Influence of anaerobic digestate and wood ash on phenanthrene bioaccessibility and mineralisation in soil.

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Abstract

The environmental persistence and ecotoxicity of polycyclic aromatic hydrocarbons (PAHs) have stimulated considerable interest in understanding their bioaccessibility and biodegradation in soils. Factors such as contact time, organic amendments, and microbial inoculation can influence these processes. This study examined how contact time, bacterial inoculation, and amendments affect the chemical extractability and biodegradation of 9-^{14}C-phenanthrene in soil. Soils were amended with anaerobic digestate (AD), wood ash (WA), or their combination (AD + WA) and monitored over 90 days. Lower recoveries of 9-^{14}C-phenanthrene activity were observed in amended soils compared with the control, particularly in AD + WA treatments, with recoveries declining over time. Both dichloromethane (DCM) and hydroxypropyl-β-cyclodextrin (HP-β-CD) extractability decreased with increasing soil – PAH contact time, with a greater reduction in HP-β-CD extractability observed in AD + WA. Inoculated systems exhibited shorter lag phases than uninoculated systems, although bacterial numbers, mineralisation rates, and extents were similar across both inoculation conditions. Lower mineralisation extents occurred in AD + WA under both inoculation conditions. This study provides new insights into how AD, WA, and AD + WA influence PAH bioaccessibility and mineralisation kinetics in soil. The findings indicate that AD and/or WA can stimulate 9-^{14}C-phenanthrene biodegradation under nutrient-limited conditions, primarily through biostimulation of indigenous microbial communities; however, bioaccessibility rather than nutrient availability or microbial abundance, ultimately constrained degradation endpoints. These results highlight the need for (bio)remediation strategies that enhance contaminant availability, rather than focusing solely on nutrient inputs or microbial abundance.

Keywords: Bioaccessibility, Digestate, Hydroxypropyl-β-cyclodextrin, Mineralisation, Polycyclic Aromatic Hydrocarbon, Wood ash

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 (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 (Semple et al., 2003; 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 (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., 2005).

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; Patel et al., 2020). 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 can degrade PAHs (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). Organic amendments have been demonstrated as suitable amendments for improving microbial degradation of PAHs in soil and for mitigating exposure and preventing possible subsequent toxicity (Sayara & Sanchez, 2020; Valizadeh et al., 2022; Li et al., 2023; Atai et al., 2023; Li et al., 2024).

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). Due to the complementary nutrients of AD and WA, their use as mixtures can be a valuable soil amendment for enhancing the biodegradation of PAHs. Ibeto et al. (2020a) demonstrated that digestate can enhance the mineralisation of ^{14}C-phenanthrene in soil. Digestate is known to contain diesel-degrading bacteria, which enabled efficient diesel removal of up to 78% (Gielnik et al., 2019a). This suggests that the microbial community present in digestate can be valuable for bioremediation purposes. For example, digestate-amended soil achieved a 74% reduction in petroleum hydrocarbons within two months, which increased to 95% when biochar-immobilized bacteria were added (Gielnik et al., 2021). In addition, digestate enhanced microbial density and diversity, promoting the enrichment of petroleum hydrocarbon degrading taxa (Gielnik et al., 2021). Similarly, the use of digestate with a bulking agent (sawdust) resulted in higher total petroleum hydrocarbon removal compared to the control in both clay and sandy soils (12.7 – 17.8%), while digestate combined with immobilized bacteria also outperformed the control (9 – 13.4%) (Gielnik et al., 2019b). Digestate supplemented with nutrients improved the removal of PAHs (phenanthrene, anthracene, fluoranthene, and pyrene) in sediment (Bianco et al., 2020). Collectively, these studies indicate that combining digestate with other amendments yields more efficient outcomes for PAH or hydrocarbon removal.

With respect to wood ash, most research has focused on the presence of metals, PAHs, and other organic contaminants in the ash and strategies to prevent potential soil and crop contamination (Jones and Quilliam, 2014; Košnář and Tlustoš, 2025). Information on the impact of wood ash on PAH extractability and mineralisation kinetics in soil remains limited. However, evidence shows that wood ash can enhance the effect of digestate on soil properties, reflected in higher crop productivity (Ibeto et al., 2020b; 2023; Zuševica et al., 2023; Rolka et al., 2025). This suggests that a combination of digestate and wood ash could improve the mineralisation kinetics of phenanthrene in soil through improving soil properties. Enhanced soil health and fertility lead to more robust microbial communities, which are key drivers of organic contaminant biodegradation (Zhu et al., 2017).

Previous studies have explored the co-application of digestate with nutrients, municipal solid waste, immobilized bacteria, biochar, and bulking agents (Gielnik et al., 2019a, 2019b, 2021; Bianco et al., 2020), demonstrating positive effects on microbial activity and PAH degradation. However, the effects of AD and WA, either individually or combined, on PAH bioaccessibility and mineralisation kinetics remain poorly understood. Specifically, there is limited information on how these amendments influence phenanthrene extractability, mineralisation lag phases, maximum rates, and cumulative extents of mineralisation, particularly when considering inoculated versus indigenous microbial communities. This present study addresses this knowledge gap by investigating how AD and WA, alone and in combination, affect phenanthrene bioaccessibility and mineralisation kinetics in soil. By linking amendment – driven changes in chemical availability to microbial degradation kinetics, this work provides a mechanistic, hypothesis – driven assessment of amendment effects. These insights are critical for sustainable soil remediation strategies, waste valorisation, and evidence-based land management, enabling more informed application of organic and mineral amendments to contaminated soils. Phenanthrene, a model PAH, was chosen as its behaviour reflect the response of other PAHs, while acknowledging that mineralisation and bioaccessibility of PAHs in soil is influenced by molecular weight, ring number, and structural properties. Therefore, responses observed in this study for phenanthrene may differ for higher-ring PAHs; however, it provides a mechanistic probe to elucidate how AD and/or WA amendments influence PAH availability and microbial degradation in soil.

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 (ca. 1.917 kg w/w; moisture content = 30%) was spiked with ^{12}C- and ^{14}C-phenanthrene (100 mg kg^{-1} and 35 kBq kg^{-1}; d/w), 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). The spiked soil was divided into four approximately equal portions (ca. 479.2 g each) using clean glass bowls and afterwards the soil was amended with AD and WA as follows: (1) $^{12/14}$C-spiked soil + AD; (2) $^{12/14}$C-spiked soil + WA; (3) $^{12/14}$C-spiked soil + AD + WA; (4) $^{12/14}$C-spiked soil with no AD and/or WA as control. Soil (ca. 479.2 g) was also weighed into a separate clean glass bowl and spiked with same amount of ^{12}C-phenanthrene for ^{12}C blank to provide 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). The soil treatments were transferred to separate pre-cleaned amber glass jars with loose Teflon–lined screw caps (to allow ambient oxygen exchange), aged in the dark for 90 d at 20 ± 2 ºC, and sampled at 0, 15, 30, 60, and 90 d.

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).

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		

<BDL = Below detectable limit.

2.4 Measurement of total ^{14}C-activity associated with 9-^{14}C-phenanthrene in amended soils.

The total 9-^{14}C-phenanthrene activity in the amended and unamended 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 w/w, n = 3) 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 from the amended soils

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

The total extractable 9-^{14}C-phenanthrene in incubated soils was carried out through an exhaustive extraction method using DCM as an extraction solvent (Reid et al., 2000; Doick et al., 2003; Reid et al., 2004; Doick et al., 2005; Rhodes et al., 2008). The soil was extracted at increasing soil-PAH contact times (0, 15, 30, 60, and 90 d). At each sampling, ca. 2.5 g (w/w, 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.

2.5.2 Hydroxypropyl-β-cyclodextrin (HP-β-CD) extraction of 9-^{14}C-phenanthrene from the amended soils

Non-exhaustive chemical extraction of 9-^{14}C-phenanthrene residues from the soil samples was carried out with HP-β-CD, to mimic bioaccessibility and estimate extents of mineralisation in the soils, at increasing soil-PAH contact times (0, 15, 30, 60, and 90 d) (Reid et al., 2000; Doick et al., 2003; Reid et al., 2004; Semple et al., 2006). Soil (ca. 1.25 g w/w; n = 3) was weighed into separate 50 ml Teflon centrifuge tubes. Afterwards, 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 labelled 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. Supernatants (6 ml) were separately sampled into clean 20 ml economy vials followed by the addition of 14 ml of Ultima Gold scintillation fluid, and quantified by liquid scintillation counting.

2.6 Mineralisation of 9-^{14}C-phenanthrene in the amended soils

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 ^{14}C-respirometric system. The mineralisation was performed in modified 250 ml Schott bottles with Teflon-lined screw-caps incorporated with metal clips (Reid et al., 2001; Couling et al., 2010). Soil (ca 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-phenanthrene distribution (Doick & Semple, 2003). Each inoculated respirometer contained 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 contained 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 °C 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 normalise the chemiluminescence effect). The ^{14}C-activity in the traps was quantified by liquid scintillation counting.

2.7 Bacterial inoculation and enumeration the amended soils

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., 2005; Couling et al., 2010). 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 °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 organic compounds such as PAHs (Hwang & Cutright, 2002; Tyagi et al., 2011). For bacteria enumeration, soil and slurry (1 ± 0.2 g w/w, each) were extracted before and after each mineralisation with one-quarter strength of Ringer's solution (1:10). An aliquot of each soil suspension (1 ml) was ten-fold serially diluted with sterile distilled water. Afterwards, 0.1 ml of each resultant solution was inoculated on separate plate count agar for heterotrophic bacteria. Control plates without inoculation were also prepared to monitor possible media contamination. Plates were incubated (25 ± 1 ºC) and distinct colonies were counted after 48 h for heterotrophic bacteria.

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 ($P < 0.05$). The comparison of the means within and across the soils was analyzed using Tukey’s HSD and LSD post-hoc tests. Pearson correlation coefficient (r) was performed to describe the relationship between HP-β-CD extractable 9-^{14}C-phenanthrene and mineralised 9-^{14}C-phenanthrene in inoculum-assisted mineralisation and uninoculated mineralisation. The residual activity was calculated as a percentage of the spiked activity while the solvent extractability and mineralisation kinetics (lag phase, maximum rate, and extent of mineralisation) were calculated from the residual activity. The lag phases were determined as the time taken to mineralised 5% of 9-^{14}C-phenanthrene at each time point, the maximum rates were calculated as the highest 9-^{14}C-phenanthrene catabolized per hour, and the extents of mineralisation as the total 9-^{14}C-phenanthrene mineralised in 14 d.

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 soil increased with increasing soil-phenanthrene contact time. Generally, there were greater losses in the AD, WA, and AD + WA amended soils as compared to the unamended soil incubations suggesting that the amendments stimulated the microbial degradation of the 9-^{14}C-phenanthrene. 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 after time zero, and WA after 30 d. As anticipated from differences in nutrient content, 9-^{14}C-phenanthrene loss was highest in AD + WA amended soil, followed by AD, then WA, and lowest in the control. Greater amounts of 9-^{14}C-phenanthrene residues were lost in soil amended with WA (30 d and 60 d) and AD (30 d, 60 d, and 90 d) compared to the control, but these differences were not statistically significant ($p > 0.05$).

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

The solvent extractability of the ^{14}C-phenanthrene was monitored for 90 d, and this was calculated as a percentage of the residual activity at each sampling time. The total amounts of DCM extractable 9-^{14}C-phenanthrene residues (%) in the soils at various contact times are shown in Figure 2. There was no significant difference ($p > 0.05$) in DCM extractability at 0, 15, and 90 d in all soil treatment conditions. Generally, the amounts of HP-β-CD-extractable 9-^{14}C-phenanthrene fractions (Figure 2) in the amended soils were lower than the control. Noticeably, AD + WA amended soil showed significantly ($p < 0.05$) lower HP-β-CD extractability than the control at 0 – 90 d (Figure 2). Higher HP-β-CD extraction was generally observed in AD amended soil compared to other soil amendment conditions, with a slightly higher HP-β-CD-extractable amount on 30 d and 90 d compared to the control. As expected, the DCM-extractable 9-^{14}C-phenanthrene fractions were higher than the HP-β-CD-extractable 9-^{14}C-phenanthrene fractions in the soil treatments due to difference in extraction strength. 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 from 0 – 90 d. 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 from 0 – 90 d. This clearly shows the difference in extractability strength of the two solvents and the impact of contact time on bioaccessibility.

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

3.3.1 Mineralisation of ^{14}C-phenanthrene in inoculum-augmented respirometry

The mineralisation of 9-^{14}C-phenanthrene in the soil incubations was monitored for 90 d to determine the lag phases, maximum rates, and extents of mineralisation. The inoculum-assisted mineralisation of the 9-^{14}C-phenanthrene is shown in Figure 3 and Table 2. The lag phases for WA and AD + WA amended soils were significantly shorter ($P < 0.05$) than the controls at time zero. Shorter lag phases were also observed in AD amended soil 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 control. Generally, AD + WA amended soil showed shorter lag phases than all soil treatment conditions (Table 2). No significant difference ($p > 0.05$) was observed in lag phases in all soil conditions from 30 d (Table 2). The rates of mineralisation were significantly higher ($P < 0.05$) than the control at time zero in WA and AD + WA amended soils. Also, the rates of mineralisation were higher in AD amended soil at time zero, as well as AD + WA at 30 d, but these were not statistically different ($P > 0.05$) from the control. No significant difference ($p > 0.05$) was observed in maximum rates in all soil conditions from 30 d (Table 2). The extents of mineralisation decreased over time in all the soil treatment conditions (Figure 3 and Table 2).

Generally, lower and higher extents of mineralisation was observed in AD + WA and AD amended soil respectively compared to other amended soils especially from 0 – 30 d (Figure 3, Table 2). However, higher extents of mineralisation were observed in soil amended with AD + WA at 60 d and 90 d compared to other amended soils. The control generally showed higher extents of mineralisation compared to amended soils.

Table 2: Mineralisation of 9-^{14}C-phenanthrene in inoculum – assisted respirometry. Values in columns followed by different letters are statistically different (Tukey's, 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 b
	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 ab
	Soil + WA	29.5 ± 5.1 a	0.2 ± 0.0 a	23.5 ± 1.3 bc
	Soil + AD + WA	32.8 ± 3.5 a	0.4 ± 0.2 a*	18.2 ± 0.8 c
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

3.3.2 Mineralisation of ^{14}C-phenanthrene in soil indigenous microbial respirometry

The mineralisation of the 9-^{14}C-phenanthrene in the incubated soils under soil indigenous microbial respirometry is shown in Figure 4 and Table 3. The lag phases did not follow a defined pattern. Lower lag phases were observed in AD amended soil at time zero and 30 d, in WA amended soil at 15 d, and in AD + WA amended soil at 60 and 90 d. The lag phases were significantly shorter ($P < 0.05$) than the control 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 these were not statistically significant ($P > 0.05$) compared to the control. The rates of mineralisation were significantly higher ($P < 0.05$) than the controls at 15 d in WA amended soil. 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 ($p > 0.05$). No significant difference ($p > 0.05$) was observed in maximum rates in all soil conditions from 30 d (Table 3). The extents of mineralisation were significantly higher ($P < 0.05$) than in all soil treatment conditions at 60 d in AD + WA amended soils. In general, lower extents of mineralisation was observed in AD + WA compared to other amended soils, with control showing higher extents of mineralisation than amended soils (Table 3). This is similar to the changes in extents of mineralisation observed in inoculum – assisted mineralisation.

Table 3: Mineralisation of 9-^{14}C-phenanthrene in soil indigenous microbial respirometry. Values in columns followed by different letters are statistically different (Tukey*'s*, 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 c
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 b
	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 b
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 b
	Soil + AD + WA	47.9± 7.1 b	0.2 ± 0.0 a	20.2±2.1 b
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 ab

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

The comparison of HP-β-CD extractable and mineralised 9-^{14}C-phenanthrene fractions (%) in the soils is given in Table 4. The HP-β-CD extractability of 9-^{14}C-phenanthrene was compared with its extent of biodegradation in soil to evaluate whether the chemically extractable fraction corresponds to the bioavailable pool accessible to microorganisms. This approach enables assessment of the predictive value of HP-β-CD extraction as a surrogate for microbial degradation potential and provides insight into the extent to which contaminant availability, rather than microbial activity, constrains biodegradation. The amounts of HP-β-CD extractable ^{14}C-phenanthrene fractions positively correlated with the mineralised ^{14}C-phenanthrene fractions in both inoculum - assisted and soil indigenous microbial respirometry at all contact times. In the inoculum - assisted and indigenous respirometry, the amounts of HP-β-CD extractable and mineralised 9-^{14}C-phenanthrene fractions were close in terms of a 1:1 proportionate relationship especially from 0 – 30 d (Table 4). HP-β-CD extractability appeared to underpredict microbial degradation endpoint in some of the soil treatment conditions in both inoculated and uninoculated mineralisation at 60 d and 90 d. However, a strong correlation was observed in both inoculated ($R^2 = 0.94$) and indigenous ($R^2 = 0.95$) mineralisation.

Table 4: Comparison of HP-β-CD extractable 9-^{14}C-phenanthrene (%) in the soil treatment conditions, and the biodegradable (%) 9-^{14}C-phenanthrene under inoculated and uninoculated soil respirometry (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

3.6 Bacterial enumeration in the amended soils

The heterotrophic bacterial numbers were monitored in the incubated soils throughout the 90 d incubation. The heterotrophic bacterial numbers were counted in the soil before mineralisation and then counted at the end of mineralisation (14 d) for inoculum-assisted and soil indigenous microbial respirometric assays. In both respirometric conditions (inoculum – assisted and soil indigenous microbes), the bacterial numbers were high throughout the experiment (0 – 90 d) with higher bacteria numbers generally observed after mineralisation in the soil slurries compared to the numbers in the soils before mineralisation (Table 5). The higher heterotrophic bacterial numbers after mineralisation was expected because as organic compounds are broken down into simpler forms, they become available to a broader range of microbial communities. This could have led to an increase in microbial activity and multiplication resulting in higher bacterial numbers. Generally, the bacteria numbers after mineralisation in soil indigenous microbial (uninoculated) respirometry and inoculum-assisted (inoculated) mineralisation were similar (Table 5). This was unexpected because the inoculated respirometry contained phenanthrene degrading inoculum (*Pseudomonas* sp.) and was expected to have significantly higher bacterial numbers after mineralisation. This observation was attributed to the inoculum trying to adapt to the new soil conditions and possible competition with indigenous soil microbes which led to a similar bacterial numbers in both respirometric conditions.

Table 5. The heterotrophic bacterial colony-forming units per gram soil (CFU g^{-1} soil) in incubated soil, inoculated, and uninoculated respirometry before and after mineralisation at increasing soil-phenanthrene contact times of 0 d, 15 d, 30 d, 60 d and 90 d; values (n = 3) represent the mean ± SEM.

Contact time (d)	Soil amendment	Heterotrophic bacterial numbers (CFU g^{-1} soil)		
		Before mineralisation	After mineralisation	
			(a) Inoculated	(b) Non-inoculated
0	Control	3.3E+06 ± 2.4E+05	1.3E+08 ± 7.2E+07	1.3E+08 ± 7.4E+07
	Soil + AD	2.2E+07 ± 2.6E+06	1.2E+07 ± 8.1E+06	1.1E+07 ± 6.2E+06
	Soil + WA	1.5E+07 ± 1.1E+07	1.5E+07 ± 8.1E+06	1.5E+07 ± 8.0E+06
	Soil + AD + WA	2.5E+07 ± 1.7E+07	1.3E+07 ± 8.1E+06	1.2E+07 ± 7.1E+06
15	Control	1.2E+07 ± 2.6E+06	1.2E+09 ± 1.5E+08	3.8E+09 ± 1.9E+09
	Soil + AD	2.5E+07 ± 1.4E+07	4.3E+09 ± 3.9E+09	5.1E+08 ± 1.0E+08
	Soil + WA	8.3E+06 ± 3.0E+06	6.6E+08 ± 9.3E+07	4.7E+08 ± 2.6E+08
	Soil + AD + WA	1.9E+07 ± 6.8E+06	5.8E+08 ± 3.8E+08	7.6E+08 ± 8.0E+07
30	Control	6.4E+06 ± 2.3E+06	1.7E+09 ± 6.1E+07	5.4E+08 ± 8.4E+07
	Soil + AD	5.2E+06 ± 2.7E+06	8.8E+08 ± 3.1E+08	3.6E+09 ± 2.7E+09
	Soil + WA	6.2E+06 ± 1.6E+06	8.0E+08 ± 1.0E+08	2.3E+09 ± 1.5E+09
	Soil + AD + WA	6.2E+06 ± 3.4E+06	8.5E+08 ± 3.6E+08	2.0E+08 ± 4.9E+07
60	Control	1.7E+07 ± 5.1E+06	2.9E+09 ± 2.4E+09	2.7E+09 ± 2.6E+09
	Soil + AD	4.3E+06 ± 4.1E+05	2.6E+08 ± 5.6E+07	2.6E+08 ± 5.2E+07
	Soil + WA	1.3E+07 ± 4.9E+06	7.4E+08 ± 1.1E+08	7.3E+08 ± 2.1E+08
	Soil + AD + WA	1.0E+07 ± 4.4E+06	2.2E+08 ± 4.0E+07	2.2E+08 ± 4.3E+07
90	Control	7.7E+07 ± 3.4E+07	5.8E+09 ± 3.4E+09	3.7E+09 ± 9.2E+08
	Soil + AD	2.0E+07 ± 3.1E+06	1.3E+09 ± 2.0E+08	1.3E+10 ± 5.2E+09
	Soil + WA	4.9E+07 ± 9.0E+06	6.5E+08 ± 1.5E+08	8.5E+09 ± 6.6E+08
	Soil + AD + WA	5.9E+07 ± 1.1E+07	9.4E+08 ± 4.4E+08	6.7E+09 ± 1.3E+08

4. Discussion

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

There was loss of 9-^{14}C-phenanthrene associated activity in all the soil incubations, particularly with the additions of AD + WA. All amended soils showed greater loss of ^{14}C-activity than the control suggesting that AD and WA could be suitable for improving the biodegradation of phenanthrene in soil. The total 9-^{14}C-phenanthrene activity in AD + WA amended soil decreased significantly from 15 d when compared to other soil treatment conditions. This suggests that the additions of AD + WA stimulated microbial degradative activity in the soil and consequently contributed to the higher biodegradation of the 9-^{14}C-phenanthrene in the soil. It is likely that the major loss process of the phenanthrene from soil was microbially mediated and further stimulated by the amendments because all amended soils generally showed lower residual activity than the control. This finding agrees with earlier studies where the biodegradation of PAHs increased following the additions of organic nutrients to soil (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 earlier 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 combined application of AD and WA exhibited a time – dependent effect on residual activity. At some time points (15 d and 60 d) the reduction exceeded the sum of the individual effects, indicating synergistic interactions, whereas at other times (30 d and 90 d) it resulted in a greater reduction in residual activity than either amendment alone, representing an additive effect, and likely complementary mechanisms where each amendment acted through distinct pathways – AD by stimulating microbial activity and WA by enhancing contaminant accessibility – thereby jointly contributing to the overall reduction. This suggests that the interaction between the amendments evolves over time, likely due to changing availability of the phenanthrene and microbial adaptation.

The DCM extraction is known to be an exhaustive method that gives the total chemically extractable PAHs in the soil (Semple et al., 2003; Doick et al., 2005). HP-β-CD extraction is used to mimic the mineralisation of PAHs or predict the extent to which PAHs can be microbially degraded in the soil (Doick et al., 2005; Papadopoulos et al., 2007; Rhodes et al., 2008). The 9-^{14}C-phenanthrene DCM extractable and HP-β-CD extractable fractions in the soils decreased as contact time increased. Similar findings have been reported in past studies (Papadopoulos et al., 2007; Rhodes et al., 2008) and have been attributed to 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 HP-β-CD extractable fraction decreased greatly between 5.6% - 1.8% at 90 d compared to 85.5% - 63.7% at time zero, therefore indicating a reduced bioaccessibility over time. The combination of AD and WA (AD + WA amended soil) showed lower extractability than other soil treatments. These observations agree with past studies which identified ageing and soil organic matter to promote a reduction in the extractability or bioaccessibility of PAHs and their nitrated analogues in soil (Doick et al., 2003; Rhodes et al., 2008; Papadopoulos et al., 2007; Chen et al., 2023). PAHs can bind to minerals, humin-like soil fractions, and organic amendments hence reducing desorption and potential bioaccessibility (Sigmund et al., 2018; Han et al., 2020).

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

The levels of $^{14}CO_2$ produced reduced significantly as the soil-PAH contact time increased. This was due to reduced bioaccessibility of 9-^{14}C-phenanthrene through ageing. The length of the soil-PAH contact time has been known to negatively impact the bioavailability of PAHs (Reid et al., 2000; Papadopoulos et al., 2007; Rhodes et al., 2008), 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 ageing. The lag phases were longer, and the mineralisation rates and extents diminished over time in both inoculated and uninoculated mineralisation. The amended soils exhibited similar mineralisation patterns to the control. Mineralisation extents were lower in AD + WA soils than in other treatments, likely due to higher amendment levels that enhanced sorption and reduced bioaccessibility, as reflected by the lower HP-β-CD extractable fraction.

In the soil incubations, amended soils exhibited lower residual activity than the control, indicating enhanced biodegradation through nutrient stimulation, with degradation greatest in AD + WA, followed by AD, and then WA. Because mineralisation kinetics was calculated from residual activity at each sampling time, the reduced total activity and bioaccessible phenanthrene fractions in AD + WA soils – resulting from greater biodegradation and subsequent sequestration – explains the lower mineralisation extents observed. In contrast, AD and WA amended soils, although showing greater biodegradation (lower residual activity) than the control, exhibited relatively similar bioaccessible and residual fractions. Under the MBS medium, which supplied all nutrients except carbon, the control, with slightly higher residual and bioaccessible fractions, achieved mineralisation extents that is slightly higher but comparable to AD and WA soils. This indicates that, in the respirometry system, reduced total activity and bioaccessibility rather than nutrient availability constrained mineralisation, thereby removing the nutrient advantage of the amendments – a distinction that would likely persist if distilled water had been used instead of MBS. Therefore, the soil incubations demonstrated that AD and WA stimulated phenanthrene degradation by supplying nutrients, with the greatest effect in AD + WA soils. Respirometry using MBS further revealed that, when nutrient limitation was removed, mineralisation was governed primarily by contaminant bioaccessibility. Together, these findings show that amendments enhance phenanthrene degradation under nutrient-limited soil conditions, but that bioaccessibility remains the ultimate constraint on mineralisation.

The lag phases in inoculated mineralisation were substantially lower than that in uninoculated mineralisation. This was due to the faster degradation of the phenanthrene by the metabolically adapted inoculum compared to the soil indigenous bacteria that are still adapting to the phenanthrene. These findings are consistent with studies where biostimulation combined with bioaugmentation enhanced the biodegradation of aged PAHs in soils, resulting in greater degradation compared to other soil incubations (Hamdi et al., 2007; Wu et al., 2020). In Tyagi et al. (2011) and Brzeszcz et al. (2020), microbial inoculation was indicated to be a better bioremediation strategy compared to biostimulation. However, the lower lag phases in inoculated mineralisation did not generally result in higher extents of mineralisation when compared to indigenous mineralisation from 0 – 30 d. This suggests that mineralisation extent was primarily governed by phenanthrene bioaccessibility rather than microbial abundance. Heterotrophic bacterial counts remained high throughout the 90-day incubation under both mineralisation conditions, indicating that the decline in mineralisation over time was attributable not to reduced bacterial abundance but to reduced bioaccessibility, as reflected by the decrease in the HP-β-CD extractable fraction. Although heterotrophic bacterial counts remained high in inoculated mineralisation, bacteria count data alone cannot distinguish between inoculated and indigenous populations. It is therefore possible that, following the initial phase of degradation, indigenous microbial communities became functionally dominant, or that competition and niche overlap limited sustained inoculum advantage. Importantly, even if inoculated bacteria persisted, their degradation potential would still be constrained by phenanthrene bioaccessibility. Higher extents of mineralisation were observed in inoculated mineralisation at 60 d and 90 d compared to uninoculated mineralisation. This shows the advantage of inoculation especially in aged soil, as the adapted inoculum could be the reason behind the slight increase in extents of mineralisation under inoculated respirometry at 60 d and 90 d. However, this difference was not significant, supporting earlier studies that reported no variation in the extents of mineralisation between inoculum-assisted and indigenous mineralisation (Doick et al., 2005; Semple et al., 2006; Allan et al., 2007).

Similar maximum rates of mineralisation were observed in both mineralisation conditions. Therefore, 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). Biostimulation has been known to provide suitable nutrients and/or conditions for soil microflora as well as PAH-degrading inoculants (Tyagi et al., 2011), and has been proposed as a suitable strategy for the removal of PAHs in aged or weathered soil (Haleyur et al., 2019). Additionally, similar extents of mineralisation were observed in inoculated and uninoculated mineralisation. This confirms that soil indigenous bacteria possess the ability to degrade PAHs (Couling et al., 2010, Picariello et al., 2020; Roszak et al, 2021), therefore artificial inoculation may not be necessary where there are indigenous microbes with required degradative repertoire. 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).

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

HP-β-CD extraction of PAHs has been demonstrated over two decades as an effective method for assessing the extent of their microbial degradation in soil and, accordingly, has been applied in recent studies to predict PAH bioaccessibility (Leech et al., 2020; Towell et al., 2021; Cao et al., 2022; Posada-Baquero et al., 2022; Jin et al., 2023). A close 1:1 relationship is expected between the HP-β-CD extractable and mineralised 9-^{14}C-phenanthrene fractions. However, in this present study, the HP-β-CD extractable 9-^{14}C-phenanthrene fractions were in close relationship with mineralised fractions at 0 – 30 d, and underpredicted the mineralised fractions at 60 – 90 d. This variation has been attributed to a slower desorption rate over time and microbial ability to attach and degrade bound fractions (Rhodes et al., 2008; Rhodes et al., 2012), as mineralisation at 60 – 90 d reflects more than just what HP-β-CD can extract at a given time. This implies that, HP-β-CD extraction closely reflected phenanthrene mineralisation during the early incubation period (0 – 30 d), when degradation was dominated by the readily desorbing fraction in rapid equilibrium with the soil solution. At longer incubation times (60 – 90 d), HP-β-CD increasingly underpredicted mineralisation because degradation progressively relied on slowly desorbing and physically sequestered phenanthrene associated with AD- and WA-amended soil, which are not efficiently captured by short-term chemical extraction. Put differently, over time, phenanthrene degradation was controlled not only by the immediately bioaccessible fraction measured by HP-β-CD, but also by processes that evolve with time, including slow desorption, diffusion from sequestered domains, and microbial adaptation or surface-associated degradation. Depletion of the labile pool over time promoted diffusion-limited desorption, while microbial adaptation and attachment to amendment – soil surfaces enabled biological access to bound fractions. As a result, the total mineralised fraction at 60 – 90 d represents the cumulative outcome of these dynamic physicochemical and biological processes and not just what HP-β-CD could extract.

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 in this study positively correlated with the mineralised 9-^{14}C-phenanthrene in the inoculated and uninoculated mineralisation. The positive correlation, high R^2-values, and near 1:1 relationship indicate that HP-β-CD extraction reliably predicts the bioaccessible concentration of ^{14}C-phenanthrene in AD and WA amended soils, supporting previous findings on its suitability for predicting PAH microbial degradation in soils (Reid et al., 2000; Allan et al., 2007; Papadopoulos et al., 2007; Rhodes et al., 2008).

5. Conclusion and real – world implications of findings

Unlike previous studies that focus primarily on nutrient supplementation and/or microbial abundance, our findings demonstrate that bioaccessibility remains the ultimate constraint on PAH mineralisation, even under nutrient-enhanced conditions. This study provides new evidence that WA can stimulate phenanthrene biodegradation in soil, addressing a gap in the existing literature where such effects have been scarcely reported. The AD + WA amendment produced the greatest stimulation of biodegradation; however, it also revealed a critical balance, where enhanced nutrient input was offset by stronger bioaccesibility effects that restricted mineralisation kinetics. Furthermore, while inoculation shortened lag phases and enhanced early biodegradation, indigenous microbial communities were ultimately capable of same effect with similar maximum rates and extents of mineralisation. By combining nutrient-focused and bioaccessibility-focused perspectives, this study deepens understanding of the mechanistic constraints of PAH (bio)remediation and emphasizes the importance of developing strategies that improve contaminant availability in aged soils, while also considering nutrient supply and microbial abundance.

The findings of this study have clear practical relevance for soil remediation and land management. By reducing phenanthrene bioaccessibility while supporting microbial mineralisation, AD and WA amendments, alone or in combination, can also help to limit contaminant mobility and exposure risks in PAH-contaminated soils. Moreover, the use of AD and WA represents a form of waste valorisation, providing a sustainable pathway for recycling organic and mineral residues into soils. Collectively, the study provides mechanistic insights that can inform evidence-based decisions for remediation strategies and sustainable land management in soils impacted by PAHs. Importantly, the findings of this study are directly applicable to soils with physicochemical characteristics comparable to those investigated here. While the observed responses provide robust insight into mineralisation dynamics and soil – amendment interactions under these conditions, soils with contrasting textures or organic matter contents may exhibit different behaviours.

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