9 b
	Soil + WA	109.7 ± 4.4 a	0.2 ± 0.0 a	10.3 ± 0.4 b
	Soil + AD + WA	74.5 ± 7.8 a+	0.1 ± 0.0 a	13.8 ± 0.7 a

3.4 Mineralisation of ^{14}C-phenanthrene in uninoculated soils

The mineralisation of the 9-^{14}C-phenanthrene in uninoculated amended and unamended soils is shown in Figure 3 and Table 4. The lag phases were significantly shorter ($P < 0.05$) than the controls with the additions of AD at time zero and WA at 15 d. Also, shorter lag phases were observed with the additions of WA at time zero, AD at 15 d and 30 d, as well as AD + WA at 60 d, but they were not statistically significant compared to the controls. The rates of

mineralisation were significantly higher ($P < 0.05$) than the controls at 15 d with the addition of WA. The higher rates of mineralisation observed with the additions of AD + WA at 15 d, as well as AD at 30 d, were not statistically significant compared to the controls ($p > 0.05$). The extents of mineralisation were significantly greater ($P < 0.05$) than the controls at 60 d in AD + WA amended soils. At time zero, 15 d, and 30 d, the control showed higher extents of mineralisation than the amended soils. The AD + WA showed lower extents of mineralisation at time zero, 15 d, and 30 d when compared to AD or WA amended soil.

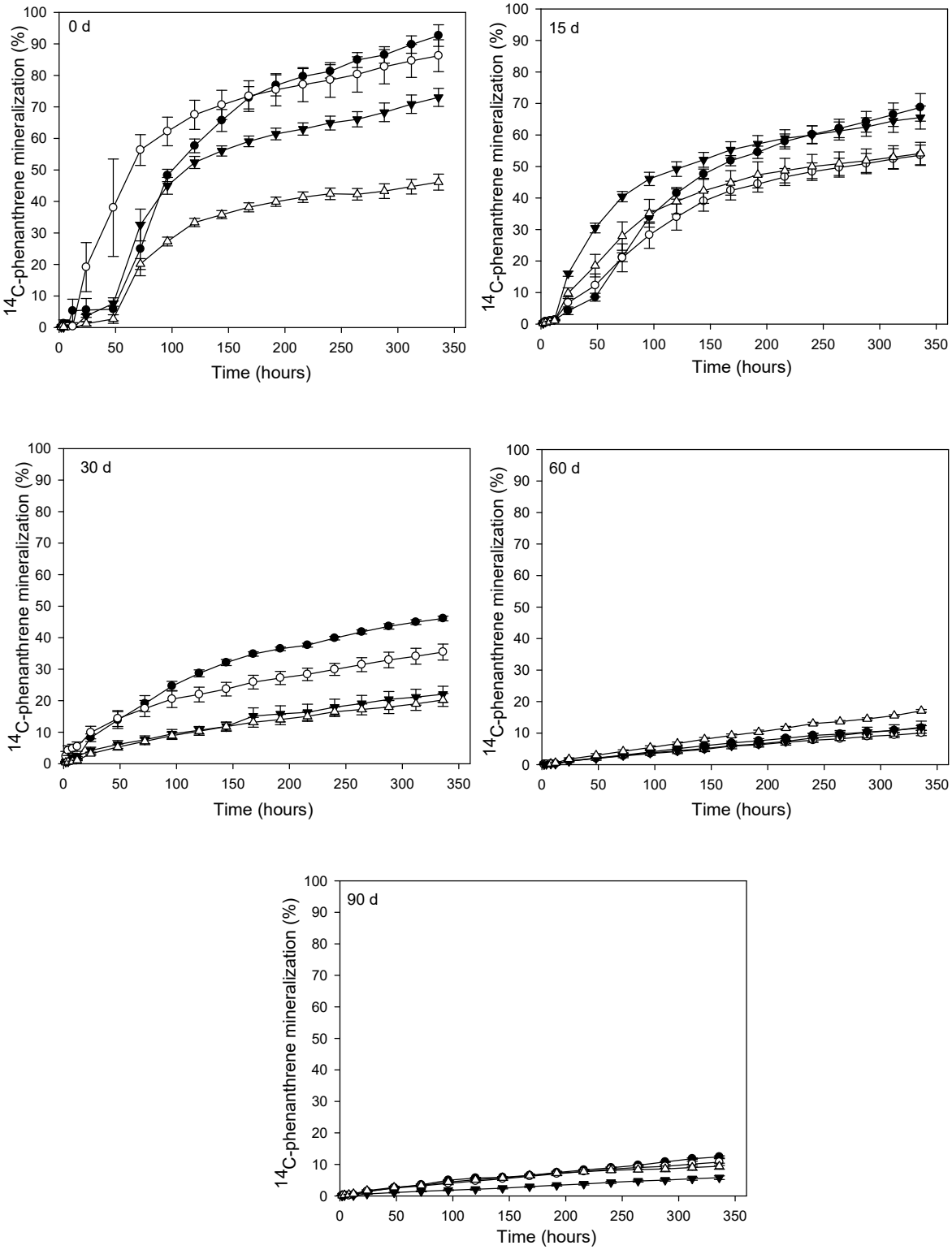

Figure 3: Mineralisation of 9-^{14}C-phenanthrene (%) in uninoculated amended and unamended soils: AD (○), WA (▼), AD + WA (△), and unamended soils (●) over 90 days, values (n = 3) represent the mean ± SEM.

Table 4: Mineralization of 9-^{14}C-phenanthrene in uninoculated amended and unamended soils. Values in columns followed by different letters are statistically different (Turkey, LSD; n = 3; $p < 0.05$); also indicated are shorter lag phases (+) and higher maximum rate (*) than the controls that are not statistically significant ($p > 0.05$); values (n = 3) represent the mean ± SEM.

Soil-PAH contact times (d)	Soil amendments	Lag phase (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
0	Control	37.4±13.9a	2.1±0.6 a	92.6±3.4 a
	Soil + AD	19.2±5.3 b	2.0±0.2 a	86.2±5.1 a
	Soil + WA	29.5±3.1a+	1.1±0.2 a	73.0±2.9 b
	Soil + AD + WA	48.4±3.6 a	0.7±0.1 b	46.1±2.5 b
15	Control	29.3±5.2 a	0.6 ± 0.1 a	68.8±4.4 a
	Soil + AD	24.3±5.5a+	0.5 ± 0.1 a	53.6±3.2 a
	Soil + WA	15.0±0.2 b	1.3 ± 0.1 b	65.5±3.7 a
	Soil + AD + WA	18.0±1.3a+	0.8 ± 0.2 a*	54.1±3.6 a
30	Control	18.2± 1.1 a	0.6 ± 0.1 a	46.1±0.7 a
	Soil + AD	8.7± 5.9 a+	1.8 ± 0.8 a*	35.4±2.6 a
	Soil + WA	37.0±10.9a	0.4± 0.2 a	22.0±2.5 a
	Soil + AD + WA	47.9± 7.1 b	0.2 ± 0.0 a	20.2±2.1 a
60	Control	120.5±10.a	0.1 ± 0.0 a	11.6±0.7 a
	Soil + AD	144.6± 9.1a	0.1 ± 0.0 a	10.0 ±0.8a
	Soil + WA	145.5±32.a	0.1 ± 0.0 a	11.9 ±1.9a
	Soil + AD + WA	87.7± 8.9a+	0.1 ± 0.0 a	17.1 ±0.4b
90	Control	85.8± 1.6 a	0.1 ± 0.0 a	12.4 ± 0.4a
	Soil + AD	107.2±10.6a	0.1 ± 0.0 a	10.7± 1.1 a
	Soil + WA	226.6±15.0b	0.1 ± 0.0 a	5.8 ± 0.6 b
	Soil + AD + WA	95.7 ± 8.5 a	0.2 ± 0.0 a	9.4 ± 1.0 b

3.5 Comparing HP-β-CD extractable and mineralized ^{14}C-phenanthrene fractions in the soils

The comparison of HP-β-CD extractable and mineralized 9-^{14}C-phenanthrene fractions (%) in the soils is given in Table 5. The amounts of HP-β-CD extractable ^{14}C-phenanthrene fractions positively correlated with the mineralized ^{14}C-phenanthrene fractions in both inoculated and uninoculated respirometry at all contact times. In the inoculated respirometric assay, using

soils amended with AD and/or WA, the amounts of HP-β-CD extractable and mineralized 9-^{14}C-phenanthrene fractions were close in terms of a 1:1 proportionate relationship. The results showed a strong correlation (R^2 = 0.94). Similarly, in most of the uninoculated respirometric assays, the HP-β-CD extractable and mineralized 9-^{14}C-phenanthrene fractions in soils amended with AD and/or WA, have a proportionately 1:1 relationship. The results showed a positive correlation (R^2 = 0.95) with increasing soil-PAH contact time.

Table 5: Comparison of HP-β-CD extractable 9-^{14}C-phenanthrene (%) and biodegradable (%) 9-^{14}C-phenanthrene in amended and unamended soils; (n = 3).

Soil-PAH contact times (d)	Amended soils	HP-β-CD extraction (%) A	HP-β-CD extraction:Biodegradation			
			Biodegradation in Inoculated soils (%) B	Ratio (A:B)	Biodegradation in uninoculated soils (%) C	Ratio (A:C)
0	Control	83.9	78.8	1.1	92.6	0.9
	Soil + AD	71.2	67.7	1.1	86.2	0.8
	Soil + WA	63.7	66.5	1.0	73.0	0.9
	Soil + AD + WA	74.2	50.1	1.5	46.1	1.6
15	Control	66.5	92.1	0.7	71.2	0.9
	Soil + AD	62.1	64.0	1.0	56.8	1.1
	Soil + WA	59.2	67.3	0.9	69.5	0.9
	Soil + AD + WA	54.7	61.1	0.9	57.4	1.0
30	Control	37.9	42.8	0.9	46.1	0.8
	Soil + AD	40.5	31.5	1.3	35.4	1.1
	Soil + WA	18.2	23.5	0.8	22.0	0.8
	Soil + AD + WA	11.2	18.2	0.6	20.2	0.6
60	Control	8.4	12.0	0.7	11.6	0.7
	Soil + AD	5.4	11.2	0.5	10.0	0.5
	Soil + WA	1.3	11.8	0.1	11.9	0.1
	Soil + AD + WA	4.6	19.1	0.2	17.1	0.3
90	Control	5.2	15.3	0.3	12.4	0.4
	Soil + AD	5.6	11.9	0.5	10.7	0.5
	Soil + WA	5.5	10.3	0.5	5.8	0.9
	Soil + AD + WA	1.8	13.8	0.1	9.4	0.2

4. Discussion

4.1 Impact of ageing and amendments on the recovery and extractability of 9-^{14}C-phenanthrene

There was loss of 9-^{14}C-phenanthrene associated activity in all the soil incubations, particularly with the additions of AD + WA. The total 9-^{14}C-phenanthrene activity in AD + WA amended soils decreased significantly from 15 d when compared to other soil treatment conditions. This suggests that the additions of AD and/or WA stimulated microbial degradative activity in the soil and consequently contributed to the biodegradation of the 9-^{14}C-phenanthrene in the soils. It is likely that the major loss process of the PAH from soils was microbially mediated (Macleod & Semple, 2000; Doick et al., 2005a) and further stimulated by the amendments. This finding agrees with earlier studies where the biodegradation of PAHs increased following the additions of organic nutrients to soils (Haritash and Kaushik, 2009; Tyagi et al., 2011; Scotti et al., 2013; Scotti et al., 2015). The loss of 9-^{14}C-phenanthrene in this study also aligns with documented studies where biodegradation was indicated as a significant process of PAH removal from the soil under favourable soil conditions (Papadopoulos et al., 2007; Lang et al., 2016; Siles & Margesin, 2018).

The DCM extraction is known to be an exhaustive method that gives the total chemically extractable PAHs in the soil (Semple et al., 2003; Allan et al., 2007). HP-β-CD extraction had been used to mimic the mineralisation of PAHs or predict the extent to which PAHs can be microbially degraded in the soil (Doick et al., 2005a; Papadopoulos et al., 2007; Rhodes et al., 2008). The 9-^{14}C-phenanthrene DCM extractable and HP-β-CD extractable fractions in soils decreased as contact time increased. Similar findings have been reported in past studies (Reid et al., 2000; Papadopoulos et al., 2007; Rhodes et al., 2008). This could be through sorption

of 9-^{14}C-phenanthrene to soil particles and amendments, and/or sequestration of 9-^{14}C-phenanthrene within the soil as the contact time increased. The DCM extractable fraction decreased from 88.9% – 40.2%, 79.1% - 41.9%, 85.6% - 36%, and 82.6% - 35.4% for Control, AD, WA, and AD + WA respectively. The HP-β-CD extractable fraction decreased from 83.5% - 5.2%, 71.2% - 5.6%, 63.7% - 5.5%, and 74.2% - 1.8% for Control, AD, WA, and AD + WA respectively. WA and/or AD appear to affect the initial extractability of 9-^{14}C-phenanthrene, and this could be attributed to sorption of 9-^{14}C-phenanthrene by the organic amendments. This observation agrees with past studies which identified ageing and soil organic matter to promote a reduction in the extractability of PAHs in soil (Reid et al., 2000; Doick et al., 2003; Semple et al., 2003; Papadopoulos et al., 2007). PAHs can bind to minerals and humin-like soil fractions hence reducing desorption and potential bioaccessibility (Han et al., 2020).

4.2 Impact of ageing, inoculant, and amendments on the mineralisation of 9-^{14}C-phenanthrene

The impact of ageing was observed on the bioaccessibility of 9-^{14}C-phenanthrene in both inoculated and uninoculated AD and/or WA amended soils. The levels of $^{14}CO_2$ produced reduced significantly as the soil-PAH contact time increased. The lag phases increased, and the mineralisation rates and extents diminished. The HP-β-CD extractable fraction also decreased greatly between 5.6% - 1.8% at 90 d, therefore indicating a reduced bioaccessibility. This observation could be because of sorption and sequestration of the 9-^{14}C-phenanthrene in soil, in addition to reduced total residual activity, as the soil-microbe-PAH interactions increased. These findings agree with earlier studies where the bioaccessibility of PAHs in PAH-contaminated soils reduced due to ageing (Macleod & Semple, 2002; Doick et

al., 2005b; Rhodes et al., 2008; Ogbonnaya et al., 2016). In addition, Chen et al. (2023) reported that ageing reduced the bioaccessibility of nitrated-PAHs in their study.

The lag phases in inoculated mineralisation were lower than that in uninoculated mineralisation. This is due to the faster degradation of the PAHs by the adapted inoculum compared to the soil indigenous bacteria that are still adapting to the PAH. This effect could also be seen in the maximum rates of mineralisation for amended inoculated soils at time zero. The inoculated soils had higher maximum rates of mineralisation at time zero and this was higher than that for uninoculated soils. These findings agree with studies (Hamdi et al., 2007; Wu et al., 2020) where biostimulation was complemented with bioaugmentation for biodegradation of aged PAHs in soils, and improved biodegradation was observed compared to other soil incubations. In Tyagi et al. (2011) and Brzeszcz et al. (2020), microbial inoculation was indicated to be a better bioremediation strategy compared to biostimulation.

The maximum rates of mineralisation stabilized for all soils treatment conditions as contact time increased, which could signify that the indigenous bacteria have adapted and begun phenanthrene catabolism. Shorter lag phases were observed for AD + WA at especially 60 d and 90 d showing that the amendment mixture provided better nutrients and aided faster PAHs microbial degradation by the inoculum with ageing. The addition of inoculum increases the effectiveness of the mineralisation of the bioaccessible PAH fraction, but in the presence of suitable indigenous bacteria, the bioaccessible fraction of PAHs will adequately be degraded. Earlier studies have demonstrated that the degradation of most PAHs is primarily constrained by their bioavailability rather than by microbial catabolic activity (Semple et al.,

2006; Allan et al., 2007). However, biostimulation has been known to provide suitable nutrients and/or conditions for soil microflora as well as PAH-degrading inoculants (Tyagi et al., 2011). In addition, biostimulation has been proposed as a suitable strategy for the removal of PAHs in aged or weathered soil (Haleyur et al., 2019).

The extent of mineralisation decreased with an increase in contact time. This wasdue to reduced bioaccessibility of 9-^{14}C-phenanthrene through ageing and reduced total activity as incubation time progressed. The length of the soil-PAH contact time has been known to negatively impact the bioavailability of PAHs (Riding et al., 2013; Umeh et al., 2017), therefore potentially reducing possible microbial degradation. In this present study, the 9-^{14}C-phenanthrene extractability and mineralisation decreased over time confirming the influence of contact time. There was no significant difference in the extents of mineralisation in inoculated and uninoculated respirometric assay. This implies that soil indigenous bacteria possess the ability to degrade PAHs (Couling et al., 2010, Okere et al., 2017; Picariello et al., 2020; Roszak et al, 2021), therefore artificial inoculation may not be necessary. The loss of 9-^{14}C-phenanthrene in this study aligns with documented studies, where biodegradation was indicated as a significant process of PAH removal from the soil, under favourable soil conditions (Papadopoulos et al., 2007; Lang et al., 2016; Siles & Margesin, 2018). In this study, lower levels of $^{14}CO_2$ (%) were produced from the mineralisation of the 9-^{14}C-phenanthrene in uninoculated mineralisations from 60 d. This implies that some relevant indigenous PAH degraders were present in the soils but were less abundant at this point (Li et al., 2009). This also shows the advantage of inoculation as the adapted inoculum and high bacteria density

could be the reason behind the slight difference. However, this difference is not significant but it's important because any reduction in contaminant concentration is beneficial.

4.3 Correlation of HP-β-CD extractability and 9-^{14}C-phenanthrene mineralisation in soil

The HP-β-CD extractability of 9-^{14}C-phenanthrene was compared with the extent of its biodegradability, both in the presence and absence of AD and/or WA. A close 1:1 relationship is expected between the HP-β-CD extractable and mineralized 9-^{14}C-phenanthrene fractions (Reid et al., 2000). However, in this present study, most of the HP-β-CD extractable 9-^{14}C-phenanthrene fractions were relatively lower than the mineralized fractions after day zero. This could be due to physical and chemical constraints. The variation has been connected to a slower desorption rate in earlier articles (Reid et al., 2000; Rhodes et al., 2008). Conversely, there are suggestions that HP-β-CD extraction may not be highly effective in predicting PAHs bioaccessibility under all conditions. Zhang et al. (2016) recommended the use of HP-β-CD in combination with components of bacteria exopolymeric substance, to be more effective than a single use of HP-β-CD. Similarly, Qin et al. (2021) reported a combination of magnetic poly(β-cyclodextrin) microparticles (Fe3O4@PCD) and HP-β-CD as a more rapid and effective extraction solvent for predicting PAHs bioaccessibility.

However, the HP-β-CD extractable 9-^{14}C-phenanthrene fractions positively correlated with the mineralized 9-^{14}C-phenanthrene, at each time point in the inoculated and uninoculated mineralisation. The positive correlation and high R^2-values indicate that HP-β-CD extraction can predict the bioaccessible concentration of PAHs in soil. Cao et al. (2022) reported that HP-

β-CD extraction is effective in predicting PAHs bioaccessibility. A similar good linear correlation was reported by Ogbonnaya et al. (2016) using ^{14}C-naphthalene. Also, the results agree with past studies where HP-β-CD extractable and mineralizable fractions of PAHs were close to a 1:1 relationship (Reid et al., 2000; Papadopoulos et al., 2007; Rhodes et al., 2008). Similarly, the findings of this study align with past studies, where HP-β-CD-extractable fractions of ^{14}C-phenanthrene were relatively lower than mineralized fractions, due to the slower desorption rates of the PAH from the soils (Rhodes et al., 2012; Ogbonnaya et al., 2016).

5. Conclusion

The ameliorating effect of WA on soil conditions and stimulating effect of AD on soil microbial activity enhanced the biodegradation of 9-^{14}C-phenanthrene in soils. The reduction in the total activity of the 9-^{14}C-phenanthrene was enhanced from the onset of the soil-PAH contact time in the presence of AD and/or WA compared to similar soils without the amendments. However, a higher reduction in total activity was observed in soil amended with AD + WA because of a better-improved rate of biodegradation. Lower lag phases and higher maximum rates of mineralisation were observed in inoculated mineralisation due to the enhanced mineralisation by the phenanthrene-adapted inoculum. The extractability and extent of mineralisation reduced as the soil-PAH interactions increased over time because of ageing. The extent of mineralisation was higher in inoculated respirometry but was similar to the results in uninoculated respirometry. The findings of this study showed that the sustainability of the optimal growth and activity of relevant PAH-degrading microorganisms, with adequate supplies of rate-limiting organic nutrients, can promote microbial accessibility and/or

degradation of PAHs. This study gives insight into the influence of ageing and organic amendment on PAHs' bioaccessibility and biodegradation. Overall, concerning the benefits of bioaugmentation, biostimulation is still an effective strategy for improving the degradation of hydrophobic organic contaminants in the soil.

6. Declaration of competing interest

The authors declare that there are no competing personal or financial relationships that could have influenced this work.

7. CRediT author statement

Adesola S. Ojo: Conceptualisation, Methodology, Visualization, Investigation, Formal analysis, Writing - Original draft **Carly J. Stevens:** Conceptualisation, Methodology, Validation, Supervision **Chisom Ejileugha:** Conceptualization, Methodology, Writing – Review & Editing **Kirk T. Semple:** Conceptualisation, Methodology, Validation, Resources, Supervision, Funding Acquisition, Writing – Review & Editing

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Catabolism of ^{14}C-phenanthrene by indigenous microbes in soil amended with anaerobic digestate and wood ash

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment. They are of concern due to their low biodegradability, environmental persistence, and inherent toxic effects on humans. However, PAHs may be degraded in soil under appropriate environmental conditions. This study investigated the impact of nutrient-rich anaerobic digestate (AD) and wood-ash (WA) on indigenous catabolism of 9-^{14}C-phenanthrene in soil over a 90-day incubation under different amendment conditions (control, soil + AD, soil + WA, and soil + AD + WA). Higher amounts of $^{14}CO_2$ (%) were produced from the mineralisation of the 9-^{14}C-phenanthrene in the presence of AD and/or WA, especially when lower amounts of AD and/or WA were amended to the soil. The mineralisation kinetics were positively influenced as shorter lag times, as well as faster rates and higher extents of mineralisation, were observed in amended soils than in similar unamended soils. Lower AD, WA, and AD + WA amendment doses had significantly ($P < 0.05$) shorter lag times and higher extents of mineralisation when compared to higher dose. This study revealed the positive influence of AD and/or WA soil amendments on stimulating indigenous microbial activity and enhancing PAH biodegradation in soil.

Keywords: digestate, mineralisation, organic amendments, polycyclic aromatic hydrocarbons, soil microflora, wood ash

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of hydrophobic organic contaminants, which are found in the environment mostly because of anthropogenic industrial processes (Macleod and Semple, 2002; Okere and Semple 2012; Lang et al., 2016; Lukić et al., 2016). The physicochemical properties of PAHs result in reduced biodegradability and increased persistence as their molecular size increases (Riding et al., 2013; Naseri et al., 2014; Yu et al., 2018). Alongside their persistent nature, PAHs exhibit toxicity and possess genotoxic and carcinogenic potential (Macleod and Semple, 2002; Lukić et al., 2016; Baldwin et al., 2020). The presence of PAHs in soil is an indication of environmental pollution, and a long-term soil exposure promotes ageing which fosters sequestration to soil matrices thereby reducing their susceptibility to degradation (Stokes et al., 2006; Riding et al., 2013; Umeh et al., 2017). However, microbial degradation can be employed to remediate PAH-contaminated soils to reduce the risk to human health (Maletić et al., 2013; Naseri et al., 2014).

Organic contaminants in soil rapidly deplete the available pools of major inorganic nutrients in the soil (especially nitrogen (N) and phosphorus (P)) and enrich its carbon (C) content (Margesin and Schinner, 2001; Stroud et al., 2007). The addition of appropriate growth-limiting mineral nutrients can restore the soil's nutrient balance and enhance its indigenous microbial activity (Naseri et al., 2014; Ou et al., 2024). Soil decontamination following stimulation of native microbiota with N, P, and potassium (K) based mineral fertilizers has been documented (Margesin and Schinner, 2001; Aghalibe et al., 2020; Udume et al., 2023). However, recurrent soil amendment with inorganic nutrients can encourage soil acidification and accumulation of heavy metal contents as well as soil layer compaction (Savci, 2012; Xu et

al., 2014; Massah and Azadegan, 2016), which can adversely affect the soil quality and microbial activity. Currently, a wide variety of low-value organic residues from bioenergy processes are applied to soil as organic fertilizers due to their recyclable and readily available micro- and macro-nutrients (Odlare et al., 2011; Colatorti et al., 2024; Mora-Salguero et al., 2025). The growing industrialization and increased recycling of organic wastes, as renewable energy sources, have led to a huge generation of organic residues with management and disposal challenges (Odlare et al., 2011; Quakernack et al., 2012). However, their addition to soil, as organic fertilizers, has been considered a suitable and sustainable alternative to chemical fertilization (Alburquerque et al., 2012a,b; Fernández-Delgado Juárez et al., 2013; Nabeela et al., 2015; Gómez-Brandón et al., 2016).

Previous studies have shown the impact of organic amendments in ameliorating soil characteristics (Medina et al., 2006; Pitman 2006; Blonska et al., 2023; Holatko et al., 2023), optimizing soil nutrients (Margesin and Schinner, 2001; Maletić et al., 2013; Glowacka et al., 2020), and stimulating soil microbial growth and activities (Haritash and Kaushik, 2009; Bougnom et al., 2012; De Corato et al., 2023). Therefore, due to the proven positive effects of organic amendments on soil microbial activity (Odlare et al., 2011; Bang-Andreasen et al., 2020; Zhao et al., 2022), they can equally enhance microbial degradation of PAHs. However, the process may require understanding and manipulating soil environmental conditions (such as pH, oxygen level, moisture content, and available nutrients) (Pontes et al., 2013) due to their impacts on microbial community composition and activity (Rousk et al., 2009; Garbini et al., 2022; De Corato et al., 2023). The level of soil's essential or growth-limiting nutrients in

terms of the C:N:P ratio is significant, due to their influence on microbial growth, activity, and hydrocarbon degradation (Stroud et al., 2007; Zam and Mustafa, 2012).

Anaerobic digestate (AD), a slurried bioenergy organic residue (Köster et al., 2014), and wood-ash (WA), a biomass combustion inorganic residue have gained more attention as soil conditioners or renewable fertilizers (Fernández-Delgado Juárez et al., 2013; García-Sánchez et al., 2015a). AD is known to be rich in organic matter (due to its residual C) and nutrients (Whelan et al., 2010; Möller and Müller, 2012; Insam et al., 2015), especially N and P (Garcia-Lopez et al., 2023), which are essential for microbial growth and activity (Alburquerque et al., 2012a; Köster et al., 2014; García-Sánchez et al., 2015a; Tiwary et al., 2015). However, the amount of AD to be added to soil should match the soil's N deficiency (Fernández-Delgado Juarez et al., 2013; Gómez-Brandón et al., 2016) to optimise its benefits as well as mitigate soil acidification, nitrate leaching, and emission of nitrous oxide (Insam et al., 2015; Monlau et al., 2016; Tampio et al., 2016; Nicholson et al., 2017). Also, due to the high alkalinity of AD, there is a propensity of N loss as volatilized ammonia (NH_3-N) within a short time of AD addition to soil which can impact microbial activity (Alburquerque et al., 2012a; García-Sánchez et al., 2015a). These facts make AD amendment dose, technique, and period essential factors of utmost consideration.

Similarly, WA contains a significant amount of macro-nutrients (calcium, magnesium, sodium, and potassium) as well as micro-nutrients (zinc, copper, lead, nickel, and arsenic) (Fernández-Delgado Juarez et al., 2013; García-Sánchez et al., 2015a; Maschowski et al., 2016; Ivezic et

al., 2021). Several studies have demonstrated that WA can be employed to correct soil acidity to a desired soil pH (Fernández-Delgado Juarez et al., 2013; García-Sánchez et al., 2015a), ameliorate soil physical, chemical, and biological properties (Ivezic et al., 2021; Blonska et al., 2023; de Oliveira et al., 2023), and immobilize heavy metals in soil (García-Sánchez et al., 2015b). However, the high pH (8 – 13) of WA coupled with its negligible amount or complete absence of N and C have limited its use for soil amendment (Demeyer et al., 2001; Fernández-Delgado Juarez et al., 2013; Köster et al., 2014; García-Sánchez et al., 2015a,b). This is due to the impact of pH, N, and C on soil microbial population, community composition, and activity (Fernández-Delgado Juarez et al., 2013; García-Sánchez et al., 2015a; Cruz-Paredes et al., 2021). The effects of WA are influenced by factors such as the amendment dose and soil type (Fernández-Delgado Juarez et al., 2013). However, excessive application of WA can disrupt the soil's physicochemical and biological properties (Perucci et al., 2006; Perucci et al., 2008; Insam et al., 2009; An & Park, 2021). A suitable amendment amount for WA has remained a challenge in its application and has been identified as a major constraint to its use as a soil bio-fertilizer (Ferreiro et al., 2011; Fernández-Delgado Juárez et al., 2013).

Previous studies have shown that amendment of soil with AD or WA can positively influence the soil physicochemical and biological properties (Demeyer et al., 2001; Insam et al., 2009; García-Sánchez et al., 2015b; Zusevica et al., 2023). However, due to the complementary nutrients (especially N, P, and K) of AD and WA, their addition to soil as a mixture can produce a potentially valuable amendment with more positive impacts on soil health and fertility (Bougnom et al., 2012). Therefore, this study investigated the biodegradation of PAHs by soil autochthonous microbes as influenced by AD and WA amendment. The biodegradation of the

9-^{14}C-phenanthrene (a model PAH) by soil indigenous microbes was quantified by measuring its mineralisation kinetics (lag time, rate of mineralisation, and extent of mineralisation) at increasing soil-PAH contact times.

2. Materials and Methods

2.1 Bioenergy residues and chemicals

Pasteurized AD was obtained as a slurry-like material from an anaerobic digestion plant in the United Kingdom (UK). It was produced from anaerobically digested household food wastes. After collection, it was stored in a dark room at 4^{o}C. The WA (fly-ash) was collected from a biomass power plant in the UK, using timber and bark as feedstocks. Fly-ash was preferred due to its higher concentrations of elements (Sharma and Kalra 2006) and low alkalinity (Noyce et al., 2016) compared to the bottom ash. The AD and WA characteristics are shown in Table 1. Chemicals used in this study include non-radiolabelled phenanthrene (>96 %, HPLC grade) and 9-^{14}C-labelled phenanthrene (55.7 mCi mmol^{-1}; >99 % purity); both were supplied by Sigma–Aldrich, UK. Gold Star multipurpose liquid scintillation cocktail and acetone (>98 %) were supplied by Meridian, UK.

Table 6: Characteristics of soil, AD, and WA; measurements were in dry weight (dw) except those marked (*) which were in wet weight (wet wt); <BDL = Below Detection Limit; values are the mean of n = 3 ± SEM.

Samples	pH	EC ($\mu S\ cm^{-1}$)	Total organic matter (%)	Total N ($mg\ kg^{-1}$)	Total C ($mg\ kg^{-1}$)	Total P ($mg\ kg^{-1}$)	PO_4-P ($mg\ kg^{-1}$)	NH_4^+-N ($mg\ kg^{-1}$)	NO_3-N ($mg\ kg^{-1}$)
Soil	6.5 ± 0.1*	36.0 ± 3.0*	5.7 ± 2.0	2.8 ± 0.1	19.0 ± 1.0	1.1 ± 0.08	0.07 ± 5.9*	0.0003 ± 0.2*	0.0014 ± 0.3*
AD	9.0 ± 0.0*	9.6 ± 0.2*	66.0 ± 2.0	43.5 ± 0.5	349.0 ± 1.0	12.9 ± 0.5	0.32 ± 0.0*	4.281 ± 0.1*	0.001 ± 0.0*
WA	12.7 ± 0.0	50.3 ± 0.5	1.0 ± 0.0	0.7 ± 0.1	13.0 ± 0.1	25.2 ± 0.0	0.027 ± 0.0	<BDL	0.032 ± 0.0

2.2 Soil sampling and bulk characterization

The soil used for the experiment was collected from a pasture field at Myerscough College, Lancashire, UK. The soil is known to be a Dystric Cambisol soil with a clayey-loam texture. The soil is rich in organic matter and Couling et al. (2010) have reported the percentage composition of the soil particles in an earlier article. Other physicochemical properties of the soil are presented in Table 1. The soil has no known history of exposure to anthropogenic petroleum hydrocarbons. After collection, the soil was sieved using a ≤2 mm sieve for the removal of plant debris and stones, as well as for homogeneity before storage at 4^{o}C in the dark. The soil's moisture content (32.1%) was determined by oven drying at 105^{o}C for 24 h (Rousk et al., 2009).

2.3 Soil spiking with 9-^{12}C-phenanthrene and amendment using AD and/or WA

Soil (3.09 kg; wet wt) was spiked with ^{12}C-phenanthrene (100 mg kg^{-1}), homogenized, and vented in a fume cupboard for 2 h to evaporate carrier solvent. Control soil (441 g; wet wt) was weighed out from the spiked soil for $^{12/14}$C blanks. Spiked soil (882 g, wet wt) was weighed out each amendment condition (AD and/or WA) and divided into four parts (220.5 g; wet wt) for each amendment amounts. The different amendment conditions were prepared as follows: soil + AD + WA; soil + AD; soil + WA. The amendment amount was varied by increasing the amendment as shown in Table 2. The mass of AD and WA used as soil amendments in this study were derived from the British agricultural practice recommendation of N-to-P (3:1) reference dose for soil amendment (AHDB, 2017) and wheat plantation (DEFRA, 2017). All amended soils were

incubated at 20 ± 2°C and sampled at defined intervals of 1 d, 15 d, 30 d, 60 d, and 90 d for respirometric assay.

Table 2: Four proportionately increasing amounts of AD and WA added to soils

Amendment Conditions	AD (g)	WA (g)	AD + WA (g)
1	0.17	0.009	AD1 + WA1
2	1.73	0.09	AD2 + WA2
3	17.3	0.94	AD3 + WA3
4	173.1	9.4	AD4 + WA4

2.4 Respirometric measurement of the 9-^{14}C-phenanthrene mineralisation in the amended soil

The mineralisation of 9-^{14}C-phenanthrene was measured in the soil treatment conditions using a respirometric assay (Reid et al., 2001). At defined sampling intervals (1 d, 15 d, 30 d, 60 d, and 90 d), soil (14.7 g; wet wt) was sampled from each microcosm into a modified pre-cleaned 250 ml Schott bottle (Reid et al., 2001). Sterilized deionized water (30 ml) was added to the Schott bottles to form a slurry. Each respirometer was spiked with 9-^{14}C-phenanthrene (50 kBq kg^{-1} soil) and ^{12}C-phenanthrene (20.1 mg kg^{-1} soil). A glass vial (7 ml) containing fresh 1 M NaOH (2 ml) was suspended (attached to the lid) inside each respirometer. Control was also set up to monitor the background ^{14}C-activity. All the respirometers were incubated at 20 ± 2°C on a flat-bed shaker (100 rpm) (SANYO Gallenkamp) for 14 d. The $^{14}CO_2$ produced from the mineralisation of the ^{14}C-phenanthrene was trapped in the 1 M $NaOH_{(aq)}$ and sampled at 2, 4, 8, 12, 24 h, and henceforth

every 24 h for 14 d (336 h). The sampled $^{14}CO_2$ traps were quantified by LSC (Canberra Packard Tri-Carb 2250CA). Respirometric data were used to calculate the changes in the lag times as well as rates and extents of the mineralisation.

2.5 Statistical analysis

Blank-corrected data were plotted with SigmaPlot 10.0. The effects of soil amendments with the AD, WA, and AD + WA on the mineralisation of 9-^{14}C-phenanthrene were analyzed using a one-way analysis of variance (ANOVA). Turkey and LSD's post-hoc tests were performed to compare the means within and across the different amended soils. Data were analyzed using SPSS (IBM SPSS version 27).

3. Results.

3.1. Mineralisation of 9-^{14}**C-phenanthrene in AD-amended soils**

The mineralisation of ^{14}C-phenanthrene in soils amended with increasing amounts of AD (0.170 g, 1.730 g, 17.31 g, and 173.1 g) was monitored (Figure 1 and Table 3). The lag times were significantly ($p < 0.05$) shorter in 0.170 g, 1.730 g, and 17.31 g amended soil with the increase in contact time compared to the control (Table 3). The lag times in 173.3 g amended soil were significantly ($p < 0.05$) longer than in the control. At the onset of the soil incubation (1 d), the addition of 173.1 g of AD resulted in a longer significant ($p < 0.05$) lag time (149.8 ± 8.2 h), which became significantly shorter as the soil-PAH contact time increased ($p < 0.05$). The 173.1 g AD amendment also had a significantly longer lag time ($p < 0.05$) from 1 d to 60 d (Table 3). The rate of mineralization in AD-amended soils including control was significantly higher ($p < 0.05$) than

the 173.1 g AD amendment. The addition of 0.17 g and 1.73 g of AD resulted in significantly higher extents of mineralisation ($p < 0.05$) compared to the higher amendments doses (17.3 g and 173.1 g). Lower extents of mineralisation were recorded in soil amended with 173.1 g of AD at every time point compared to other amendment doses (Figure 1 and Table 3). The extents of mineralisation in 173.1 g amended soil were lower ($p < 0.05$) than the control (Figure 1 and Table 3).

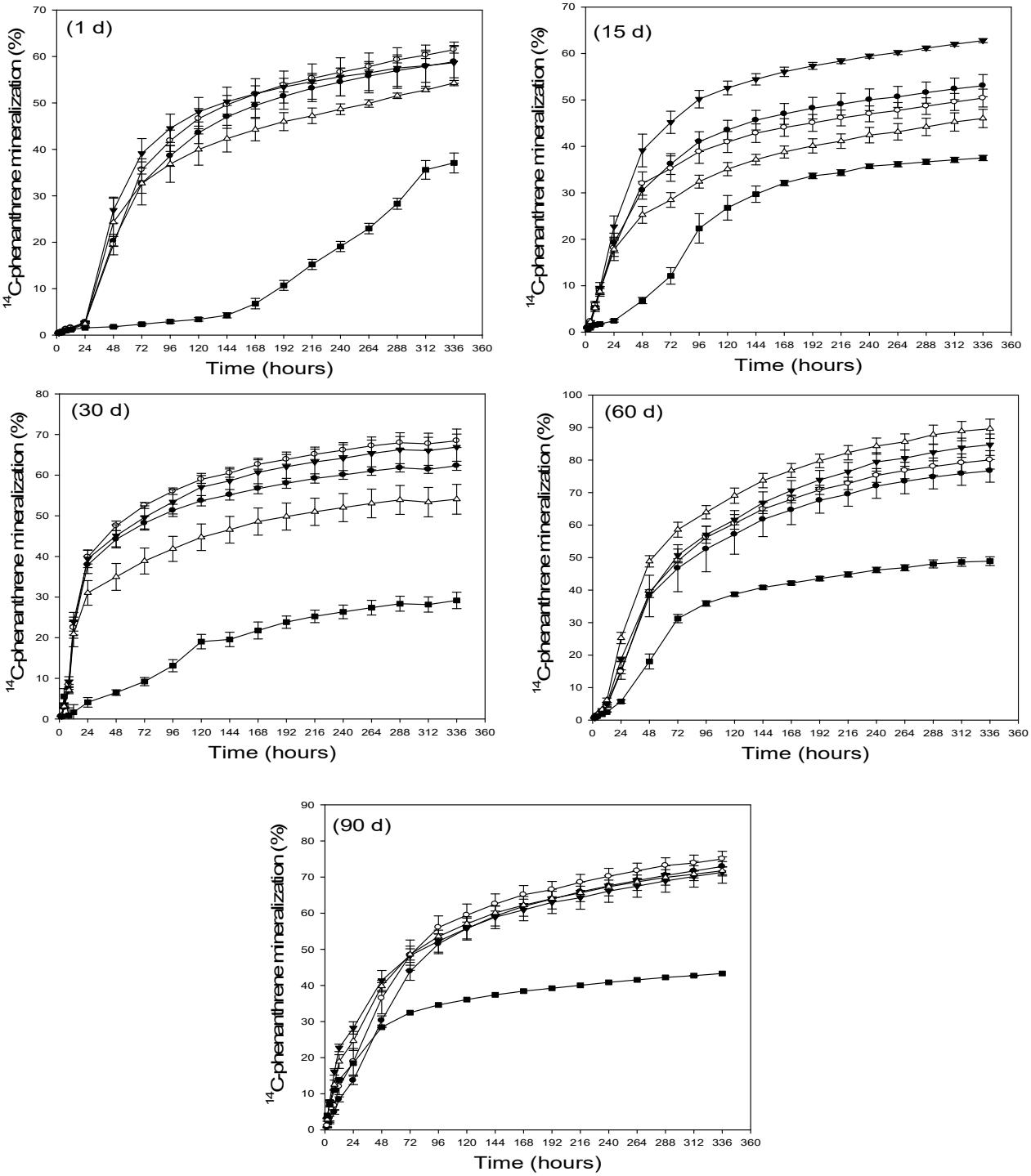

Figure 1: The extents of mineralisation in unamended soils (controls) (●); soils amended with proportionately increasing amounts of AD: 0.170 g (○); 1.730 g (▼); 17.310 g (△); 173.100 g (■) at increasing soil-PAH contact time; values are the means of n = 3 ± SEM

Table 3: Lag times, maximum rates, and extents of mineralisation in AD-amended soils. Values in columns followed by different letters are statistically different (Turkey, LSD; n = 3; P < 0.05): table also shows shorter lag times (†) and higher mineralisation (*) that were not statistically significant (p > 0.05) compared to controls; values are the mean of n = 3 ± SEM

Soil-PAH contact time (d)	Soil + AD (g)	Lag time (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1	Control	27.0 ± 0.4 A	1.4 ± 0.6 A	58.9 ± 3.5 A
	0.170	27.4 ± 0.5 A	0.8 ± 0.0 A	61.5 ± 0.7 B
	1.730	26.4 ± 0.4 A†	1.0 ± 0.1 A	58.7 ± 4.4 A
	17.31	27.4 ± 1.5 A	0.9 ± 0.2 A	54.3 ± 0.6 A
	173.1	149.8 ± 8.2 B	0.3 ± 0.0 B	37.1 ± 2.1 C
15	Control	7.7 ± 0.2 A	1.1 ± 0.1 A	53.1 ± 2.4 A
	0.170	7.1 ± 0.6 A†	1.2 ± 0.1 A*	50.4 ± 1.9 A
	1.730	8.0 ± 1.2 A	1.3 ± 0.1 A*	62.8 ± 0.4 B
	17.31	7.8 ± 0.45 A	1.3 ± 0.1 A*	46.0 ± 2.0 A
	173.1	36.8 ± 2.4 B	0.6 ± 0.1 B	37.5 ± 0.6 C
30	Control	5.2 ± 0.1 A	3.7 ± 0.3 A	62.3 ± 1.1 A
	0.170	5.0 ± 0.2 A	3.5 ± 0.4 A	68.5 ± 1.7 A*
	1.730	4.7 ± 0.2 A†	3.7 ± 0.2 A	66.9 ± 4.5 A*
	17.31	5.2 ± 0.2 A	3.4 ± 0.6 A	54.1 ± 3.7 A
	173.1	10.6 ± 7.3 A	2.5 ± 1.0 A	29.2 ± 2.0 A
60	Control	12.3 ± 1.0 A	1.2 ± 0.2 A	76.7 ± 3.5 A
	0.170	12.6 ± 0.5 A	1.1 ± 0.0 A	80.0 ± 2.8 A*
	1.730	11.7 ± 0.6 A†	1.2 ± 0.0 A	84.7 ± 3.3 A*
	17.31	10.7 ± 0.7 A†	1.7 ± 0.1 B	89.6 ± 3.0 B
	173.1	21.3 ± 1.6 B	0.7 ± 0.0 C	48.9 ± 1.3 C
90	Control	7.9 ± 0.6 A	0.9 ± 0.0 A	72.9 ± 2.6 A
	0.170	6.1 ± 1.1 A†	1.3 ± 0.3 A*	75.0 ± 2.1 A*
	1.730	2.7 ± 0.0 B	2.4 ± 0.1 B	71.3 ± 3.0 A
	17.31	2.9 ± 0.3 B	2.2 ± 0.2 B	71.7 ± 1.1 A
	173.1	2.4 ± 0.1 B	2.1 ± 0.0 B	43.3 ± 0.0 B

3.2 Mineralisation of 9-^{14}C-phenanthrene in soils amended with WA

The effects of additions of increasing amounts of WA (0.009 g, 0.090 g, 0.940 g, and 9.400 g) on the mineralisation of ^{14}C-phenanthrene in soils were monitored (Figure 2 and Table 4). The lag times at 1 d were significantly longer ($p < 0.05$) than for the other contact times (Table 4). The soil amended with 9.4 g of WA showed significantly longer lag times ($p < 0.05$) than other WA amended soils. The lag times in 9.4 g amended soil were significantly ($p < 0.05$) longer than in the control. The lower amendment amounts (0.009 g, 0.090 g, and 0.940 g) showed higher mineralisation rates than in the 9.4 g amended soil. The extents of mineralisation were higher ($p < 0.05$) in the soil amended with lower WA amounts (0.009 g, 0.090 g, and 0.940 g) compared to 9.4 g amended soil (Figure 2 and Table 4). The extents of mineralisation in all the levels of WA amendment (except 9.4 g) were higher than in the control (Table 4). The extents of mineralisation in 9.4 g amended soil was significantly lower ($p < 0.05$) than in the control.

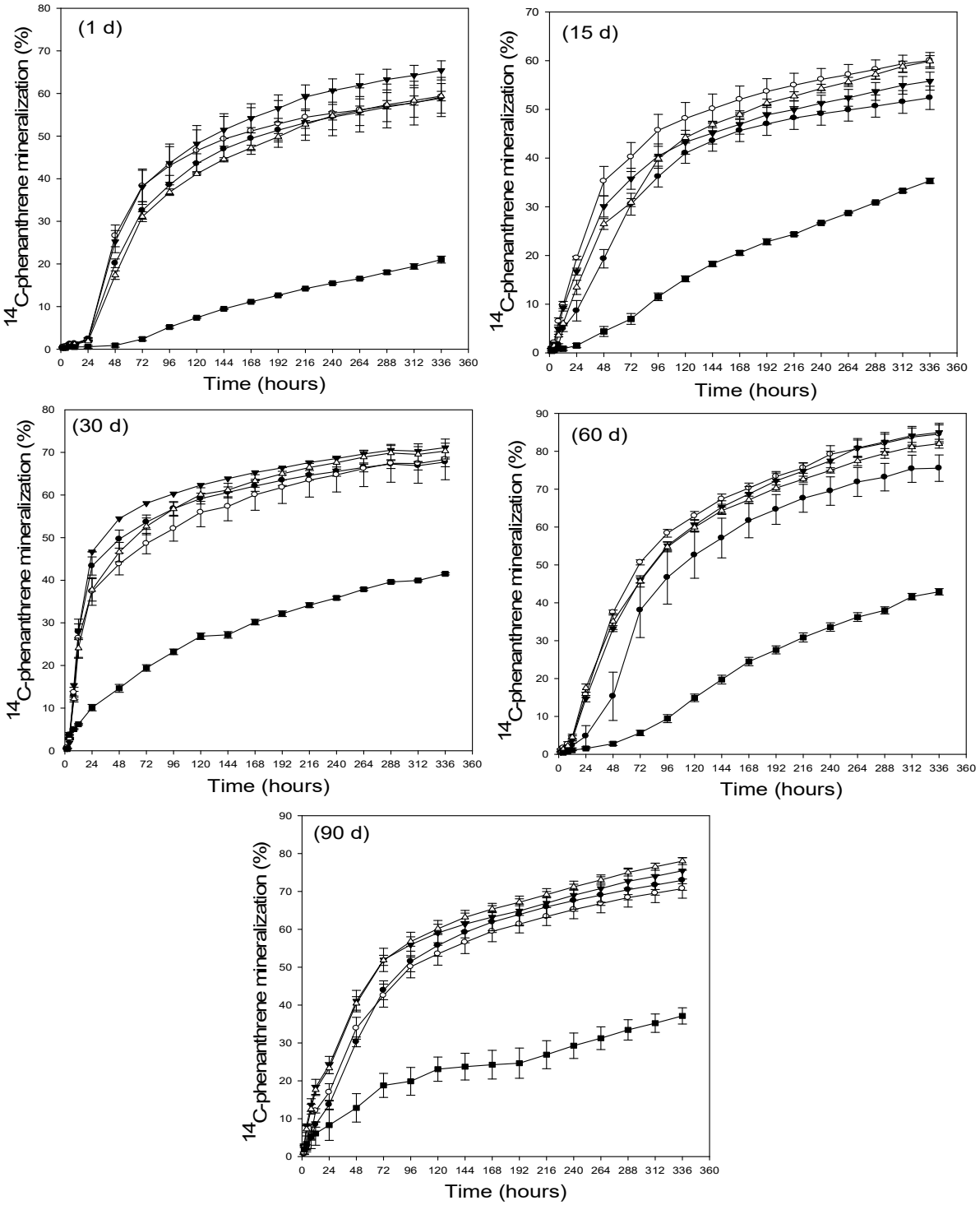

Figure 2: The extents of mineralisation in unamended soils (controls) (●); soils amended with proportionately increasing amounts of WA: 0.009 g (○); 0.090 g (▼); 0.940 g (△) and 9.400 g (■) at increasing soil-PAH contact time; values are the means of n = 3 ± SEM

Table 4: Lag times maximum rates and extents of mineralisation in WA amended soils. Values in columns followed by different letters are statistically different (Turkey, LSD; n = 3; $p < 0.05$): table also shows shorter lag times (†) and higher mineralisation (*) that were not statistically significant ($p > 0.05$) compared to controls; values are the mean of n = 3 ± SEM

Soil-PAH contact time (d)	Soil + WA (g)	Lag time (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1	Control	27.0 ± 0.4 A	0.7 ± 0.0 A	58.9 ± 3.5 A
	0.009	26.4 ± 0.5 A†	1.0 ± 0.1 A*	59.2 ± 4.6 A*
	0.090	26.5 ± 0.6 A†	0.1 ± 0.1 A	65.4 ± 2.3 A*
	0.940	28.3 ± 0.4 A	0.6 ± 0.0 A	59.4 ± 1.1 A*
	9.400	91.8 ± 2.6 B	0.2 ± 0.0 B	21.0 ± 0.8 B
15	Control	7.7 ± 0.2 A	1.0 ± 0.1 A	52.4 ± 2.4 A
	0.009	6.7 ± 0.6 A†	1.1 ± 0.1 A*	60.0 ± 1.6 B
	0.090	8.0 ± 0.4 A	1.1 ± 0.1 A*	55.8 ± 1.8 A*
	0.940	10.0 ± 0.2 A	0.7 ± 0.0 A	59.9 ± 1.2 A*
	9.400	52.7 ± 9.6 B	0.2 ± 0.0 B	35.3 ± 0.5 C
30	Control	4.9 ± 0.0 A	3.7 ± 0.3 A	71.5 ± 1.1 A
	0.009	4.5 ± 0.2 A†	3.4 ± 0.6 A	68.4 ± 4.8 A
	0.090	4.9 ± 0.0 A	3.4 ± 0.0 A	68.6 ± 0.0 A
	0.940	4.9 ± 0.0 A	3.0 ± 0.4 A	72.5 ± 1.9 A*
	9.400	7.8 ± 0.3 B	1.7 ± 0.1 B	71.1 ± 0.2 A
60	Control	12.3 ± 1.0 A	1.0 ± 0.2 A	75.5 ± 3.5 A
	0.009	12.1 ± 1.2 A†	1.0 ± 0.0 A	84.5 ± 2.4 B
	0.090	13.2 ± 0.2 A	0.9 ± 0.1 A	84.9 ± 2.4 C
	0.940	12.4 ± 0.4 A	1.1 ± 0.1 A	82.0 ± 1.2 A*
	9.400	41.3 ± 3.4 B	0.2 ± 0.0 B	42.9 ± 0.8 D
90	Control	7.9 ± 0.6 A	0.9 ± 0.0 A	72.9 ± 2.6 A
	0.009	7.1 ± 1.0 A†	1.6 ± 0.1 A*	70.7 ± 2.5 A
	0.090	2.7 ± 0.0 A†	2.7 ± 0.1 B	75.5 ± 3.4 A*
	0.940	2.9 ± 0.3 A†	2.2 ± 0.2 C	78.0 ± 0.9 A*
	9.400	13.1 ± 8.5 A	1.2 ± 0.5 A*	37.1 ± 2.1 B

3.3. Mineralisation of 9-^{14}C-phenanthrene in soils amended with AD + WA

The effects of additions of mixtures of AD and WA on the mineralisation of ^{14}C-phenanthrene in soils were monitored from 1 – 90 d (Figure 3 and Table 5). The addition of mixtures of 173.1 g of AD and 9.4 g of WA consistently showed significantly longer ($p < 0.05$) lag times compared to

other amendment conditions (Table 5). It also showed significantly ($p < 0.05$) longer lag times than the control. Shorter lag times were also observed in all the other AD + WA additions from 15 d to 90 d compared to 1 d, but they were not significant ($p > 0.05$) from the control. The maximum rates of ^{14}C-phenanthrene mineralisation were higher with the addition of mixtures of 17.31 g of AD and 0.94 g of WA, 0.17 g of AD and 0.009 g of WA, and 1.730 of AD and 0.090 of WA, but this difference was not significant ($p > 0.05$) when compared to 173.1 g of AD and 9.4 g of WA. The difference was not also significant ($p < 0.05$) when compared to the control. All AD + WA amendment conditions (except 173.1 g AD + 9.4 g WA) had higher extents of mineralisation than control. They also showed significantly ($p < 0.05$) higher extents of mineralisation than a mixture of 173.1 g of AD and 9.4 g of WA (Figure 3 and Table 5).

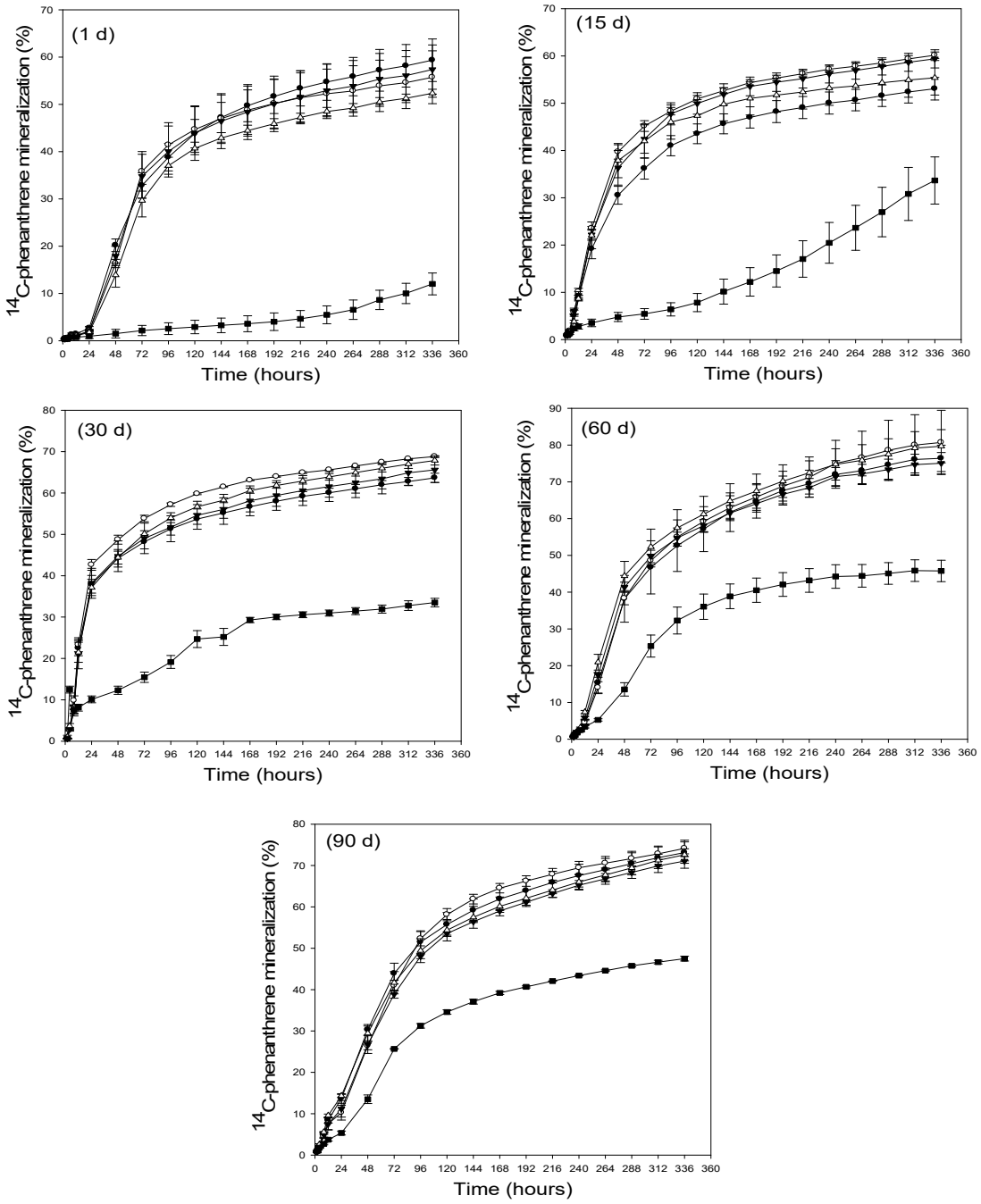

Figure 3: The extents of mineralisation of ^{14}C-phenanthrene in unamended soils (controls) (●); soils amended with AD + WA: 0.170+0.009 (○); 1.730+0.090 (▼); 17.310+0.940 (△); 173.100+9.400 (■) at increasing soil-PAH contact time; values are the means of n = 3 ± SEM.

Table 5: Lag times maximum rates and extents of mineralisation in AD + WA-amended soils. Values in columns followed by different letters are statistically different (Turkey, LSD; n = 3; $p < 0.05$): table also shows reduced lag times (†) and higher mineralisation (*) that were not statistically significant ($p > 0.05$); values are the mean (n = 3) ± SEM.

Soil-PAH contact time (d)	Soil + AD + WA (g)	Lag time (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1	Control	27.0 ± 0.4 A	0.7 ± 0.0 A	59.3 ± 4.5 A
	0.170 + 0.009	28.7 ± 0.2 A	0.8 ± 0.1 A*	55.7 ± 5.6 A
	1.730 + 0.090	28.4 ± 0.8 A	0.7 ± 0.1 A	57.4 ± 5.2 A
	17.31 + 0.940	30.5 ± 1.3 A	0.6 ± 0.1 A	52.3 ± 0.8 A
	173.1 + 9.400	208.0 ± 38.6 B	0.2 ± 0.0 B	12.0 ± 2.3 B
15	Control	7.7 ± 0.2 A	1.0 ± 0.1 A	53.1 ± 2.4 A
	0.170 + 0.009	7.1 ± 0.3 A†	1.2 ± 0.0 A*	60.2 ± 0.7 A*
	1.730 + 0.090	7.1 ± 0.6 A†	1.2 ± 0.0 A*	59.4 ± 1.9 A*
	17.31 + 0.940	8.9 ± 0.2 A	1.3 ± 0.1 B	55.4 ± 3.7 A*
	173.1 + 9.400	66.3 ± 36.4 B	0.5 ± 0.1 C	33.6 ± 5.0 B
30	Control	5.2 ± 0.1 A	3.3 ± 0.3 A	63.7 ± 1.1 A
	0.170 + 0.009	5.0 ± 0.1 A†	3.4 ± 0.1 A*	68.8 ± 0.2 A*
	1.730 + 0.090	5.2 ± 0.2 A	3.4 ± 0.6 A*	65.6 ± 3.0 A*
	17.31 + 0.940	4.7 ± 0.3 A†	3.3 ± 0.6 A	67.9 ± 1.1 A*
	173.1 + 9.400	2.7 ± 0.1 B	5.9 ± 0.4 B	33.5 ± 1.0 B
60	Control	12.3 ± 1.0 A	1.0 ± 0.2 A	76.4 ± 3.6 A
	0.170 + 0.009	12.6 ± 0.8 A	1.0 ± 0.0 A	80.7 ± 8.7 A*
	1.730 + 0.090	11.0 ± 0.5 A†	1.1 ± 0.0 A*	75.0 ± 2.9 A
	17.31 + 0.940	9.7 ± 0.2 A†	1.1 ± 0.1 A*	79.7 ± 4.5 A*
	173.1 + 9.400	21.7 ± 1.6 B	0.5 ± 0.0 A	45.8 ± 2.9 B
90	Control	7.9 ± 0.6 A	0.9 ± 0.0 A	73.1 ± 2.6 A
	0.170 + 0.009	8.9 ± 1.0 A	0.9 ± 0.1 A	74.1 ± 2.0 A*
	1.730 + 0.090	8.4 ± 1.5 A	0.9 ± 0.1 A	71.1 ± 1.7 A
	17.31 + 0.940	6.9 ± 0.1 A†	1.0 ± 0.1 A*	72.6 ± 1.5 A
	173.1 + 9.400	19.2 ± 2.4 B	0.6 ± 0.0 A	47.5 ± 0.6 B

4. Discussion

4.1 Impact of AD amendment on 9-^{14}C-phenanthrene mineralisation in soil.

The addition of proportionately increasing amounts of AD to soil showed shorter lag times, as well as increased rates and extents of mineralisation of ^{14}C-phenanthrene than unamended soils as the soil-PAH contact time increased. The enhanced mineralisation kinetics were indicative of the potential of AD in stimulating the indigenous microbial community in the soil. This promoted successful biodegradation of the ^{14}C-phenanthrene by the indigenous phenanthrene-degrading microbial populations (Gielnik et al., 2019). Digestate improved soil microbial population, nutrient content, and ^{14}C-phenanthrene mineralization (Ibeto et al., 2020). These observations imply that the indigenous microbes can adapt to the addition of the AD or utilize the nutrients in the AD for proliferation. However, in this study, the stimulating effect of AD was consistent in the soils with lower amounts of AD compared to the soils with a higher amount of AD. The lag times were longer at the onset of incubations, with a higher effect in the soils with a higher amount of AD, showing the probable adverse effect of a large amount of AD on soil biological activity.

According to Pranckietiene et al. (2023) soil amendment with digestate should be based on soil agrochemical properties and plant needs especially N needs. This recommendation should also be employed in contaminated soils based on microbial needs to achieve enhanced contaminant mineralisation. However, as the soil-PAH contact time increased, the lag time in the soils with a higher amount of AD became shorter. This depicts improved biological activity as the interactions of the soil and AD nutrients increased. The delayed effect at the incubation onset suggests that the microorganisms were adapting to the phenanthrene and AD, and/or withstanding any

possible unfavorable effect(s), as the soil has no previous history of PAH contamination. This delayed effect aligns with previous findings that spiking soil with phenanthrene reduces soil nutrients and microbial populations (Ibeto et al., 2020). It also supports the notion that a specific period is necessary for the activation of microbial catabolic functions in indigenous PAH-degrading bacteria (Semple et al., 2001). Meanwhile, evidence in the literature highlights the benefits of AD as a soil amendment. AD has been shown to enhance soil physicochemical properties and plant yield (Glowacka et al., 2020), restore and improve soil fertility (Badagliacca et al., 2020), and boost plant biomass yield and nutritional value (Holatko et al., 2023). These findings suggest that AD is an effective amendment for promoting soil health and fertility.

The effect of the addition of AD was observed on the rates and extents of mineralisation as the soil-PAH contact time increased. The addition of AD (0.17 g, 1.73 g, and 17.31 g) showed higher rates of mineralisation. The addition of AD increased the extents of mineralisation when compared to unamended soil except for higher amount of AD (173.1 g) which had the lowest extents of mineralisation. An increase in phenanthrene mineralisation indicates enhanced microbial activity, as microbial catabolism drives higher levels of mineralisation. Therefore, amendments that boost soil microbial activity can effectively promote PAH mineralisation. AD demonstrates this potential, as its addition to soil has been shown to significantly enhance microbial activity, including biomass and physiological diversity, both in the short and long term (García-Sánchez et al., 2015a). The application of sewage sludge digestate to petroleum hydrocarbon-contaminated soil enhanced microbial gene concentrations and achieved 74% degradation of petroleum hydrocarbons within two months (Gielnik et al., 2021). Anaerobic

digestate (AD) has been shown to influence microbial community structure, abundance, and characteristics in soil (Garbini et al., 2022; De Corato et al., 2023). While digestate generally increases microbial abundance (Garbini et al., 2022), its effect on soil microbial activity varies depending on soil properties and the type of digestate feedstock used (Doyeni et al., 2021).

Additionally, the prolonged lag times and reduced extents of mineralisation in soils amended with higher doses of AD may result from the excess carbon readily available to indigenous soil microbes compared to phenanthrene. This abundance of carbon can shift microbial preference toward more accessible sources, thereby hindering the degradation of the target contaminant. Previous studies have shown that AD provides significant amounts of macro- and micronutrients, which are easily utilized by soil microorganisms (Alburquerque et al., 2012a; Fernández-Delgado Juárez et al., 2013; Koszel and Lorencowicz, 2015). AD has been reported to supply essential nutrients such as N, P, K, Ca, and Mg to amended soils (Garcia-Lopez et al., 2023). Higher AD doses have been found to negatively impact microbial enzyme activity, microbial biomass, soil carbon, and phosphorus cycling (Garcia-Lopez et al., 2023), which may also explain the reduced mineralisation observed at higher AD amendments (173.1 g) in this study. However, Supronienе et al. (2022) noted that AD amendment might not significantly alter the diversity of the soil prokaryotic community, suggesting that other factors and soil properties should be considered in these scenarios.

4.2 Impact of WA amendment on 9-^{14}C-phenanthrene mineralisation in soil

The addition of WA to soil affected the lag times as well as the rates and extents of mineralisation with increasing soil-PAH contact time. The changes in lag times as the soil-PAH contact time progressed did not follow a consistent fixed pattern. However, as the soil-PAH contact time increased, the lag times became shorter in the soils amended with lower amounts of WA. WA addition to soil has been indicated to be maximally beneficial at lower amounts, and possibly toxic at higher amounts (Pitman, 2006). Soil amended with a higher amount of WA (9.4 g) had longer lag times and lower extents of mineralisation. Longer lag times (observed mainly under 9.4 g WA amendment) were observed to be associated with lower extents of mineralisation. Higher doses of WA added to PAH-contaminated soil may hinder mineralisation by affecting soil health and fertility. Earlier studies have shown that WA indirectly impacts soil microbial processes by altering soil pH and other physicochemical properties (Bougnom and Insam, 2009; García-Sánchez et al., 2015a). Low-dose WA additions are considered safe for soil microbiota (Cruz-Paredes et al., 2021). Bacteria, which play a crucial role in PAH mineralisation, are particularly sensitive to WA-induced pH changes, often experiencing reduced activity with increased pH. In contrast, fungi appear to be affected only at higher WA doses (Cruz-Paredes et al., 2021). This microbial sensitivity to pH shifts and higher WA dose may explain the diminished mineralisation observed with higher WA amendments. Perucci et al. (2006) had earlier reported that the addition of a lower amount of WA (5 t ha^{-1}) increased soil microbial activity, while the addition of a higher amount of WA (20 t ha^{-1}) resulted in reduced microbial activity after four (4) months of treatment. Lower doses of WA is beneficial and sustainable as soil amendment for improving soil properties but higher dose appear to cause negative effect (An & Park, 2021). Neimane et al.

(2021) observed no benefit from a high WA dose and recommended its addition at low doses to achieve the desired result and eliminate environmental concerns.

The increased mineralisation observed with the addition of WA suggests enhanced microbial activity. This is supported by García-Sánchez et al. (2015b), who reported a rise in soil microbial biomass following WA application. Similarly, Campos et al. (2018) observed positive microbial responses, including increased dehydrogenase activity and soil oxygen consumption, in soils amended with up to 20% WA. Fernández-Delgado Juárez et al. (2013) also documented improved microbial activity, as indicated by higher basal respiration and microbial biomass carbon, in WA-treated soils. Additionally, studies by Rocha et al. (2023) and de Oliveira et al. (2023) highlighted the benefits of WA in enhancing soil health and fertility, which translated into improved agronomic performance of plants. WA also boosts bacterial abundance by enhancing soil physicochemical properties (Bang-Andreasen et al., 2020). However, in a study by García-Sánchez et al. (2015a), there were no significant effects observed on the microbial activity in WA-amended soils. Similarly, Bougnom et al. (2012) and Noyce et al. (2016) reported lack of significant effects on microbial processes in soils after amendment with WA. The various effects observed in WA-amended soils might be due to soil type, WA pre-treatment, WA amendment amount, length of the experiment, parameters of analysis, as well as sampling time. Studies have reported that these factors affect the impact of WA in amended soil (Kuba et al., 2008; Fernando-Jaurez et al., 2013; Gómez-Brandón et al., 2016). A recent study by Joseph et al. (2022) concluded that WA's impact on soil quality is limited and inconsistent, therefore there is no benefit or disadvantage of using WA in forest soil.

4.3 Impact of AD + WA amendment on 9-^{14}C-phenanthrene mineralisation in soil

Soil amendment with mixtures of AD and WA enhanced the mineralisation of ^{14}C-phenanthrene in the soil to a similar extent as did the separate additions. The influence of high amendment amounts as observed in the separate additions was also evident in the combined amendment. A mixture of the amendments (AD and WA) at high amounts had longer lag times and lower extents of ^{14}C-phenanthrene mineralisation. In this study, AD + WA amendment had faster rates, higher extents of mineralisation, and shorter lag times when compared to unamended soil. AD and WA can complement each other in nutrient and has the potential as a good soil amendment for contaminant mineralisation when applied together. WA improved the impact of digestate on soil properties which makes it an appropriate booster for soil nutrients in soil amended with organic fertilizer (Ibeto et al., 2022).

WA contains variable amounts of P and K (Kuba et al., 2008; Ivezic et al., 2021), and a negligible amount of N (due to ammonia volatilization during combustion) (Perucci et al., 2006; Whelan et al., 2010; Möller and Müller, 2012; Köster et al., 2014). However, AD contains considerable amounts of N and P, as well as degradable organic matter (Alburquerque et al., 2012a; Fernández-Bayo et al., 2017). Considering these properties, the combined addition of AD and WA can potentially supply sufficient essential nutrients to soil than their separate additions. Information in the literature indicates that AD + WA can improve soil health and fertility; such effect can be utilized in the mineralisation of PAHs in contaminated soil. In a study by Fernández-

Delgado Juárez et al. (2013), soil amendment with AD + WA increased NH_4^+ as well as total C and P contents of the soil. According to Kuba et al. (2008), soil amendment with mixtures of organic wastes and WA increased the soil's available macronutrients. A combination of straw and WA was better in improving soil microbial biomass carbon (143.33%) than a single use of straw (120.23%) or wood ash (13.89%) (Zhao et al., 2022). Their combination was also better at improving soil bacterial diversity and enzymatic activity. Similarly, a combination of AD and WA improved plant growth and leaf photosynthesis (Zusevica et al., 2023).

5. Conclusions

The findings in this study indicate that soil amendment with AD and/or WA can potentially enhance the biodegradation of PAHs by indigenous microbes. The shorter lag times observed in the presence of AD and/or WA suggests the development of earlier microbial adaptation and activation of microbial catabolic activity, which are significant processes for the successful biodegradation of PAHs. The results of this study suggest that soil amendments with AD and/or WA, can positively influence indigenous soil microbial function, and consequently enhance biodegradation of PAHs. Significant reductions in the kinetics of mineralisation were found where higher amounts of AD (173.1 g) and WA (9.400 g), as well as their mixtures (173.1 g of AD + 9.400 g of WA), were added to soils. This observation could be due to a switch to an alternative more available source of carbon in AD, due to a reduced bioavailability of phenanthrene through sorption/sequestration by WA, or due to unfavourable impacts of AD and/or WA on soil

properties. Therefore, caution is required when using AD and/or WA as soil amendments, as excessive amounts may not be beneficial and could negatively impact soil health and fertility.

CRediT author statement

Adesola S. Ojo: Conceptualisation, Methodology, Investigation, Formal analysis, Data Curation, Writing - Original draft **Chisom Ejileugha:** Methodology, Visualization, Writing – Review & Editing **Carly J. Stevens:** Conceptualisation, Methodology, Validation, Supervision and **Kirk T. Semple:** Conceptualisation, Methodology, Validation, Resources, Supervision, Funding Acquisition

Declaration of conflicting interest

Authors declare no conflicting financial, personal, or professional interest

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