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Effects of soil amendment with anaerobic digestate and wood-ash (bioenergy residues) on biodegradation of polycyclic aromatic hydrocarbons in soil.

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Declaration

I hereby declare that the body of work presented in this research thesis is my original work, and no part of the work in the same whole form has been submitted elsewhere for the award of a higher degree.

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Abstract

Many organic contaminants found in the soil are associated with industrial emissions and/or spills; these include petroleum hydrocarbons and polycyclic aromatic hydrocarbons (PAHs), in particular. PAHs accumulate in soil due to their low aqueous solubility, volatility, hydrophobicity and recalcitrant chemical structures. The environmental persistence and risks associated with human and environmental health emphasize the importance of the treatment of PAH-contaminated soil. Various physical and chemical technologies known to have been employed in remediating PAH-contaminated soils are expensive and have further environmental challenges compared to microbial degradation which is less costly and environmentally sustainable. However, the stimulation of microbial activity with recurrent addition of mineral nutrients is known to damage the soil quality, while soil amendment with the residues of renewable energy production becomes a suitable option due to their nutrient contents, environmental sustainability and economic feasibility. This thesis evaluates the environmental fate and impact of PAHs in the soil and the biodegradation of PAHs in contaminated soils. It further investigates the effects of soil amendment with organic residues, particularly anaerobic digestate (AD) (a semi-solid biogas residue), wood-ash (WA) (a timber combustion residue) and their mixtures on indigenous microbial activity, and how the effects influence the indigenous biodegradation of PAHs. The findings provide insights into the implications of microbial degradation of PAHs in soils lacking in nutrients, the effects of soil amendment with AD and/or WA on biodegradation of PAHs, and the correct amounts of AD and WA that could be used as a combined soil amendment to stimulate indigenous biodegradation of PAHs.

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Abbreviations

PAH - Polycyclic Aromatic Hydrocarbons

AD - Anaerobic digestate

WA - Wood-ash

LMW - Low molecular weight

HMW - High molecular weight

C - Carbon

SOM – Soil organic matter

K_p - Partition coefficients

K_{oc} - Soil organic carbon normalized partition coefficient

K_{ow} - Octanol-water partition coefficient

K_d - Soil/sediment-water distribution coefficient

CFUs - Colony Forming Units

N-P-K – Nitrogen-Phosphorus-Potassium

TPH - Total petroleum hydrocarbon

HOC - Hydrophobic organic contaminants

DCM – Dichloromethane

HP-β-CD - Hydroxypropyl-β-cyclodextrin

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1. Introduction

1.1 Polycyclic aromatic hydrocarbons (PAHs) and soil contamination

Many organic contaminants found in the soil are associated with industrial emissions and/or spills (Lukić *et al.*, 2016). These contaminants include petroleum hydrocarbons, which consist of complex mixtures of organic chemicals, including polycyclic aromatic hydrocarbons (PAHs). PAHs are part of the constituents of fossil fuels and are generated during processes involving incomplete combustion of organic materials, such as coal, oil, petrol, and wood (Kanaly & Harayama, 2000; Megharaj *et. al.*, 2011; Abdel-Shafy and Mansour, 2016; Yu *et. al.*, 2018; Roslund *et. al.*, 2018). The environmental distribution of PAHs is known to be influenced by changes in their sources of emission or transport pathways (Kuppusamy, *et. al.*, 2017). Previous studies have identified a variety of PAH sources, which include natural and anthropogenic activities. Furthermore, these PAHs are commonly grouped into pyrogenic, petrogenic and biogenic PAHs (Lukić *et. al.*, 2016; Abdel-Shafy and Mansour, 2016; Kuppusamy, *et. al.*, 2017).

Pyrogenic PAHs are formed under high temperature (350 °C to more than 1200 °C) combustion processes, with low or without oxygen (pyrolysis), of which the natural processes include volcanic eruptions and wildfires (Quilliam *et al.*, 2012; Abdel-Shafy and Mansour, 2016; Baldwin *et al.*, 2020). Also, pyrogenic PAHs are generated during industrial-related anthropogenic processes (Okere and Semple 2012; Baldwin *et al.*, 2020) involving thermal decomposition of organic materials (pyrolysis), and their subsequent recombination (Weinstein *et al.*, 2017; Duca *et al.*, 2018). Pyrogenic PAHs are emitted into the atmosphere as gases or particulate materials, and the atmospheric partitioning between the gaseous and particulate forms is

known to influence their fate, transport and soil deposition (Lima *et. al.*, 2005; Abdel-Shafy and Mansour, 2016). However, the anthropogenic sources of pyrogenic PAHs are known to be predominant (Okere and Semple 2012; Quilliam *et al.*, 2012), and as the PAHs accumulate in the soil they become environmentally ubiquitous (Lima *et al.*, 2005; Crampon *et al.*, 2014; Baldwin *et al.*, 2020).

Many PAH-contaminated soils are associated with industrial petrogenic processes involving low temperatures from refined petroleum products, such as asphalt, diesel, gasoline, lubricants, as well as unprocessed coal and crude oil (Kanaly & Harayama, 2000; Baldwin *et al.*, 2020). Such industrial processes include petroleum refining, oil spills, illicit disposal of industrial effluents and disposal of fossil fuels or fossil-fuel-derived products (like coal tar and carbon black coal tars) (Peng *et. al.*, 2011; Lang *et. al.*, 2016). Also, naturally occurring biogenic organic compounds are formed through biological processes (Wang *et. al.*, 2015) in certain plants and bacteria, or during degradation of vegetative matter (Abdel-Shafy and Mansour, 2016). However, the biosynthesis of biogenic organic compounds is indicated to be a localized source with little impact on the global scale (Lima *et. al.*, 2005). Generally, the amounts of PAHs in the soil could vary from 1 µg to 300 g kg^{-1} soil, dependent on the contamination sources (Megharaj *et. al.*, 2011). The distribution of PAHs in the soil could provide information for assessing their occurrence, identifying their sources of emission and evaluating their associated risks of environmental exposures (Peng *et al.*, 2011).

Generally, PAHs are known to be of significant concerns because of their adverse effects on human and ecological health upon sufficient exposures (Marini and Frapiccini, 2013), their persistence in the soil and/or environment at elevated concentrations (Stokes *et. al.*, 2006; Semple *et. al.*, 2007; Xiong *et. al.*, 2017), as

well as their bioaccumulation potential (Xiong *et. al.*, 2017). The effects are known to cause or contribute to mutations in human and other ecological organisms, and even death in extreme cases (Das and Chandran, 2011). However, previous studies on soils confirm that several microorganisms can utilize different types of PAHs as carbon (C) sources, depending on the physico-chemical characteristics of the soil and PAHs (Maletić *et. al*., 2013; Naseri *et. al.*, 2014; Lang *et al.*, 2016; Roslund *et. al.*, 2018; Siles and Margesin, 2018). Also, the soil-PAH contact time and/or interaction influences the mineralisation of PAHs in the soil, while the sequestered PAH fractions within the soil particles may not be microbially available and/or degradable compared to the labile PAHs (Crampon *et al.*, 2014). Therefore, the development of cost-effective method(s) for remediating PAH-contaminated soils remains a top priority.

In past studies, soil amendments with the addition of nutrients (Alburquerque *et. al.*, 2012a; Fernández-Delgado Juárez *et. al.*, 2013; Maletić *et. al.*, 2013; Gómez-Brandón *et. al.*, 2016) and modification of environmental parameters (Leal *et al.*, 2017) were successfully employed to stimulate microbial activity (Margesin and Schinner, 2001; Bougnom *et. al.*, 2012; Naseri *et. al.*, 2014) and optimise microbial degradation of PAHs. The recurrent soil amendment with mineral nutrients has been known to deteriorate the soil quality (Savci, 2012). However, soil amendment with organic materials has been proven to improve the soil physico-chemical properties as well as enhance the soil microbial activity and, consequently, the degradation of soil contaminants (Haritash and Kaushik, 2009; Odlare *et. al.*, 2011; Bougnom *et. al.*, 2012; Koszel and Lorencowicz 2015). For example, studies showed that the additions of AD (Köster *et al.*, 2014; García-Sánchez *et. al.*, 2015a) and WA (Ferreiro *et. al.*, 2011; Fernández-Delgado Juárez *et. al.*, 2013; García-Sánchez *et al.*, 2015a) as well as their mixture (due to their complementary nutrient contents)

(Bougnom *et. al.*, 2012) improved the soil biological activities (Margesin and Schinner, 2001; García-Sánchez *et. al.*, 2015a; Koszel and Lorencowicz 2015). In addition, studies on biostimulation of soils with organic materials have been known to be cost-effective and environmentally sustainable (Tiwary *et. al.*, 2015; Gómez-Brandón *et. al.*, 2016; Ning *et. al.*, 2017).

1.2 Physico-chemical properties of PAHs and the implications on soil

Homologous PAHs consist of 2 or more fused benzene rings with only carbon and hydrogen atoms (ATSDR, 2013). They are bonded in linear, cluster, or angular configurations (Abdel-Shafy and Mansour, 2016), and the variations in their molecular structure indicate the differences in their properties (ATSDR, 2013) (Figure 1). Structurally, homologous PAHs are non-polar organic compounds categorized as either low (with up to 2 - 3 benzene rings) or high (4 or more benzene rings) molecular weight compounds (Lang *et. al.*, 2016; Demeter *et. al.*, 2017; Kuppusamy, *et al.*, 2017).

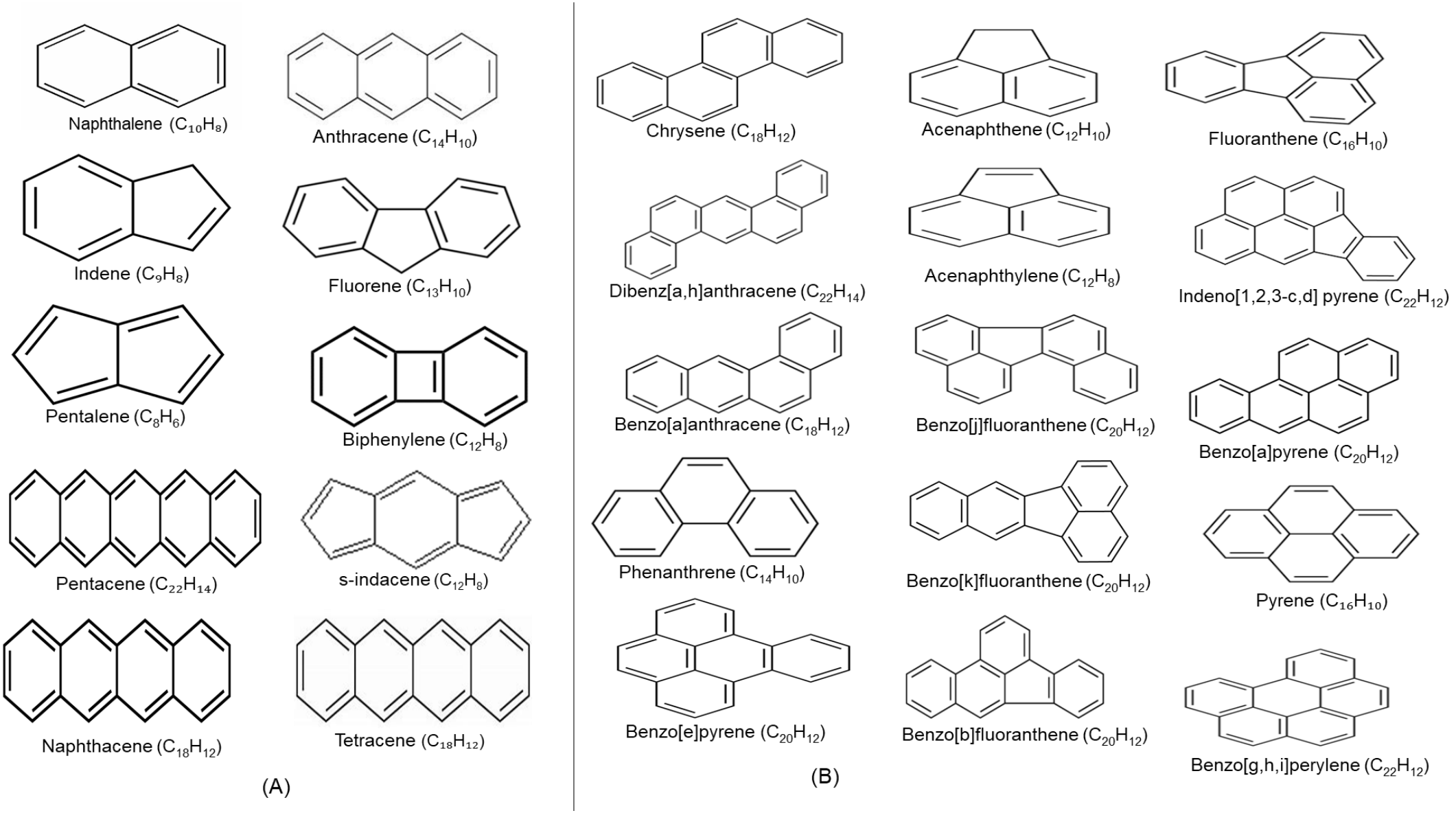

Figure 1: Chemical structures of selected linear (A), as well as angular and clustered (B) polycyclic aromatic hydrocarbons (PAHs) (IARC, 1983; PubChem, 2020).

The configuration of the aromatic rings is known to affect the stability of PAHs; for example, linear PAHs are more unstable than the clustered PAHs (Abdel-Shafy and Mansour, 2016). Most PAHs appear as colourless, white/pale yellow solids in water, with low aqueous solubility and volatility (at room temperature) as well as high melting and boiling points. (Haritash and Kaushik, 2009; Abdel-Shafy and Mansour, 2016). As the molecular weight of PAHs increase, their vapour pressure and aqueous solubility tend to decrease (Haritash and Kaushik, 2009; Abdel-Shafy and Mansour, 2016). The physico-chemical nature of the PAHs has a significant influence on their microbial availability and/or degradation. For example, Crampon *et. al.* (2014) studied the biodegradation of seven selected PAHs by indigenous microorganisms in five different soils; low molecular weight (LMW) PAHs were observed degraded in less than 2 – 3 months with a significant presence of relevant phenanthrene-degrading bacteria; high molecular weight (HMW) PAHs were less bioavailable and the abundance of the relevant phenanthrene-degrading microorganisms was lower. Some physical and chemical properties of selected PAHs are shown in Table 1.

Table 1: The physical and chemical properties of selected polycyclic aromatic hydrocarbons (PAHs), including halve lives (days) (IARC, 1983; Environment Agency, 2003; Ghosal *et al.*, 2016; PubChem, 2020).

Name	Physical appearance	Melting point (°C)	Vapour pressure (Pa at 25 °C)	Aqueous solubility (mg $^{-l}$ at 25°C)	*Log K_{ow}*	*Log K_{oc}*	*Log K_d* (in Water)	Half-lives (days) (estimated)
Naphthalene	White, volatile, solid	80.2	6.53 - 111.0	12.5 - 137.35	3.01 - 4.70	2.66 - 3.91	7.50E-10	5.66
Phenanthrene	Colourless, monoclinic crystals	99.2	0.0127 - 0.464	0.0446 - 11.25	3.60 - 5.92	3.58 - 6.12	-	14.97
Anthracene	White crystals or flakes	215.0 - 218.0	3.87E-07 - 0.095	0.030 - 0.551	4.15 - 4.73	2.96 - 5.76	7.74E-10	123
Fluoranthene	Colourless solid, often pale yellow	107.8	1.65E-04 - 1.79	0.19 - 1.43	4.78 - 6.50	4.0 - 6.38	6.35E-10	191.4
Benzo[a]anthracene	Colourless-to-yellow brown fluorescent	155 - 162	3.87E-07 - 6.06E-04	0.0086 - 0.044	5.48 - 7.50	4.0 - 7.3	9.00E-10	343.8
Pyrene	flakes/powder; Pale yellow or colourless solid	150.62 - 151.2	1.70E-04 - 0.0119	0.032 - 1.56	4.45 - 6.70	3.11 - 6.51	7.24E-10	283.4
Benzo[k]fluoranthene	Yellow crystals	217	5.20E-08 - 6.70E-05	7.00E-03 - 0.008	6.0 - 7.20	4.0 - 7.0	5.56E-10	284.7
Benzo[b]fluoranthene	Colourless crystals	168 - 168.4	5.0E-07 - 6.70E-05	0.0015 - 0.014	5.78 - 6.57	5.70 - 6.09	5.56E-10	284.7
Benzo(e)pyrene	Colourless crystals or white crystalline solid	175 - 179	$5.7X10^{-9}$	0.0063	6.44	-	9.00E-10	-
Benzo[a]pyrene	Pale yellow needles	176.5 - 179.0	8.53E-10 - 2.53E-05	1.7E-03 - 0.008	4.05 - 8.50	4.0 - 8.30	9.00E-10	421.6
Dibenzo[a,h]anthracene	Colourless crystalline powder	267 - 269.5	4.25E-10 - 1.33E-08	5.0E-04 - 0.0025	5.80 - 7.19	5.0 - 7.80	5.18E-10	511.4
Benzo[ghi]perylene	Pale yellow-green crystals	278 - 278.3	1.40E-08 - 2.25E-05	2.6E-04 - 2.6E-04	6.50 -7.10	5.61 - 6.26	-	517.1
Indeno[1,2,3-cd]pyrene	Yellow crystals	163.6 - 164	1.30E-08 - 1.33E-08	2.2E-05 - 2.30E-05	6.58 - 6.65	6.20 - 6.54	5.66E-10	349.2
Acenaphthene	White-to-beige crystals	93 - 95	0.122 - 4.02	2.42 - 7.37	3.32 - 4.49	3.59 - 5.38	7.69E-10	18.77
Acenaphthylene	Colourless crystalline solid.	89.4 - 92.5	0.893 - 4.14	2.94 - 16.1	3.55 - 4.08	3.4 - 3.83	-	30.7
Benzo[j]fluoranthene	Yellow crystals	165.2 - 166	$2.7X10^{-8}$	-	6.11	-	-	-
Chrysene	Colourless-to-beige crystals/powder	254 - 258.2	5.7E-07 - 1.07E-04	1.02E-03 - 0.017	5.01 - 7.10	3.66 - 6.90	6.21E-10	343.8
Fluorene	Small, white, crystalline plates	114.76 - 114.8	0.08 - 1.66	1.50 - 10.98	3.91 - 4.47	3.76 - 5.47	7.88E-10	15.14

1.3 Human and environmental health risks of PAHs

Many PAHs are toxic and harmful to human health as well as other organisms upon sufficient exposures; such harmful effects include carcinogenic, mutagenic and teratogenic changes (Abdel-Shafy and Mansour, 2016; Demeter *et. al.*, 2017; Baldwin *et. al.*, 2020), as well as respiratory, haematological, neurological and immunological problems (Roslund *et. al.*, 2018). The ubiquitous environmental occurrence and persistence of PAHs enhance their exposure risks to human and environmental health (Yu *et. al.*, 2018). LMW PAHs, such as naphthalene, fluorene, phenanthrene and anthracene, are known to be less toxic compared to the HMW PAHs such as fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[a]pyrene, dibenz[a,h]anthracene (Kanaly & Harayama, 2000), which are identified as environmentally recalcitrant and genotoxic to humans (Kuppusamy, *et. al.*, 2017). The environmental agencies including Environment Canada, U.S. Environmental Protection Agency (USEPA), European Union and Agency for Toxic Substances and Disease Registry have classified some PAHs (Table 1) as contaminants of concern, due to their toxic and mutagenic properties (Luster-Teasley *et. al.*, 2009; ATSDR, 2013; Marini and Frapiccini, 2013; Demeter *et. al.*, 2017; Han *et al.*, 2017; Roslund *et. al.*, 2018). Consequently, the classified PAHs are environmentally regulated and kept under routine monitoring with legislated disposal methods (Marini and Frapiccini, 2013; Richter-Brockmann and Achten, 2018).

Benzo(a)pyrene is known as the most potent carcinogen; therefore, it had been identified as an environmental indicator for PAHs (ATSDR, 2013). However, elevated HMW PAHs are of significant ecological concern and public health, due to their environmental persistence and high risk of a genotoxic effect on human (Johnsen *et. al.,* 2005; Stokes *et. al.*, 2006; Semple *et. al.*, 2007). HMW PAHs have higher toxicity than LMW PAHs (Ghosal *et. al.*, 2016), and the relative solubility of HMW PAHs in water and organic solvents

enhance their capacities to be absorbed by human and to accumulate in soil organisms (Abdel-Shafy and Mansour, 2016). This, in turn, leads to biomagnification across the trophic levels due to their lipophilic nature (Kanaly & Harayama, 2000; Semple *et al.,* 2003; Rhodes *et al.*, 2010).

The routes of human exposure to PAHs include ingestion, inhalation and dermal absorption (Chen and Liao, 2006). The short-term effects of the exposure on human health are known to depend on the route, level and length of exposure, as well as toxicity of the PAHs. The long-term (or chronic) exposure to PAHs has been known to adversely affect the functioning of some organs and systems of the human body (Abdel-Shafy and Mansour, 2016; Duca *et. al.*, 2018). Occupational studies of individuals with long-term exposure to certain PAHs revealed their carcinogenic potential on human, and harmful effects on neonates upon sufficient prenatal exposure (Weinstein *et. al.*, 2017; Duca *et. al.*, 2018). Laboratory studies involving a long-term exposure of animals to high levels of certain PAHs revealed the occurrence of lungs, stomach and skin cancer, as well as embryotoxic effects (Abdel-Shafy and Mansour, 2016). The intermediate metabolites of some PAHs are known to be more soluble, as well as toxic than the parent compounds (ATSDR, 2013). The exposure risks of PAHs to human and environmental health have contributed to the growing scientific passion for continuous studies on remediation of PAH-contaminated soils/lands.

1.4 The fate of polycyclic aromatic hydrocarbons (PAHs) in soil

The environmental fate of PAHs is mostly controlled by several factors. One important factor is their physico-chemical properties which include; aqueous solubility, hydrophobicity and molecular structures. Other factors include soil characteristics, environmental parameters such as temperature, and finally the natural loss processes occurring during

the soil-PAH interactions (Semple *et. al.*, 2007; Lima *et. al.*, 2005; Riding *et al.*, 2013). The strong affinity of PAHs to soil organic matter (SOM) is known to make soils (and sediment) the major sinks for PAHs (Yang *et. al.*, 2010; Okere and Semple 2012; Kuppusamy, *et. al.*, 2017). Also, the weak aqueous solubility and higher hydrophobicity of PAHs enhance their sorption to SOM (Lima *et al.*, 2005; Haritash and Kaushik, 2009), as well as sequestration within the soil's mineral and organic matter fractions, as the soil-PAH contact time increases (a process termed ageing) (Semple *et. al.*, 2003; Stokes *et. al.*, 2006). These make PAHs less bioaccessible and more persistent in soil, especially with higher molecular weight PAHs (Semple *et. al.*, 2004; Peng *et. al.*, 2011; Riding *et. al.*, 2013; Naseri *et. al.,* 2014; Yu *et. al.*, 2018).

In a study by Northcott and Jones (2001), the amounts of extractable ^{14}C-phenanthrene, ^{14}C-pyrene and ^{14}C-benzo[a]pyrene recovered (through organic solvents and base saponification extractions) from sterile sewage sludge-amended arable soils decreased as they aged, and became increasingly non-extractable as their molecular weights increased. Similarly, in sediments, PAHs can become increasingly persistent by strongly absorbing onto the settling particles in aquatic systems, which eventually affects their bioavailability (Lima *et. al.*, 2005). Also, in a study by Włodarczyk-Makuła (2012), the persistence and concentration of PAHs in stored biotic and abiotic sewage sludges were observed dependent on time exposition; although, a significant decrease of PAHs were found in the presence of microorganisms in sewage sludges after 10 weeks of storage.

Notably, soil-PAH interactions and soil contaminant concentrations are affected by the amount and nature of SOM and inorganic factors such as pore size and structure (Semple *et. al.*, 2001; Semple *et al.*, 2003). However, the nature of PAHs, soil properties, microbial activity, soil-PAH interactions and prevailing environmental conditions significantly influence the fate of PAHs in soil (Semple *et. al.*, 2003; Hwang and Cutright, 2002; Semple

et al., 2007; Hofman *et al.*, 2014). Also, in the soil, the loss or persistence of PAHs are determined by several physico-chemical, biological and environmental processes (Stokes *et al.*, 2006; Umeh *et. al.*, 2017), by which the labile fractions of PAHs might be removed at different rates or levels. Such environmental processes include adsorption, volatilization, photolysis, leaching and biodegradation (Figure 2) (Semple *et al.*, 2003; Stokes *et al.*, 2006; Haritash and Kaushik, 2009). However, considerable amounts of PAHs can remain or sequester within the soil materials, part of which may accumulate within the soil biota (Semple *et al.*, 2003; Stokes *et al.*, 2006).

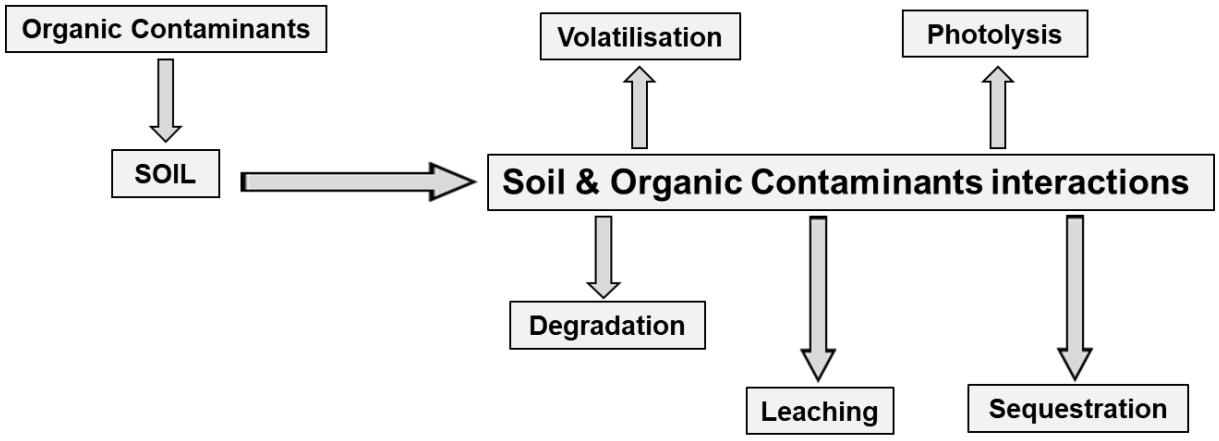

Figure 2: The natural loss processes of organic contaminants in soil (Semple *et al.*, 2003; Stokes *et al.*, 2006).

The sorption of organic contaminants has been known to occur largely by partition or dissolution into SOM (Chiou *et. al*, 1998). The major biphasic kinetic stages identified to be exhibited by PAHs during their interactions with the soil materials involves (1) an initial stage of rapid PAH sorption on to SOM as well as similar desorption, followed with a period of slower desorption (Stokes *et. al.*, 2006; Rhodes *et al.*, 2008; Abdel-Shafy and Mansour, 2016); and (2) then a gradual sorption or partitioning of the retained PAH fractions within the soil matrix over time, where they are occluded from abiotic and biotic loss processes and, consequently become less bioaccessible with increasing contact time with the soil

(Figure 3) (Northcott and Jones 2001; Semple *et al.*, 2003; Semple *et. al.*, 2004; Stokes *et. al.*, 2006; Abdel-Shafy and Mansour, 2016; Umeh *et. al.*, 2017).

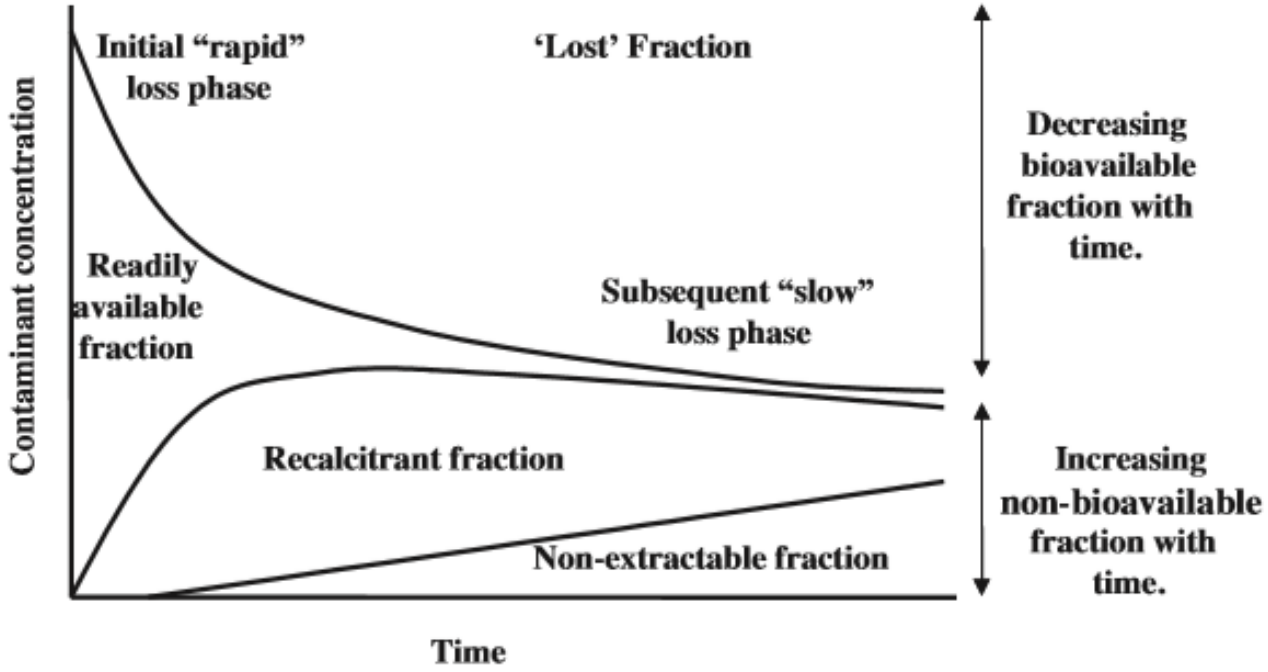

Figure 3: The loss of organic contaminants in soil. The Figure illustrates changes in organic contaminants' availability with increasing contaminants-soil contact time (Riding *et al.*, 2013).

The pattern of loss of PAHs from the soil have been described in three models (Figure 4), whereby the labile portions of the PAHs are rapidly lost while the rates, as well as extents of the loss diminish as the soil-PAH contact time increases and/or the non-bioavailable fractions remain persistent in the soil (Semple *et. al.*, 2003; Stokes *et. al.*, 2006).

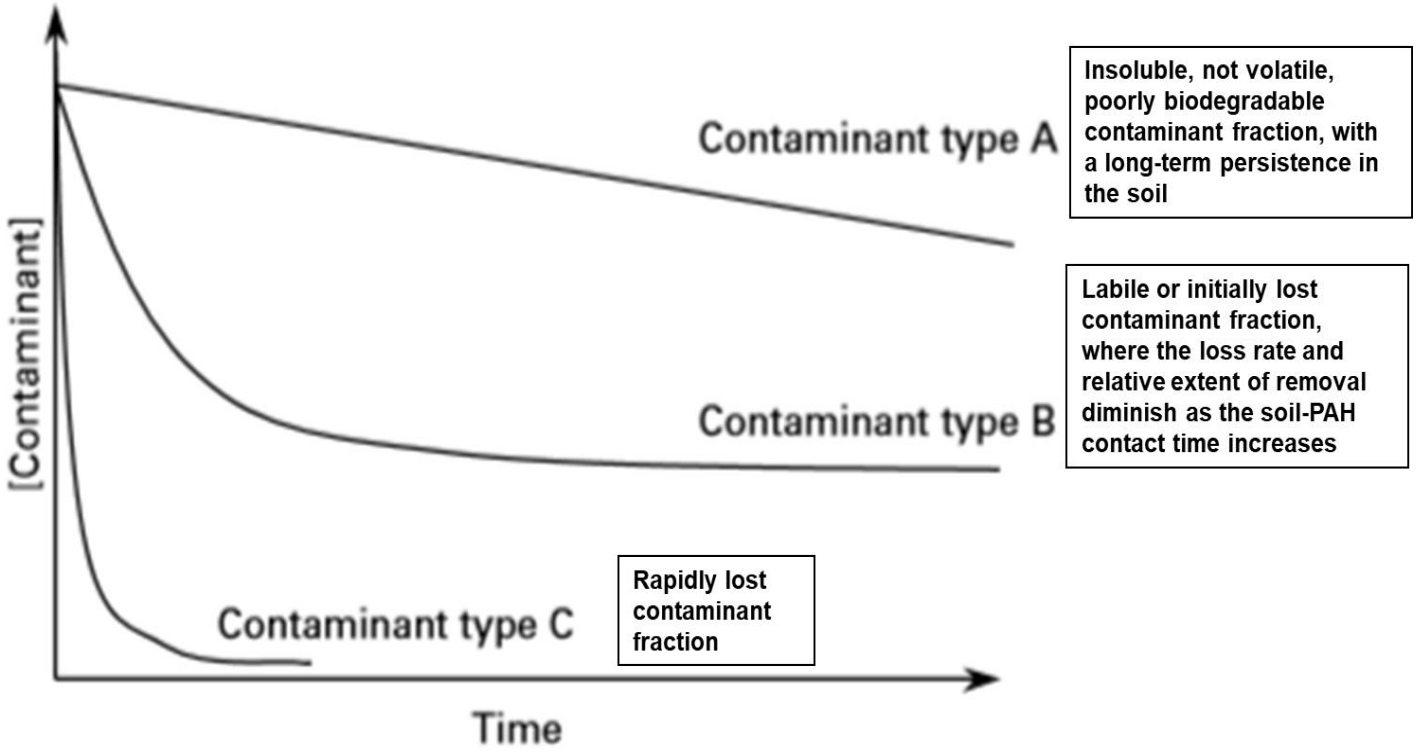

Figure 4: The loss patterns for different contaminant types in the soil; adapted from Stokes *et al.* (2006).

Furthermore, the persistent diffusion and partitioning of the adsorbed PAHs into the micro- and nano-interstitial spaces of the soil organic or mineral matters increase their desorption-resistance (Figure 5) (Okere and Semple 2012; Riding *et. al.*, 2013; Umeh *et. al.*, 2017). The adsorbed PAHs, consequently, bind strongly to SOM and become recalcitrant with reduced bioavailability (Stokes *et. al.*, 2006; Riding *et al.*, 2013; Yu *et. al.*, 2018). However, part of the PAHs that strongly bound to the soil organic materials may be chemically extractable while the remnant remains irreversibly soil-bound or non-extractable (Stokes *et. al.*, 2006; Riding *et. al.*, 2013).

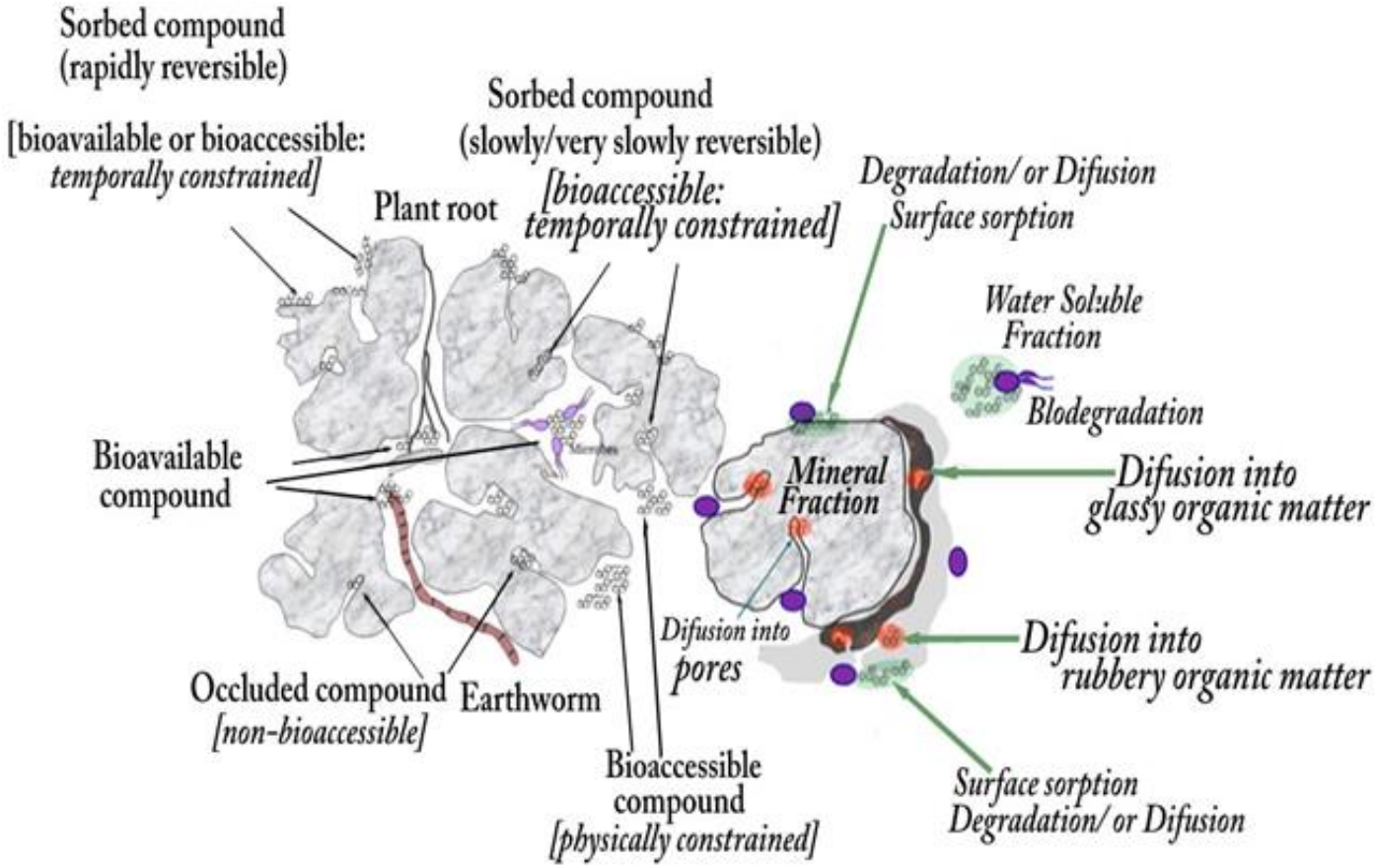

Figure 5: A summary of a contaminant's physical behaviour within the soil (Semple *et al.*, 2004).

Further, studies show the significance of determining the soil/sediment–water partitioning of PAHs, as it shows the extent of sorption of PAHs to soil/sediment and, consequently, influences the transport and fate of PAHs in the environment (Chiou *et. al*, 1998; Sun *et. al.*, 2017). The knowledge of the concentration levels, partitioning and sources of organic contaminants can be useful in determining the appropriate treatment method, for PAH-contaminated soils, and quality criteria (Sun *et. al.*, 2017). In studies, the partitioning properties of PAHs between the soil/sediment and water are evaluated and related to the octanol-water partition coefficient (K_{ow}) (Yu *et. al.*, 2009; Guo *et. al.*, 2011; Kim *et al.*, 2014; Cao *et. al.*, 2015). For instance, Cao *et. al.* (2015) determined the partition coefficients (K_p) and partition model for some PAHs by correlating the soil organic C normalized partition coefficient (K_{oc}) to the octanol–water partition coefficient (K_{ow}). The K_{oc} evaluates the tendency of PAHs to adsorb onto the SOM and the degree of the partitioning of the PAHs, while K_{ow} relates to the soil/sediment-water distribution coefficient (K_d) of the

PAHs, which is dependent on the soil/sediment organic matter or C content (Chiou *et. al*, 1998; Sun *et. al.*, 2017). Invariably, the soil/sediment organic matter or C content, PAH-contamination sources, as well as the physicochemical properties of PAHs, such as K_{ow}, can affect the partitioning of PAHs, and can be employed to predict the distribution of PAHs in the soil/sediment (Guo *et. al.*, 2011). Therefore, a good log relationship between the K_{oc} and K_{ow} suggests the accumulation/adsorption of PAHs in the soil/sediment (Guo *et. al.*, 2011).

1.5 Microbial degradation of PAHs in soil

Microorganisms play significant roles in the maintenance of ecologically balanced environments (Fantroussi and Agathos, 2005). Soil microflora consists of diverse and synergistic or antagonistic microbial communities (Megharaj *et al.*, 2011), which include bacteria, algae, fungi, protozoa, actinomycetes and archaea. They play vital roles in nutrient cycling, organic matter turnover and stabilization of soil structure (Odlare *et. al.*, 2011). Soil microorganisms have diverse capabilities to attack hydrocarbons (Maletić *et. al.*, 2013; Lang *et. al.*, 2016; Siles and Margesin, 2018), and have been employed in the degradation of PAHs (Rhodes *et. al.*, 2008; Maletić *et. al.*, 2013; Naseri *et al.*, 2014; Lang *et. al.*, 2016). Previous studies confirm that bacteria, fungi, algae and archaea have catabolic abilities that could be employed to remediate PAH contaminated soils (Lang *et. al.*, 2016; Siles and Margesin, 2018). Microbial diversity, catabolic activity, adaptability to varying environments (Fantroussi and Agathos, 2005) and ability to interact with both natural and anthropogenic compounds (Semple *et. al.*, 2001) are indicated to be due to their cellular membrane modifications, production of surface-active compounds and cellular effluxing of toxic compounds (Tyagi *et. al.*, 2011).

Bacteria are reported to be actively involved in the degradation of organic contaminants, due to their metabolic versatility (Watanabe and Baker, 2000; Haritash and Kaushik, 2009; Ghosal *et. al.*, 2016). Studies show that several species of bacteria known to degrade PAHs have been successfully isolated from hydrocarbon-contaminated soils or sediments (Haritash and Kaushik, 2009; Megharaj *et al.*, 2011; Patowary *et. al.*, 2016; Truskewycz *et al.*, 2019). Therefore, bacteria are commonly used in biodegradation studies and for remediation of PAH-contaminated soils (Ghosal *et. al.*, 2016). Various bacterial strains have been studied for their catabolic capabilities and observed with the metabolic routes required for degradation of PAHs, including the species of *Pseudomonas*, *Mycobacterium*, *Haemophilus*, *Rhodococcus*, *Paenibacillus* and *Ralstonia* (Kanaly & Harayama, 2000; Tyagi *et. al.,* 2011). *Mycobacterium* species are reported to have a good catabolic efficiency towards PAHs with up to five benzene rings and have established exceptional lipophilic surfaces for removal of bound contaminants from soil particles (Haritash and Kaushik, 2009).

The occurrence of PAHs in the soil is known to increase the carbon-to-nitrogen (C/N) ratio of the soil, and may also induce adverse environmental conditions, which consequently reduce microbial growth and activity (Lee *et al.*, 2008; Margesin and Schinner, 2001; Warr *et al.*, 2013). Similarly, the increased interactions of soil materials with PAH molecules contribute to the reduced bioavailability of PAHs (Megharaj *et al.*, 2011). Also, the nonpolar hydrophobic nature of PAHs and partitioning into the soil (Figure 4) (Lang *et al.*, 2016) reduce the loss of PAHs through the natural processes such as photolysis, leaching, volatilization, or biodegradation (Figure 1) (Stokes *et al.*, 2006; Haritash and Kaushik, 2009), and promote their accumulation in the soil (Johnsen *et al.*, 2005). The assessments of the PAH fractions that can be metabolized or transformed by microorganisms, or move freely in/on to microbial degraders (termed bioavailable fractions) are significant (Semple *et al.*, 2003; Semple *et al.*, 2004; Semple *et al.*, 2007). They help to understand the risks of

exposures and means of successful remediation of PAH-contaminated soils (Semple *et al.*, 2003).

Microbial degradation is thought to be a significant mechanism for removing PAHs from the soil due to the microbial ability to interact with organic compounds and catabolic potential to attack them (Semple *et. al.*, 2001; Couling *et. al.*, 2010; Maletić *et al.*, 2013; Lang *et. al.*, 2016; Siles and Margesin, 2018). However, the process is known to depend on the bioavailability of PAHs, indigenous microbial catabolic ability and population of relevant resident microbes (Lee *et. al.*, 2008; Couling *et. al.*, 2010; Maletić *et. al.*, 2013). Past studies have confirmed the capabilities of microbes to degrade PAHs (Rhodes *et. al.*, 2008; Lang *et. al.*, 2016) to simpler molecules (which can be utilized for microbial cells' functioning) and environmentally harmless inorganic end-products (carbon dioxide, methane and water) (Maletić *et. al,* 2013; Naseri *et. al.,* 2014). This biodegradation process is comparatively cheap and environmentally sustainable (Alvarez *et al.*, 2011; Maletić *et. al.,* 2013), such that it is applicable *in situ* (Haritash and Kaushik, 2009). The ease and cost-effective nature of biodegradation are comparatively advantageous compared to physical and/or chemical methods such as; chemical inactivation, photolysis, soil vapour extraction, combusting and soil washing, which are environmentally and economically costly (Naseri, *et. al.*, 2014; Siles and Margesin, 2018).

Successful indigenous biodegradation of PAHs depends on their microbial adaptation to mineralize the PAHs (Patowary *et. al.*, 2016). The adaptation process involves the activation of microbial catabolic activity, and studies suggest that this can be controlled by pre-exposures of indigenous microorganisms to PAHs (Semple *et. al.*, 2003; Macleod and Semple, 2006). Microbial degradation in the soils previously exposed to PAHs has shown reduced or no lag phases. For example, Kanaly & Harayama (2000) documented past studies where faster and extensive mineralisation occurred in soils freshly inoculated with

pure cultures of microorganisms isolated by enrichment from previously contaminated soils. Similarly, recent studies have reported a reduction in the lag phases and higher degradation of PAHs in soils inoculated with microbes isolated from previously contaminated sites (Megharaj *et. al.*, 2011; Patowary *et al.*, 2016; Truskewycz *et al.*, 2019). Therefore, microbial pre-exposure to PAHs can contribute to the improvement of the survival and metabolic potential of PAH-degrading microorganisms (Couling *et al.*, 2010; Megharaj *et al.*, 2011). In addition, a greater level of microbial activity and degradation of PAHs have been reported in soils with increased PAHs' interaction (Rhodes *et al.,* 2008). However, a threshold of PAH concentration or specific time is required for catabolic induction in the resident microbes possessing the degradation potentials (Semple *et al.*, 2001).

Furthermore, the population size of the microbial degraders has a significant influence on biodegradation efficiency (Lee *et. al.*, 2008; Maletić *et. al.*, 2013). Research suggests that the absence or reduced abundance of microbial degraders in soil may be caused by an introduction of either highly concentrated and specifically pre-adapted exogenous pure microbial strain or a consortium and/or genetically engineered microbial variants (a process termed bioaugmentation) (Ruberto *et. al.*, 2009; Han *et. al.*, 2017; Leal *et. al.*, 2017). These are to enhance mineralisation, especially during the lag periods, and/or to extend the range of contaminants to be metabolized (Atlas, 1995).

The microbial inoculants possess relevant metabolic activities, and the soil properties may be modified to enable the survival and expression of the metabolic abilities of the inoculants (Atlas 1995; Tyagi *et. al.*, 2011). The genetically engineered variants possess improved enzymatic and/or metabolic pathways (Megharaj *et. al.*, 2011), and the mechanism involves packaging the relevant genes into the vectors that are conjugated into indigenous bacteria (Tyagi *et. al.*, 2011). However, the vector introduction approach is indicated to be affected by the microbial cells' fitness and extra energy demand, as well as

the risk of conjugating undesirable microorganisms (Megharaj *et. al.*, 2011). Generally, both aerobic and anaerobic degradation of PAHs are identified in studies, with aerobic catabolism as the most common form of microbial degradation (Kanaly & Harayama, 2000; Ghosal *et. al.*, 2016) (Figure 6).

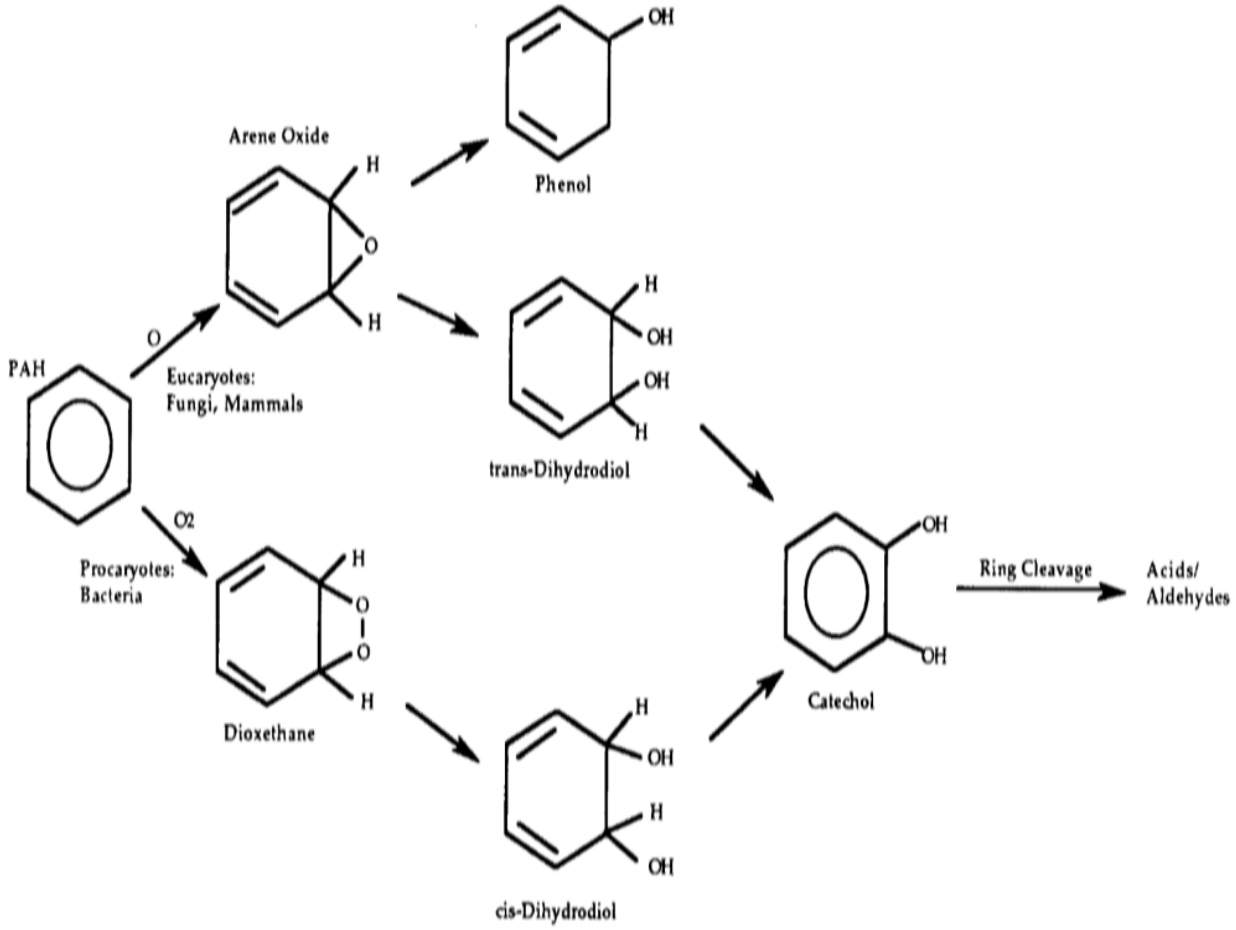

Figure 6: Microbial Degradation of polycyclic aromatic hydrocarbons (PAHs) in soil (obtained from Wilson and Jones, 1993).

Previous studies suggest that the biochemical pathway for aerobic biodegradation shows the biodegradability of LMW PAHs compared to HMW PAHs (Couling *et. al.*, 2010; Demeter *et. al.*, 2017). This is due to the greater suitability of LMW PAHs as a sole C source (Figure 7) for microbial degraders (Couling *et. al.*, 2010). However, the presence of more easily utilizable or enzymatically degradable alternative C-sources for microbial degraders can limit the biodegradation of PAHs (Abdel-Shafy and Mansour, 2016). Furthermore, the rates and extents of degradation of different PAHs can be different due to their physico-chemical properties, which include molecular size and structure, hydrophobicity as well as aqueous solubility (Couling *et. al.*, 2010).

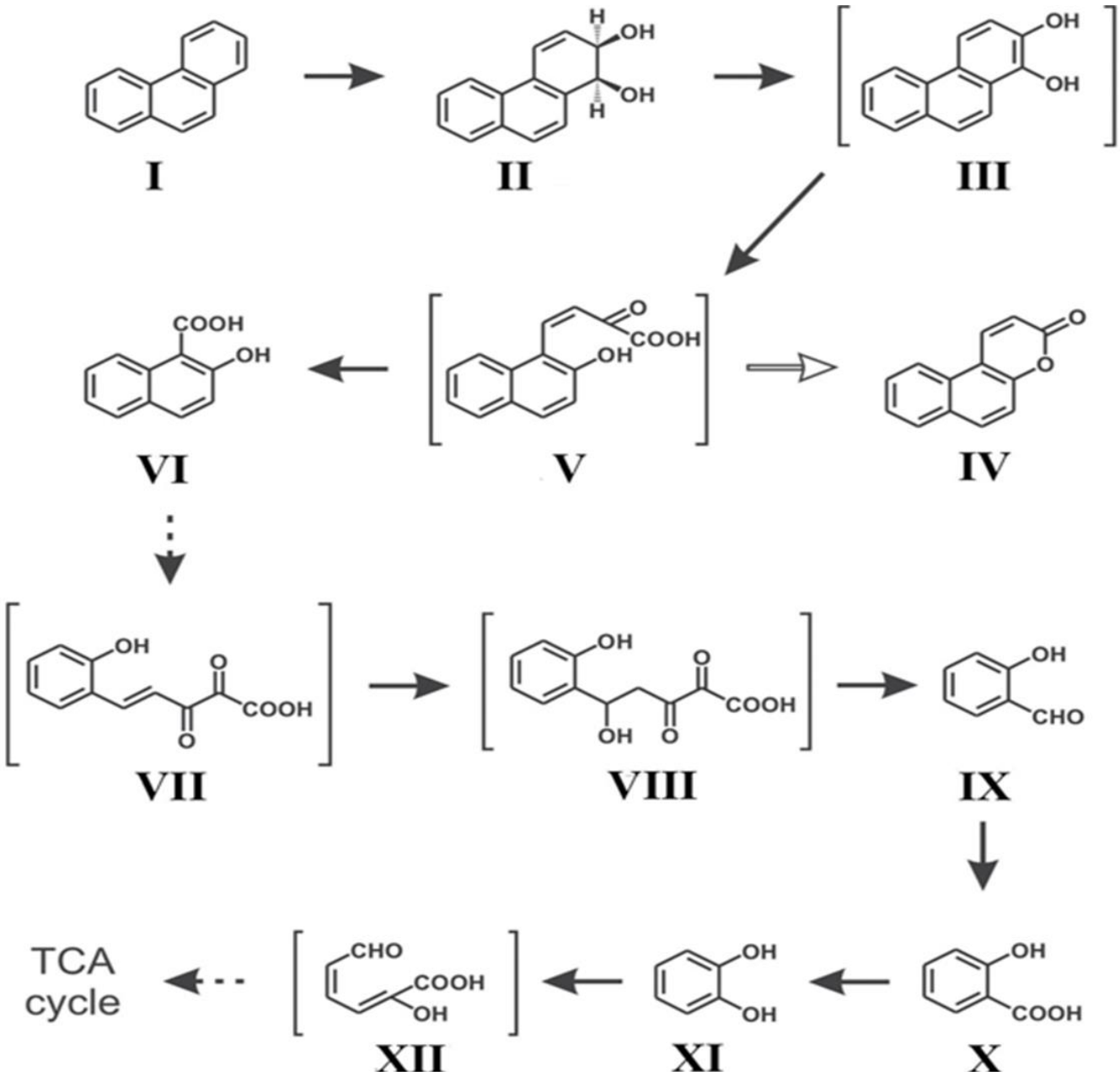

Figure 7: Proposed biodegradation pathway for phenanthrene by *Ochrobactrum* sp. strain PWTJD (obtained from Ghosal *et al.*, 2016).

1.6 Soil physicochemical characteristics and biodegradation of PAHs

Soils vary in terms of physical and chemical properties due to their parent materials and prevailing environmental conditions such as temperature, pH, moisture content, available nutrients and biota. The soil characteristics influence the activity of microbial populations and bioavailability of organic contaminants (Rhodes *et. al.*, 2008; Haritash and Kaushik, 2009). Also, microbial distribution and activities are significantly influenced by soil

environmental conditions and organic matter (Watanabe and Baker, 2000; Semple *et. al.*, 2001). Microbial activity and biomass promptly respond to environmental stress, therefore, they are considered useful tools for assessing the effects of contaminants on soil microbial community (Boucard *et. al.*, 2008).

Biodegradation of organic contaminants has been proven effective at sites where environmental conditions favour optimum microbial growth and expressions of degradation-associated enzyme activities. However, biodegradation efficiency depends on the soil physicochemical conditions, microbial ecology (including microbial diversity, the population size of relevant microorganisms and prior exposure to contaminants), as well as the nature, concentrations and bioavailability of the contaminants (Lee *et. al.*, 2008; Maletić *et. al.*, 2013; Ghosal *et. al.*, 2016) (Figure 8).

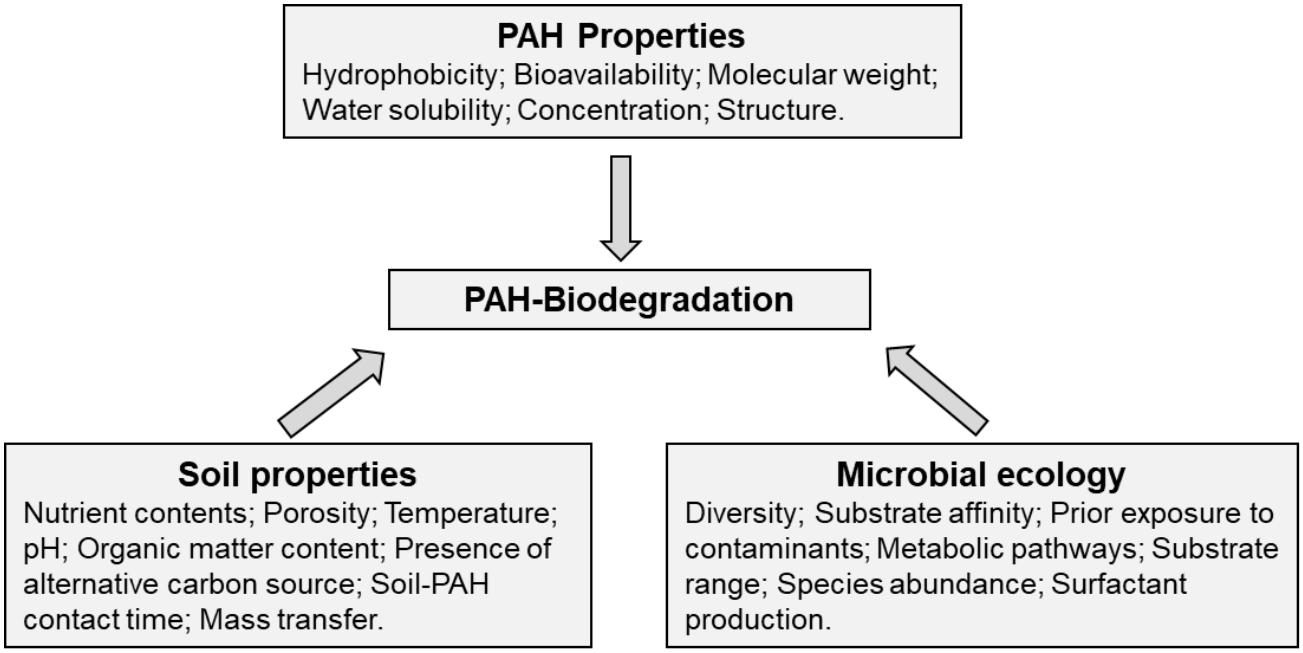

Figure 8: Factors influencing biodegradation of polycyclic aromatic hydrocarbons (PAHs) in soil (adapted from Ghosal *et al.*, 2016).

The recommended microbial population required for successful and optimal biodegradation of organic contaminants are known to be between 10^4 to 10^7 Colony Forming Units (CFUs), while a lower than 10^3 CFUs per gram of soil indicates toxicity occurrence, which might be due to the concentration of the present contaminant (Maletić *et. al.*, 2013). Microbial population, activity and diversity are known to be essential parts of soil quality, and there are optimal environmental conditions required for microbial growth and degradation of hydrocarbons (Maletić *et. al.*, 2013) (Table 3).

Table 3: Optimal conditions for microbial growth and hydrocarbon biodegradation (adapted from Maletić *et al*., 2013).

Parameters	Microbial growth	Hydrocarbon (HC) biodegradation
Water holding capacity	25-28	40-80
pH	5.5-8.8	6.5-8.0
Temperature (^{o}C)	10-45	20-30
Oxygen (air-filled pore space)	10%	10-40 %
C:N:P	100:10:1(0.5)	100:10:1(0.5)
Contaminants	Not too toxic	5-10% of dry weight of soil
Heavy metals	<2000 ppm	<700 ppm

Environmental factors (such as temperature, pH, moisture content, available nutrients) might be relevantly customized to stimulate soil microbial activity and enhance the bioaccessibility of PAHs (Semple *et. al.*, 2004). For example, the desorption rates of contaminants from soil particles can be increased by thermal or chemical pre-treatment of contaminated soils before microbial remediation (Haritash and Kaushik, 2009). Also, the addition of higher concentrations of organic solvents can increase the mass transfer rate of organic contaminants in soil (Haritash and Kaushik, 2009). Similarly, in a bioaugmentation approach, the physicochemical conditions of the soil may need to be modified to support the survival of inoculant(s) and enhance their activity for optimal degradation of contaminants (Tyagi *et. al.*, 2011).

Temperature is another example of an environmental factor that has been identified to have a profound effect on the rates of organic matter decomposition in soil, as well as microbial growth, activity and metabolism of PAHs (Liu *et. al.*, 2009). An increase in temperature has been shown to affect the chemical composition and/or enhance the solubility of PAHs, which consequently increases their bioavailability (Tyagi *et al.*, 2011). The adsorption of contaminants to soil particles decreases as the temperature rises, and this makes the organic contaminants more bioavailable (Liu *et. al.*, 2009). In a study by Siles and Margesin (2018), soil fertilization with N-P-K was accompanied by temperature

increase which led to higher rates of biodegradation of total petroleum hydrocarbon (TPH) compared to natural attenuation. The soil–water partition coefficient is indicated to decrease with increasing temperature, which consequently increases the diffusion rate of contaminants through liquid pores and SOM (Haritash and Kaushik, 2009). The partition-coefficient of PAHs is known to decrease by 20 - 30 % at every 10 ^{o}C rise in temperature (Haritash and Kaushik, 2009), while the maximum rates of oxidation of PAHs and optimum bacterial growth are about 30 ^{o}C (Haritash and Kaushik, 2009). However, extremely high temperatures can negatively affect soil microbial community structure (Tyagi *et. al.*, 2011), and induce biotransformation of some organic contaminants to intermediate products that are often toxic and recalcitrant than the parent compounds (Ghosal *et. al.*, 2016).

1.7 Addition of nutrients to soils

Contaminated soils are often characterized by a low amount of organic matter (Lee *et al.*, 2008), which is known to be significant for improving soil quality, as it aids the retention of nutrients and water in soil (Smebye *et. al.*, 2016). Also, the presence, compositions and/or concentrations of organic contaminants in soil usually prompt adverse environmental conditions that reduce indigenous microbial activities (Lee *et. al.*, 2008). Addition of sources of nutrients to the soil and/or modifications of environmental parameters (such as temperature, pH and moisture content) have been known to contribute to overcoming rate-limiting factor(s) of PAH biodegradation (Leal *et. al.*, 2017). The addition of nutrients to PAH contaminated soils have been proven to enhance microbial degradation (Hollender *et. al.*, 2003).

1.7.1 Soil amendments with inorganic nutrients

Previous studies show that soil amendment with correct amounts of growth-limiting nutrients (a process called biostimulation) can stimulate indigenous microbial growth and degradation activity (Ruberto *et. al.*, 2009; Scotti *et. al.*, 2013, Scotti *et. al.*, 2014). The absence of fresh substrates in the soil can influence the microbial biomass carbon (Joergensen and Wichern, 2018). However, the addition of inorganic nutrients enhances microbial growth and/or activity (Ravanipour *et. al.*, 2015). Due to their significance in microbial activity and cell proliferation, the mineral nutrients commonly used for biostimulation are known to be rich in essential nutrients, such as nitrogen, phosphorus, potassium and carbon (Ruberto *et. al.*, 2009; Naseri *et. al.*, 2014). Such minerals include ammonium, urea, different types of phosphates (K_2HPO_4 and $MgNH_4PO_4$) and nitrates (KNO_3, $NaNO_3$, NH_3NO_3) (Megharaj *et. al.*, 2011; Naseri *et. al.*, 2014). In a study by Ruberto *et. al.* (2009), the addition of mineral N and P to PAH-contaminated Antarctic soil increased its total heterotrophic aerobic and hydrocarbon-degrading bacterial counts. Their results on the biostimulation study further improved microbial degradation of hydrocarbon-contaminants compared to their results on inoculation with hydrocarbon-degrading bacteria. Similarly, in a study by Siles and Margesin (2018), soil fertilization with inorganic (N-P-K) nutrients coupled with increased temperature, stimulated indigenous microbial activity and higher rates of biodegradation of total petroleum hydrocarbons compared to natural attenuation.

Although biostimulation is a viable and effective method of biodegradation, a variety of factors need to be evaluated to assess the effectiveness of the biostimulation process, and these include soil properties, prevailing environmental conditions and physico-chemical characteristics of organic contaminants in the soil (Semple *et. al.*, 2001; Semple *et. al.*, 2003; Stroud *et. al.*, 2007). Furthermore, the long term application of inorganic nutrients

often lead to or increase soil acidification, which tends to influence soil quality and microbial community (Savci, 2012; Xu *et. al.*, 2014), and consequently reduce the formation of organic matter in the soil (Sapp *et. al.*, 2015). Similarly, soil acidification can influence soil biota and biogeochemical processes (Xu *et. al.*, 2014), which can affect microbial growth and degradation activities. Other than the direct impacts on soil, the environmental emission of nitrous oxide (N_2O) may increase in acidified soils, and thereby contributes to wider scale impacts like climate change (Xu *et. al.*, 2014; Ning *et. al.*, 2017). Also, the mining, extraction and processing of P rock are environmentally damaging, while the transportation of P-based fertilizers is known to be environmentally costly (Sapp *et. al.*, 2015).

1.7.2 Soil amendments with organic materials

The incipient agricultural and industrial developments have led to an increased generation of different organic wastes (García-Sanchez *et. al.*, 2015). In the United Kingdom, around 14 million tonnes of food wastes were reported to be generated each year, and about 8 million tonnes of biodegradable wastes are being disposed of the landfill yearly (Nicholson *et. al.*, 2017). These enormous wastes can have a negative impact on the soil and environmental health if they are not adequately managed (Alburquerque *et. al.*, 2012a). The legislative trends in the field of wastes management have been based on integrated management that involves adding value to the wastes, which had led to the sustainable recycling of the biodegradable wastes as soil biofertilizers for agricultural and/or ecological improvement (Alburquerque *et. al.*, 2012a).

The management of organic wastes, as soil amendments, has reinforced their environmental sustainability as sources of organic C and nutrients (Martins *et. al.*, 2018) for the improvement of soil physico-chemical properties and biological processes (microbial

activity, biomass, community structure and diversity) (Rigby and Smith, 2013; Insam *et. al.*, 2015; Gómez-Brandón *et al.*, 2016Ning *et. al.*, 2017). A range of different wastes that are locally available for soil amendments include agricultural wastes, combustion by-products (from agricultural wastes), mineral originated products, anaerobic digestion products (of farm wastes, industrial wastes, municipal and sewage sludges) (Table 4).

Table 4: A range of waste types/organic amendments that could be applied to soils (Fernández-Delgado Juarez *et. al.*, 2013; Insam *et. al.*, 2015; Sapp *et. al.*, 2015; Papafilippaki *et. al.*, 2015; García-Sánchez *et. al.*, 2015b; Gómez-Brandón *et. al.*, 2016; Monlau *et. al.*, 2016; Tampio *et. al.*, 2016; Demiraj *et. al.*, 2017; Nicholson *et. al.*, 2017; Gao, 2019; Yan *et. al.*, 2019; Larkin, 2020).

Waste types/soil amendments	**Examples**	**Potential benefits and risks associated with their use in soil remediation**	**Regulations and guidelines on their use.**
Agricultural wastes	straws, manure, compost	- supply large amounts of organic matter and a variety of micronutrients / essential trace elements such as copper (Cu), zinc (Zn), lead (Pb) and cadmium (Cd) to soil; - improve soil health through their favourable effects on soil properties	- They can be potential risks to human health and the environment due to the presence of heavy metals; - They must be properly treated to avoid harmful effects on soil properties and reduce environmental or health hazards associated with raw wastes.
Combustion by-products from agricultural wastes	biochar, plant biomass ash	- High pH - Porous in structure - Rich in phosphorus (P), potassium (K) and other nutrients - Good material for remediation of degraded soil and improvement in soil structure - Biochar reduces nitrogen (N) loss, by reducing both N_2O and NH_3 emissions - Biochar enhances the adsorption of NO_3−N and effectively reduces nitrogen (N) leaching.	- As of present, there is no legislation regarding soil application of biochar and plant biomass ash.
Mineral originated products	zeolite, apatite, lime, bentonite, polyacrylamide	- Increase most nutrient element concentrations and microbial respiration - Bentonite and polyacrylamide reduce nitrogen (N) loss; - Bentonite reduces NH_3-N volatilization, N_2O-N emission and N leaching	- As of present, there is no published legislation regarding soil application of mineral originated products.
Anaerobic digestion products of farm wastes, industrial wastes, municipal wastes and sewage sludge	anaerobic digestates (AD)	- Vital source of organic matter, considerable amounts of macronutrients such as nitrogen (N), phosphorus (P) and potassium (K), as well as micro-nutrients such as copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) - Digestates enhance soil organic matter (SOM) formation and biological activities, due to their significant amount of residual organic C and N compounds - Excessive application causes soil acidification, nitrate leaching and emission of nitrous oxide (N_2O).	- Certain quality characteristics which involve biological stability and hygiene must be satisfied - Amount of anaerobic digestate (AD) to be applied should match the soil's nitrogen (N) deficiency

Several studies have documented the positive effects of soil amendment with organic materials on soil's physico-chemical and biological properties (Alburquerque *et. al.*, 2012a;

Fernández-Delgado Juárez *et. al.*, 2013; García-Sánchez *et. al.*, 2015a), as well as biodegradation of organic contaminants (Haritash and Kaushik, 2009; Kallenbach and Grandy, 2011; Odlare *et. al*., 2011; Bougnom *et. al.*, 2012; Das and Dkhar, 2012; Koszel and Lorencowicz, 2015; Heijboer *et. al.*, 2016; Lukić *et. al.*, 2016; Han *et. al.*, 2017). In Gaind *et. al.* (2006), the soil amended with P-enriched organic materials showed improved biological activities compared to similar soils amended with inorganic nutrients. PAH-contaminated soils amended with spent mushroom composts showed higher available nutrients, enhanced microbial growth (with PAH-degrading efficiency of 82 %) and reduced toxicity (Haritash and Kaushik, 2009). In a study by Lukić *et. al.* (2016), the soils amended with buffalo manure, activated sewage sludge, mixtures of food and kitchen wastes and mixtures of fruit and vegetables wastes significantly decreased the concentrations of low molecular weight (LMW) PAHs, high molecular weight HMW PAHs, and total PAHs in soils, while LMW PAHs were observed with the highest dissipated contaminants.

In another study by Kallenbach and Grandy (2011), soils amended with animal and plant manure-based organic materials showed increased microbial biomass C and N than similar soils amended with inorganic fertilizers. Similarly, a study by Das and Dkhar (2012) showed a positive response in the soil physicochemical properties, microbial populations and biomass C following soil amendments with plant compost, vermicompost, farmyard manure and integrated plant compost. Heijboer *et. al.* (2016) reported an increase in the soil microbial biomass and activity, along with improved microbial community composition in the soils amended with organic nutrients. In a study by Han *et. al.* (2017), the soils amended with wheat stalk, mushroom cultivation substrate waste and cow manure showed accelerated dissipation of aged PAHs, with a significant increase in the abundance of PAH-degrading bacterial genes and improved microbial community structure.

Pyrolyzed agricultural residues (also referred to as biochars) have been observed to improve soil quality (Smebye *et. al.*, 2016) as well as stimulate microbial growth and degradation of PAHs (Ogbonnaya and Semple, 2013; Quilliam *et. al.*, 2012; Smebye *et. al.*, 2016) due to their considerable amounts of macro- and micro-nutrients (Zielińska *et. al.*, 2015) and high adsorptive capacity (Zielińska, and Oleszczuk, 2016). The higher aromatic C compounds in biochars further differentiate them from the other types of organic matters (Zielińska *et. al.*, 2015). Also, the immobilizing mechanisms of biochars are known to include alkalization (due to its alkaline pH) (Zielińska *et. al.*, 2015), enhanced ion exchange capacity as well as physical sorption and precipitation (Pukalchik *et al.*, 2018). The ability of the pyrolyzed carbon materials to adsorb onto PAHs has been established (Rhodes *et. al.*, 2012), and the adsorption of PAHs have been confirmed to reduce the exposure and associated risks of PAHs exposure (Xiong *et. al.*, 2017).

Biochar-amended soils have been observed with ameliorated properties, increased nutrients retention, pH increase (in acidic soils), as well as positive changes in microbial population and community structure (Lehmann *et. al.*, 2011; Xu *et. al.*, 2014; Zielińska *et. al.*, 2015; Smebye *et. al.*, 2016; Zielińska, and Oleszczuk, 2016; Pukalchik *et al.*, 2018). Also, environmental emission was observed reduced due to biochar's increased C sequestration in the soil (Lehmann *et. al.*, 2011; Monlau *et. al.*, 2016; Smebye *et. al.*, 2016). The positive effects of soil amendment with pyrolyzed organic materials on soil's physico-chemical and biological properties as well as biodegradation of PAHs were reported in studies (Kallenbach and Grandy 2011; Das and Dkhar, 2012; Ogbonnaya and Semple, 2013; Heijboer *et. al.*, 2016; Smebye *et al.*, 2016; Han *et.. al.*, 2017; Lukić *et. al.*, 2017; Sadegh-Zadeh *et. al.*, 2018). In a study by Xu *et. al.* (2014), biochar addition to soil (with a pH of 4.48) increased the soil pH, C and N contents, as well as C-to-N ratio along with the soil cation exchange capacity, while the nitrification and denitrification processes were also stimulated.

In Lukić *et. al.* (2017), the soils amended with centrifuged activated sewage sludge removed priority-listed PAHs which include pyrene, relative to similar unamended soils. Similarly, in Bao *et. al.* (2020), soil amendment with mixtures of biochar and mushroom residue, as well as biochar and corn straw enhanced the removal of PAHs and increased the soil C content compared to the addition of biochar alone. Also, sewage sludge-derived biochar contains significant amounts of micro- and macro-elements which increase its nutrient value and optimize soil microbial activity following addition to soil (Zielińska, and Oleszczuk, 2016). In Sadegh-Zadeh *et. al*. (2018), the addition of biochars to calcareous sandy soils increased the soil pH and EC, while the addition of mixtures of biochars and compost increased the amounts of N, P and K contents, as well as the level of micronutrients (such as iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn). Studies on biochar-amended soils with enhanced sequestration of organic contaminants were also reviewed in Ogbonnaya and Semple (2013).

However, biochar-sorbed PAHs become less bioaccessible with increasing contact time (Xiong *et. al.*, 2017), and consequently cause accumulation and long-term persistence of PAHs in soil (Quilliam *et al.*, 2012; Ogbonnaya and Semple, 2013). For example, in a study by Quilliam *et. al.* (2012), the soils amended with rice husk and wood biochars were observed with reduced degradation of PAHs compared to similar unamended soils. Biochar addition to soil may influence the soil microbial diversity through increased carbon-flux and dissolved organic matter (Smebye *et. al.*, 2016). In Pukalchik *et. al.* (2018), soil amendment with biochar was observed less effective in immobilizing Cu, lead (Pb) and Zn (trace elements) compared to soil amendment with WA (due to its high soluble concentrations of carbonates and phosphates). Also, the addition of sewage sludge-derived biochar to soil can introduce considerable amounts of trace metals (due to pyrolysis) (Zielińska, and Oleszczuk, 2016).

1.7.3 Soil amendments with renewable bioenergy residues

Bioenergy residues consist of higher amounts of organic matter and nutrient elements, and they are inexpensive and ecologically friendly compared to chemical fertilizers. Therefore, their addition to soil, as sources of nutrients, has gained widespread acceptance as economically feasible and environmentally sustainable, compared to conventional chemical fertilizers (Tiwary *et. al.*, 2015; Ning *et. al.*, 2017). In studies, the addition of various bioenergy residues to soil has proven to improve soil conditions and/or physicochemical properties (such as water holding capacity, aeration, pH, and ion exchange capacity) (Medina *et. al.*, 2006; Pezzolla *et al.*, 2012). These residues further stimulate microbial activities (Lee *et al.*, 2008), microbial biomass, basal respiration and enzyme activities (García-Sánchez *et al.*, 2015a & García-Sánchez *et. al.*, 2015b); and also aide the recycling of nutrients that would have been lost from the soil system (Medina *et. al.*, 2006; Pezzolla *et. al.*, 2012).

Recurrent soil amendments with bioenergy residues can ensure a perpetual supply of essential nutrients and maintenance of soil nutrients-balance (Alburquerque *et. al.*, 2012a). Consequently, these will maintain the soil health/quality (Alburquerque *et. al.*, 2012a) and improve the physicochemical characteristics of the soil, with a positive impact on the soil-contaminant interactions (Haritash and Kaushik, 2009). The sustainable development of soil amendment with bioenergy residues can bring about additional environmental advantages which include: the conservation of natural resources used in the manufacture of mineral fertilizers (Alburquerque *et. al.*, 2012a); the sustainability of renewable energy production (Gómez-Brandón *et. al.*, 2016); and the mitigation of environmental emissions of greenhouse gases (GHG). These GHGs include such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) (Whelan *et. al.*, 2010; Pezzolla *et. al.*, 2012). Soil amendment with bioenergy residues will also reduce the release of leachates to surface

and underground waters (Holm-Nielsen *et. al.*, 2009; Alburquerque *et. al.*, 2012a) and the environmental emissions are peculiar to land-fill disposal sites (Palmiotto *et. al.*, 2014).

However, excessive addition of bioenergy residues to the soil can cause some adverse ecological/environmental effects. Some of them are unfavourable changes in the soil pH (Cuske *et. al.*, 2016) and salinization, which could reduce microbial cell proliferation and degradation activities, an increase in emissions of GHG (Whelan *et. al.*, 2010; Pezzolla *et. al.*, 2012) and, leaching of nutrients and organic matter (Holm-Nielsen *et. al.*, 2009; Whelan *et. al.*, 2010). Other consequences are the occurrence of some negative effects on soil properties (such as aeration and structures) (Liu *et. al.*, 2009), especially with excessive N loads (Insam *et. al.*, 2015), which could reduce microbial growth and degradation activities. Also, the accumulation of heavy metals (known to be present in trace amounts in the residues) (Ning *et. al.*, 2017), which can be toxic to soil biota and/or transcend the trophic levels can be a consequence of excessive soil amendment with bioenergy residues (Cuske *et. al.*, 2016). The absence of significant effect and observation of adverse effects of soil amendment with bioenergy residues have been reported (Lee *et. al.*, 2008).

1.8 Additions of anaerobic digestate (AD) and wood-ash (WA) to soil and their effects on biodegradation of PAHs

Intensive (pastoral and arable) farming systems lead to enormous generation and accumulation of biodegradable wastes (Holm-Nielsen *et al.*, 2009; Alburquerque *et. al.*, 2012a; Gómez-Brandón *et. al.*, 2016). Sustainable recycling of wastes for energy recovery (Holm-Nielsen *et. al.*, 2009) was realized in a bid to reduce the biodegradable fraction of wastes to land-fills (Whelan *et. al.*, 2010). However, the increased on-farm and industrial productions of renewable energy (biogas), from the anaerobic digestion of biodegradable wastes, have led to a huge generation and accumulation of residual wastes, also called

anaerobic digestate (AD) (Insam *et. al.*, 2015; Tiwary *et. al.*, 2015). The management and disposal of AD have become increasingly challenging (Odlare *et. al.*, 2011; Quakernack *et. al.*, 2012) due to their potential risks of adverse environmental emissions (Holm-Nielsen *et. al.*, 2009; Gómez-Brandón *et. al.*, 2016).

1.8.1 Anaerobic digestate (AD) and its addition to soil

AD can be a vital source of organic matter (García-Sánchez *et al.*, 2015b; Tiwary *et. al.*, 2015), considerable amounts of macronutrients including N, P and K, as well as micro-nutrients (Sapp *et. al.*, 2015) such as copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) (Vaneeckhaute *et al.*, 2019). Also, much of the N content of the AD is in ammoniacal forms as ammonium (NH_4^+) (Alburquerque *et. al.*, 2012b; Möller and Müller, 2012) and ammonia (NH_3) (Whelan *et. al.*, 2010). AD addition enhances SOM formation and biological activities, due to its significant amount of residual organic C (Insam *et al.*, 2015; García-Sánchez *et. al.*, 2015b) and N compounds (Whelan *et al.*, 2010; Möller and Müller, 2012; García-Sánchez *et. al.*, 2015a). Studies have documented the positive effect of soil amendment with AD on soil properties and microbial activity (Alburquerque, *et. al*, 2012a; Walsh *et. al.*, 2012; Johansen *et. al.*, 2013; García-Sánchez *et. al.*, 2015a; Koszel and Lorencowicz 2015; Fernández-Bayo *et. al.*, 2017).

In studies, soil amendment with AD increased the soil microbial biomass as well as N and P contents, which are significant for optimal biodegradation of PAHs (Johansen *et. al.*, 2013; Fernández-Bayo *et. al.*, 2017). Alburquerque, *et. al.* (2012a) reported a rapid development of microbial activity following the addition of AD to the soil. Similarly, significantly improved microbial and enzymatic activities, as well as biomass and community structure have been reported in AD-amended soils (García-Sánchez *et. al.*, 2015a; García-Sánchez *et al.*, 2015b). Significantly improved microbial activity, biomass

and physiological diversity have been reported both within a short application, and after a long-term soil amendment with AD (García-Sánchez *et. al.*, 2015a). Similarly, in a study by Fernández-Delgado Juárez *et al.* (2013), improved microbial communities were reported in AD-amended soils, while in Walsh *et. al.* (2012), consistently higher microbial growth was observed in AD-amended soils compared to similar unamended soils. All these positive microbial responses and/or microbial community development following AD addition to soil are indications of possible enhanced biodegradation of PAHs in the soils amended with renewable bioenergy residues.

However, changes in soil pH and EC have been noticed in AD-amended soils but the pH changes are known to stabilize around the pH value of similar unamended soils (Bougnom *et. al.*, 2012; Koszel and Lorencowicz 2015) due to the soil buffering capacity (Alburquerque *et. al.*, 2012a). The increase in soil pH following the soil amendment with organic materials is known to contribute to indigenous biostimulation in soil (Mahmood *et al.*, 2003; Jokinen *et. al.*, 2006). In practice, the amount of AD to be added to soil should match the N deficiency of the soil (Fernández-Delgado Juarez *et al.*, 2013; Gómez-Brandón *et al.*, 2016). This will help to mitigate excessive environmental emissions, such as excess nitrates which can erode to surface waters, leach to underground waters, as well as denitrify into gaseous form and emit as GHG (Demeyer *et al.*, 2001; Fernández-Delgado Juarez *et al.*, 2013; Gómez-Brandón *et al.*, 2016; Monlau *et al.*, 2016).

On the other hand, excessive addition of AD can negatively affect the rate of ammonia oxidation in the soil, due to the ammonia-oxidising inhibitory compounds found in AD (depending on the application dose) (Gómez-Brandón *et al.*, 2016). Therefore, particular quality requirements or characteristics of the AD relating to stability and hygiene should be satisfied before its application to soil in order to ensure the maintenance of the soil quality (Alburquerque *et al.*, 2012a; Pezzolla *et al.*, 2012; Fernández-Delgado Juarez *et al.*, 2013).

Figure 9: A picture showing application of anaerobic digestate (AD) to soil.

1.8.2 Wood-ash and its addition to soil

Wood-ash (WA) (a wood combustion residue) is also a source of macro- and micronutrients (Richard *et al.*, 2018). The macronutrients include calcium (Ca), magnesium (Mg), K and P (Pukalchik *et al.*, 2018). P is known to be present in variable amounts (Kuba *et al.*, 2008), while N is completely absent or present in a negligible amount due to volatilization during combustion (Demeyer *et al.*, 2001; Fernández-Delgado Juarez *et al.*, 2013; Köster et al., 2014; García-Sánchez *et al.*, 2015a). The micro-nutrients in WA include Fe, Mn, Zn, and Cu (Kuba *et al.*, 2008; Fernández-Delgado Juárez *et al.*, 2013; Richard *et al.*, 2018).

Figure 10: A picture showing a small quantity of wood-ash (WA) inside a container.

WA has been considered a good supplement to soil due to its recyclable nutrients and their concentrations (Perucci *et al.*, 2006; Fernández-Delgado Juárez *et al.*, 2013; Pukalchik *et al.*, 2018), as well as alkalinity (Bougnom and Insam, 2009; Bougnom *et al.*, 2010; Fernández-Delgado Juárez *et al.*, 2013; Pukalchik *et al.*, 2018). However, soil amendment with WA can be limited due to its low N and C contents, as well as high pH, which may influence the soil microbial activity (García-Sánchez *et al.*, 2015a).

Studies documented the positive effects of WA on soil physico-chemical properties, as well as microbial activity and biomass (Bougnom and Insam 2009; Insam *et. al.*, 2009; Bougnom *et al.*, 2010; Fernández-Delgado Juárez *et al.*, 2013; García-Sánchez *et al.*, 2015a; Nabeela *et al.*, 2015). In studies, WA addition to soil increased the soil pH and available nutrients, as well as stimulated microbial activity (García-Sánchez et al., 2015a; Fernández-Delgado Juárez *et al.*, 2013). García-Sánchez *et al.* (2015a), observed no impact on microbial activity following WA addition to soil; however, some chemical

variables of the soil were positively affected. In a study by Bougnom and Insam (2009), improved microbial community and activity were observed in the soils amended with the compost-AD mixture to 8 % of WA, while similar soils amended with the compost-AD mixture to 16 % of WA showed improved microbial community without enhanced microbial activity. The stimulatory effect of WA on microbial activity has been reported to be demonstrated at lower amounts or concentrations (Nabeela *et al.*, 2015).

WA has an acid-neutralizing effect due to its high pH (8 to 13) and causes a short period of increase in soil pH after addition (Bougnom *et al.*, 2010; Fernández-Delgado Juarez *et al.*, 2013; García-Sánchez *et al.*, 2015a; García-Sánchez *et al.*, 2015b). WA is known to affect soil microbial processes through changes in soil physico-chemical properties (García-Sánchez et al., 2015a). For example, enhancement of soil microbial activity and biomass in WA-amended soils is induced by pH changes and increased concentration of dissolved organic C (Bougnom and Insam, 2009). The effects are thought to be governed by the soil type, WA application rates, length of the experiment and WA pre-treatment (Pitman 2006; Fernando-Jaurez *et al.*, 2013; Pukalchik *et. al.*, 2018); while the effect on soil microorganisms is identified to depend on WA pre-treatment and application rates (Kuba *et al.*, 2008).

On the other hand, excessive amounts of WA may affect the soil texture, aeration, water holding capacity and salinity (Perucc*i et al.*, 2008). For example, WA particles may block soil pores as they swell when in contact with water and cause a decrease in soil aeration and loss of contaminants (Demeyer *et al.*, 2001; Reid *et. al.*, 2004). Also, WA alkalinity may increase soil electrical conductivity (EC) and influence the soil elements' solubility (Demeyer *et al.*, 2001; Bougnom and Insam, 2009; Bougnom *et al.*, 2010). The trace amount of heavy metals in WA may accumulate in soil (Ning *et al.*, 2017; Richard *et al.*, 2018), and consequently, inhibit microbial growth and/or degradation activity.

1.8.3 Addition of mixtures of anaerobic digestate and wood-ash to soil

In studies, separate additions of AD (García-Sánchez *et al.*, 2015a; Koszel and Lorencowicz, 2015) and WA (Demeyer *et al.*, 2001; Bougnom and Insam, 2009; Bougnom *et al.*, 2010) to soil positively improved soil biological properties (Alburquerque *et al.*, 2012a; Fernández-Delgado Juárez *et al.*, 2013; García-Sánchez *et al.*, 2015a). The complementary nutrients of AD and WA suggest that their combined addition can mitigate soil nutrients deficiencies and, consequently, improve microbial growth (Bougnom *et al.*, 2010; Fernández-Delgado Juárez *et al.*, 2013) and degradation of PAHs compared to their separate additions. For instance, WA is known to contain variable amounts of P and negligible amount or complete absence of N, while AD addition results in better N recovery (Perucci *et al.*, 2006; Kuba *et al.*, 2008; García-Sánchez *et al.*, 2015a). This thought is in agreement with studies where mixtures of AD and WA were used to improve the soil physico-chemical and biological properties.

In a study by Bougnom and Insam (2009), soil amendment with WA admixture to composts changed the microbial community levels or physiological profiles. Also, Fernández-Delgado Juárez *et al.* (2013) studied WA admixture to enriched N-based organic wastes, digestate and manure, and observed higher amounts of nutrients (NH_4^+, total C and P) irrespective of the amounts of WA added. Therefore, it is envisaged that soil amendments with a mixture of AD and WA (AD-WA) can improve the soil conditions, increase the availability of macro- and micro-nutrients in the soil (Fernández-Delgado Juárez *et al.*, 2013; García-Sánchez *et al.*, 2015b) and stimulate soil microbial growth and activities, and consequently, enhance indigenous biodegradation of PAHs. Furthermore, it is hypothesised that they may be an environmentally friendly alternative to mineral fertilization in acidic soils and constitute efficient ways of recycling their nutrients (Bougnom *et al.*, 2010; Fernández-Delgado Juárez *et al.*, 2013).

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2. The experimental design of the study/thesis

2.1 Aims and objectives

Amendment of soil with AD and WA, as well as their mixtures, has been reported to positively improve soil physico-chemical and biological properties (Demeyer *et al.*, 2001; Insam *et al.*, 2009; García-Sánchez *et al.*, 2015b). It is envisaged that the addition of mixtures of AD and WA can potentially produce a more positive impact on soil conditions, and consequently, enhance indigenous biodegradation of PAHs. In this study, AD and WA were added to the soils pre-exposed to ^{12}C-phenanthrene (as sources of nutrients) to enhance indigenous microbial degradation of freshly added ^{14}C-phenanthrene. Therefore, this thesis aimed to:

(1) Review available literature on

(a) the environmental fate and impact of PAHs;

(b) the biodegradation of PAHs in contaminated soils as relayed in past studies;

(c) the effects of soil amendments with organic amendments, especially AD and/or WA, on indigenous microbial activity; and

(d) how the effects influence indigenous biodegradation of PAHs;

(2) Investigate the development of indigenous biodegradation of ^{14}C-phenanthrene (a radiolabelled PAH) in AD- and/or WA-amended soils under different amendment conditions, with increasing soil-PAH contact time, using a ^{14}C-respirometric system, and without analyzing the microbial components;

(3) Investigate the effect of soil amendment with mixtures of AD and WA (under different amendment conditions) on the development of indigenous catabolism of ^{14}C-phenanthrene;

(4) Investigate the development of indigenous catabolic evolution of ^{14}C-phenanthrene in soils with separate pre-exposure to multiple additions and a single application of higher

concentrations of ^{12}C-phenanthrene, in the presence as well as the absence of AD and/or WA.

2.2 A schematic diagram of the experimental design

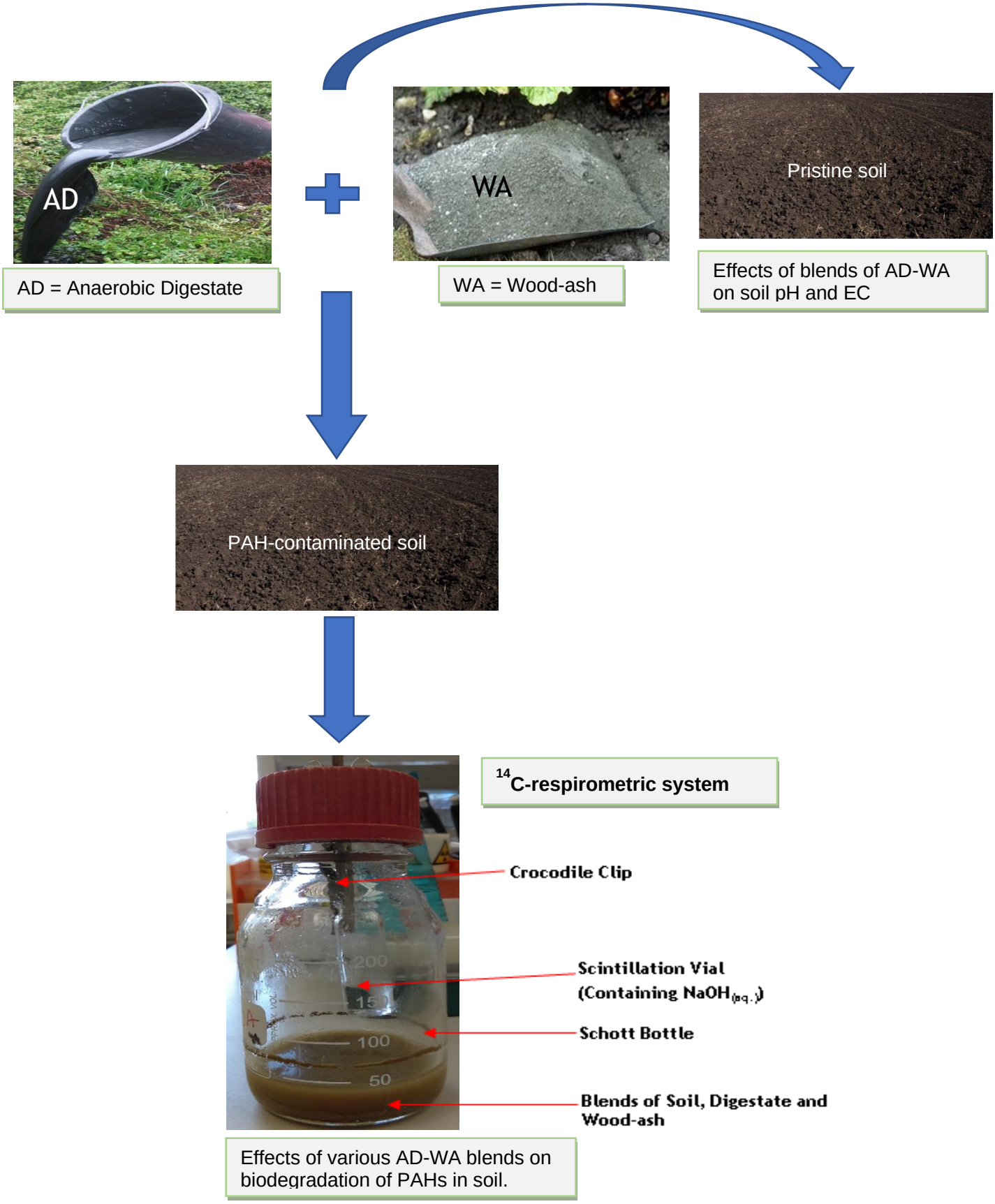

Figure 11: A schematic diagram of the **i**nvestigation of the influence of addition of blends of anaerobic digestate (AD) and wood-ash (WA) on biodegradation of polycyclic aromatic hydrocarbons in soil.

3. Summary of papers

3.1 Indigenous catabolic evolution of phenanthrene in anaerobic digestate- and wood ash-amended soils.

Preliminary studies were carried out on the influence of the addition of mixtures of AD and WA on the soil pH and EC. After the addition of AD and WA mixtures, there was a slight increase in the soil pH (6.3) and EC (257.9 µS cm^{-1}), compared to the similar unamended soil pH (6.15) and EC (103.6 µS cm-1); there were no extreme changes as the values remained close or within the neutral pH range and EC threshold level of the pristine soil. Also, the indigenous catabolic evolution of ^{14}C-phenanthrene was assessed in soil only, AD only as well as in the mixtures of AD and WA incubations. There was a higher percentage of catabolically evolved $^{14}CO_2$ (%) in the soil only incubations (ranging from 56.0 ± 6.3 % to 84.8 ± 1.9 %) compared to the AD (ranging from 4.6 ± 0.6 % to 14.7 ± 8.4 %) as well as the mixtures of AD and WA (ranging from 5.7 ± 0.9 % to 20.0 ± 5 %).

In the biodegradation study, most of the AD- and WA-amended soils showed significantly ($P < 0.05$) shorter lag phases, as well as faster rates and greater extents of mineralisation than observed in similar unamended soils. Addition of AD (0.170 g, 1.730 g, 17.31 g and 173.1 g kg^{-1} soil) significantly shortened the lag phases, as well as increased the rates and extents of mineralisation. However, this result was consistent in the soils with lower additions of AD (0.170 g, 1.730 g, 17.31 g kg^{-1} soil), while there was no significant effect relative to the highest amounts (173.1 g kg^{-1} soil) of AD across all the time points except after 90 d where the mineralisation rates were significantly higher than the controls. Similarly, soils with additions of low amounts of WA (0.009 g, 0.090 g, 0.940 g kg^{-1} soil) showed significantly greater extents of mineralisation than those with higher amount (9.400 g) of WA, where no significant effect was observed. In the soils amended with mixtures of

AD and WA (0.170 g AD /0.009 g WA; 1.730 g AD / 0.090 g WA; 17.310 g AD / 0.940 g WA; 173.100 g AD / 9.400 g WA kg^{-1} soil), shorter lag phases were observed earlier compared to AD-amended soils, while no significant effect was observed in the WA-amended soils. Similarly, the rates and extents of mineralisation were increased in most of the ^{12}C-phenanthrene-exposed soils amended with the mixtures of AD and WA compared to similar soils with separate additions of AD and WA, as the soil-PAH contact time increased.

3.2 The effects of combined additions of anaerobic digestate and wood-ash on the development of indigenous phenanthrene catabolism in soil.

In this study, the soils amended with mixtures of proportionately increasing amounts of AD (0.170 g, 1.730 g, 17.31 g and 173.1 g kg^{-1} soil) and a single amount of WA (0.94 g kg^{-1} soil) significantly shortened the lag phases, as well as increased the rates and extents of mineralisation than the similar unamended soils (controls), as the soil-PAH contact time increased. Also, the single amount of WA (above-indicated) significantly reduced the lag phases, as well as increased the rates and extents of mineralisation, as the soil-PAH contact time increased. It was noted that mineralisation was further improved with additions of mixtures of proportionately increasing amounts of WA (0.009 g, 0.090 g, 0.940 g and 9.400 g kg^{-1} soil) and a single amount of AD (17.31 g kg^{-1} soil), which were also observed as significantly shorter lag phases and increased rates and extents of mineralisation, compared to the similar unamended soils. However, these results were observed in the soils amended with the mixtures of low amounts of WA (0.009 g, 0.090 g and 0.940 g kg^{-1} soil) and a single amount of 17.31 g kg^{-1} soil of AD. Generally, the combined additions of the AD and WA showed higher levels of mineralisation than their separate additions. The findings provided information on the appropriate amounts of AD and WA that can be

employed as a combined soil amendment to stimulate and/or enhance indigenous biodegradation of PAHs.

3.3 Influence of multiple additions of ^{12}C-phenanthrene on the development of ^{14}C-phenanthrene catabolism in anaerobic digestate- and wood-ash-amended soils.

In this study, the influence of soil pre-exposures to multiple additions and higher applications of ^{12}C-phenanthrene in the absence and presence of AD and/or WA on catabolic evolution of ^{14}C-phenanthrene was investigated. The results showed higher percentage of catabolically evolved $^{14}CO_2$ from the mineralisation of the freshly added ^{14}C-phenanthrene across all the kinetics of mineralisation, with significantly shorter lag phases, as well as higher rates and greater extents of mineralisation than the soils with a single exposure to ^{12}C-phenanthrene only. These mineralisation results suggest an occurrence of enhanced indigenous microbial activity, which have a significant implication on the biodegradation of the ^{14}C-phenanthrene. The effect of the nutrients additions, as AD, WA as well as the mixtures of AD and WA, improved the development of the catabolic evolution of ^{14}C-phenanthrene in the ^{12}C-phenanthrene-exposed soils compared to the similar unamended soils. However, a higher occurrence of biodegradation was predominant with the addition of the mixtures of AD and WA, with a higher number of significantly shorter lag phases as well as increased rates and extents of mineralisation across the soil-PAH ageing period (0 d to 90 d) compared to their separate additions.

Similarly, the effect of the addition of AD and/or wood-ash WA on the microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) of the ^{12}C-phenanthrene-exposed soils was determined after 0, 60 and 90 d incubations (20 ± 2 ^{o}C), with chloroform fumigation method. There were higher microbial biomass C and N, analysed as the total organic C and N (mg kg^{-1} soil), respectively, in the ^{12}C-phenanthrene-exposed soils in the

presence of AD and/or WA, than observed in the soils with a single exposure to ^{12}C-phenanthrene only (controls). This effect also suggests a possibility of improved indigenous microbial activity, and consequently, PAH biodegradation following the additions of AD and WA. In previous studies, soil microbial biomass was used to indicate the occurrence of soil contamination (Joergensen and Wichern, 2018); changes in soil microbial biomass have been traced to the nutrients availability as well as microbial growth and death processes (Fujita *et al.*, 2019; Ren *et al.*, 2019).

Also, the microbial community structure of the ^{12}C-phenanthrene-exposed soils, both in the presence and absence of the AD and/or WA, as the concentrations of phospholipid fatty acids (PLFAs) in the soils, were determined after 90 d aged incubation at 20 ± 2 ^{o}C. The soils that were pre-exposed to multiple additions and higher applications of ^{12}C-phenanthrene showed greater changes in terms of higher amounts of PLFAs compared to the soils with a single exposure to ^{12}C-phenanthrene (controls). However, the additions of AD and/or WA to the ^{12}C-phenanthrene-exposed soils further contributed to the increase in the amounts of the PLFAs in some of the incubations, compared to the similar unamended soils and the controls. In previous studies, PLFA analysis has been used for quantitative assessment of microbial community compositions and the monitoring of soil responses to environmental changes (Kaiser *et al.*, 2010; Buyer and Sasser, 2012).

3.4 The influence of contact time on biodegradation and extraction of ^{14}C-phenanthrene in anaerobic digestate- and wood-ash-amended soils.

In this study, the influence of contact time on indigenous and inoculum-induced biodegradation and chemical extraction of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with nutrient-rich AD (a biogas residue) and/or WA (a biomass combustion residue) was investigated. Before mineralisation, the recovered ^{14}C-phenanthrene activities

(dpm) were higher in the absence of AD and/or WA from 0 d to 15 d, and gradually reduced in the presence of AD and/or WA with increasing soil-PAH contact time until after 90 d. Also, the ^{14}C-activities recovered from the inoculated mineralisation were lower ($P < 0.05$) than the controls' in the presence of the mixtures of AD and WA, as well as WA only after 0 d and with additions of WA and AD as the soil-PAH contact time increased ($P > 0.05$). However, in the uninoculated mineralisation, the recovered ^{14}C-activities were lower ($P < 0.05$) than the controls after 0 d with addition of AD and after 60d with additions of WA as well as the mixtures of AD and WA.

The amounts of ^{14}C-phenanthrene in the uninoculated ^{12}C-phenanthrene-exposed soils amended and unamended with AD and/or WA were extracted with Dichloromethane (DCM); while the non-extractable fractions were recovered from the soil pellets by combustion in a sample oxidizer (Table 2). Most of the DCM-extractable fractions were higher in the absence of AD and/or WA (controls) from 0 d to 90 d but became lower ($P < 0.05$) in the presence of WA after 30 d and 60 d as well as with the addition of AD after 60 d. Non-extractable residues recovered from the soil pellets were significantly lower than the controls after 15 d with the additions of WA, as well as the mixtures of AD and WA, and after 60 d with separate additions of AD and WA. Both extractable and non-extractable fractions and their total amounts were significantly lower than the controls after 60 d with the additions of AD and/or WA. However, their total amounts gradually reduced with increasing soil-PAH contact time irrespective of their separate amounts.

The HP-β-CD-extractable and non-extractable ^{14}C-phenanthrene fractions (%) in the uninoculated ^{12}C-phenanthrene-exposed soils amended with AD and/or WA were determined. The amounts of HP-β-CD-extractable ^{14}C-phenanthrene were observed lower ($P < 0.05$) than the controls after 0 d in the presence of AD and/or WA. Similarly, the HP-β-CD-extractable fractions were lower ($P < 0.05$) than the controls as the soil-PAH contact

time increased from 15 d in the presence of the mixtures of AD and WA, as well as after 30 d with WA addition. However, from 60 to 90 d, the HP-β-CD-extractable fractions reduced ($P < 0.05$) in the presence and absence of AD and/or WA without any corresponding increase in the non-extractable residues. Generally, the non-extractable residues were lower ($P < 0.05$) than the controls' after 0 d in the presence of AD and/or WA, particularly the mixtures of AD and WA.

In addition, the indigenous and inoculum-induced mineralisation of the ^{14}C-phenanthrene in the ^{12}C-phenanthrene-exposed soils amended and unamended with AD and/or WA were studied at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d. In inoculated ^{12}C-phenanthrene-exposed soils, the addition of WA as well as mixtures of AD and WA significantly reduced the lag phases and increased the mineralisation rates from 0 d and with increasing soil-phenanthrene interaction compared to the similar unamended soils. The mineralisation rates increased relative to the population size of the PAH-degraders.

In uninoculated mineralisation with the addition(s) of AD and/or WA, there were significantly shorter lag phases, as well as higher rates of mineralisation and microbial density than observed in similar unamended soils. However, some of the levels of mineralisation were independent of the bacterial number. In inoculated AD- and WA-amended soils, bacterial counts increased as the mineralisation rates increased, but this was not the case with the extents of mineralisation (at the onset of the soil-PAH contact time). Similarly, the population size of the inoculant (*Pseudomonas* species) increased after mineralisation than the initial inoculum dilution/density ($\sim 10^6 – 10^7$ CFUs g^{-1} soil).

4. The comparisons of the results of the studies on biodegradation of PAHs in AD and/or WA amended soils

This present study evaluated the influence of soil amendment with anaerobic digestate (AD) and wood-ash (WA) on the indigenous biodegradation of PAHs in soils. The soils' pre-exposures to non-labelled ^{12}C-phenanthrene before their mixtures with the AD and/or WA contributed to the adaptation of the indigenous microbial population to metabolize and/or degrade the freshly added ^{14}C-phenanthrene (Macleod and Semple, 2002; Macleod and Semple, 2006; Patowary *et al.*, 2016). The findings of the preliminary studies showed higher indigenous catabolic activity in the soil compared to the residues, before their applications as soil amendments; this indicated that the microbial degradation of the ^{14}C-phenanthrene in throughout this present study was predominantly indigenous. The results of all the four biodegradation investigations in this present study showed improved mineralisation with different changes in the catabolic behaviour of the AD-, WA- and AD-WA-amended and unamended soils.

In the first biodegradation study, the indigenous catabolic evolution of the freshly added ^{14}C-phenanthrene in the AD- and WA-amended soils, under different amendment conditions was studied. The addition of the nutrient-rich AD and WA contributed to the production of a greater level of $^{14}CO_2$ (%) with the mineralisation kinetics positively influenced as shorter lag phases as well as faster rates and greater extents of mineralisation than similar unamended soils. However, these findings were mostly observed in the soils with the lower amounts of AD and/or WA as well as the soils with the additions of the mixtures of AD and WA. A further investigation was made into the effects of soil amendment with combined addition of AD and WA on the development of indigenous catabolism of ^{14}C-phenanthrene. The findings also revealed reduced lag periods as well as increased rates and extents of mineralisation, which were predominant

in the soils with the small concentrations of the residues compared to those with higher concentrations. Generally, the mineralisation was higher with the combined applications of AD and WA compared to their separate soil applications. The findings suggested WA admixture to AD potentially supplied the required types and sufficient amounts of essential nutrients to the soils than their separate additions.

For instance, AD has a high proportion of mineral nitrogen (N) in addition to other essential elements (P, Ca, Mg and K) while WA is also direct source of major elements such as P, Ca, Mg and K, with the absence of N (Alburquerque, *et. al.*, 2012a; Johansen et al., 2013; García-Sánchez *et al.*, 2015a; García-Sánchez *et al.*, 2015b; Fernández-Bayo *et. al.*, 2017). Therefore, the combined addition of AD and WA potentially impacted on the soil conditions as well as microbial populations and functioning. The findings agreed with past studies that reported occurrence of higher amounts of nutrients (NH_4^+, total C and P) in the soils amended with the mixture of AD and WA (Fernández-Delgado Juárez *et. al.* (2013). The findings gave insights into the appropriate amounts of AD and WA that could be co-applied to soil to stimulate indigenous mineralisation of PAHs. The insight will be helpful in mitigating excessive addition of the residues to the soil thereby avoiding soil acidity, nutrient leaching, surface water eutrophication and inhibition to optimal biological activities (Fernández-Delgado Juarez *et. al.*, 2013; Maletić *et. al.*, 2013; Naseri *et. al.*, 2014; Ning *et. al.*, 2017; Richard *et. al.*, 2018). Similarly, excessive additions of the residues to soil can be hazardous to environmental health (Perucc*i et. al.*, 2008; Gómez-Brandón *et. al.*, 2016).

The effects of soil pre-exposures to multiple additions and/or additions of higher concentrations of ^{12}C-phenanthrene on the development of indigenous catabolism of freshly added ^{14}C-phenanthrene both in the presence and absence of AD and/or WA were investigated. The findings showed positive effects both in the presence and absence of the AD and WA compared to similar soils with a single exposure to ^{12}C-phenanthrene.

However, there was a significantly higher percentage of catabolically evolved $^{14}CO_2$ (%) from the mineralisation of the ^{14}C-phenanthrene in the soils with multiple exposures to ^{12}C-phenanthrene than similar soils with a single exposure to ^{12}C-phenanthrene. Also, more positive effects were noticed in the presence of AD and/or WA, especially as a combined amendment. Similarly, significant changes were observed in the composition of the microbial community in terms of higher phospholipid fatty acid (PLFA) in the soils with multiple and higher exposures to ^{12}C-phenanthrene, especially with the addition of mixtures of AD and WA, than similar soils with a single exposure to ^{12}C-phenanthrene in the absence of AD and/or WA.

The influence of contact time on the chemical extraction as well as indigenous and inoculum-induced biodegradation of ^{14}C-phenanthrene in the presence and absence of AD and/or WA was investigated. Significantly low ^{14}C-phenanthrene activity was recovered in most of the AD- and/or WA-amended soils, particularly those with the mixtures of AD and WA, before and after mineralisation compared to similar unamended soils. Also, the amounts of the chemically extractable fractions of the ^{14}C-phenanthrene were low in the soils with the additions of AD and/or WA. For instance, the dichloromethane (DCM)- and hydroxypropyl-β-cyclodextrin (HP-β-CD)-extractable ^{14}C-labelled fractions in the AD- and/or WA-amended soils were low compared to the unamended soils. Also, the extractable amounts declined with increasing soil-PAH contact time in both chemical extractions while there was a nearly 1:1 relationship between the HP-β-CD-extractable and mineralised fractions. Greater levels of $^{14}CO_2$ (%) were produced from the mineralisation of the ^{14}C-phenanthrene in both inoculated and uninoculated mineralisation (with little difference in-between them) while the positive effect was predominant in the soils with the additions of mixtures of AD and WA. As the soil-PAH contact time increased, the amount of the $^{14}CO_2$ (%) declined. Heterotrophic bacteria were higher in number at the incubation onset than at the end of mineralisation while the inoculant's number was higher at the end

of the mineralisation. The levels of mineralisation (%) observed in some of the uninoculated soil incubations suggested the mineralisation was independent of the bacterial density.

Generally, all the findings of this present study provided insights into the (1) implications of microbial degradation of PAHs in the soils lacking in nutrients, (2) effects of soil amendments with AD and/or WA on biodegradation of PAHs, (3) correct amounts of AD and WA that could be mixed as a soil amendment to stimulate indigenous biodegradation of PAHs.

5. Discussion

In PAH-contaminated soils, nutrients availability is a major concern due to the enhanced carbon-to-nitrogen ratio at the expense of other essential nutrients, as a result of the presence of PAHs (Margesin and Schinner, 2001; Stroud *et al.*, 2007; Warr *et al.*, 2013). The availability of sufficient nutrients influences the indigenous catabolic activity and, consequently, the biodegradation of PAHs in the soil. In past studies, indigenous microbial activity was enhanced by the additions of inorganic nutrients (Scotti *et al.*, 2013; Ravanipour *et al.*, 2015; Scotti *et al.*, 2014) and organic nutrients (Heijboer *et al.*, 2016; Lukić *et al.*, 2016; Han *et al.*, 2017; Martins *et al.*, 2018). However, organic soil amendments were widely preferred due to their readily available micro- and macro-nutrients, low cost, local availability and environmental sustainability (Odlare *et al.*, 2011; Fernandez-Delgado Juarez *et al.*, 2012; Martins *et al.*, 2018).

Past studies showed the positive effects of nutrients' availability and additions of organic amendments on soil characteristics (Medina *et al.*, 2006; Pitman 2006; Maletić *et al.*, 2013), as well as their impacts on indigenous catabolic activity (Haritash and Kaushik, 2009; Odlare *et al.*, 2011; Bougnom *et al.*, 2012). Several low-value organic residues from renewable bioenergy processes have been used for soil amendments (Haritash and Kaushik, 2009). In the present study, nutrient-rich AD and WA were added to soil to enhance indigenous biodegradation of freshly added ^{14}C-phenanthrene (a model PAH), which have not been reported in studies to the best knowledge of the authors.

Both AD and WA are known to have a high pH, and their addition to soil is expected to influence the soil pH and EC. However, there were no extreme changes in the pH and EC of the AD- and WA-amended soils, which is important because microorganisms are pH-sensitive. Variations in soil pH and salinity beyond optimum levels can impair microbial

cellular functions, reduce microbial activity and biomass, as well as cause changes in microbial community structure (Haritash and Kaushik, 2009; Silva and Fay, 2012; Fernández-Delgado Juarez *et al.*, 2013; Naseri *et al.*, 2014; Gómez-Brandón *et al.*, 2016). Neutral pH is known to mostly favour the catabolism of PAHs (Maletić *et al.*, 2013). For instance, the maximum rates of oxidation of PAHs and optimum bacterial growth have been reported to be at pH 7.0, and the biodegradation rate of PAHs is known to increase under low salinity (Haritash and Kaushik, 2009). The findings in this preliminary study agree with past studies that revealed an increase in soil pH following the addition of organic materials, and the stabilization of the pH around that of similar unamended soil, due to the soil buffering capacity (Bougnom *et al.*, 2012; Koszel and Lorencowicz, 2015; Wang *et al.*, 2015). However, the slight increase in pH is suspected to have influenced the development of indigenous microbial activity (Jokinen *et al.*, 2006; Perucci *et al.*, 2006).

The indigenous catabolism of the ^{14}C-phenanthrene was enhanced across all the mineralisation kinetics in the presence of the AD- and/or WA-amendments compared to the unamended soils. The observations suggest that the nutrients-rich AD and WA supplied essential nutrients that might have been deficient in the ^{12}C-phenanthrene-exposed soils due to the presence of the ^{14}C-phenanthrene (an organic contaminant). This observation was also supported by the results from the additions of the mixtures of AD and WA, whereby more mineralisation was observed compared to their separate additions. For instance, shorter lag phases were observed earlier than found in AD only incubations while no significant effect was observed in WA only incubations. Similarly, faster rates of mineralisation were noticed earlier in the soils amended with mixtures of AD and WA than their separately amended soils. However, the positive effects observed on soil microbial activity following the addition of AD and WA as soil amendments agree with past studies in terms of the stimulation of indigenous microbial activity (Insam *et al.*, 2009; Alburquerque, *et al.*, 2012a; Fernández-Delgado Juárez *et al.*, 2013; García-Sánchez *et al.*, 2015b). Also,

the complementary nutrients (especially N, P and K) of the AD and WA have implications as a mixture, on soil conditions and microbial performance (Bougnom *et al.*, 2012).

The influence of soil's pre-exposure to PAHs on indigenous mineralisation of PAHs has been reported in studies (Macleod and Semple, 2006; Rhodes *et al.*, 2008; Couling *et al.*, 2010). In this present study, the mineralisation of ^{14}C-phenanthrene was significantly enhanced in the soils with multiple additions of ^{12}C-phenanthrene and the soils with higher concentrations of ^{12}C-phenanthrene in the presence of AD and/or WA from 0 d and as the soil-PAH contact time increased (compared to the similar unamended soils and the soils with a single ^{12}C-phenanthrene-exposure only). These results further showed the significance of the availability of sufficient nutrients for optimal microbial activity. The findings are in agreement with past studies where microbial adaptation to mineralize PAHs is related to the exposure history of the soil to the PAHs or their analogues, with emphases on the concentration of the PAHs, interaction of the PAHs with indigenous microbial population and length of the soil-PAH contact time (Macleod and Semple, 2006; Rhodes *et al.*, 2008; Couling *et al.*, 2010).

Soil-PAH contact time has been known to influence biodegradation, and the impact of which was also investigated in ^{12}C-phenanthrene-exposed soils amended with AD and/or WA, using the indigenous soil microorganisms and a catabolic inoculum. The extractable ^{14}C-phenanthrene before and after mineralisation from the AD- and WA-amended soils significantly declined with increasing soil-PAH contact time. The observation is similar to the findings in indigenous and inoculum-induced mineralisation where the percentage of $^{14}CO_2$ produced from the mineralisation of ^{14}C-phenanthrene also significantly declined with increasing soil-PAH contact time, due to ageing, both in the presence and absence of AD and/or WA. Similarly, the total DCM-extractable ^{14}C-phenanthrene declined gradually with increasing soil-PAH contact time, both in AD- and/or WA-amended as well as

unamended soils, due to ageing. This observation agrees with past studies where the increase in soil-contaminant interaction reduces the bioavailability and chemical extractability of the contaminant (Reid *et. al.*, 2000; Papadopoulos *et. al.*, 2007).

However, the recovered ^{14}C-phenanthrene concentrations were lower in the AD- and WA-amended soils, especially in the soils amended with the mixtures of AD and WA compared to the similar unamended soils. These observations suggest that the nutrient elements supplied by the AD and WA have been utilized by the soil microorganisms and, consequently influenced the biodegradation of the ^{14}C-phenanthrene. Also, the HP-β-CD-extractable ^{14}C-phenanthrene gradually declined in both AD- and/or WA-amended, as well as in unamended soils, with increasing soil-PAH contact time. HP-β-CD-extractable ^{14}C-phenanthrene is known to mimic the indigenous mineralisable ^{14}C-phenanthrene and predict the extent of intrinsic microbial degradation (Doick *et. al.*, 2005). In the results, biodegradable and cyclodextrin-extractable ^{14}C-phenanthrene fractions were close both in the inoculated and uninoculated AD- and/or WA-amended soils (Reid *et. al.*, 2000), as studies link cyclodextrin-extractable phenanthrene to the mineralisable phenanthrene in soils (Reid et. al., 2000; Reid *et. al.*, 2004; Doick *et. al.*, 2005).

6. Papers (1 – 4)

6.1 Paper 1: Indigenous catabolic evolution of phenanthrene in anaerobic digestate- and wood ash-amended soils.

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are present ubiquitously in the environment. They are of concern due to their low biodegradability and persistence, as well as inherently toxic and mutagenic properties. However, it is known that PAHs might be degraded in the soil under appropriate environmental conditions. This study investigated the impact of soil amendment with nutrient-rich anaerobic digestate (AD) and wood-ash (WA) on indigenous catabolism of ^{14}C-labelled phenanthrene (a PAH) over a 90-day incubation, under different amendment conditions. A greater level of $^{14}CO_2$ (%) was produced from the mineralisation of the ^{14}C-phenanthrene in the presence of AD and/or WA, especially in their lower amounts, and the mineralisation kinetics were positively influenced as shorter lag phases as well as faster rates and greater extents of mineralisation compared to the similar unamended soils. The AD-WA mixture slightly increased the soil pH and electrical conductivity (EC) before incubation; also, a greater level of $^{14}CO_2$ was produced from indigenous catabolism of ^{14}C-phenanthrene in pristine soils only compared to the AD and AD-WA incubations. The study showed the influence of soil amendment with AD and WA on stimulation of indigenous microbial activity and enhancement of biodegradation of PAHs.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of hydrophobic organic contaminant, which are found in the environment mostly as a result of anthropogenic industrial processes (Macleod and Semple, 2002; Okere and Semple 2012; Lang *et al.*, 2016; Lukić *et al.*, 2016). Due to the physico-chemical properties of PAHs, their biodegradability decreases, and their persistence increases with increasing molecular size (Riding *et al.*, 2013; Naseri *et al.,* 2014; Yu *et. al.*, 2018). Coupled with their persistent characteristics, PAHs can be toxic, and genotoxic in particular (Macleod and Semple, 2002; Lukić *et al.*, 2016; Baldwin *et al.*, 2020) The presence of these hydrophobic contaminants in soil is an indication of environmental pollution, and possibly a long-term reservoir from prolonging ageing could foster sequestration to soil matrices thereby reducing their susceptibility to degradation (Stokes *et al.*, 2006; Riding *et al.*, 2013; Umeh *et. al.*, 2017).

Microbial degradation may be employed to remediate PAH-contaminated soils to reduce the risk to human health (Maletić *et al.*, 2013; Naseri *et al.*, 2014). It involves breaking down PAHs into simpler molecules, which could further be utilized as sources of carbon and energy to produce environmentally harmless compounds (such as carbon dioxide and water), mineral salts and new microbial cellular constituents (Boopathy, 2000; Debosz *et al.*, 2002; Vidali 2001; Griffiths *et al.*, 2012; Maletić *et al.*, 2013; Naseri *et al.*, 2014). One approach is to use indigenous microbial populations in the soil to degrade the PAHs, a process called biostimulation (Atlas and Cerniglia, 1995; Watanabe and Baked, 2000; Haritash and Kaushik 2009). Another approach involves the addition of environmental-adapted PAH-degrading microorganisms to PAH-contaminated soils to improve the rates and extents of biodegradation of PAHs, a process known as bioaugmentation (Watanabe and Baker, 2000; Naseri *et al.*, 2014).

The input of organic contaminants into the soil is known to rapidly deplete the available pools of major inorganic nutrients of the soil, especially nitrogen (N) and phosphorus (P), and enrich its carbon (C) content (Margesin and Schinner, 2001; Stroud *et al.*, 2007). Addition of appropriate growth-limiting mineral nutrients can restore the soil's nutrient balance and enhance its indigenous microbial activity (Naseri *et al.*, 2014). Soil decontamination following stimulation of native microbiota with N, P and potassium (K) based chemical fertilizers have been documented (Margesin and Schinner, 2001; Vidali, 2001). However, recurrent soil application of inorganic nutrients can encourage soil acidification, the accumulation of their heavy metal contents in the soil and occurrence of soil layer compaction (Savci, 2012; Xu *et al.*, 2014; Massah and Azadegan, 2016). These can adversely affect 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; Fernandez-Delgado Juarez *et al.*, 2012). The growing industrialization and increased recycling of organic wastes, as renewable energy sources, have led to a huge generation of these organic residues, with management and disposal challenges (Odlare *et al.*, 2011; Quakernack *et al.*, 2012). However, their application to soil, as biological fertilizers, have been considered a suitable and sustainable alternative to chemical fertilization (Alburquerque et al., 2012a; Alburquerque *et al.*, 2012c; 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), optimizing soil nutrients (Margesin and Schinner, 2001; Maletić *et al.*, 2013) and stimulating soil microbial growth and activities (Haritash and Kaushik, 2009; Bougnom *et al.*, 2012). Therefore, due to the proven positive effects of organic amendments on soil microbial activity (Odlare *et al.*, 2011) it is thought

they can equally enhance microbial degradation of PAHs. However, the process may require understanding and manipulating the environmental conditions of the soil, (such as pH, oxygen level, moisture content and available nutrients) (Vidali 2001; Pontes *et al.*, 2013), owing to their impacts on microbial community composition and activity (Boucard *et al.*, 2008; Rousk *et al.*, 2009). The level of the soil's essential/growth-limiting nutrients, in terms of C:N:P ratio (Stroud *et al.*, 2007), is significant due to their influence on microbial growth, activity and hydrocarbon degradation (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 in recent years (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) (Whelan *et al.*, 2010; Möller and Müller, 2012; Insam *et al.*, 2015) and nutrients, especially N and P, which are essential for microbial growth and/or 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 applied should match the soil's N deficiency (Fernández-Delgado Juarez *et al.*, 2013; Gómez-Brandón *et al.*, 2016), in order to optimise its benefits as well as mitigate soil acidification, nitrate leaching and emission of nitrous oxide (N_2O) (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) in AD-amended soils within a short time of application (Alburquerque *et al.*, 2012a; García-Sánchez *et al.*, 2015a), which can impact on microbial activity. Therefore, AD application dose, technique and period are crucial.

Similarly, WA contains a significant amount of macro-nutrients such as calcium (Ca), magnesium (Mg), sodium (Na) and potassium (K), as well as micro-nutrients (such as zinc

(Zn), copper (Cu), lead (Pb), nickel (Ni) and arsenic (As)) (Fernández-Delgado Juarez *et al.*, 2013; García-Sánchez *et al.*, 2015a; Maschowski *et al.*, 2016). Several studies have demonstrated that WA can be used for (1) correcting soil acidity to a desired soil pH (Fernández-Delgado Juarez *et al.*, 2013; García-Sánchez *et al.*, 2015a); (2) ameliorating soil physical, chemical and biological properties; (3) stimulating soil microbial activity; and (4) immobilizing heavy metals in soil (García-Sánchez *et al.*, 2015c). However, the high pH (8 to 13) of the 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; García-Sánchez *et al.*, 2015c). This is due to the impact of pH, N and C on soil microbial population and/or community composition and activity (Fernández-Delgado Juarez *et al.*, 2013; García-Sánchez *et al.*, 2015a). The effect of WA has been mentioned to be governed by its application dose and soil type (Fernández-Delgado Juarez *et al.*, 2013), while its excessive addition can affect the soil's physico-chemical and biological properties (Perucci *et al.*, 2006; Perucci *et al.*, 2008; Insam *et al.*, 2009). Therefore, the application of the correct amount of WA had remained uncertain, and this 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 soil amendment with AD and WA positively influenced the physico-chemical and biological properties of the soil (Demeyer *et al.*, 2001; Insam *et al.*, 2009; García-Sánchez *et al.*, 2015c). However, due to the complementary nutrients (especially N, P and K) of the AD and WA, their addition to soil as a mixture, can produce a potentially valuable amendment with more positive impacts on the soil conditions, as well as indigenous microbial functioning (Bougnom *et al.*, 2012). To the best of our knowledge, no study has examined the influence of these two organic residues on indigenous microbial degradation of PAHs in contaminated soils.

Therefore, this study investigated the development of indigenous biodegradation (or mineralisation) of ^{14}C-phenanthrene (a model radiolabeled PAH) in ^{12}C-phenanthrene-exposed soils amended with AD- and/or WA, under different amendment conditions over 90 days. The biodegradation of the ^{14}C-phenanthrene was quantified by measuring the mineralisation kinetics (lag phases, mineralisation rates and extents) in the soils at the increasing soil-PAH contact times 1 d, 15 d, 30 d, 60 d and 90 days (d). The study showed the influence of soil amendment with AD and WA on stimulation of indigenous microbial activity and enhancement of biodegradation of PAHs.

2. Materials and Methods

2.1 Bioenergy residues and chemicals

Pasteurized anaerobic digestate (AD) was obtained as a semi-liquid residue from an anaerobic digestion plant in the United Kingdom (UK). It was produced from anaerobically digested household food wastes. It was pasteurized at the source before collection (as a legislative requirement of organic wastes disposal/recovery). After collection, it was stored in a dark room under 4 ^{o}C. Wood-ash (WA) (fly-ash) was collected from a biomass power plant in the UK; it was generated in a full-scale heat and power plant, with timber and bark as the input materials. Fly-ash was preferred due to its higher concentrations of elements (Sharma and Kalra 2006; Omil et al. 2013) and low alkalinity (Noyce et al., 2016) compared to the bottom ash. AD and WA characteristics are briefly described in Table 1 below.

Chemicals used in this study include non-radiolabelled (>96 %, HPLC grade) and ^{14}C-labelled phenanthrene (55.7 mCi mmol^{-1}; >99 % purity), both of which were supplied by Sigma–Aldrich, Poole, UK. Also, Gold Star multipurpose liquid scintillation cocktail, acetone (>98 %), sodium hydroxide (NaOH) (1 M) and concentrated sulphuric acid (H_2SO_4) (1 M) were supplied by Meridian, UK. Other materials used include amber glass bottles and 7 ml glass vials.

Table 1: Characteristics of soil, anaerobic digestate (AD) and wood-ash (WA); measurements were in dry weight (d/w), except those marked (*) which were in wet weight (w/w); <BDL = Below Detection Limit; values are the mean of n = 3 ± standard error of the mean (SEM).

Samples	pH	EC (µS/cm)	Total organic matter (LOI) (%)	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 this 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, and has no record of previous exposure to PAHs. The soil is rich in organic matter content , and consists of clay (19.5 % ± 0.7), silt (20.0 % ± 0.9), and sand (60.4 % ± 1.2, with 0.12 % ± 0.01 coarse, 6.9 % ± 0.1 medium, and 53.3 % ± 0.6 fine particles) (Couling*, et al.*, 2010). Other measured physico-chemical characteristics of the soil are presented in Table 1 above. The soil has no known history of exposure to anthropogenic petroleum hydrocarbons.

After collection, the soil was homogenized at its natural humidity with ≤2 mm sieve, during which the plants debris, worms and stones were removed before storage in a dark room at 4 ^{o}C until subsequent usage (Doick et al., 2003; Joergensen and Wichern, 2018). The soil's moisture content (32.1 %) was determined by oven drying at 105 ^{o}C and weighing 24 hourly until a constant weight was achieved (Couling *et al.*, 2010), while the soil pH (6.5) and EC (35.7 µS cm^{-1}) were determined following the standard methods (Rousk *et al.*, 2009). The soil's carbon-to-nitrogen (C:N) was 6.7±0.3 (Couling, et. al., 2010).

2.3 Soil amendment with renewable bioenergy residues

The amounts of the AD (17.31 g w/w) and WA (0.94 g d/w) used for the soil amendments 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).

2.4 Determination of pH and electrical conductivity (EC) in anaerobic digestate (AD) and wood-ash (WA)-amended soils

Soil pH was measured in a mixture of soil and distilled water, 1:2.5 (weight/volume), using a glass electrode pH meter (Rousk et al., 2009). Soil (10 g w/w) (≤ 2 mm) and deionized water (25 ml) were mixed end-to-end on an orbital shaker (100 rpm) for 30 minutes (min) and left to settle for another 30 min. The pH probe was calibrated to pH 4, 7 and 10 (following the manufacturer's guide). Similarly, the soil EC was measured using an EC electrode (calibrated with a standard solution of known conductivity).

2.5 Microbial activity in the soil and renewable bioenergy residues

2.5.1. Soil and renewable bioenergy residues spiking

AD (220.5 g w/w), a mixture (220.5 g; w/w) of AD (17.3 g AD w/w) and WA (0.94 g WA d/w) (AD-WA) and pristine soil (220.5 g w/w) were separately spiked with ^{12}C-phenanthrene (100 mg kg^{-1} soil), using acetone as the carrier solvent. The mixtures were homogenized and left in the fume cupboard for 2 hours, to allow the acetone to volatilize. Afterwards, they were separately aged (in triplicates) in pre-cleaned, labelled dark amber glass jars, which were loosely sealed (for ambient gas exchange) and incubated (20 ± 2 ^{o}C) in the dark for 90 d (Macleod and Semple, 2002; Macleod and Semple, 2006).

2.5.2. Mineralisation of ^{14}C-phenanthrene in the soil and renewable bioenergy residues

The mineralisation of ^{14}C-phenanthrene in pristine soil (control), AD, and mixtures of AD and WA were carried out using a ^{14}C-respirometric system. At defined intervals of 1, 15, 30

and 90 d of incubating the soil, AD, and a mixture of anaerobic digestate and wood-ash (AD-WA), each microcosm was sampled (14.7 g w/w) in modified pre-cleaned Schott bottle (250 ml size) (respirometer) (Reid et al., 2001). The soils were slurried with sterilized deionized water (30 ml). Each of the respirometric microcosms was spiked with 5 μl ^{14}C-phenanthrene (50 kBq kg^{-1} soil) and 20.1 mg kg^{-1} soil of ^{12}C-phenanthrene. A glass vial (7 ml) containing fresh NaOH (2 ml) was suspended (attached to the lid) inside each respirometer. Blank microcosms for AD, AD-WA and soil were also set up to monitor the background ^{14}C-activity. All the respirometers were incubated (20 ± 2 ^{o}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 $NaOH_{(aq)}$ 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 a liquid scintillation counter (Canberra Packard Tri-Carb 2250CA scintillation counter).

2.6 Indigenous catabolism of ^{14}C-phenanthrene in the soils amended with renewable bioenergy residues

Sieved soil (2.87 kg w/w) was spiked with ^{12}C-phenanthrene (100 mg kg^{-1} soil), using acetone as the delivery solvent. After homogenizing the mixtures, they were left in the fume cupboard for 2 hours to allow the acetone to volatilize. Control soil (220.5 g w/w) was separated and 3 types of soil amendments (882 g w/w each) were prepared as follow: (1) soil + AD + WA (2) soil + AD (3) soil + WA. Four proportionately increasing amounts of AD and WA were used at every addition of the AD and WA, and the resultant amendments were, henceforth, referred to as ‘conditions’ (Table 2).

Table 2: Four proportionately increasing amounts of anaerobic digestate (AD) and wood-ash (WA) added to the soils, henceforth referred to as 'conditions'.

Soil amendments (conditions)	AD Concentrations (w/wt, g)	WA Concentrations (d/w, g)
1	0.17	0.009
2	1.73	0.09
3	17.3	0.94
4	173.1	9.4

All amended soils were incubated at 20 ± 2 ^{o}C and sampled at defined intervals of 1 d, 15 d, 30 d, 60 d and 90 d soil-amendments contact times, for respirometric assays (previously described). The ^{14}C-activity in the trapped $^{14}CO_2$ (produced from the mineralisation of the ^{14}C-phenanthrene) was quantified by liquid scintillation counting (using Canberra Packard Tri-Carb 2250CA scintillation counter). The respirometric data were used to calculate the changes in the lag times, as well as the rates and extents of the mineralisation.

2.7 Statistical Analysis

Blank-corrected data were plotted with SigmaPlot 10.0. The effects of the soil amendments with the AD, WA and the mixtures of AD and WA on mineralisation of the ^{14}C-phenanthrene were analyzed using a one-way analysis of variance (ANOVA), at 95 % confidence level ($P < 0.05$), to determine the least significant difference. Also, Turkey and LSD's Post-hoc tests (SPSS) were performed to compare the means within and across the different amended soils.

3. Results

3.1. Changes in pH and electrical conductivity (EC) in anaerobic digestate and wood-ash (AD-WA)-amended soils

The impact of soil amendment with the mixtures of AD and WA on soil pH and EC were monitored over 36 h period. The results showed a slight increase in the pH (6.3) (Figure 1) and EC (257.9 µS cm^{-1}) (Figure 2) of the AD-WA-amended soils compared to the pH (6.15) and EC (103.6 µS cm^{-1}) of the unamended soils. However, the pH of the AD-WA-amended soils remained within the neutral and/or optimal pH range for microbial growth (5.5 - 8.8) and hydrocarbon degradation (6.5 - 8.0) (Maletić *et al.*, 2013). Similarly, the EC remained within the EC threshold level of the unamended soils (2 ds m^{-1} or 2000 µS cm^{-1}) (Herrero and Pérez-Coveta, 2005; Fernández-Delgado Juárez *et al.*, 2013; Gómez-Brandón *et al.*, 2016).

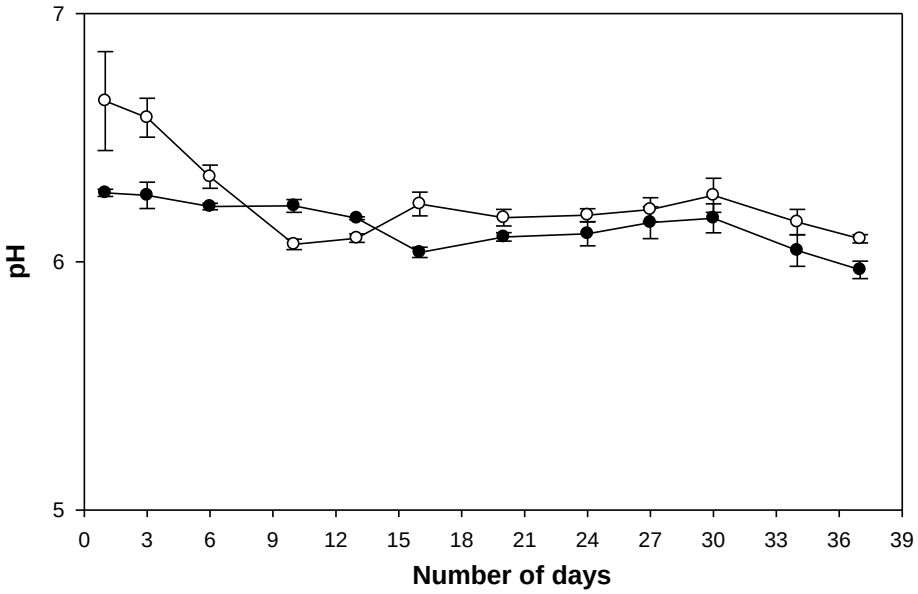

Figure 1: Changes in pH of unamended (●) and anaerobic digestate and wood-ash (AD-WA)-amended (○) soils over 37 days; values are the mean of *n = 3* ± SEM.

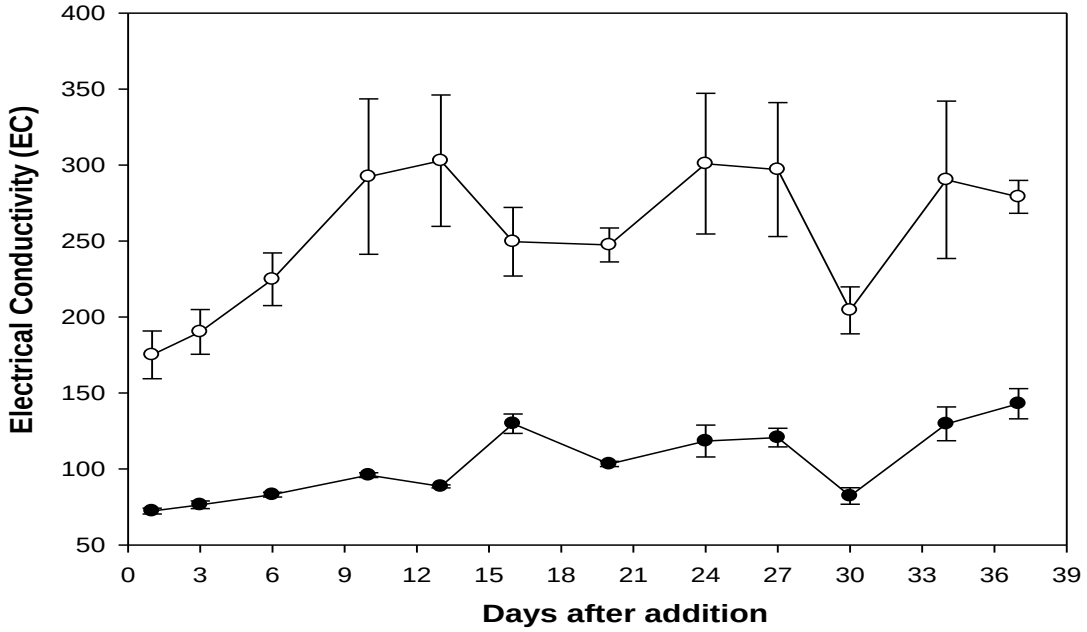

Figure 2: Changes in electrical conductivity (EC) of unamended (●) and anaerobic digestate and wood-ash (AD-WA)-amended (○) soils over 37 days; values are the mean of $n = 3$ ± SEM.

3.2 Microbial catabolic activity in soil and renewable bioenergy residues

Indigenous catabolic evolution of ^{14}C-phenanthrene was assessed in AD, mixtures of AD and WA and soil only (control). The occurrence of catabolic activities in the AD, mixtures of AD and WA and soil only incubations were monitored for 14 d at intervals of 1 d, 15 d, 30 d and 90 d soil-PAH contact time (Figure 3 and Table 3). The amount of $^{14}CO_2$ (%) produced at each time point was considered as the measures of the indigenous microbial activity that occurred in the AD, mixtures of AD and WA, as well as soil incubations. The lag phases were significantly ($P < 0.05$) shorter in the soil only incubations, ranging from 14.2 ± 0.2 h to 8.1 ± 0.2 h, than in the AD (313.9 ± 22.1 h to 154.9 ± 91.1 h), as well as mixtures of AD and WA (252.3 ± 42.9 h to 41.2 ± 17.4 h) as their interactions with the PAH increased.

Also, the mineralisation rates were higher ($P < 0.05$) in soil only incubations (1.1 ± 0.2 % h^{-1} to 1.9 ± 0.3 % h^{-1}) compared to the AD (0.1 ± 0.0 % h^{-1} to 0.3 ± 0.0 % h^{-1}) as well as mixtures of AD and WA (0.1 ± 0.0 % h^{-1} to 0.4 ± 0.1 % h^{-1}). Similarly, the extents of mineralisation were greater ($P < 0.05$) in soil only incubations (56.0 ± 6.3 % to 84.8 ± 1.9 %) than AD (4.6 ± 0.6 % to 14.7 ± 8.4 %) and AD-WA (5.7 ± 0.9 % to 20.0 ± 5.0 %), with increasing contact time of the PAH and AD, mixtures of AD and WA as well as soil (Table 3).

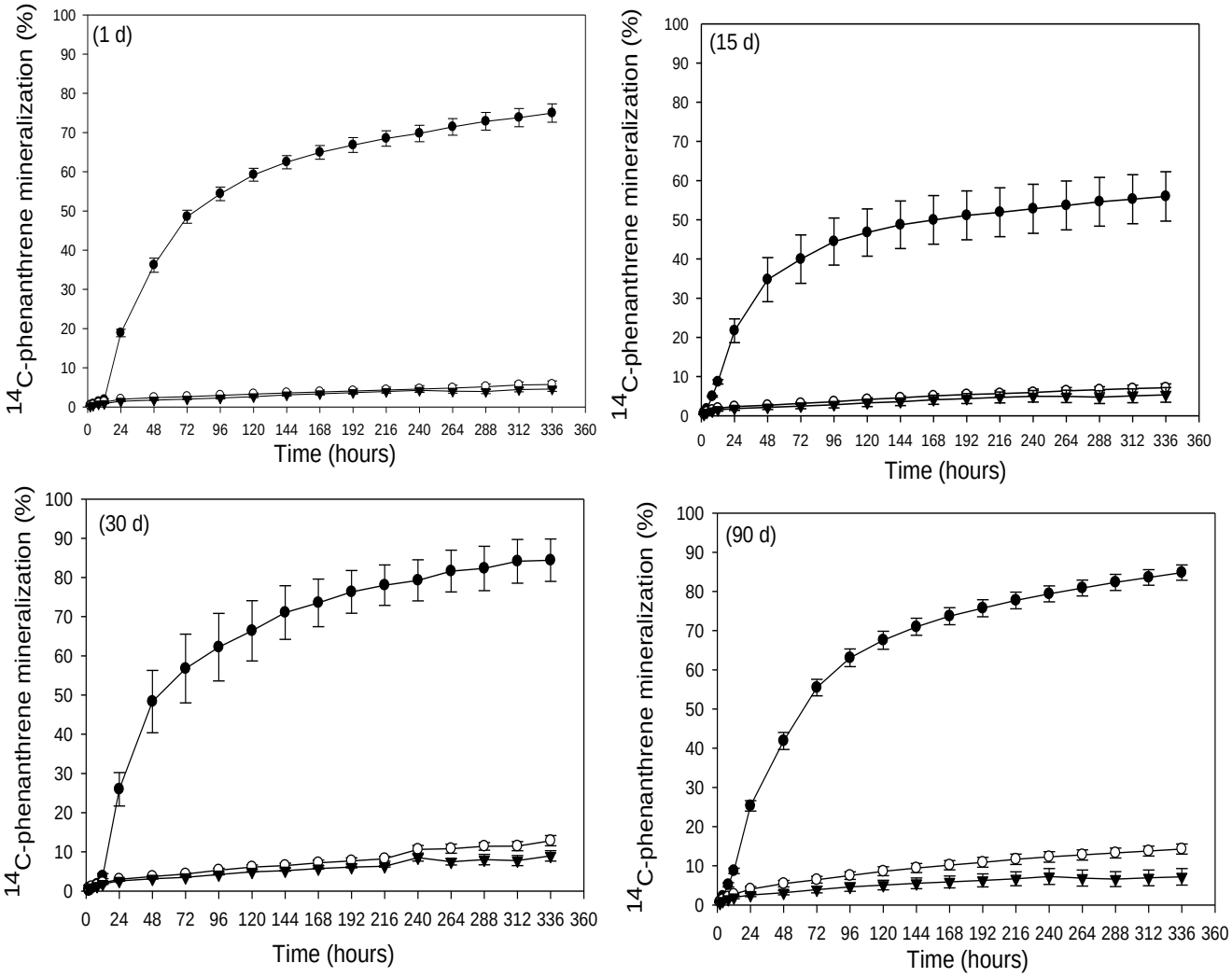

Figure 3: The mineralisation of ^{14}C-phenanthrene in the ^{12}C-phenanthrene-spiked soils (controls) (●), mixture of anaerobic digestate and wood-ash (AD-WA) (○) and anaerobic digestate (AD) (▼) at increasing AD-, AD-WA- and soil-PAH contact times 1, 15, 30 and 90 d; values are the mean of *n=3* ± standard error of the mean (SEM).

Table 3: The phases of mineralisation of ^{14}C-phenanthrene in the ^{12}C-phenanthrene-spiked soils (controls), mixture of anaerobic digestate and wood-ash (AD-WA) and anaerobic digestate (AD); values in columns followed by different letters are statistically different (Turkey, LSD; *n = 3*; *P < 0.05*); Values are the mean of *n = 3* ± standard error of the mean (SEM).

Soil-PAH contact time (d)	**Amendment type**	**Lag Phase (h)**	**Maximum Rate (% h^{-1})**	**Overall extent $\sum^{14}CO_2$ (%)**
1 d	Control (soil +^{14}C-PAH)	14.2 ± 0.2 ^{A}	1.4 ± 0.1 ^{A}	75.0 ± 2.3 ^{A}
	AD + WA	237.5 ± 50.1 ^{B}	0.1 ± 0.0 ^{B}	5.7 ± 0.9 ^{B}
	AD	296.1 ± 38.4 ^{C}	0.1 ± 0.0 ^{B}	4.6 ± 0.6 ^{C}
15 d	Control	8.1 ± 0.2 ^{A}	1.1 ± 0.2 ^{A}	56 ± 6.3 ^{A}
	AD + WA	188.4 ± 38.6 ^{B}	0.3 ± 0.1 ^{B}	7.2 ± 1.0 ^{B}
	AD	218.8 ± 58.8 ^{C}	0.3 ± 0.0 ^{B}	5.3 ± 1.9 ^{C}
30 d	Control	12.6 ± 0.5 ^{A}	1.9 ± 0.3 ^{A}	84.4 ± 5.4 ^{A}
	AD + WA	252.3 ± 42.9 ^{B}	0.1 ± 0.0 ^{B}	12.8 ± 1.3 ^{B}
	AD	313.9 ± 22.1 ^{C}	0.1 ± 0.0 ^{B}	9.0 ± 1.3 ^{C}
90 d	Control	7.8 ± 0.6 ^{A}	1.4 ± 0.1 ^{A}	84.8 ± 1.9 ^{A}
	AD + WA	41.2 ± 17.4 ^{B}	0.4 ± 0.1 ^{B}	20.0 ± 5.0 ^{B}
	AD	154.9 ± 91.1 ^{C}	0.3 ± 0.0 ^{B}	14.7 ± 8.4 ^{C}

3.3 Indigenous catabolism of ^{14}C-phenanthrene in soils amended with renewable bioenergy resources.

The kinetics of mineralisation of the ^{14}C-phenanthrene in the AD- and/or WA-amended soils were measured at 1 d, 15 d, 30 d, 60 d and 90 d soil-PAH contact times (Figures 4 to 6). The length of the lag phases, as well as the rates and extents of mineralisation of the ^{14}C-phenanthrene were measured to quantify the impacts of each amendment on the mineralisation over time (Tables 4 to 6).

3.3.1. Mineralisation of ^{14}C-phenanthrene in anaerobic digestate (AD)-amended soils

The mineralisation of the ^{14}C-phenanthrene in the ^{12}C-phenanthrene-exposed soils amended with increasing amounts of AD (0.170 g, 1.730 g, 17.31 g and 173.1 g) were quantified (Figure 4 and Table 4). The lag phases were significantly ($P < 0.05$) shorter after 90 d with the additions of 1.73 g of AD (2.7 ± 0.0 h), 17.3 g of AD (2.9 ± 0.3 h) and 173.1 g of AD (2.4 ± 0.1 h) compared to the controls (7.9 ± 0.6 h). Also, shorter lag phases were observed with the additions of 1.73 g of AD (1 d, 30 d and 60 d) and 0.17 g of AD (15 d and 90 d) but they were not statistically different ($P > 0.05$) from the controls. The addition of 173.1 g of AD resulted in longer ($P < 0.05$) lag phases (149.8 ± 8.2 h) at the onset of the soil incubation (1 d), however there were significant reductions as the soil-PAH contact time increased.

The rates of mineralisation were higher ($P < 0.05$) than the controls with the additions of 1.73 g AD after 90 d (2.4 ± 0.1 % h^{-1}), 17.31 g AD after 60 d (1.7 ± 0.1 % h^{-1}) and 90 d (2.2 ± 0.2 % h^{-1}), as well as 173.1 g AD after 90 d (2.1 ± 0.0 % h^{-1}). Mineralisation rates were also increased with the additions of 0.17 g AD after 15 d (1.2 ± 0.1 % h^{-1}) and 90 d

(1.3 ± 0.3 % h^{-1}) but they did not statistically differ ($P > 0.05$) from the controls. Generally, soil amendments with 0.17 g, 1.73 g and 17.31 g of AD consistently resulted in higher rates from 1 d to 60 d of soil-PAH contact time.

The extents of ^{14}C-phenanthrene mineralisation were also monitored over time. At 1 d, the addition of 0.17g of AD resulted in a significantly higher extent of mineralisation compared to the other amendment conditions and controls. There were no observed significant differences ($P > 0.05$) among the amendment conditions, as well as between the amendment conditions and controls in most of the time points throughout the study. However, the greatest extents of mineralisation were found after 60 d with 1.73 g (89.6 ± 3.0 %); while the lowest extents were recorded with 173.1 g of AD (29.2 ± 2.0 %, 30 d). Also, lower extents of mineralisation were generally observed with the addition of 173.1 g of AD in all the time points compared to the other amendment conditions; however, they were significantly lower than the controls. Noticeably, all the AD amendments, apart from 173.1g, showed greater extents of mineralisation.

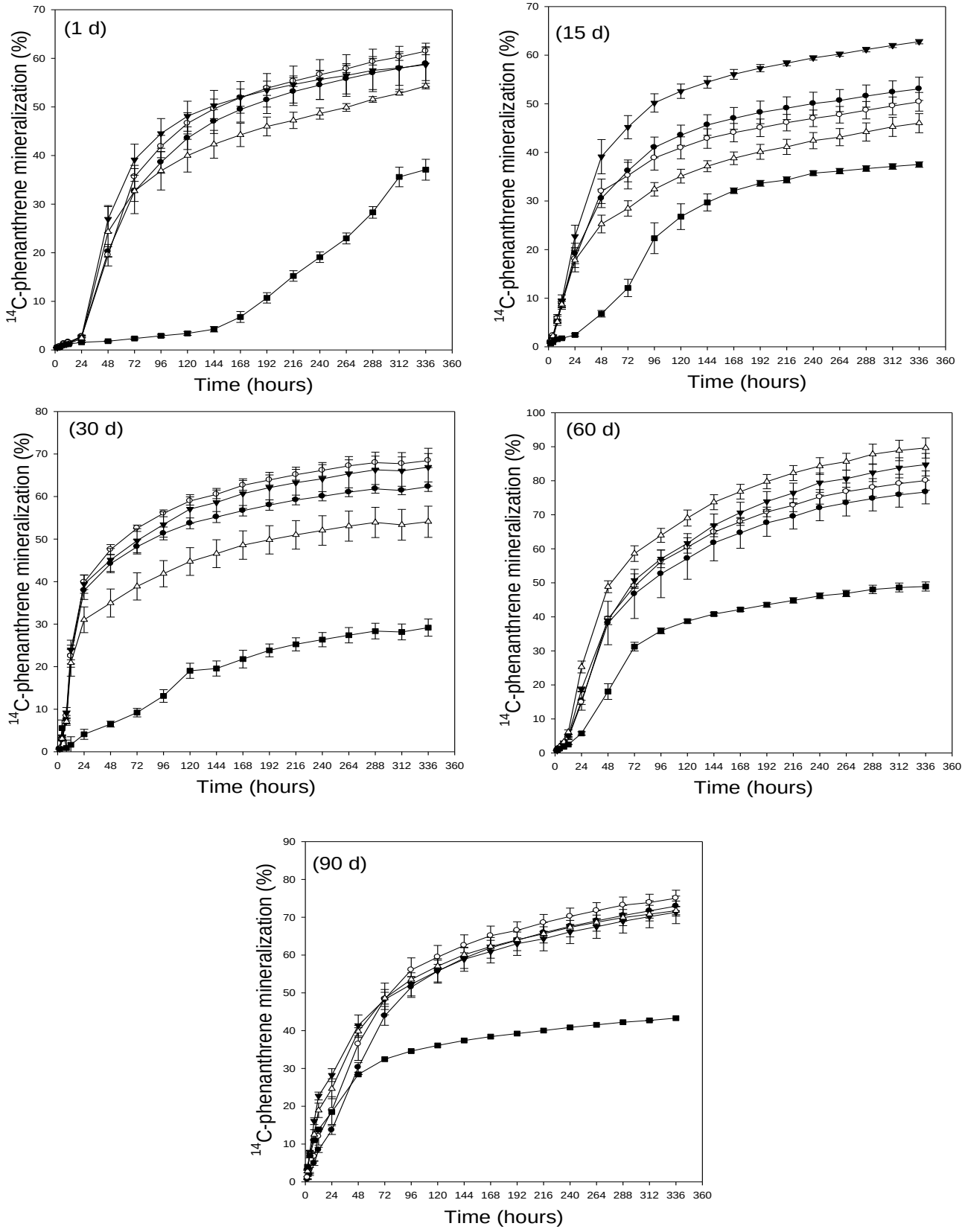

Figure 4: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in unamended ^{12}C-phenanthrene-exposed soils (controls) (●) and ^{12}C-phenanthrene-spiked soils amended with proportionately increasing amounts of anaerobic digestate (AD): 0.170 g (○); 1.730 g (▼); 17.310 g (△); 173.100 g (■) at increasing soil-PAH contact time 1, 15, 30, 60 and 90 d; values are the means of n = 3 ± SEM.

Table 4: The phases of mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-spiked soils amended with anaerobic digestate (AD); values in columns followed by different letters are statistically different (Turkey, LSD; *n = 3*; *P < 0.05*): also showed reduced lag times (†) and higher mineralisation (*) that were not statistically significant *(P > 0.05)*; values are the mean of *n = 3* ± SEM.

Soil-PAH contact time (d)	Soil + AD	Lag time (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1 d	Control (Soil + ^{14}C-PAH)	27.0 ± 0.4 A	1.4 ± 0.6 A	58.9 ± 3.5 A
	0.170 g	27.4 ± 0.5 A	0.8 ± 0.0 A	61.5 ± 0.7 B
	1.730 g	26.4 ± 0.4 A†	1.0 ± 0.1 A	58.7 ± 4.4 A
	17.31 g	27.4 ± 1.5 A	0.9 ± 0.2 A	54.3 ± 0.6 A
	173.1 g	149.8 ± 8.2 B	0.3 ± 0.0 B	37.1 ± 2.1 C
15 d	Control	7.7 ± 0.2 A	1.1 ± 0.1 A	53.1 ± 2.4 A
	0.170 g	7.1 ± 0.6 A†	1.2 ± 0.1 A*	50.4 ± 1.9 A
	1.730 g	8.0 ± 1.2 A	1.3 ± 0.1 A*	62.8 ± 0.4 B
	17.31 g	7.8 ± 0.45 A	1.3 ± 0.1 A*	46.0 ± 2.0 A
	173.1 g	36.8 ± 2.4 B	0.6 ± 0.1 B	37.5 ± 0.6 C
30 d	Control	5.2 ± 0.1 A	3.7 ± 0.3 A	62.3 ± 1.1 A
	0.170 g	5.0 ± 0.2 A	3.5 ± 0.4 A	68.5 ± 1.7 A*
	1.730 g	4.7 ± 0.2 A†	3.7 ± 0.2 A	66.9 ± 4.5 A*
	17.31 g	5.2 ± 0.2 A	3.4 ± 0.6 A	54.1 ± 3.7 A
	173.1 g	10.6 ± 7.3 A	2.5 ± 1.0 A	29.2 ± 2.0 A
60 d	Control	12.3 ± 1.0 A	1.2 ± 0.2 A	76.7 ± 3.5 A
	0.170 g	12.6 ± 0.5 A	1.1 ± 0.0 A	80.0 ± 2.8 A*
	1.730 g	11.7 ± 0.6 A†	1.2 ± 0.0 A	84.7 ± 3.3 A*
	17.31 g	10.7 ± 0.7 A†	1.7 ± 0.1 B	89.6 ± 3.0 B
	173.1 g	21.3 ± 1.6 B	0.7 ± 0.0 C	48.9 ± 1.3 C
90 d	Control	7.9 ± 0.6 A	0.9 ± 0.0 A	72.9 ± 2.6 A
	0.170 g	6.1 ± 1.1 A†	1.3 ± 0.3 A*	75.0 ± 2.1 A*
	1.730 g	2.7 ± 0.0 B	2.4 ± 0.1 B	71.3 ± 3.0 A
	17.31 g	2.9 ± 0.3 B	2.2 ± 0.2 B	71.7 ± 1.1 A
	173.1 g	2.4 ± 0.1 B	2.1 ± 0.0 B	43.3 ± 0.0 B

3.3.2 Mineralisation of ^{14}C-phenanthrene in wood-ash (WA)-amended soils

The effects of the additions of increasing amounts of WA (0.009 g, 0.090 g, 0.940 g and 9.400 g) on mineralisation of ^{14}C-phenanthrene were monitored (Figure 5 and Table 5). After 1 d, the addition of 9.4 g of WA showed longer ($P < 0.05$) lag phases (91.8 ± 2.6 h) which significantly reduced with increasing soil-PAH contact time. The addition of 0.009 g of WA resulted in consistently shorter lag phases as the soil-PAH contact time increased but they were not statistically significant. There were similar results with WA additions of 0.090 g after 1 d and 90 d, as well as 0.94 g after 90 d. Lag phases were significantly shortened with all the WA additions after 15 d, which continued as the soil-PAH contact time increased compared to 1 d.

The mineralisation rates were significantly higher after 90 d with the additions of 0.090 g of WA (2.7 ± 0.1 % h^{-1}) and 0.94 g of WA (2.2 ± 0.2 % h^{-1}) than the controls (0.9 ± 0.0 % h^{-1}). Also, higher rates were observed with the addition of 0.009 g of WA after 1 d, 15 d and 90 d but they were not statistically significant. The extents of mineralisation were greater ($P < 0.05$) with the addition of 0.009 g of WA after 15 d and 60 d (60.0 ± 1.6 % and 84.5 ± 2.4 %) than the controls (52.4 ± 2.4 % and 75.5 ± 3.5 %), respectively. However, the extents of mineralisation in all the WA additions increased with increasing soil-PAH contact time except 9.40 g of AD. The enhancement effects were more pronounced after 15 d and as the soil-PAH contact time increased compared to 1 d.

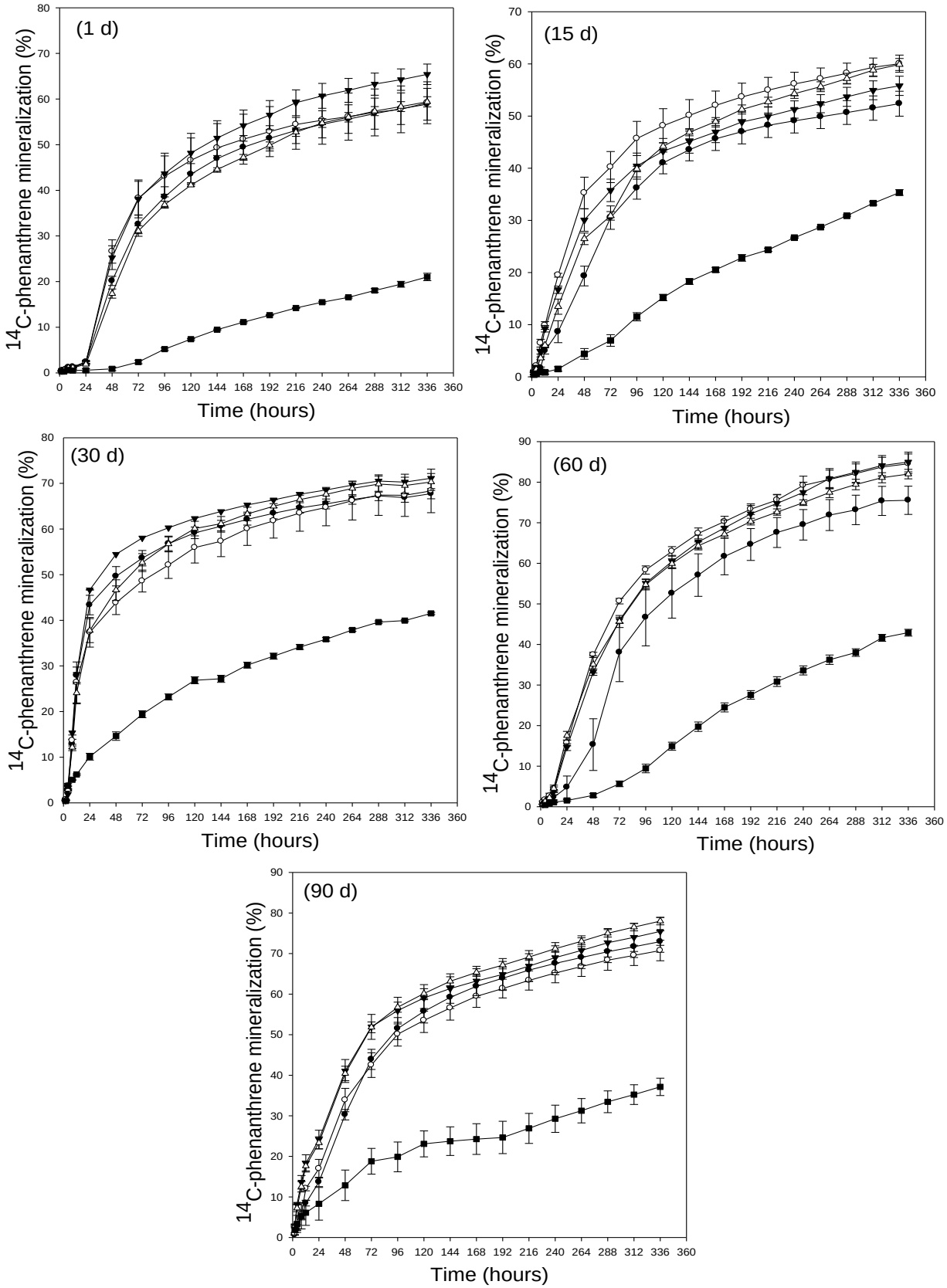

Figure 5: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in unamended ^{12}C-phenanthrene-exposed soils (controls) (●) and ^{12}C-phenanthrene-spiked soils amended with proportionately increasing amounts of wood-ash (WA): 0.009 g (○); 0.090 g (▼); 0.940 g (△) and 9.400 g (■) at increasing soil-PAH contact time 1, 15, 30, 60 and 90 d; values are the mean of n = 3 ± SEM

Table 5: The phases of mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-spiked soils amended with wood-ash (WA); values in columns followed by different letters are statistically different (Turkey, LSD; *n = 3*; *P < 0.05*): also showed reduced lag times (†) and higher mineralisation (*****) that were not statistically significant *(P > 0.05)*; values are the mean of *n = 3* ± SEM.

Soil-PAH contact time (d)	Soil + WA	Lag time (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1 d	Control (Soil + ^{14}C-PAH)	27.0 ± 0.4 A	0.7 ± 0.0 A	58.9 ± 3.5 A
	0.009 g	26.4 ± 0.5 A†	1.0 ± 0.1 A*	59.2 ± 4.6 A*
	0.090 g	26.5 ± 0.6 A†	0.1 ± 0.1 A	65.4 ± 2.3 A*
	0.940 g	28.3 ± 0.4 A	0.6 ± 0.0 A	59.4 ± 1.1 A*
	9.400 g	91.8 ± 2.6 B	0.2 ± 0.0 B	21.0 ± 0.8 B
15 d	Control	7.7 ± 0.2 A	1.0 ± 0.1 A	52.4 ± 2.4 A
	0.009 g	6.7 ± 0.6 A†	1.1 ± 0.1 A*	60.0 ± 1.6 B
	0.090 g	8.0 ± 0.4 A	1.1 ± 0.1 A*	55.8 ± 1.8 A*
	0.940 g	10.0 ± 0.2 A	0.7 ± 0.0 A	59.9 ± 1.2 A*
	9.400 g	52.7 ± 9.6 B	0.2 ± 0.0 B	35.3 ± 0.5 C
30 d	Control	4.9 ± 0.0 A	3.7 ± 0.3 A	71.5 ± 1.1 A
	0.009 g	4.5 ± 0.2 A†	3.4 ± 0.6 A	68.4 ± 4.8 A
	0.090 g	4.9 ± 0.0 A	3.4 ± 0.0 A	68.6 ± 0.0 A
	0.940 g	4.9 ± 0.0 A	3.0 ± 0.4 A	72.5 ± 1.9 A*
	9.400 g	7.8 ± 0.3 B	1.7 ± 0.1 B	71.1 ± 0.2 A
60 d	Control	12.3 ± 1.0 A	1.0 ± 0.2 A	75.5 ± 3.5 A
	0.009 g	12.1 ± 1.2 A†	1.0 ± 0.0 A	84.5 ± 2.4 B
	0.090 g	13.2 ± 0.2 A	0.9 ± 0.1 A	84.9 ± 2.4 C
	0.940 g	12.4 ± 0.4 A	1.1 ± 0.1 A	82.0 ± 1.2 A*
	9.400 g	41.3 ± 3.4 B	0.2 ± 0.0 B	42.9 ± 0.8 D
90 d	Control	7.9 ± 0.6 A	0.9 ± 0.0 A	72.9 ± 2.6 A
	0.009 g	7.1 ± 1.0 A†	1.6 ± 0.1 A*	70.7 ± 2.5 A
	0.090 g	2.7 ± 0.0 A†	2.7 ± 0.1 B	75.5 ± 3.4 A*
	0.940 g	2.9 ± 0.3 A†	2.2 ± 0.2 C	78.0 ± 0.9 A*
	9.400 g	13.1 ± 8.5 A	1.2 ± 0.5 A*	37.1 ± 2.1 B

3.3.3. Mineralisation of ^{14}C-phenanthrene in the soils amended with mixtures of anaerobic digestate and wood-ash (AD-WA)

The effects of the additions of the mixtures of AD and WA on the mineralisation of ^{14}C-phenanthrene were monitored (Figure 6 and Table 6). The addition of mixtures of 173.1 g of AD and 9.4 g of WA consistently showed longer lag phases at every time point when compared to the other conditions and controls; however, there were significant reductions over time. The longest (208 ± 38.6 h) and shortest (2.7 ± 0.1 h) lag phases were observed with the addition of mixtures of 173.1 g of AD and 9.4 g of WA after 1 d and 30 d, respectively. Shorter lag phases were also observed in all the other AD additions from 15 d to 90 d compared to 1 d incubations but they were not statistically different ($P > 0.05$) from the controls.

The rates of ^{14}C-phenanthrene mineralisation were significantly higher with the addition of mixtures of 17.31 g of AD and 0.94 g of WA after 15 d, as well as 173.1 g of AD and 9.4 g of WA after 30 d compared to the controls. Also, higher rates were observed with the additions of mixtures of 0.17 g of AD and 0.009 g of WA (1 d, 15 d and 30 d), 1.73 g of AD and 0.009 g of WA (15 d, 30 d and 60 d), as well as 17.31 g of AD and 0.94g of WA (60 d and 90 d) but they were not statistically significant.

The extents of mineralisation were greater than the controls with the addition of mixtures of 0.170g of AD and 0.009 g of WA after 15 d to 90 d but they were not statistically different ($P > 0.05$) from the controls. Similar results were observed with the addition of mixtures of 17.31 g of AD and 0.940 g of WA after 60 d. Also, greater extents of mineralisation were observed with the additions of all the mixtures of AD and WA after 15 d but they were not statistically significant compared to the controls. The greatest extents of mineralisation were observed with the addition of mixtures of 0.17 g of AD and 0.009 g

of WA (80.7 ± 8.7 h), while the lowest extents were observed with the mixtures of 173.1 g of AD and 9.400 g of WA, compared to the controls.

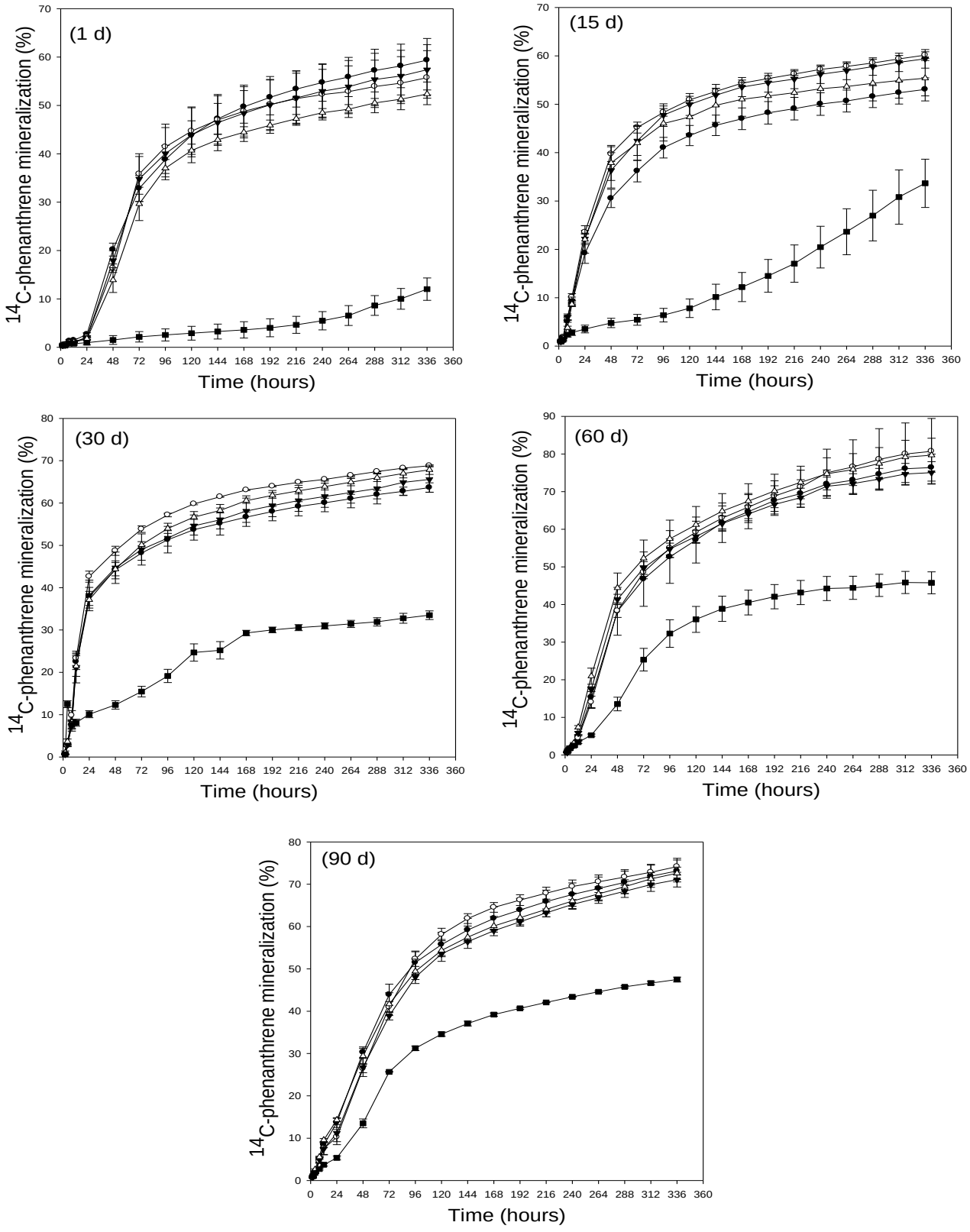

Figure 6: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in unamended ^{12}C-phenanthrene-exposed soils (controls) (●) and ^{12}C-phenanthrene-spiked soils amended with mixtures of anaerobic digestate and wood-ash (AD-WA): 0.170 g anaerobic digestate (AD)/0.009 g wood-ash (WA) (○); 1.730 g anaerobic digestate (AD)/0.090 g wood-ash (WA) (▼); 17.310 g anaerobic digestate (AD)/0.940 g wood-ash (WA) (△); 173.100 g AD/9.400 g wood-ash (WA) (■) at increasing soil-PAH contact time 1, 15, 30, 60 and 90 d; values are the means *of* n = 3 ± SEM.

Table 6: The phases of mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-spiked soils amended with mixtures of anaerobic digestate and wood-ash (AD-WA); values in columns followed by different letters are statistically different (Turkey, LSD; *n = 3*; *P < 0.05*): also showed 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	Lag time (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1 d	Control (^{14}C-PAH + soil)	27.0 ± 0.4 A	0.7 ± 0.0 A	59.3 ± 4.5 A
	0.170g / 0.009 g	28.7 ± 0.2 A	0.8 ± 0.1 A*	55.7 ± 5.6 A
	1.730 g / 0.090 g	28.4 ± 0.8 A	0.7 ± 0.1 A	57.4 ± 5.2 A
	17.31 g / 0.940 g	30.5 ± 1.3 A	0.6 ± 0.1 A	52.3 ± 0.8 A
	173.1 g / 9.400 g	208.0 ± 38.6 B	0.2 ± 0.0 B	12.0 ± 2.3 B
15 d	Control	7.7 ± 0.2 A	1.0 ± 0.1 A	53.1 ± 2.4 A
	0.170g / 0.009 g	7.1 ± 0.3 A†	1.2 ± 0.0 A*	60.2 ± 0.7 A*
	1.730 g / 0.090 g	7.1 ± 0.6 A†	1.2 ± 0.0 A*	59.4 ± 1.9 A*
	17.31 g / 0.940 g	8.9 ± 0.2 A	1.3 ± 0.1 B	55.4 ± 3.7 A*
	173.1 g / 9.400 g	66.3 ± 36.4 B	0.5 ± 0.1 C	33.6 ± 5.0 B
30 d	Control	5.2 ± 0.1 A	3.3 ± 0.3 A	63.7 ± 1.1 A
	0.170g / 0.009 g	5.0 ± 0.1 A†	3.4 ± 0.1 A*	68.8 ± 0.2 A*
	1.730 g / 0.090 g	5.2 ± 0.2 A	3.4 ± 0.6 A*	65.6 ± 3.0 A*
	17.31 g / 0.940 g	4.7 ± 0.3 A†	3.3 ± 0.6 A	67.9 ± 1.1 A*
	173.1 g / 9.400 g	2.7 ± 0.1 B	5.9 ± 0.4 B	33.5 ± 1.0 B
60 d	Control	12.3 ± 1.0 A	1.0 ± 0.2 A	76.4 ± 3.6 A
	0.170g / 0.009 g	12.6 ± 0.8 A	1.0 ± 0.0 A	80.7 ± 8.7 A*
	1.730 g / 0.090 g	11.0 ± 0.5 A†	1.1 ± 0.0 A*	75.0 ± 2.9 A
	17.31 g / 0.940 g	9.7 ± 0.2 A†	1.1 ± 0.1 A*	79.7 ± 4.5 A*
	173.1 g / 9.400 g	21.7 ± 1.6 B	0.5 ± 0.0 A	45.8 ± 2.9 B
90 d	Control	7.9 ± 0.6 A	0.9 ± 0.0 A	73.1 ± 2.6 A
	0.170g / 0.009 g	8.9 ± 1.0 A	0.9 ± 0.1 A	74.1 ± 2.0 A*
	1.730 g / 0.090 g	8.4 ± 1.5 A	0.9 ± 0.1 A	71.1 ± 1.7 A
	17.31 g / 0.940 g	6.9 ± 0.1 A†	1.0 ± 0.1 A*	72.6 ± 1.5 A
	173.1 g / 9.400 g	19.2 ± 2.4 B	0.6 ± 0.0 A	47.5 ± 0.6 B

4. Discussion

4.1 Changes in soil pH and electrical conductivity (EC) in AD and WA amended soils

Soil amendments with AD and WA may cause changes in the soil pH and other physico-chemical properties (Bougnom and Insam, 2009; García-Sánchez *et al.*, 2015a). Excessive variations in soil pH or salinity can affect the microbial activity, biomass and community structure (Fernández-Delgado Juarez *et al.*, 2013; Naseri *et al.*, 2014; Gómez-Brandón *et al.*, 2016), which can, consequently, affect the optimal biodegradation of PAHs. In this study, the soils amended with a mixture of AD and WA did not show extreme changes in pH and EC compared to the pH of similar unamended soils. The pH buffer capacity (pHBC) of the soil might have maintained the pH of the soils amended with the mixtures of AD and WA within the optimal range of microbial growth and hydrocarbon degradation (Wang *et al.*, 2015; Maletić *et al.*, 2013). The results in this study showed increase in soil pH and EC following the combined addition of AD and WA. Further, the new pH was around the pH value of the similar unamended soils. This tendency of pH rise in AD-WA-amended soils and the pH stabilization around the pH of similar unamended soils have been reported in previous studies (Perucci *et al.*, 2006; Jokinen *et al.*, 2006; Perucci *et al.*, 2008; Bougnom *et al.*, 2012; Koszel and Lorencowicz 2015; Gómez-Brandón *et al.*, 2016). Also, it has been reported that the increase in soil pH following the additions of organic amendments may positively influence microbial activity (Mahmood *et al.*, 2003; Jokinen *et al.*, 2006; Perucci *et al.*, 2006), which can positively affect the biodegradation of PAHs.

4.2 Catabolic activity in the soil and renewable bioenergy residues

Indigenous microbial activity in the soil, AD, as well as mixtures of AD and WA was studied. This step helps to assess the levels of indigenous microbial activity in the soil and residues before the biodegradation study. This assessment helps to avoid introducing exogenous microbial population into the soils through the residues. Both the soil and the residues were pre-exposed to non-labelled (^{12}C-) phenanthrene to mimic occurrence of previous exposures (Macleod and Semple, 2006; Semple *et al.*, 2006) before the addition of the fresh organic contaminant (^{14}C-phenanthrene), at the beginning of the respirometric assays. The experimental conditions were enhanced and the ^{14}C-phenanthrene is assumed to be metabolised by the available microbial populations in the ^{12}C-phenanthrene-exposed and AD- and/or WA-amended soils. The amount of the catabolically evolved $^{14}CO_2$ (%) at a given time was sampled and measured at defined intervals of 2, 4, 6, 8, 12 and 24 hours and henceforth 24 hourly for 14 d. After 14 d, the mineralisation was observed plateaued; the cumulative percentage of the $^{14}CO_2$ produced at each defined interval, or sampling time, is taken as the measure or level of the catabolic activity in the soils and residues at the time (Semple *et al.*, 2006).

The AD used in this study had been pasteurized to remove potential pathogens, as well as to meet the legislative criteria for the management and disposal of AD (Directive 2008/98/EC, 2008). Also, the WA was obtained from a full-scale heat and power process. Therefore, significant catabolic activity was not expected in either residue. However, low levels of catabolic activity were observed in the AD (4.6 ± 0.6 % to 14.7 ± 8.4 %), as well as the mixtures of AD and WA (5.7 ± 0.9 % to 20.0 ± 5.0 %). This was expected due to the ubiquitous nature of PAHs (Lima *et al.*, 2005; Okere and Semple 2012; Baldwin *et al*., 2020), and the susceptibility of PAHs to degradation by microbial populations (Lang *et al.*, 2016; Roslund *et al.*, 2018; Siles and Margesin, 2018), especially the LMW PAHs

(Couling *et al.*, 2010; Crampon *et al.*, 2014; Demeter *et al.*, 2017). However, the higher biological activity observed in the soil (56.0 ± 6.3 % to 84.8 ± 1.9 %) is expected because soil contains abundant and diversified microorganisms (Maletic *et al.*, 2013). Microorganisms play significant roles in nutrients cycling and organic matter turnover, as well as stabilisation of soil structure with diverse capacity for metabolising hydrocarbons (Maletic *et al.*, 2013). The biological properties of the soil have contributed to the successful biodegradation of the ^{14}C-phenanthrene. This step of investigating the indigenous catabolic activity of the soil, and the residues prior to their soil applications, agrees with several studies where the indigenous catabolic activity of the soil was determined through the mineralisation of ^{14}C-PAHs in the soil (Reid *et al.*, 2001; Rhodes *et. al.*, 2008, Macleod and Semple, 2002, 2006; Semple *et. al.*, 2006). The findings from the present study indicate that the microbial degradation of the ^{14}C-phenanthrene in the ^{12}C-phenanthrene-exposed soils amended with AD and/or WA, was predominantly indigenous; although, the PAH-degrading microbial populations were not analysed.

4.3 Indigenous catabolism of ^{14}C-phenanthrene in AD- and/or WA-amended and unamended soils

The entry of organic contaminants into the soil can increase the soil carbon-to-nitrogen ratio (C:N), as well as induce adverse soil conditions such as elevated pH and salinity, which can affect indigenous microbial activity (Lee *et al.*, 2008; Margesin and Schinner, 2001; Silva and Fay, 2012; Warr *et al.*, 2013; Naseri *et al.*, 2014). The soils used in this study were pre-exposed to non-labelled (^{12}C-) phenanthrene before the addition of the AD and/or WA in order to enhance the indigenous microbial adaptation to mineralize the ^{14}C-phenanthrene freshly added. This agrees with previous studies where soil microbes with previous exposures to organic contaminants degraded freshly added contaminants faster

compared to the similar soils with no exposure history (Macleod and Semple, 2002; 2006).

In past studies, indigenous microbial pre-exposures to PAHs were reported to influence the microbial catabolic activity and successful microbial adaptation to mineralise PAHs (Macleod and Semple, 2006; Patowary *et al.*, 2016). The amount of organic matter and clay contents in the soil may significantly impact on the microbial adaptation to mineralise PAHs in soil (Macleod and Semple, 2002, 2006; Rhodes *et. al.,* 2008). This is due to the potential of the organic contaminants to sorb on to the SOM and clay minerals which, consequently, limit the bioavailability and/or biodegradation of the organic contaminants in the soil (Stokes *et al.*, 2006; Yang *et al.*, 2010; Okere and Semple 2012; Riding *et al.*, 2013; Umeh *et. al.*, 2017). In this present study, changes in catabolic behaviour differed in the AD-, WA- and AD-WA-amended and unamended soils. Also, the lack of differences between the AD- and/or WA-amended soils could have been due to the ability of the indigenous microorganisms to outcompete any exogenous microorganisms from the bioenergy residues. A similar finding was observed in Fernández-Delgado Juarez *et al.* (2013), where no significant differences were observed between the AD- and WA-amended and unamended soils, and this was also mentioned to be due to the ability of the soil microflora to outcompete the exogenous microbes contained in the soil additives.

4.3.1 Mineralisation of ^{14}C-phenanthrene in AD-amended soils.

Additions of proportionately increasing amounts of AD to soil showed shorter lag phases, as well as increased rates and extents of mineralisation of ^{14}C-phenanthrene when compared to the unamended soils, as the soil-PAH contact time increased. The positive effects observed in the results were indicative of the potential of the AD and/or WA to stimulate indigenous microbial functioning of PAH-contaminated soils, and the successful

biodegradation of the ^{14}C-phenanthrene by the indigenous phenanthrene-degrading microbial populations. These observations imply that the indigenous microbes were able to adapt to the addition of the AD and utilize the nutrients supplied to the soil for their activity. However, these findings were consistent in the soils with lower amounts or additions of AD compared to the soils with the higher amount of AD. The lag phases were significantly longer at the onset of the incubations in the soils with the higher amount of AD. This might be due to the quantity of the AD added to the soils, which may have altered the soil biological or physico-chemical properties. However, the lag phases became significantly shorter as the soil-PAH contact time increased, which indicated an improved biological activity. The delayed effect at the onset of the incubation suggests the microorganisms were adapting to the addition/use of the AD and/or withstanding any possible unfavourable effect(s) induced by the residues on addition to the soil. This observation agrees with the consideration of a specific time being required for the catabolic function of the indigenous PAH-degrading bacteria in the soil (Semple *et al.*, 2001).

Also, the effect of the addition AD was observed on the rates and extents of mineralisation as the soil-PAH contact time increased. The addition of a higher amount of AD was observed with higher rates of mineralisation of aged ^{14}C-phenanthrene. However, consistent observations of higher rates were also made in the soils with the lower amounts or additions of AD (0.17 g, 1.73 g and 17.31 g) from the incubation onset and as the soil-PAH contact time increased.

Previous studies documented successful biostimulation in AD-amended soils. In a study by Alburquerque, *et al.* (2012a), the addition of AD to soil increased the N and P contents of the soil, which positively influenced the soil biological properties (microbial biomass and enzyme activities) compared to the corresponding soils amended with cattle manure and

mineral fertilizer. In a soil biosolarization study by Fernández-Bayo *et al.* (2017), soil amendment with AD significantly increased the C, P and K contents of the soils, which are essential nutrients for optimal microbial activity and mineralisation of PAHs. In another study by García-Sánchez *et al.* (2015a), significantly increased microbial activities (including biomass and physiological diversity) were observed in the soil shortly after the addition of AD, which increased on a long-term addition of AD. AD is known to contain considerable amounts of macro- and micro-nutrients, which are readily utilizable by the soil microorganisms (Alburquerque *et al.*, 2012a; Fernández-Delgado Juárez *et al.*, 2013; Koszel and Lorencowicz, 2015). These have been a significant influence on the indigenous microbial PAH-degraders of the soils in the present study, where the ^{14}C-phenanthrene mineralisation had been enhanced.

4.3.2 Mineralisation of ^{14}C-phenanthrene in WA-amended soils.

Similarly, WA addition to soil positively affected the lag phases, as well as the rates and extents of mineralisation, with increasing soil-PAH contact time. There was no effect on mineralisation at the onset of the soil-incubations. However, as the soil-PAH contact time increased, the lag phases were shorter in the ^{12}C-phennanthrene-exposed soils amended with lower amounts of WA, though they were not statistically significant and the shorter lag phases were not relative to the amounts of the WA added. However, the observation connotes the occurrence of an enhanced microbial activity. Also, the addition of lower amounts of WA resulted in greater extents of mineralisation as the soil-PAH contact time increased.

The biostimulatory effect of the WA in this study also agrees with past studies. In a study by García-Sánchez *et al.* (2015b), soil microbial biomass was increased after WA addition. Also, in a study by Perucci *et al.* (2006), the addition of a lower amount of WA (5

t ha^{-1}) increased the soil microbial activity, while the addition of a higher amount of WA (20 t ha^{-1}) resulted in reduced microbial activity after 4 months of treatment. Similarly, Campos *et al.* (2018) observed positive microbial responses (dehydrogenase activity and soil oxygen consumption) in the soils amended with lower percentages (up to 20 %) of WA. In addition, Fernández-Delgado Juárez *et al.* (2013) observed positive effects in microbial activity (assessed by basal respiration and microbial biomass C) in WA-treated soils, and the effects observed were not proportional to the amounts of the WA added to the soils.

Therefore, WA addition to soil has been indicated to be maximally beneficial at lower application rates, and possibly toxic at higher applications (Pitman, 2006). Studies indicated WA indirectly influences soil microbial processes by inducing changes on the pH and other physico-chemical properties of the soil (Bougnom and Insam, 2009; García-Sánchez *et al.*, 2015a). This suggests a reason why excessive addition of WA to soil can inhibit optimal microbial functioning and degradation of PAHs. For example, soil microorganisms are sensitive to pH and salinity, while any variation beyond the optimum level of these parameters can affect microbial growth/activity, biomass and community structure (Silva and Fay, 2012; Fernández-Delgado Juarez *et al.*, 2013; Maletić *et al.*, 2013; Naseri *et al.*, 2014).

On the contrary, in a study by García-Sánchez *et al.* (2015a), no significant effect was observed on the microbial activity of WA-amended soils. Similar results where no significant effect was observed in soil microbial processes after soil amendment with WA are reported in Bougnom *et al.* (2012) and Noyce *et al.* (2016). However, the various effects observed in WA-amended soils have been indicated might be due to the soil types, WA pre-treatment and application doses, length of the experiments, parameters of analysis, as well as the sampling time (Fernando-Jaurez *et al.*, 2013; Gómez-Brandón *et*

al., 2016). Studies show that sampling time may influence the soil pH, EC, nutrient contents (total N, $NH4^+$, and NO_3^-) and microbial community structure (Fernando-Jaurez et al., 2013; Gómez-Brandón *et al.*, 2016), while WA pre-treatment and application rates can affect the soil microflora (Kuba *et al.*, 2008).

4.3.3 Mineralisation of ^{14}C-phenanthrene in the soils amended with the mixtures of AD and WA.

Soil amendment with the mixtures of AD and WA showed more positive effects on the mineralisation of ^{14}C-phenanthrene in soil, as observed in the length of the lag phases, as well as the rates and extents of mineralisation. For example, shorter lag phases were observed in most of the ^{12}C-phenqanthrene-exposed soils amended with the mixtures of AD and WA compared to those separately amended with AD and WA. Similar results were observed with the rates of mineralisation as the combined addition of AD and WA increased the rates compared to their separate additions. WA contains variable amounts of P (Kuba *et al.*, 2008) and 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; Johansen et al., 2013; Fernández-Bayo *et al.*, 2017). Combined addition of AD and WA can potentially supply sufficient essential nutrients to soil than their separate additions. This could have contributed to their early biostimulatory effect upon addition to soils in the present study.

Similar observations were made in past studies where combined additions of AD and WA were used for soil amendment. In a study by Fernández-Delgado Juárez *et al.* (2013), the addition of mixtures of AD and WA increased the $NH4^+$ as well as the total C and P contents of the soil. Also, in Kuba *et al.* (2008), soil amendment with the mixtures of

organic wastes and WA increased the soil's available macronutrients. Generally, soil amendment with the mixtures of AD and WA can constitute an alternative effective organic soil amendment for enhancing microbial activity and degradation of PAHs. This is environmentally friendly and can constitute a sustainable option for the recycling of the residues (Fernando-Jaurez *et al.*, 2013; Tiwary *et al.*, 2015; Ning *et al.*, 2017).

5. Conclusion

The findings in this study indicate that soil amendments with AD and WA can potentially enhance indigenous biodegradation of PAHs. The shorter lag phases observed in the presence of the AD and/or WA suggests the development of earlier microbial adaptation and the activation of microbial catabolic activity, which are significant processes for successful biodegradation of PAHs. The results of this study suggest that soil amendments with AD and/or WA can positively influence the indigenous soil microbial function and, consequently, enhance the biodegradation of PAHs. Most importantly, reduced lag phases and fastest rates of mineralisation were observed with the addition of mixtures of AD and WA compared to their separate additions. Significant reductions in the kinetics of mineralisation were found where higher concentrations of the 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 applied to soils. The two investigated residues (AD and WA) should be explored for their contributions to successful stimulation of indigenous microbial activity and biodegradation of PAHs in contaminated soil.

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6.2 Paper 2: The effects of combined additions of anaerobic digestate and wood-ash on the development of indigenous phenanthrene catabolism in soil.

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are mostly associated with anthropogenic industrial processes relating to petroleum-containing materials or their derivatives. Their recalcitrance to biodegradation and risk of exposures to humans and other ecological receptors have promoted their environmental concerns. The improvement of soil conditions with appropriate additions of organic nutrients is considered amenable to enhancing indigenous biodegradation of PAHs to environmentally safe levels. This study investigated and compared the effects of soil amendments with single and combined additions of two different bioenergy residues, which are anaerobic digestate (AD) and wood-ash (WA) (under different amendment conditions), on the development of indigenous catabolism of ^{14}C-labelled phenanthrene (a model PAH) in ^{12}C-phenanthrene-exposed soils. Findings revealed that the ^{12}C-phenanthrene-exposed soils amended with lower amounts of AD and WA, both as separate and combined additions, reduced the lag phases, as well as increased the rates and extents of mineralisation. However, the combined addition of AD and WA generally increased the level of mineralisation compared to their separate additions. The findings in this study provided information on the appropriate amounts of AD and WA that can be added to soil to stimulate indigenous biodegradation of PAHs.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in the environment due to a variety of natural and anthropogenic activities (Macleod and Semple, 2002; Haritash and Kaushik, 2009; Okere and Semple 2012; Lukić *et al.*, 2016). PAHs are environmental persistent because of their low aqueous solubility, ability to adsorb on to SOM, as well as less bioavailability, especially those with higher molecular weights (HMW) (Doick *et al.*, 2005; Macleod and Semple, 2000; Crampon *et al.*, 2014). Some of these hydrophobic organic contaminants (HOCs) have a great risk of exposure to human and other environmental contacts, due to their characteristic toxicity and mutagenicity (Marini and Frapiccini, 2013; Lukić *et al.*, 2016; Demeter *et al.*, 2017; Baldwin *et al.*, 2020), as well as ability to transcend various trophic levels (Semple *et al.*, 2001). Increased awareness of their environmental impacts arouses the perpetual need to abate their persistence to environmentally safe levels.

Past studies have revealed the capability of soil microflora to remove or degrade PAHs to environmentally safe levels under favourable soil conditions (Vidali, 2001; Lang *et al.*, 2016; Siles and Margesin, 2018). However, in past studies, the occurrence of PAHs in the soil affected the carbon-to-nitrogen (C:N) ratio of the soil and, consequently, limit the soil microbial growth and activity (Margesin and Schinner, 2001; Warr *et al.*, 2013). In past studies, soil amendments with suitable mineral nutrients and application of site-specific strategies improved the soil conditions and, consequently, enhanced the indigenous microbial growth and/or activity (Boopathy, 2000; Margesin and Schinner, 2001). On the contrary, long-term addition of inorganic nutrients to soil is known to induce adverse effects on soil health, due to the accumulation of heavy metals, radiolabelled molecules and residual salts (Savci, 2012); while the perpetual use of site-

specific strategies involving mechanical operations can negatively affect the top-soil (Weisskopf *et al.*, 2010; Massah and Azadegan 2016; Ning *et al.*, 2017).

Alternatively, residues of renewable energy production are presently being considered for soil amendment due to their nutrients and high organic matter contents, which are essential for soil microbial growth and/or activity (Odlare *et al.*, 2011; Bougnom *et al.*, 2012). Organic and inorganic residues are currently being generated in large amounts due to the growing industrialization and demands for renewable energy sources (Perruci *et al.*, 2006; Odlare *et al.*, 2008; García-Sánchez *et al.*, 2015a). Consequently, the management and/or disposal of the residues have become increasingly difficult because of the cost and potential environmental impact (Odlare *et al.*, 2011; Ochecova *et al.*, 2014). Therefore, the addition of the residues to soil, as bio-fertilizers or soil conditioners, has become a suitable management option for the wastes' disposal (Gómez-Brandón *et al.*, 2016). This approach recovers the residues' nutrients, improves the soil conditions (García-Sánchez *et al.*, 2015c), mitigates environmental ammonia emissions (typical of wastes' landfills) (Palmiotto *et al.*, 2014; Insam *et al.*, 2015) and sustains renewable energy production (Gómez-Brandón *et al.*, 2016).

Anaerobic digestate (AD), a slurry based organic residue of biogas production (Köster *et al.*, 2014) and wood-ash (WA), (a biomass rich combustion residue) (Ferreiro *et al.*, 2011; Fernández-Delgado Juárez *et al.*, 2013; Richard *et al.*, 2018) have gained growing interests in recent years as soil amendments. AD is known to be a vital source of organic carbon (C) and nutrients (Insam *et al.,* 2015) and its application to soil results in considerable amounts of P, as well as N in form of ammonium (NH_4^+) and ammonia (NH_3) (Odlare et al., 2008; Odlare et al., 2011; Möller and Müller, 2012). Similarly, WA is known as a good supplement to soil due to its available macronutrients, which include calcium (Ca) magnesium (Mg), K and P (Fernández-Delgado Juárez *et al.*, 2013;

García-Sánchez *et al.*, 2015a; García-Sánchez et al. 2015c; Nabeela *et al.*, 2015), as well as micronutrients (such as iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), aluminium (Al), lead (Pb), nickel (Ni) and chromium (Cr)) (Kuba *et al.*, 2008; Bougnom and Insam, 2009; Fernández-Delgado Juárez *et al.*, 2013; Richard *et al.*, 2018). Also, WA has a high acid-neutralizing (liming) capacity due to its high pH (8 to 13) (Bougnom *et al.*, 2010; Fernández-Delgado Juárez *et al.*, 2013), and the effect of WA is known to be governed by its application dose and soil type (Pitman 2006; Fernández-Delgado Juárez *et al.*, 2013). In previous studies, soil amendment with AD and/or WA positively improved the soil physico-chemical and biological properties (Demeyer *et al.*, 2001; Insam *et al.*, 2009; García-Sánchez *et al.*, 2015c). However, due to the complementary nutrients of the AD and WA (Bougnom *et al.*, 2012), their combined addition to soil, as an amendment, can be a promising approach to stimulate microbial degradation of PAHs but this has not been explored specifically.

Therefore, this study aimed to investigate the effect of soil amendment with mixtures of AD and WA, under different amendment conditions, on the development of indigenous catabolism of ^{14}C-phenanthrene over time. Two different mixtures of AD and WA were separately used for soil amendment as follow: (i) proportionately increasing amounts of AD and a single amount of WA, and (ii) proportionately increasing amounts of WA and a single amount of AD. The effects of the two different mixtures of AD and WA on the mineralisation of ^{14}C-phenanthrene in the soil is determined over time by using a ^{14}C-respirometric system (Reid *et al.*, 2001).

2. Materials and Methods

2.1 Materials

Anaerobic digestate (AD) was collected as a semi-solid residue of anaerobically digested household food wastes from an anaerobic digestion plant in United Kingdom. The WA was a fly-ash component of a biomass combustion residue, with timber and bark as the feedstocks; it was collected from a biomass power plant in the UK. Further information about the AD and WA, as well as other materials and chemicals used for this study have been stated in Paper 1, Section 2.1 above.

2.2 Soil sampling and characterization

The soil (Dystric Cambisol) used for this study was collected from an agricultural field in Myerscough, Lancashire, United Kingdom. More information about the soil and its physico-chemical characteristics (Table 1) are mentioned in Paper 1, Section 2.2 above.

Table 1: Characteristics of wood-ash (WA), anaerobic digestate (AD) and Myerscough soil; measurements were in dry weight (d/w), except those marked (*) which were in wet weight (w/w); <BDL = below the detection limit; values are the mean of n = 3 ± standard error of the mean (SEM).

Variables	Wood-ash	Digestate	Soil
pH *	12.7 ± 0.0	9.0 ± 0 .0	6.5 ± 0.1
EC * (mS cm^{-1})	50.3 ± 0.5	9.6 ± 0.2	35.7 ± 3.0
Dry matter (%)	99.9±0.0	4.2 ± 3.2	78 ± 0.0
Organic matter (LOI) (%)	1.0 ± 0.0	66.2 ± 2.0	5.7 ± 2.0
Total N (g kg^{-1})	0.7 ± 0.1	43.5 ± 0.5	2.8 ± 0.1
Total C (g kg^{-1})	13.0 ± 0.1	349 ± 1.0	19.0 ± 1.0
Total P (g kg^{-1})	25.2 ± 0.0	12.9 ± 0.5	1.1 ± 0.08
Water Soluble PO_4-P * (g kg^{-1})	0.027 ± 0.0	0.316 ± 0.0	0.0736 ± 5.9
NH_4^+-N * (g kg^{-1})	< BDL	4.281 ± 0.1	0.0003 ± 0.2
NO_3-N * (g kg^{-1})	0.032 ± 0.0	0.001 ± 0.0	0.0014 ± 0.3

2.3 Application of anaerobic digestate (AD) and wood-ash (WA) to soil

The amounts of the AD (0.17 g, 1.73 g, 17.31 g and 173.1 g kg^{-1} soil) and WA (0.009 g, 0.09 g, 0.94 g and 9.4 g kg^{-1} soil) applied to the soils were derived from the British agricultural practice recommendation reference dose of nitrogen-to-phosphorus (N:P) (3:1) for agricultural soil amendment (AHDB, 2017) and wheat plantation (DEFRA, 2017).

2.4 Soil spiking and microcosms

Soil (2.87 kg w/w) was spiked with non-labelled (^{12}C) phenanthrene (100 mg kg^{-1} soil), using acetone as the carrier solvent. The mixtures were homogenized and kept in the fume cupboard for 2 h to allow the acetone to volatilize (Macleod and Semple, 2002). The spiked soils were equally divided for the 2 sets of soil amendments, as described in Table 2. In the first type of soil amendment, four proportionately increasing amounts of anaerobic digestate (AD) were separately mixed with a single amount of wood-ash (WA) (Table 3); while in the second type of amendment, four proportionately increasing amounts of wood-ash (WA) were separately mixed with a single amount of anaerobic digestate (AD) (Table 4). Soil (441 g w/w) without additions of AD and/or WA (control) was also prepared. Both the amended and unamended (control) soils were separately aged *(n = 3)* in pre-cleaned and labelled amber glass bottles, and incubated at 20 ± 2 ^{o}C for 1, 15, 30, 60 and 90 d (Macleod and Semple, 2002), with the lids loosely closed for ambient gas exchange.

Table 2: Two different types of soil treatments with anaerobic digestate (AD) and wood-ash (WA)

Description	First type of soil treatments	Second type of soil treatments
1	soil + proportionately increasing amounts of AD + a single amount of WA	soil + proportionately increasing amounts of WA + a single amount of AD
2	soil + proportionately increasing amounts of AD	soil + proportionately increasing amounts of WA
3	soil + a single amount WA	soil + a single amount AD

Table 3: The amounts of anaerobic digestate (AD) and wood-ash (WA) added to the soils in the first type of the treated soils

Soil amendment description	Amount of AD (g kg^{-1} soil w/w)	Amount of WA (g kg^{-1} soil d/w)
1	0.17	
2	1.73	0.94
3	17.31	
4	173.1	

Table 4: The amounts of anaerobic digestate (AD) and wood-ash (WA) added to the soils in the second type of the treated soils

Soil amendment description	Amount of WA (g kg^{-1} soil d/w)	Amount of AD (g kg^{-1} soil w/w)
1	0.009	
2	0.09	17.31
3	0.94	
4	9.4	

2.5 Mineralisation of ^{14}C-phenanthrene in anaerobic digestate- and/or wood-ash-amended soils

The mineralisation of ^{14}C-phenanthrene in AD- and/or WA-amended soils was carried out using a ^{14}C-respirometry system. At defined intervals of 1 d, 15 d, 30 d, 60 d and 90 d. Both amended and unamended soil incubations were sampled (14.7 g w/w each) into separate pre-cleaned and labelled modified Schott bottles (250 ml size)

(respirometers) (Reid et al., 2001). The soil samples were slurried with sterile deionized water (30 ml) for respirometric assays. Each respirometric microcosm was spiked with 5 μl of the mixture of ^{14}C-phenanthrene (50 Bq g^{-1} soil) and ^{12}C-phenanthrene (20.1 mg kg^{-1} soil) excluding the blanks. A glass vial (7 ml) containing fresh sodium hydroxide ($NaOH_{(aq)}$) (2 ml) was suspended (by attaching to the lid) inside each respirometer. The lids were securely screwed and the respirometers were incubated (20 ± 2 ^{o}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 captured by the $NaOH_{(aq)}$ in the suspended vials. These were sampled and refreshed at 2, 4, 8, 12 and 24 h, and henceforth every 24 h for 14 d (336 h). 5 ml of liquid scintillation fluid (Ultima Gold) was added to each spent vial and allowed to rest in the dark for 12 h before quantifying their ^{14}C-activities with liquid scintillation counting (using Canberra Packard Tri-Carb 2250CA scintillation counter).

2.6 Statistical Analysis

The data obtained from the mineralisation assays were blank-corrected and plotted with SigmaPlot 10.0. One-way analysis of variance (ANOVA) (SPSS) was employed to analyze the significant differences in the mineralisation kinetics (lag phases, maximum rates and cumulative extents) between the different AD- and/or WA-amended soil conditions, alongside similar unamended soils (controls), at 95% confidence level. Also, Turkey and LSD's Post-hoc tests (SPSS) were carried out to compare the differences within the amendment conditions and against the controls.

3. Results

3.1 Mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with a single amount of anaerobic digestate and/or wood-ash.

The mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with a single amount of AD (17.310 g kg^{-1} soil) and/or WA (0.94 g kg^{-1} soil) was studied at defined intervals of 1 d, 15 d, 30 d, 60 d and 90 d soil-PAH contact time (Figure 1 and Table 5). The lag phases were shorter ($P < 0.05$) after 1 d and 15 d with AD additions than WA amendments and controls. Most of the time points showed similar lag phases between the AD and WA amendments, as well as the controls. Significant reductions in the lag phases were observed with WA additions after 60 d and 90 d soil-PAH contact times.

The rates of ^{14}C-phenanthrene mineralisation were higher ($P < 0.05$) than the controls with AD additions after 1 d and as the soil-PAH contact time increased (15 d to 90 d); while mineralisation rates were higher ($P < 0.05$) than the controls after 60 and 90 d with WA amendment. The cumulative extents of mineralisation were enhanced after 1 d, 15 d and 60 d but they were not statistically different ($P > 0.05$) from the controls. Similar result of cumulative extents of mineralisation were observed with WA amendment after 1 d and 15 d.

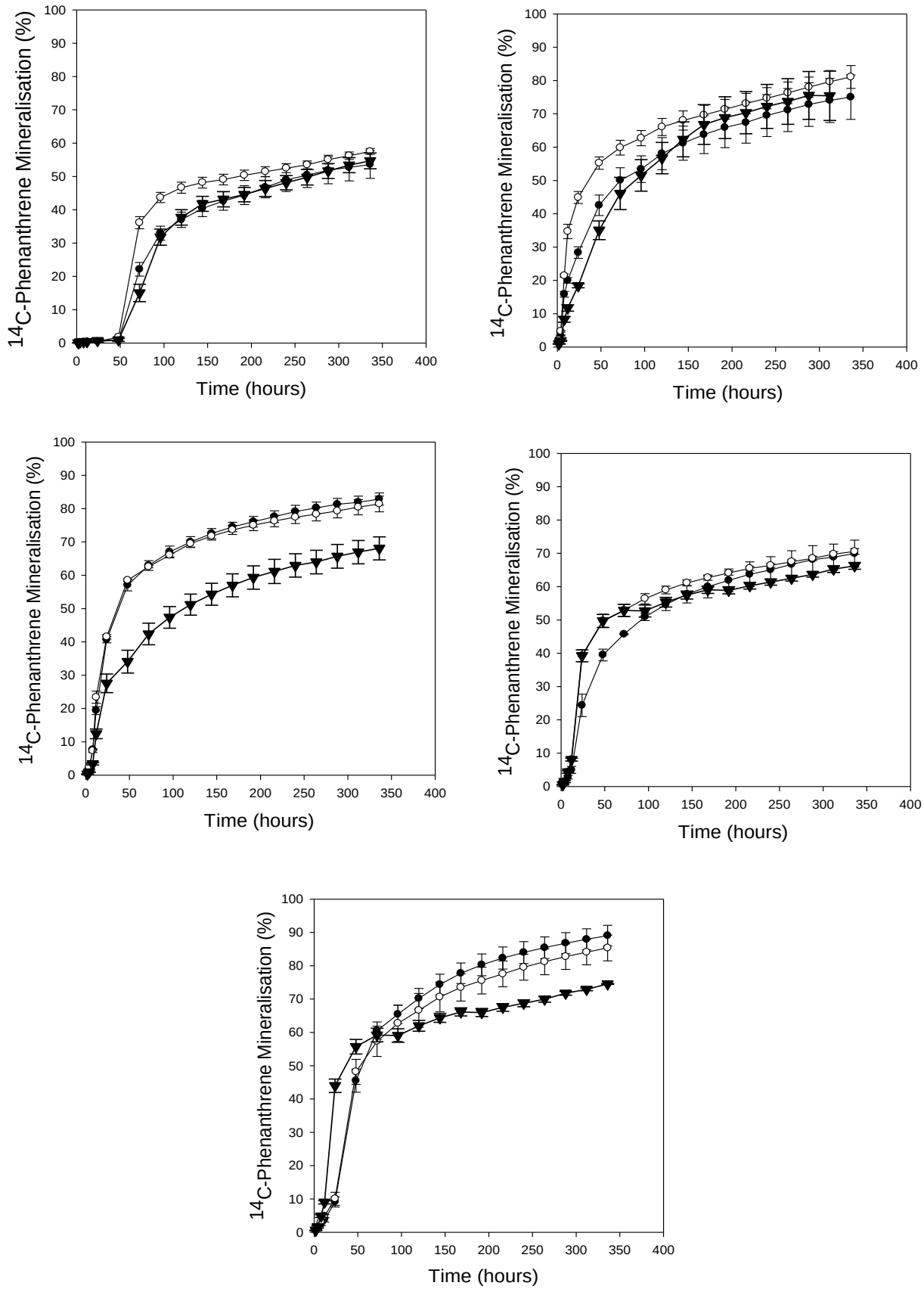

Figure 1: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils with a single amount of 17.31 g (w/w) of anaerobic digestate (AD) (○) and 0.94 g (d/w) of wood-ash (WA) (▼), and unamended soils (controls) (●) with increasing soil-PAH contact times 1, 15, 30, 60 and 90 d. Values are mean ± standard error of the mean (SEM; n = 3).

Table 5: The kinetic of mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils separately amended with a single amount of 17.31 g (w/w/) of anaerobic digestate (AD) and 0.94 g (d/w) of wood-ash (WA). Values in columns followed by different letters are statistically different (Turkey, LSD; Values are mean ± standard error of the mean (SEM; n = 3; $P < 0.05$).

Soil-PAH contact time (d)	Soil treatments	Lag time (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1-day	Controls (soil + ^{12} & ^{14}C-PAH)	52.7 ± 0.4A	0.9 ± 0.1A	53.6 ± 4.1A
	17.31 g AD	46.1 ± 0.6B	1.4 ± 0.1B	57.5 ± 0.9A*
	0.94 g WA	55.6 ± 2.1A	0.7 ± 0.0A	54.8 ± 2.4A*
15-day	Controls	4.4 ± 0.0A	3.1 ± 0.2A	75.0 ± 6.7A
	17.31 g AD	4.0 ± 0.1B	4.2 ± 0.1B	81.1 ± 3.4A*
	0.94 g WA	6.0 ± 0.3C	1.61 ± 0.7C	79.0 ± 7.6A*
30-day	Controls	6.1 ± 0.1A	2.9 ± 0.3A	82.9 ± 1.8A
	17.31 g AD	6.2 ± 0.3A	4.0 ± 0.4B	81.4 ± 2.4A
	0.94 g WA	8.8 ± 0.3A	2.3 ± 1.0A	68.1 ± 3.5B
60-day	Controls	11.7 ± 1.1A	1.6 ± 0.2A	69.9 ± 4.0A
	17.31 g AD	8.7 ± 0.4B	2.6 ± 0.1B	70.6 ± 0.9A*
	0.94 g WA	8.7 ± 0.4B	2.6 ± 0.1B	65.8 ± 1.0A
90-day	Controls	15.7 ± 2.4A	1.5 ± 0.1A	89.0 ± 3.1A
	17.31 g AD	13.5 ± 2.1A*	1.6 ± 0.2A*	85.3 ± 3.9A
	0.94 g WA	8.1 ± 0.4B	2.9 ± 0.2B	74.5 ± 0.1A

3.2 Mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with proportionately increasing amounts of anaerobic digestate

The effects of the additions of proportionately increasing amounts of AD (0.170 g, 1.730 g, 17.310 g and 173.1 g kg^{-1} soil) on the mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils were studied at defined intervals of 1 d, 15 d, 30 d, 60 d and 90 d soil-PAH contact time (Figure 2 and Table 6). The lag phases significantly ($P < 0.05$) reduced in both controls and with the AD amendments throughout the study compared to the 1 d incubations. However, the addition of 173.1 g of AD consistently resulted in longer lag phases compared to the other AD amendments and controls, except after 90 d which were still not statistically different ($P > 0.05$) from the controls. Shortest lag phases were consistently recorded with the additions of 17.31 g of AD from 30 d to 90 d, although they were not different ($P > 0.05$) from the controls after 60 d and 90 d.

The rates of mineralisation were higher ($P < 0.05$) than the controls with the additions of 0.17 g and 1.73 g of AD after 1 d and 30 d, as well as 17.3 g of AD after 15 d, 30 d and 60 d. Also, higher rates were observed after 60 d with the additions of 0.17 g and 1.73 g of AD, as well as after 90 d with the additions of 17.31 g and 173.1 g of AD, but they were not statistically significant. Cumulative extents of mineralisation were greater than the controls after 1 d with the addition of 0.17 g of AD but were not statistically significant. Also, higher extents were observed in all the AD additions after 60 d and 90 d but they were not statistically different ($P > 0.05$) from the controls. The lowest extents of mineralisation were found with the addition of the largest amount of the AD (173.1 g kg^{-1} w/w).

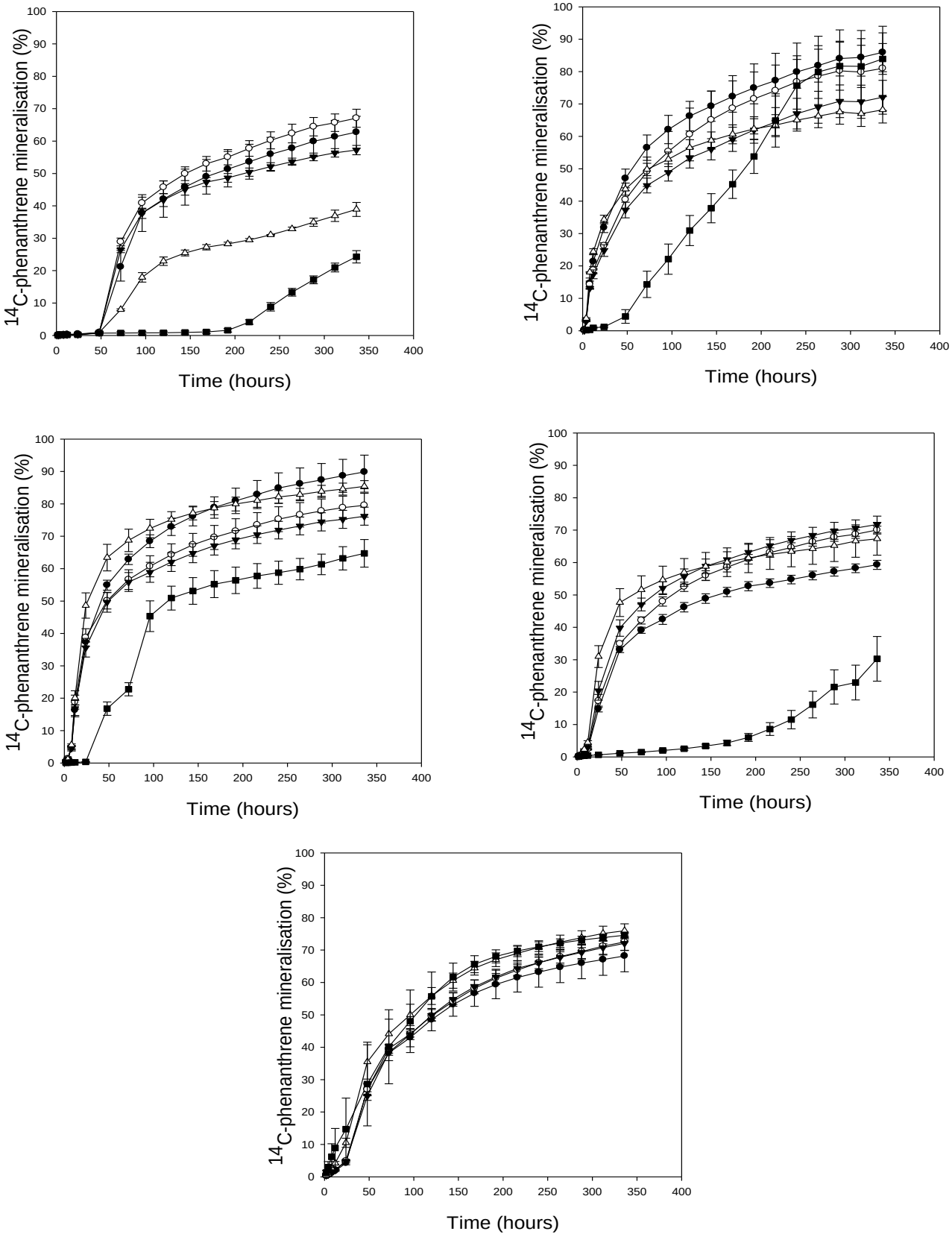

Figure 2: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with proportionately increasing amounts of anaerobic digestate (AD): 0.170 g (○); 1.730 g (▼); 17.310 g (Δ); 173.1 g (■) and unamended soils (controls) (●) at increasing soil-PAH contact times 1, 15, 30, 60 and 90 d. Values are mean ± standard error of the mean (SEM; n = 3).

Table 6: The kinetic of mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with proportionately increasing amounts of anaerobic digestate (AD). Values in columns followed by different letters are statistically different (Turkey, LSD; mean ± SEM; n = 3; $P < 0.05$)

Soil-PAH contact time (d)	Soil + AD	Lag phase (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1 d	Controls (soil + ^{14}C-PAH)	53.4 ± 1.4 A	0.9 ± 0.2 A	62.9 ± 4.9 A
	0.17 g	51.6 ± 0.2 A▪	1.2 ± 0.1 B	67.2 ± 2.8 A
	1.73 g	51.8 ± 0.1 A▪	1.1 ± 0.0 B	57.4 ± 1.4 B
	17.31 g	58.8 ± 3.0 A	0.4 ± 0.1 C	39.1 ± 2.2 B
	173.1 g	220 ± 4.6 B	0.2 ± 0.0 C	24.4 ± 1.9 B
15 d	Controls	4.7 ± 0.1 A	2.9 ± 0.4 A	88.5 ± 6.0 A
	0.17 g	4.7 ± 0.1 A	2.8 ± 0.2 A	83.5 ± 13.0 A
	1.73 g	5.8 ± 0.2 A	2.6 ± 0.1 A	74.6 ± 5.2 A
	17.31 g	4.9 ± 0.1 A	3.6 ± 0.0 B	70.8 ± 4.1 A
	173.1 g	53.7 ± 15.2 B	0.6 ± 0.1 A	86.5 ± 4.7 A
30 d	Controls	8.0 ± 0.2 A	2.9 ± 0.3 A	89.9 ± 5.2 A
	0.17 g	8.0 ± 0.3 A	3.5 ± 0.4 B	79.6 ± 3.7 A
	1.73 g	7.9 ± 0.50 A	3.1 ± 0.4 B	76.2 ± 2.8 A
	17.31 g	5.4 ± 1.3 B	3.6 ± 0.5 B	85.4 ± 1.8 A
	173.1 g	30.9 ± 1.0 C	1.0 ± 0.1 C	64.7 ± 4.3 A
60 d	Controls	14.6 ± 0.2 A	1.0 ± 0.1 A	59.4 ± 1.4 A
	0.17 g	14.3 ± 0.4 A	1.2 ± 0.2 A*	70.1 ± 1.2 A*
	1.73 g	13.5 ± 0.4 A▪	1.5 ± 0.2 A*	71.8 ± 2.6 A*
	17.31 g	12.1 ± 0.4 A▪	2.2 ± 0.3 B	67.4 ± 5.1 A*
	173.1 g	183.4 ± 20.1 B	0.3 ± 0.1 C	30.4 ± 6.9 A
90 d	Controls	24.5 ± 0.9 A	0.9 ± 0.1A	68.2 ± 4.9A
	0.17 g	24.0 ± 0.2 A	0.9 ± 0.1A	72.5 ± 2.6A*
	1.73 g	23.8 ± 1.0 A	0.8 ± 0.0A	72.1 ± 2.2A*
	17.31 g	13.8 ± 1.2 A▪	1.0 ± 0.2A*	76.0 ± 2.1A*
	173.1 g	16.5 ± 7.4 A▪	1.1 ± 0.4A*	74.6 ± 1.0A*

3.3 Mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with proportionately increasing amounts of wood-ash

The mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with proportionately increasing amounts of WA (0.009 g, 0.090 g, 0.940 g and 9.4 g kg^{-1} soil) was studied at defined intervals of 1 d, 15 d, 30 d, 60 d and 90 d soil-PAH contact time (Figure 3 and Table 7). After 1 d, the lag phases were shorter with the addition of 0.009 g of WA, although they were not statistically different ($P > 0.05$) from the controls and the other amendment conditions. However, the higher the amounts of WA added, the longer the lag phases observed (9.4 < 0.94 < 0.09 < 0.009 g). Also, lag phases were shorter ($P < 0.05$) from 15 d to 90 d compared to 1 d both in the absence and presence of WA. Addition of 9.4 g of WA resulted in longer lag phases after 1 d which became shorter as the soil-PAH contact time increased.

The rates of mineralisation were higher in 0.009 g of WA after 1 d and 30 d than the controls and other WA-treatments. Although, faster rates of mineralisation were observed in the absence of WA than with its additions after 60 d and 90 d, however, they were not statistically different ($P > 0.05$) from the WA additions. The rates of mineralisation were lower ($P < 0.05$) with the addition of 9.4 g of WA compared to the other WA-treatments and similar unamended soils (controls). The cumulative extents of mineralisation were greater ($P < 0.05$) than the controls after 30 d and 60 d with the additions of 0.009 g and 0.94 g of WA. Noticeable increase in extents of mineralisation were observed with the additions of 0.009 g, 0.090 g and 0.940 g of WA after 1 d but they were not statistically different from the controls. Also, the greater extents of mineralisation observed after 15 d, 60 d and 90 d in the controls were not statistically different ($P > 0.05$) from the WA amendments.

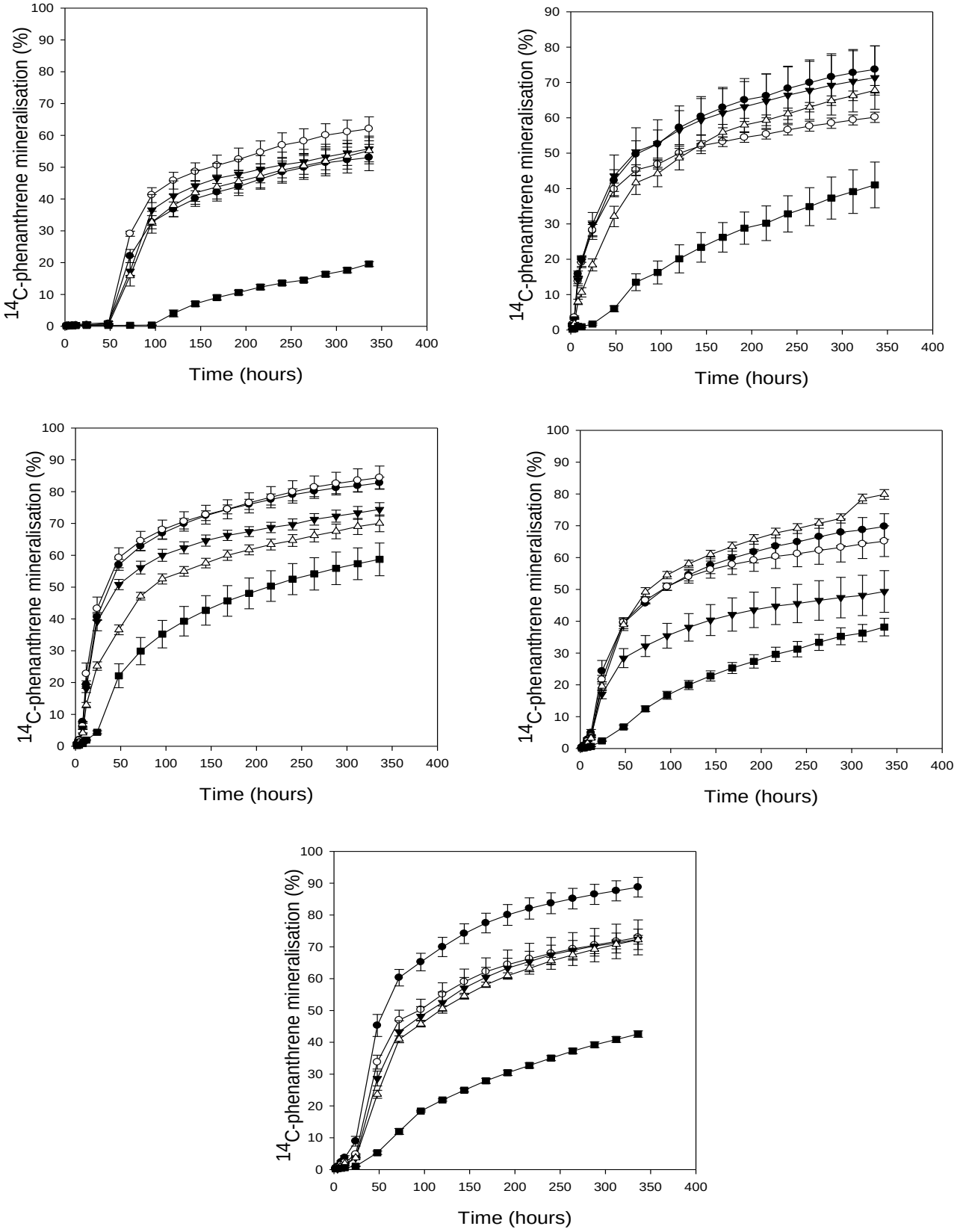

Figure 3: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed unamended soils (controls) (●), and ^{12}C-phenanthrene-exposed soils amended with proportionately increasing amounts of wood-ash (WA): 0.009 g (○); 0.090 g (▼); 0.940 g (∆); 9.4 g (■) at increasing soil-PAH contact time 1, 15, 30, 60 and 90 d. Values are mean ± standard error of the mean (SEM; n = 3).

Table 7: The kinetic of mineralization of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with proportionately increasing amounts of wood-ash (WA). Values in columns followed by different letters are statistically different (Turkey, LSD; mean ± SEM; n = 3; $P < 0.05$).

Soil-PAH contact time (d)	Soil + WA	Lag phase (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1 d	Controls (soil + ^{14}C-PAH)	52.7 ± 0.4 A	0.9 ± 0.1 A	53.2 ± 4.1 A
	0.009 g	51.5 ± 0.1 A▪	1.2 ± 0.0 B	62.2 ± 3.8 A*
	0.09 g	55.2 ± 2.91 A	0.8 ± 0.1 A	55.9 ± 4.0 A*
	0.94 g	55.0 ± 0.1A	0.8 ± 0.1 A	55.4 ± 4.1 A*
	9.4 g	126.7 ± 6.1 B	0.2 ± 0.0 C	19.8 ± 0.8 B
15 d	Controls	4.4 ± 0.0 A	3.1 ± 0.2 A	74.3 ± 6.7 A
	0.009 g	4.4 ± 0.3 A	2.6 ± 0.3 A	60.8 ± 1.4 A
	0.09 g	4.8 ± 0.2 A	2.8 ± 0.2 A	72.0 ± 8.9 A
	0.94 g	5.2 ± 0.5 A	1.5 ± 0.2 B	68.4 ± 1.4 A
	9.4 g	40.8 ± 3.6 B	0.3 ± 0.1 B	41.7 ± 6.5 B
30 d	Controls	6.1 ± 0.1 A	2.9 ± 0.3 A	82.9 ± 1.8 A
	0.009 g	6.9 ± 0.7 A	4.1 ± 0.6 B	84.5 ± 3.7 B
	0.09 g	7.4 ± 0.20 A	3.1 ± 0.3 A*	74.6 ± 2.1 C
	0.94 g	8.4 ± 0.0 A	2.2 ± 0.2 A	59.7 ± 2.5 C
	9.4 g	25.1 ± 1.0 B	0.7 ± 0.1 C	58.9 ± 5.1 C
60 d	Controls	11.7 ± 1.1 A	1.6 ± 0.2 A	70.0 ± 4.0 A
	0.009 g	12.8 ± 0.3 A	1.5 ± 0.1 A	65.4 ± 4.8 A
	0.09 g	13.1 ± 0.4 A	1.1 ± 0.1 A	49.6 ± 6.5 A
	0.94 g	13.3 ± 0.3 A	1.4 ± 0.1 A	80.1 ± 1.5 B
	9.4 g	38.7 ± 2.2 B	0.2 ± 0.0 B	38.4 ± 2.8 C
90 d	Controls	15.7 ± 2.4 A	1.5 ± 0.1 A	88.9 ± 3.1 A
	0.009 g	23.9 ± 0.4 A	1.2 ± 0.1 A	73.1 ± 5.5 A
	0.09 g	25.2 ± 0.1 A	1.0 ± 0.1 A	72.5 ± 3.2 A
	0.94 g	25.5 ± 0.4 A	0.8 ± 0.1 A	72.4 ± 1.4 A
	9.4 g	46.2 ± 2.5 B	0.3 ± 0.0 B	42.7 ± 1.0 B

3.4 Mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with the mixtures of proportionately increasing amounts of anaerobic digestate and a single amount of wood-ash.

The effects of the additions of mixtures of proportionately increasing amounts of AD (0.170 g, 1.730 g, 17.31 g and 173.1 g kg^{-1} soil) and a single amount of WA (0.94 g kg^{-1} soil) on the mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils were studied after 1 d, 15 d, 30 d, 60 d and 90 d soil-PAH contact times (Figure 4 and Table 8). Shorter lag phases were observed with additions of mixtures of 0.17 g of AD and 0.94 g of WA after 1 d and 60 d but they were not significantly different from the controls. Similarly, noticeable increase were observed with the additions of 1.73 g of AD and 0.94 g of WA as well as 17.31 g of AD and 0.94 g of WA compared to the controls after 60 d but they were not statistically significant. There were significantly longer lag phases with the addition of mixtures of 173.1 g of AD and 0.94 g of WA compared to the other amendments and controls in all the time points, however, they became gradually reduced as the soil-PAH continued. Generally, lag phases were observed shorter as the soil-PAH contact time increased compared to 1 d, and the reduction were not significantly different in all the additions of the AD-WA mixtures ($P > 0.05$) except the mixtures of 173.1 g of AD and 0.94 g of WA.

The maximum rates of mineralisation were higher ($P < 0.05$) than the controls after 60 d with the addition of the mixtures of 17.31 g AD and 0.94 g WA. Also the mineralisation rates were higher from 1 d to 30 d in the presence of all the AD-WA mixtures but they were not significantly different from the controls. Overall, there were no significant differences among the amendment conditions in most of the time points. The cumulative extents of mineralisation were significantly greater ($P < 0.05$) in most of the time points compared to

the 1 d soil-PAH contact time. Mineralisation was generally lower in the mixtures of 173.1 g of AD and 0.94 g of WA compared to the other AD-WA mixtures and controls until after 90 d, where the mineralisation extents were significantly higher than the controls as well as the other mixtures of AD and WA.

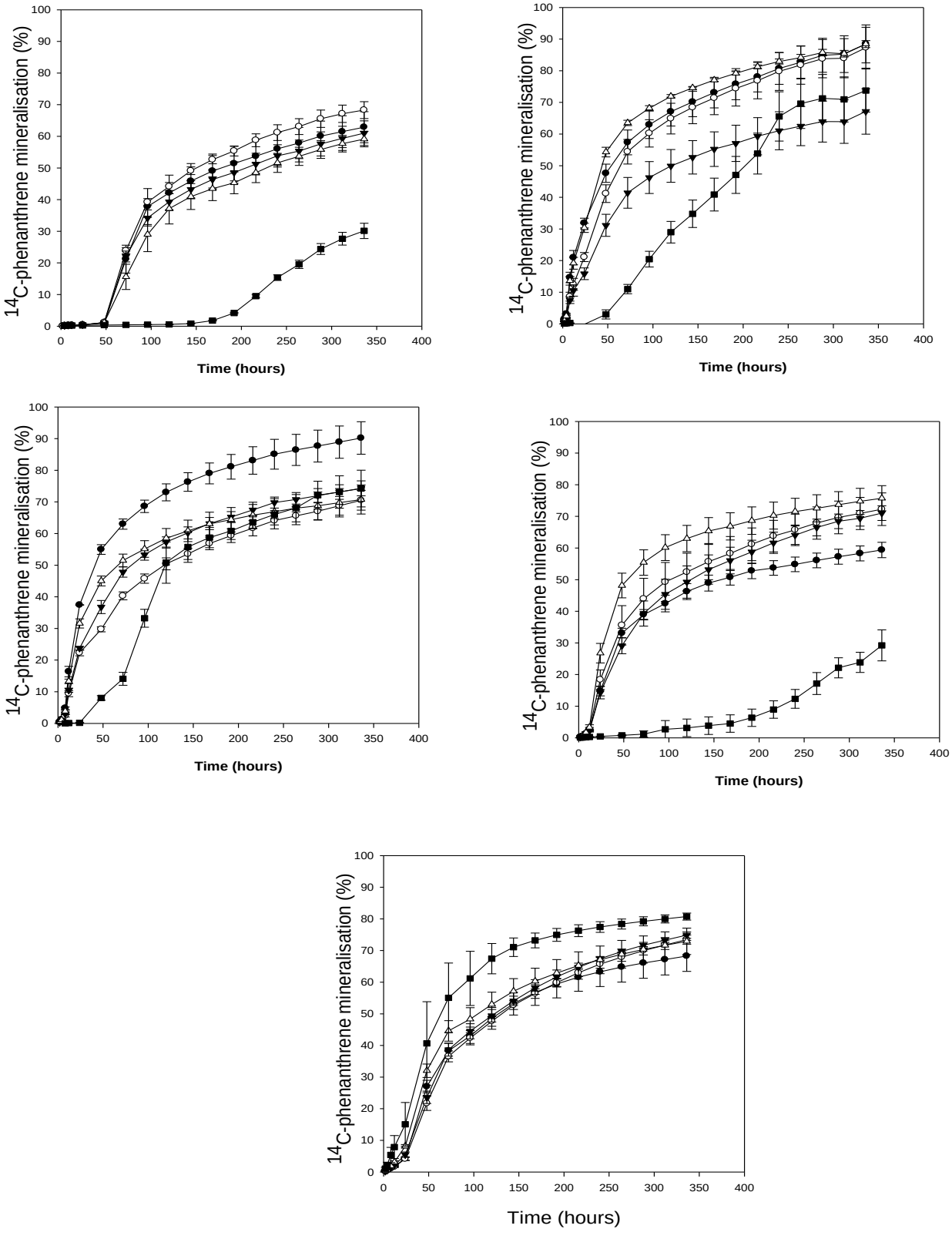

Figure 4: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with mixtures of proportionately increasing amounts of anaerobic digestate (AD) and a single amount of wood-ash (WA): 0.170 g anaerobic digestate (AD) / 0.940 g wood-ash (WA) (○); 1.730 g anaerobic digestate (AD) / 0.940 g wood-ash (WA) (▼); 17.31 g anaerobic digestate (AD) / 0.94 g wood-ash (WA) (Δ); 173.1 g anaerobic digestate (AD) / 0.94 g wood-ash (WA) (■) and unamended soils (controls) (●) at increasing soil-PAH contact times 1, 15, 30, 60 and 90 d. Values are mean ± standard error of the mean (SEM; n = 3).

Table 8: The kinetic of mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with mixtures of proportionately increasing amounts of anaerobic digestate (AD) and a single amount of wood-ash (WA). Values in columns followed by different letters are statistically different (Turkey, LSD; mean ± SEM; n = 3; $P < 0.05$).

Soil-PAH contact time (days) (d)	Soil + AD + WA	Lag phase (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1 d	Controls (soil + ^{14}C-PAH)	53.4 ± 1.4 A	0.9 ± 0.2 A	62.9 ± 4.9 A
	0.17 g / 0.94 g	52.6 ± 29.8 A▪	0.9 ± 0.0 A	68.3 ± 2.7 A*
	1.73 g / 0.94 g	53.2 ± 29.9 A	0.9 ± 0.1 A	61.0 ± 3.9 A
	17.31 g / 0.94 g	60.7 ± 30.1 A	0.7 ± 0.1 B	59.1 ± 2.4 A
	173.1 g / 0.94 g	195.0 ± 114.1 B	0.2 ± 0.0 B	30.1 ± 2.4 B
15 d	Controls	4.7 ± 0.1 A	2.9 ± 0.4 A	88.5 ± 6.0 A
	0.17 g / 0.94 g	6.1 ± 0.4 A	1.8 ± 0.3 A	87.3 ± 6.5 A
	1.73 g / 0.94 g	6.4 ± 0.4 A	1.4 ± 0.2 A	67.2 ± 7.2 A
	17.31 g / 0.94 g	5.0 ± 0.2 A	2.8 ± 0.3 A	88.4 ± 1.1 A
	173.1 g / 0.94 g	53.0 ± 4.8 B	0.5 ± 0.1 B	73.8 ± 6.8 A
30 d	Controls	8.0 ± 0.2 A	2.9 ± 0.3 A	90.2 ± 5.1 A
	0.17 g / 0.94 g	9.6 ± 0.3 A	1.8 ± 0.2 A	70.5 ± 3.1 A
	1.73 g / 0.94	9.1 ± 0.2 A	1.9 ± 0.1 A	74.3 ± 2.3 A
	17.31 g / 0.94 g	8.5 ± 0.1 A	2.4 ± 0.1 A	70.8 ± 4.6 A
	173.1 g / 0.94 g	38.8 ± 1.6 B	0.8 ± 0.2 A	74.2 ± 5.8 A
60 d	Controls	14.6 ± 0.2 A	1.0 ± 0.1A	59.4 ± 1.4 A
	0.17 g / 0.94 g	13.9 ± 0.5 A▪	1.3 ± 0.1A*	72.3 ± 3.0 B
	1.73 g / 0.94 g	14.8 ± 0.4 A	1.0 ± 0.1A	71.0 ± 1.3 B
	17.31 g / 0.94 g	12.9 ± 0.3 A▪	2.0 ± 0.1B	75.7 ± 2.3 B
	173.1 g / 0.94 g	159.2 ± 34.7 B	0.3 ± 0.0C	29.7 ± 2.5 C
90 d	Controls	24.5 ± 0.9 A	0.9 ± 0.1 A	68.3 ± 4.9 A
	0.17 g / 0.94 g	25.2 ± 1.0 A	0.7 ± 0.1 A	73.5 ± 1.5 A*
	1.73 g / 0.94 g	22.5 ± 2.0 A▪	0.8 ± 0.0 A	74.9 ± 1.0 A*
	17.31 g / 0.94 g	16.6 ± 1.2 A▪	1.0 ± 0.1 A*	72.9 ± 4.2 A*
	173.1 g / 0.94 g	13.5 ± 6.9 B	1.3 ± 0.2 A*	80.7 ± 1.1 B

3.5 Mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with the mixtures of proportionately increasing amounts of wood-ash and a single amount of anaerobic digestate

The mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with the mixtures of proportionately increasing amounts of WA (0.009 g, 0.090 g, 0.940 g and 9.4 g kg^{-1} soil) and a single amount of AD (17.31 g kg^{-1} soil) was studied at defined intervals of 1 d, 15 d, 30 d, 60 d and 90 d soil-PAH contact time (Figure 5 and Table 9). After 1 d, the addition of mixtures of 17.31 g of AD and 9.4 g of WA resulted in significantly ($P < 0.05$) longer lag phases than the other AD-WA mixtures and controls. However, significant differences were not observed among the other AD-WA mixtures and also, compared to the controls. Lag phases were significantly reduced with increasing soil-PAH interactions from 15 d, compared to 1 d soil-PAH contact time. Also, the data showed that the additions of mixtures of 17.31g of AD and 0.009 g of WA produced shorter lag phases in most of the time points throughout the study.

The mineralisation rates were higher than the controls from 15 d in all the mixtures of the AD and WA, irrespective of the amounts of the WA, as the soil-phenanthrene contact time increased than observed in 1 d aged incubations. After 15 d and 30 d, the addition of mixtures of 17.31g AD and 0.009 g WA resulted in higher ($P < 0.05$) maximum rates of mineralisation, followed with the mixtures of 17.31g AD and 0.09 g WA and then the mixtures of 17.31 g AD and 0.94 g WA. However, the rates were significantly higher than the controls after 30 d and 60 d with the addition of mixtures of 17.31 g AD and 0.94 g WA followed with the mixtures of 17.31g AD and 0.009 g WA. The extents of mineralisation significantly increased ($P > 0.05$) both in the absence and presence of all the AD-WA mixtures. The mixtures of 17.31g AD and 0.009 g WA displayed the greatest amount of

$^{14}CO_2$ (%) from the mineralisation of the ^{14}C-phenanthrene in most of the sampling time points. However, the results were not statistically significant ($P > 0.05$) compared to the controls, as well as the other mixtures of AD and WA.

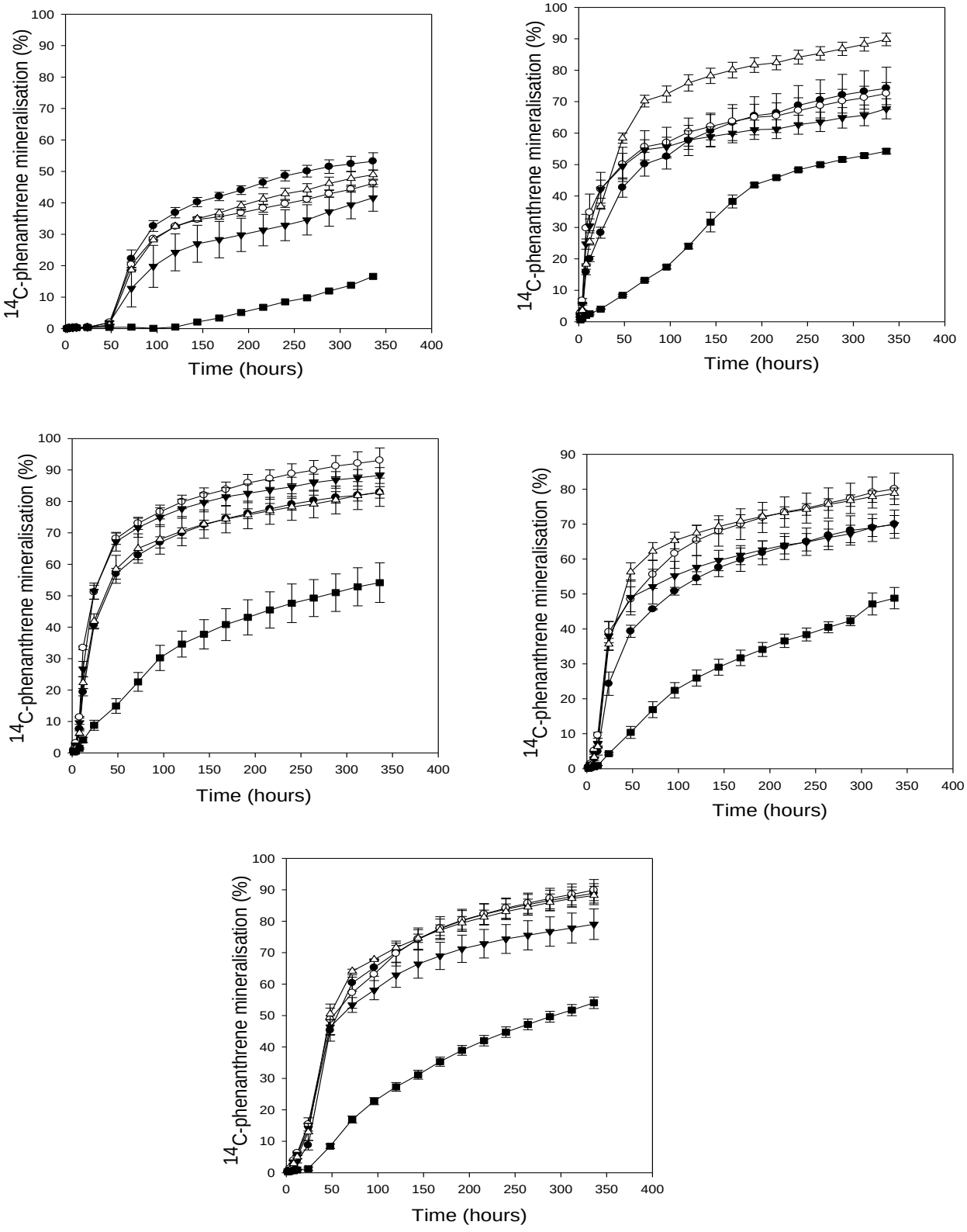

Figure 5: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with mixtures of proportionately increasing amounts of wood-ash (WA) and a single amount of anaerobic digestate (AD): 0.009 g wood-ash (WA) / 17.31 g anaerobic digestate (AD) (○); 0.09 g wood-ash (WA) / 17.31 g anaerobic digestate (AD) (▼); 0.94 g wood-ash (WA) / 17.31 g anaerobic digestate (AD) (∆); 9.4 g wood-ash (WA) / 17.31 g anaerobic digestate (AD) (■) and unamended soils (controls) (●) at increasing soil-PAH contact times 1, 15, 30, 60 and 90 d. Values are mean ± standard error of the mean (SEM; n = 3).

Table 9: The kinetic of mineralization of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with mixtures of proportionately increasing amounts of wood-ash (WA) and a single amount of anaerobic digestate (AD). Values in columns followed by different letters are statistically different (Turkey, LSD; mean ± SEM; n = 3; $P < 0.05$).

Soil-PAH contact time (d)	Soil + AD + WA	Lag phase (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
1 d	Controls (soil + ^{14}C-PAH)	52.7 ± 0.4 A	0.9 ± 0.1 A	53.2 ± 4.1 A
	17.31 g / 0.009 g	52.2 ± 1.3 A	0.8 ± 0.1 A	46.2 ± 2.7 A
	17.31 g / 0.09 g	54.7 ± 0.9 A	0.5 ± 0.1 A	41.6 ± 1.1 A
	17.31 g / 0.94 g	56.0 ± 4.4 A	0.8 ± 0.2 A	49.0 ± 4.3 A
	17.31 g / 9.4 g	196.1 ± 22.1 B	0.1 ± 0.0 B	16.6 ± 1.4 B
15 d	Controls	4.4 ± 0.0 A	3.1 ± 0.2 A	74.3 ± 6.7 A
	17.31 g / 0.009 g	2.5 ± 0.1 B	5.8 ± 1.0 B	72.6 ± 3.5 A
	17.31 g / 0.09 g	3.7 ± 0.1 A▪	4.8 ± 0.4 B	67.7 ± 3.2 A
	17.31 g / 0.94 g	4.3 ± 0.1 A▪	3.7 ± 0.1 A*	89.8 ± 2.0 B
	17.31 g / 9.4 g	28.4 ± 0.8 C	0.4 ± 0.0 C	54.2 ± 0.8 C
30 d	Controls	6.1 ± 0.1 A	2.9 ± 0.3 A	82.9 ± 1.8 A
	17.31 g / 0.009 g	4.9 ± 0.0 A▪	5.5 ± 0.2 B	93.1 ± 3.9 A*
	17.31 g / 0.09 g	5.4 ± 0.2 A▪	4.3 ± 0.5 B	88.3 ± 2.5 A*
	17.31 g / 0.94 g	7.0 ± 0.5 B	4.0 ± 0.3 B	82.9 ± 4.5 A
	17.31 g / 9.4 g	15.2 ± 2.1 B	0.7 ± 0.2 C	49.2 ± 5.8 B
60 d	Controls	11.7 ± 1.1 A	1.6 ± 0.2 A	70.0 ± 4.0 A
	17.31 g / 0.009 g	7.9 ± 0.3 A▪	2.5 ± 0.2 B	80.1 ± 4.5 A*
	17.31 g / 0.09 g	9.3 ± 0.6 A▪	2.2 ± 0.5 A*	69.9 ± 2.6 A
	17.31 g / 0.94 g	10.3 ± 0.2 A▪	2.4 ± 0.1 B	78.8 ± 1.6 A*
	17.31 g / 9.4 g	28.0 ± 3.0 B	0.3 ± 0.0 C	48.8 ± 3.1 B
90 d	Control	15.7 ± 2.4 A	1.5 ± 0.1 A	88.9 ± 3.1 A
	17.31 g / 0.009 g	10.2 ± 0.8 B	1.4 ± 0.1 A	89.9 ± 3.4 A*
	17.31 g / 0.09 g	10.6 ± 0.7 B	1.3 ± 0.0 A	79.1 ± 4.9 A
	17.31 g / 0.94 g	12.5 ± 1.2 A▪	1.6 ± 0.2 A*	88.2 ± 3.0 A
	17.31 g / 9.4 g	36.2 ± 2.0 C	0.4 ± 0.0 B	54.1 ± 1.8 B

4. Discussion

4.1 Mineralisation of ^{14}C-phenanthrene in soils amended with a single and increasing amounts of anaerobic digestate

In this study, soil amendment with AD positively influenced microbial activity, and hence degradation of ^{14}C-phenanthrene, freshly added to the ^{12}C-phenanthrene-exposed soils amended with AD. A single addition of 17.31 g kg^{-1} soil of AD significantly reduced the microbial adaptation times and increased the mineralisation rates, as the soil-PAH-contact time increased. However, the effects were not relative to the amounts of the AD added especially as there were no significant differences with the additions of its proportionately increasing amounts (0.17 g, 1.73 g, 17.31 g and 173.1 g kg^{-1} soil). In the results, lower amounts of AD reduced the lag phases and increased the mineralisation rates with increasing soil-PAH contact time, compared to the similar soils with higher amounts of AD and similar unamended soils. For example, the shortest lag phases and fastest rates of mineralisation were observed to be consistent after the addition of 17.31 g of AD, while the lowest extents of mineralisation were found with the addition of 173.1 g kg^{-1} soil.

The addition of AD to soil is known to supply readily available nutrients in the short-term, especially N, to the soil micro-organisms (Alburquerque *et al.*, 2012; García-Sánchez *et al.*, 2015a). This may suggest the reason for the early effects observed on the lag times and mineralisation rates during the experiment. These findings imply the indigenous microbial activity was supported at the early stages of the soil-phenanthrene contact time due to the readily available nutrients from the AD addition. This result is in agreement with previous studies relating to the stimulatory effect of the AD on soil microbial activity and biomass, as well as soil quality by the addition of organic matters from the AD (Insam *et al.*, 2015;

Gómez-Brandón *et al.*, 2016). For example, in a study by García-Sánchez *et al.* (2015a), the soil microbial activity, biomass and physiological diversity were significantly improved within a short time of addition of AD. In addition, in a study by Alburquerque, *et al.* (2012), the addition of AD to soil resulted to a short-term increase in NH^{+}_{4}-N and P contents, with more positive effects on soil microbial biomass C and N compared to the similar unamended soils. Also, in a study by Walsh *et al.* (2012), consistently higher bacterial growth was observed in AD-amended soils compared to the similar unamended soils. In García-Sánchez *et al.* (2015b), soil amendment with AD significantly increased the C and N contents of the soils, as well as enhanced the microbial activity, biomass, community structures and physiological diversity of the soils. Similarly, in García-Sánchez *et al.* (2015a), AD addition improved the microbial activity and physiological diversity of the soil. Also, Fernando-Juárez *et al.* (2013) observed positive changes in the microbial community structure of AD-amended soils, while Fernández-Bayo *et al.* (2017) observed significantly increased available nutrients, which include C, P and K in AD-amended soils. Therefore, the abundance of N and the presence other essential nutrients, as well as a considerable amount of microbial degradable organic matter in the AD are significant in the stimulation of the microbial activity and, consequently, degradation of the ^{14}C-phenanthrene in the AD-amended soils. On the contrary, in a study by Gómez-Brandón *et al.*, 2016, AD addition to soil did not have any significant effect on the microbial activity and biomass (but only increased the nitrification rates) compared to the unamended soils.

4.2 Mineralisation of ^{14}C-phenanthrene in soils amended with a single and increasing amounts of wood-ash

The addition of WA to soils positively influenced the indigenous mineralisation of ^{14}C-phenanthrene, as there were noticeable changes compared to the controls throughout the

study. The soils amended with a single amount of 0.94 g kg^{-1} soil of WA showed enhanced mineralisation, in terms of significantly shorter lag phases, as well as higher rates and greater extents of mineralisation as the soil-PAH contact time increased. This suggests that soil amendment with the WA supplied readily available nutrients, including higher P content compared to the amount available in the ^{14}C-PAH-contaminated soil, and consequently enhanced the microbial activity. Also, it is known that soil amendment with the right amount of WA can induce positive effects on soil physico-chemical and biological properties (García-Sánchez *et al.*, 2015a), which can stimulate microbial degradation of PAHs. In past studies, the addition of WA positively changed the soil microbial processes and chemical properties. For example, in a study carried out by García-Sánchez *et al.* (2015b), soil amendment with WA significantly increased the soil microbial biomass, while Noyce *et al.* (2016) noticed positive alterations in the microbial composition/communities of WA-amended soils. Similarly, in Fernández-Delgado Juárez *et al.* (2013), soil amendment with WA positively influenced the microbial activity and biomass C, irrespective of the amounts of WA added to the soil.

In this present study, the mineralisation of ^{14}C-phenanthrene observed in the presence of WA did not correspond to the proportionately increasing amounts of WA (0.009 g, 0.09 g, 0.94 g and 9.4 g kg^{-1} soil d/w) added to the soils. The rates and extents of mineralisation were increased with the lower additions of WA, particularly 0.09 g and 0.94 g, as the soil-phenanthrene contact time increased, while the higher addition (9.4 g kg^{-1} soil d/w) reduced the rates and extents of mineralisation. These results suggest that lower amounts of WA support microbial activity and can stimulate microbial degradation of PAHs in PAH-contaminated soils. Although the shorter lag periods observed in some of the soils with lower amounts of WA were not statistically significant, the results similarly imply an enhancement of microbial activity. These findings also suggest that biostimulation with WA

is independent of the increasing/higher amounts. This observation agrees with past studies where soil amendment with lower amounts of WA significantly improved the soil biological properties. In a study by Perucci *et al.* (2006), soil microbial activity and biomass were significantly increased at a lower application rate of 5 t ha^{-1} of WA, while a higher application rate of 20 t ha^{-1} of WA reduced the microbial activity and biomass. In Nabeela *et al.* (2015), significant microbial counts were observed in the soils amended with 1 to 10 g kg^{-1} soil of WA compared to the soils amended with higher amounts of WA. Also, an increase in soil microorganisms following WA addition to soils have been reported (Kuba *et al.*, 2008).

García-Sánchez *et al.* (2015b) observed differences in the enzymatic activities of WA-amended soils according to the amounts of WA added. Also, Fernando-Juárez *et al.* (2013) reviewed studies where the microbiological properties of WA-treated soils were influenced in proportional to the amounts of WA added to the soils. However, the various disparities observed in studies regarding WA-amended soils are indicated might be due to the differences in the soil properties, length of the experiments, analysis parameters, as well as WA pre-treatment and application amounts (Fernando-Jaurez *et al.*, 2013). The results obtained in this study suggests lower amounts of WA can positively influence the soil biological properties and, consequently, the biodegradation of PAHs compared to its higher application (Perucci *et al.*, 2006; Nabeela *et al.*, 2015; Monlau *et al.*, 2016).

4.3 Mineralisation of ^{14}C-phenanthrene in the soils amended with mixtures of anaerobic digestate and wood-ash

The results from this study showed that the addition of mixtures of proportionately increasing amounts of AD (0.17 g, 1.73 g, 17.31 g and 173.1 g kg^{-1} soil w/w) and a single amount of WA (0.94 g kg^{-1} soil d/w) contributed to the enhancement of the indigenous

mineralisation of ^{14}C-phenanthrene in the soils. Similarly, the addition of mixtures of proportionately increasing amounts of WA (0.009 g, 0.090 g, 0.940 g and 9.4 g kg^{-1} soil) and a single amount of AD (17.31 g kg^{-1} soil) significantly enhanced the mineralisation compared to their separately single additions, as well as the similar unamended soils. However, the findings of these investigations showed that the enhancement of the mineralisation of the ^{14}C-phenanthrene was not proportional to the increasing amounts of the residues added to the soils. For example, the additions of mixtures of AD with lower amounts, particularly 0.17 g and 17.31 g kg^{-1} soil (w/w) and 0.94 g kg^{-1} soil (d/w) of WA increased the mineralisation rates and extents compared to the mixtures of the higher amount of AD (173.1 g kg^{-1} soil w/w) and 0.94 g kg^{-1} soil d/w of WA. Similar results were observed in the cumulative extents of mineralisation, which were significantly greater in most of the time points compared to the 1 d aged incubations. This study showed that the AD-WA mixtures with the lower amounts of AD and WA resulted in shorter lag phases with a corresponding increase in the rates and extents of mineralisation. These results were more pronounced with the additions of mixtures of lower amounts of AD and WA (17.31 g of AD and 0.009 g of WA, 17.31g of AD and 0.09 g of WA as well as 17.31 g of AD and 0.94 g of WA). These findings complement the earlier observations of stimulation of microbial activity and degradation of the ^{14}C-phenanthrene added to soils.

Similar observations were also reported in past studies; for example, in a study by Kuba *et al.* (2008), where soil amendment with mixtures of organic wastes and lower amounts (up to 16 %) of WA resulted in higher available macronutrients. Also, in Fernando-Juárez *et al.* (2013), the addition of mixtures of AD and lower amounts of WA (0, 1, and 3 t ha^{-1}) increased the total C and P as well as $NH4^+$ in the soils; however, there were no significant changes in the microbial activity regardless of the amounts of WA applied. On the contrary, in Bougnom *et al.* (2012), soil amendment with the mixtures of WA and anaerobic sludge

reduced the soil biological activities in proportional to the amounts of WA added to the soils, (while the increase in soil pH corresponded to the amounts of WA applied).

In this present study, the effects of both AD and WA did not correspond to their amounts added to the soils. AD addition to the soils enhances the microbial processes due to its considerable amount of organic C and readily microbial utilizable nutrients (García-Sánchez *et al.* (2015b) most of which are also available in the WA. Hence, the combination of AD and WA as soil amendments in PAH-contaminated soils can potentially augment some nutrients (P, N, K, Ca and Mg) which are deficient in the soil (Tiwary *et al.*, 2015; Maschowski *et al.*, 2016).

5. Conclusion

The effects of a single addition and combined application of AD and/or WA to soil, as soil amendments, were investigated on the mineralisation of ^{14}C-phenanthrene in the soil. The study revealed that soil amendment with lower amounts of AD and/or WA, both as a single and combined addition enhanced the biodegradation of the ^{14}C-phenanthrene. This was supported with shorter lag phases, as well as higher rates and greater extents of mineralisation, and the mineralisation kinetics were not proportionate to the increasing amounts of the AD and/or WA. It was observed that the lowest amounts of the amendment conditions showed more positive effects in both single and combined applications, and in the ageing amended soils, the lag phases were reduced while the rates and extents of mineralisation increased. The findings in the present study suggest that soil amendments with AD and WA can stimulate indigenous microbial degradation by supplying microbial growth- or rate-limiting nutrients. Amending PAH-contaminated soils with the mixtures of the correct amounts of AD and WA should be considered for indigenous biodegradation of PAHs and other organic contaminants.

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6.3 Paper 3: Influence of multiple additions of ^{12}C-phenanthrene on the development of ^{14}C-phenanthrene catabolism in anaerobic digestate- and wood-ash-amended soils.

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Abstract

The retention and persistence of Polycyclic Aromatic Hydrocarbons (PAHs) in soil, as organic contaminants, are known to be enhanced by their chemical structure as well as the properties of their host soil. Effective biodegradation of PAHs in soil has been linked to the pre-exposure of the soil to PAHs and/or their naturally occurring analogues. The exposures are thought to drive indigenous mineralisation of organic contaminants in soil. This study investigated the effects of soil exposures to multiple additions and higher concentrations of ^{12}C-phenanthrene on the development of ^{14}C-phenanthrene catabolism in the presence of two different bioenergy residues, which are anaerobic digestate (AD) and/or wood-ash (WA). Findings revealed a significantly higher percentage of catabolically evolved $^{14}CO_2$ from the mineralisation of the ^{14}C-phenanthrene in the ^{12}C-phenanthrene-exposed soils amended with AD and/or WA than in similar unamended soils. In addition, significant changes were observed in the composition of the microbial community found in the ^{12}C-phenanthrene-exposed soils amended with the mixtures of AD and WA-compared to the similar unamended soils. Also, the microbial community structure of the 90 d aged incubations revealed significant changes compared to the controls. The outcomes of this study provide insights into the impact of the pre-exposure of soils to the contaminants of study in the presence of organic nutrients and an evaluation of the implication of microbial remediation of PAH-contaminated soils lacking in nutrients.

1. Introduction

PAHs are generated by natural and anthropogenic activities involving incomplete combustion of organic matter (Megharaj *et al.*, 2011; Lang *et al.*, 2016; Yu *et. al.*, 2018; Roslund *et al.*, 2018). However, the anthropogenic inputs of PAHs from various industrial activities have led to their ubiquitous presence in the environment (Haritash and Kaushik, 2009; Couling *et al.*, 2010; Okere and Semple 2012; Lang *et al.*, 2016). Studies revealed the retention and persistence of PAHs in the soil occur as a result of their physico-chemical characteristics and soil properties (Reid *et al.*, 2000; Semple *et al.*, 2003; Reid et al., 2004). The labile fractions of PAHs are known to be lost naturally through leaching, volatilization or biodegradation (Stokes *et al.*, 2006) but considerable concentrations are retained in the soil due to their non-polar hydrophobic nature (Lang *et al.*, 2016).

In addition, the soil-PAH interaction reduces the bioavailability and/or bioaccessibility of PAHs (Reid *et al.*, 2000; Macleod and Semple, 2002). Many PAHs exhibit toxic, carcinogenic, mutagenic and teratogenic properties following exposure to humans and other biotas, and are consequently categorized as priority contaminants by Environmental Agencies, including the European Union, United States Environmental Protection Agency (USEPA) and Environment Canada (Marini and Frapiccini, 2013; Demeter *et al.*, 2017; Han *et al.*, 2017; Roslund *et al.*, 2018). Consequently, the remediation of PAH-contaminated soils remains a top priority for abating their environmental persistence and exposure risks, as well as sustainable recovery of PAH-contaminated soils.

Microorganisms are known to have catabolic capabilities to naturally degrade PAHs in soils, and this is known to be dependent on the physico-chemical properties of the soil, the nature of the PAHs and a variety of environmental factors (Watanabe and Baker, 2000; Semple *et*

al., 2001; Rhodes *et al.*, 2008; Lang *et al.*, 2016). In past studies, the successful adaptation of microorganisms for PAH mineralisation was linked to the pre-exposure of the microorganisms to PAHs or their naturally occurring analogues (Semple *et al.*, 2003; Macleod and Semple, 2006). The exposure level is further believed to affect the potential of the microbes to extensively degrade fresh PAHs faster (Macleod and Semple 2006; Rhodes *et al.*, 2008; Couling *et al.*, 2010). According to Couling *et al.* (2010), the microbial adaptation to mineralize PAHs has been thought to be controlled by the concentration of PAHs, the interaction of PAHs with indigenous microbial population and the length of the soil-PAHs contact time. Appropriate soil adaptation is believed to involve specific microbial enzymatic activities and/or genetic changes, as well as selective stimulation of environmentally relevant PAH-degraders (Semple *et al.*, 2003; Macleod and Semple 2002; Macleod and Semple 2006).

However, the entry of PAHs into the soil elevates the soil organic carbon (C) content beyond other essential nutrients, especially nitrogen (N) and phosphorus (P), and adversely affect microbial activity (Rojas-Avelizapa *et al.*, 2000; Warr *et al.*, 2013). In past studies, indigenous microbial activity was successfully stimulated through the addition of inorganic nutrients (Margesin and Schinner, 2001; Vidali, 2001; Semple *et al.*, 2003; Macleod and Semple 2006). However, it is known from past studies that continuous application of inorganic nutrients can harm soil health, with significant impact on the structure and activity of the microbial community (Savci, 2012; Sapp *et al.*, 2015). Also, recurrent application of N-based fertilizers to soils increases nitrous oxide (N_2O) emission (Xu *et al.*, 2014), while the mining, production and transport of P-based fertilizers are costly (Sapp *et al.*, 2015).

Recently, soil conditions have been enhanced with a wide variety of residues from on-farm and industrial production of renewable energy (Margesin and Schinner, 2001; Maletić *et al.*,

2013), due to their high organic matter content, as well as recyclable and readily available nutrient element contents (Insam *et al.*, 2015; Scotti *et al.*, 2014; Gómez-Brandón *et al.*, 2016; Leal *et. al.*, 2017). Their economic feasibility (due to local availability in large amounts), and environmental sustainability (due to reduced environmental emission) (Köster *et al.*, 2014; Palmiotto *et al.*, 2014; Tiwary *et al.*, 2015; Gómez-Brandón *et al.*, 2016; Tampio *et al.*, 2016; Ning *et al.*, 2017) have contributed to their soil application as a suitable option and sustainable alternative to mineral fertilizers (Alburquerque *et al.*, 2012; Insam *et al.*, 2015).

In this study, AD (a biogas residue) and WA (a biomass combustion residue) were added to soil as sources of nutrients. AD and WA are rich in readily available nutrients which are utilisable for microbial activities, and previous studies confirm the positive effect of soil amendment with AD and WA on the physico-chemical properties, microbial biomass and enzyme activities of the soil (Demeyer *et al.*, 2001; Bougnom and Insam 2009; Bougnom *et al.*, 2010; Alburquerque, *et al*, 2012; Walsh *et al.*, 2012; Fernández-Delgado Juárez *et al.*, 2013; Johansen *et al.*, 2013; García-Sánchez *et al.*, 2015a; García-Sánchez *et al.*, 2015c; Koszel and Lorencowicz 2015; Nabeela *et al.*, 2015; Fernández-Bayo *et al.*, 2017). Therefore, it is hypothesized that the addition of AD and WA, as well as their mixtures (due to their complementary N, P and K contents) to soils pre-exposed to PAHs can further enhance indigenous microbial degradation of freshly added PAHs. To the authors' knowledge, this approach has not been reported in the literature.

Therefore, this study aimed to investigate the development of indigenous catabolic evolution of ^{14}C-phenanthrene in the soils with previous exposures to multiple additions of ^{12}C-phenanthrene and the soils with a single application of higher concentrations of ^{12}C-phenanthrene, in the presence and absence of AD and/or WA. At defined intervals of 0 d,

15 d, 30 d, 60 d and 90 d soil-PAH contact time, the effects of the soil-exposures to ^{12}C-phenanthrene and the subsequent amendment(s) with the AD and/or WA were assessed by quantifying the percentage $^{14}CO_2$ produced from the mineralisation of the fresh ^{14}C-phenanthrene added to the soils, at specific respirometry time points. Also, the microbial biomass C and N of all the ^{12}C-phenanthrene-exposed soils with and without addition(s) of the AD and/or WA were assessed at 0 d, 60 d and 90 d time points and compared to the microbial biomass C and N of the soils with a single exposure to ^{12}C-phenanthrene in the absence of AD and/or WA. Further, the microbial community composition of the 90 d AD- and WA-amended soils was monitored to see any significant changes compared to the similar (aged) unamended soils (controls). The outcome of the study provided insights into the biodegradation of PAHs in the presence of added nutrients, and also illustrates the implications of microbial remediation of PAH-contaminated soils lacking in nutrients.

2. Materials and Methods

2.1 Materials

The chemicals and other materials used for this study have been previously listed in Paper 1, Section 2.1 above.

2.2 Sampling of soil and renewable bioenergy residues

The soil used for this experiment was collected from research and agricultural field in Myerscough, United Kingdom (UK). More information about the soil and its physico-chemical properties (Table 1) have been mentioned in Paper 1, Section 2.2 above. The anaerobic digestate (AD) used in this experiment was a pasteurized semi-liquid residue, while the wood-ash (WA) (fly-ash) originated from a biomass power plant (with timber and bark as the feedstocks) (Table 1). More information about the AD and WA have been mentioned in Paper 1, Section 2.1 above.

Table 1: Characteristics of Myerscough soil, anaerobic digestate (AD) and wood-ash (WA); measurements were in dry weight (d/w), except those marked (*) which were in wet weight (w/w); values *(n = 3)* relative standard deviation (RSD). <BDL = below detection limit.

Variables	Soil	Anaerobic digestate	Wood-ash
pH *	6.5 ± 0.1	9.0 ± 0 .0	12.7 ± 0.0
EC * (mS cm^{-1})	35.7 ± 3.0	9.6 ± 0.2	50.3 ± 0.5
Dry matter * (%)	78 ± 0.0	4.2 ± 3.2	99.9 ± 0.0
LOI (%)	5.7 ± 2.0	66.0 ± 0.0	0.95 ± 0.0
Total C (g kg^{-1})	19.0 ± 1.0	349.0 ± 1.0	13.0 ± 0.1
Total N (g kg^{-1})	2.8 ± 0.1	43.5 ± 0.5	0.7 ± 0.1
Total P (g kg^{-1})	1.1 ± 0.08	12.9 ± 0.5	25.2 ± 0.0
Water Soluble PO4-P * (g kg^{-1})	0.0736 ± 5.9	0.316 ± 0.0	0.027 ± 0.0
NH4+-N * (g kg^{-1})	0.0003 ± 0.2	4.281 ± 0.1	<BDL
NO3-N * (g kg^{-1})	0.0014 ± 0.3	0.001 ± 0.0	0.032 ± 0.0

2.3 Soil preparation and spiking with ^{12}C-phenanthrene

Two sets of soil (2.75 kg w/w each) were exposed to ^{12}C-phenanthrene (100 mg kg^{-1}) at various concentrations (Table 2) with a moisture content of 23.8 %, over 21 days (d). During the soil spiking period, the ^{12}C-phenanthrene standards were prepared in acetone and spiked into the soils in separate pre-cleaned glass bowls as explained in Doick *et al.* (2003). The homogenized mixtures were left in the fume cupboard to allow the acetone to volatilize for 2 h before incubation (20 ± 2 ^{o}C) in a dark temperature controlled room for the subsequent spiking and ageing.

Table 2: Soil spiking with single or multiple application(s) of ^{12}C-phenanthrene (100 mg kg^{-1}) and the spiking intervals

Concentrations of ^{12}C-phenanthrene [mg kg^{-1}] added to soil		**Soil spiking intervals (days)**
1st soil treatment	**2nd soil treatment**	
1 x 100	1 x 100	21st
2 x 50	2 x 100	14th & 21st
4 x 25	4 x 100	0, 7th, 14th & 21st

2.4 Soil treatment with renewable bioenergy residues

The amounts of the AD (17.31g) and WA (0.94g) applied to the soils were based on the agricultural practice recommendations of nitrogen-to-phosphorus (N:P) (3:1) reference doses for soil amendments (AHDB, 2017) and wheat plantation (DEFRA, 2017). After 21 d of the soils exposure to ^{12}C-phenanthrene, 17.31g of AD and/or 0.94g of WA was/were added to each of the two sets of soils (Table 2). The spiking conditions include: ^{12}C-phenanthrene-exposed soil + AD + WA; ^{12}C-phenanthrene-exposed soil + AD; ^{12}C-phenanthrene-exposed soil + WA; and ^{12}C-phenanthrene-exposed soils without additions of AD and/or WA. After each addition of the AD and WA, mixtures were homogenized

manually (Doick et al., 2003) and aged in separate labelled loosely closed (for ambient gas exchange) amber glass jars in triplicates at controlled temperature of 20 ± 2^{o}C in a dark room for the period of study (Macleod and Semple, 2002; Couling *et al.*, 2010).

2.5 Mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with renewable bioenergy residues

The mineralisation of freshly added ^{14}C-phenanthrene in the soil microcosms to $^{14}CO_2$ was carried out using a ^{14}C-respirometry system. At 1, 15, 30, 60 and 90 d soil-PAH contact times, the soil incubations were separately sampled (13.1 g w/w) into modified 250 ml Schott bottles (respirometers) and slurried with 30 ml of sterilized distilled water (soil:liquid ratio of 1:3) to ensure even $^{12/14}$C-PAH distribution (Doick and Semple 2003). Each respirometric microcosm was spiked with 5 µl solution of ^{14}C-phenanthrene (50 kBq kg^{-1} soil) and ^{12}C-phenanthrene (30.6 mg kg^{-1} soil) using acetone as a carrier solvent. A 7ml scintillation vial containing 1 M sodium hydroxide ($NaOH_{(aq)}$) was suspended inside each respirometer (with a metal clip incorporated on to the screw cap). Respirometers were incubated at 20 ± 2 ^{o}C on flat-bed orbital shakers (SANYO Gallenkamp) at 100 rpm for 14 d. The $^{14}CO_2$ produced from the mineralisation of the ^{14}C-phenanthrene was trapped in the solution in the vials, which were removed and replaced with fresh ones at 2, 4, 8, 12, 24 h, and then replaced every 24 h till 336 h (14 d). 5 ml of liquid scintillation fluid (Ultima Gold) was added and mixed with each spent $^{14}CO_2$-trap; the vials were wipe-cleaned with an acetone-moistened tissue and allowed to rest in the dark (to prevent chemoluscence effect) for 12 h (to prime). The ^{14}C-activities in the $^{14}CO_2$-traps were quantified by liquid scintillation counting (using Canberra Packard Tri-Carb 2250CA scintillation counter). Cumulative mineralisation of the ^{14}C-phenanthrene was determined, and the amount of $^{14}CO_2$ produced

from the catabolic evolution of the ^{14}C-phenanthrene, at each sampling time, was considered a measure of its bioaccessibility.

2.6 Microbial biomass carbon and nitrogen in ^{12}C-phenanthrene-exposed soils in the presence and absence of anaerobic digestate and/or wood-ash

The microbial biomass C and N of the ^{12}C-phenanthrene-exposed soils amended and unamended with AD and/or WA was measured after 0 d, 60 d and 90 d incubations (20 ± 2 ^{o}C), using chloroform fumigation method (Joergensen *et. al.*, 2011). Two sets of fresh soil sub-samples (5 g w/w, each) were weighed into separate pre-cleaned Teflon tubes (50ml size). The first set was fumigated in a vacuum with chloroform for 24 h followed by extraction with 0.5 M K_2SO_4 (25 ml) (pH 6.8 - 7) on an orbital shaker (100 rpm) with an end-to-end mixing for 30 minutes, while the second set was similarly extracted with the same extractant without fumigation. The extract-solutions were filtered using Whatman Grade 6 filter paper, and the filtrates were analysed for total available C (TC) and N (TN) with a total organic C and N analyzer (TOC-L/TN Series Analyzer, Shimadzu, Japan). The microbial biomass C and N were calculated as the differences of the TC (g^{-1} $_{soil}$) and TN (g^{-1} $_{soil}$) concentrations between the fumigated and non-fumigated samples, with a conversion factor of 0.45 (Joergensen *et. al.*, 2011).

2.7 Soil microbial community structures of ^{12}C-phenanthrene-exposed soils amended with renewable bioenergy residues

This experiment studied the influence of nutrient additions to soils, on microbial community structures or compositions of the ^{12}C-phenanthrene-exposed soils in the presence and absence of AD and/or WA. It was monitored in the 90 d incubations at 20 ± 2 ^{o}C. The

method of determination employed was phospholipid fatty acid (PLFA) analysis. The PLFA extractions were carried out by exposing freeze-dried by exposing known portions (1 – 1.5 g w/w) of the freeze-dried soil samples to a single-phase mixture of chloroform, methanol, and water in an initial ratio of 1:2:0.8, as described in Vestal and White (1989). After 2 hours of extraction, chloroform and citrate buffer (citric acid + Milli-Q water) were added to separate the lipid phases. Then the chloroform, the lipid fraction in the lower chloroform phase was fractionated into neutral, glyco- and phospholipids; the phospholipids were trans-esterified (fatty acid methyl esters) and quantified by gas chromatography (GC) (Hewlett Packard GC series 6890 with flame ionization detectors), relative to an internal standard (Willers *et al.*, 2015).

2.8 Statistical analysis

Blank-corrected data was plotted using SigmaPlot 10.0. The effects of the soils exposures to ^{12}C-phenanthrene, in the absence and presence of AD and/or WA, were analysed against the unamended soils (controls) by using a one-way analysis of variance (ANOVA), at 95 % confidence level ($P < 0.05$), to determine the least significant difference. Also, Turkey and LSD's Post-hoc tests (SPSS) were used to analyze the differences in the means within and across the ^{12}C-phenanthrene-exposed soils amended with AD, WA and mixtures of AD and WA. The occurrence of group mean differences were considered statistically significant if $P < 0.05$ and statistically non-significant if $P > 0.05$.

3. Results

3.1 Mineralisation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils in the presence and absence of renewable bioenergy residues

3.1.1 Mineralisation of ^{14}C-phenanthrene in the soils exposed to 1 x 100 mg kg^{-1} of ^{12}C-phenanthrene in the presence and absence of renewable bioenergy residues

The mineralisation of ^{14}C-phenanthrene in the soils pre-exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene, in the presence and absence of AD and/or WA was studied at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d (Figure 1 and Table 3). After 0 d, the addition of mixtures of AD and WA reduced ($P < 0.05$) the lag phases compared to the controls. After 60 d soil-PAH contact time, the addition of AD and WA, as well as their mixtures shortened ($P < 0.05$) the lag phases compared to the controls. Also, similar results were observed with the AD and mixtures of AD and WA after 90 d, although they were not statistically different ($P > 0.05$) from the controls. All the amendment conditions showed significant reductions in the lag phases from 30 d to 90 d compared to the 0 d incubations.

The maximum rates of mineralisation were higher ($P < 0.05$) with the addition of mixtures of AD and WA after 0 d and as the soil-PAH contact time increased (60 to 90 d), while their separate addition significantly increased the mineralisation rates than the controls after 60 d. The extents of ^{14}C-phenanthrene mineralisation were greater ($P < 0.05$) than the controls with the addition of mixtures of AD and WA throughout the study, except after 15 d where all the soil amendments' conditions were statistically similar and not different the controls. Similarly, the extents of mineralisation were greater ($P < 0.05$) than the controls with the

addition of AD after 30 d and 60 d, while WA addition resulted in greater extents of mineralisation after 60 and 90 d but were not statistically different ($P > 0.05$) from the controls.

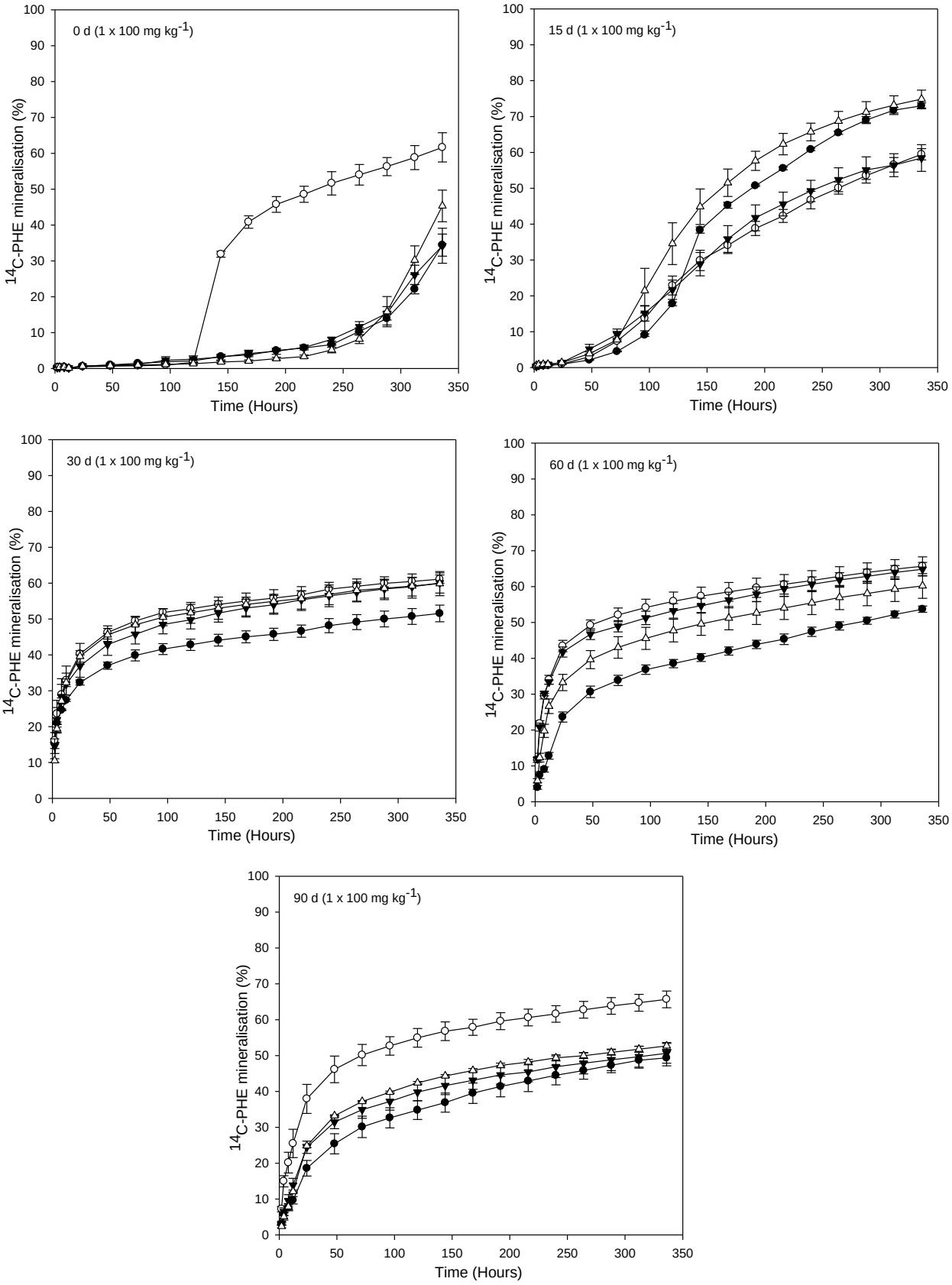

Figure 1: Amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene in the presence of mixtures of anaerobic digestate and wood-ash (○), anaerobic digestate (AD) (▼), wood-ash (WA) (∆) and absence of anaerobic digestate and wood-ash (controls 1) (●) at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values are the mean (n=3) ± standard error of the means (SEM).

Table 3: The parameter of mineralisation of ^{14}C-phenanthrene in the soils exposed to 1 x 100 mg kg^{-1}soil of ^{12}C-phenanthrene in the absence and presence of anaerobic digestate (AD) and/or wood-ash (WA) (controls 1), at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values in the columns followed by different letters are statistically different (Turkey, LSD; *n = 3*; *P < 0.05*); also indicated are shorter lag phases (▪) and higher mineralisation (*) than the controls which were not statistically significant *(P > 0.05)*; values are the mean *(n = 3)* ± SEM.

Soil-PAH contact times (d)	Concentrations of ^{12}C-PAH (mg kg^{-1})	Soil amendments	Lag phases (h)	Maximum rates (% h^{-1})	Cumulative extents (%)
0 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	192.2 ± 4.1 A	0.5 ± 0.1 A	34.4 ± 3.1 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	122.6 ± 0.1 B	1.3 ± 0.0 B	61.6 ± 4.1 B
		soil + $^{12\,\&\,14}$C-PAH + AD	184.6 ± 17.5 A▪	0.5 ± 0.2 A	34.2 ± 4.9 A
		soil + $^{12\,\&\,14}$C-PAH + WA	235.7 ± 6.6 A	0.7 ± 0.1 A*	45.3 ± 4.4 A*
15 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	74.4 ± 0.8 A	0.9 ± 0.0 A	77.2 ± 4.9 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	94.5 ± 8.1 A	0.4 ± 0.0 A	49.0 ± 3.0 A
		soil + $^{12\,\&\,14}$C-PAH + AD	73.7 ± 5.4 A▪	0.4 ± 0.1 A	56.8 ± 3.6 A
		soil + $^{12\,\&\,14}$C-PAH + WA	97.6 ± 4.8 A	0.4 ± 0.1 A	57.6 ± 4.6 A
30 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	0.6 ± 0.0 A	7. 8± 0.0 A	51.5 ± 2.3 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.6 ± 0.1 A	8.1 ± 1.1 A*	61.1 ± 1.2 B
		soil + $^{12\,\&\,14}$C-PAH + AD	0.7 ± 0.1 A	7.3 ± 1.0 A	60.0 ± 2.8. B
		soil + $^{12\,\&\,14}$C-PAH + WA	1.0 ± 0.0 A	5.3 ± 0.3 A	59.9 ± 3.4 B
60 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	2.7 ± 0.3 A	2.1 ± 0.2 A	53.7 ± 0.9 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.9 ± 0.1 B	5.9 ± 0.3 B	65.7 ± 2.6 B
		soil + $^{12\,\&\,14}$C-PAH + AD	0.8 ± 0.0 B	6.0 ± 0.2 B	64.8 ± 1.9 B
		soil + $^{12\,\&\,14}$C-PAH + WA	1.7 ± 0.2 B	3.2 ± 0.4 B	60.2 ± 3.4 A*
90 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	3.4 ± 0.3 A	1.6 ± 0.1 A	49.3 ± 2.2 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	1.4 ± 0.2 A▪	3.9 ± 0.4 B	65.7 ± 2.3 B
		soil + $^{12\,\&\,14}$C-PAH + AD	3.3 ± 0.5 A▪	1.8 ± 0.2 A*	50.6 ± 2.8 A*
		soil + $^{12\,\&\,14}$C-PAH + WA	4.2 ± 0.7 A	1.5 ± 0.2 A	52.7 ± 1.0 A*

3.1.2 Mineralisation of ^{14}C-phenanthrene in the soils exposed to 2 x 50 mg kg^{-1} of ^{12}C-phenanthrene in the presence and absence of renewable bioenergy residues.

The mineralisation of ^{14}C-phenanthrene in soils with previous exposure to 2 x 50 mg kg^{-1} soil concentration of ^{12}C-phenanthrene, in the presence and absence of AD and/or WA, was studied at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d (Fig. 2 and Table 4). In the absence of AD and/or WA, lag phases were shorter ($P < 0.05$) in the soils exposed to 2 x 50 mg kg^{-1} soil concentration of ^{12}C-phenanthrene (controls 2) from 0 d to 90 d soil-PAH contact time than the soils exposed to 1 x 100 mg kg^{-1}$_{l}$ of ^{12}C-phenanthrene spiked soil (controls 1). Addition of mixtures of AD and WA reduced ($P < 0.05$) the lag phases from 0 d to 90 d soil-PAH contact time than both controls. Similarly, lag phases were significantly shorter than the two controls with additions of AD after 0 d to 60 d, while they were not statistically different from the two controls after 90 d.

In the absence of AD and/or WA, the soils exposed to 2 x 50 mg kg^{-1} soil concentration of ^{12}C-phenanthrene (controls 2) showed higher ($P < 0.05$) mineralisation rates after 60 d soil-PAH contact time than the soils exposed to 1 x 100 mg kg^{-1} soil concentration of ^{12}C-phenanthrene (controls 1). Additions of mixtures of AD and WA significantly increased the mineralisation rates higher than both controls from 30 d to 90 d soil-PAH contact time. AD addition was observed to significantly enhance the mineralisation rates than both controls from 60 d to 90 d soil-PAH contact time. The extents of mineralisation were higher both in the absence and presence of AD and/or WA in the soils exposed to 2 x 50 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 2) than the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1) after 0 d and from 30 d to 90 d soil-PAH contact time.

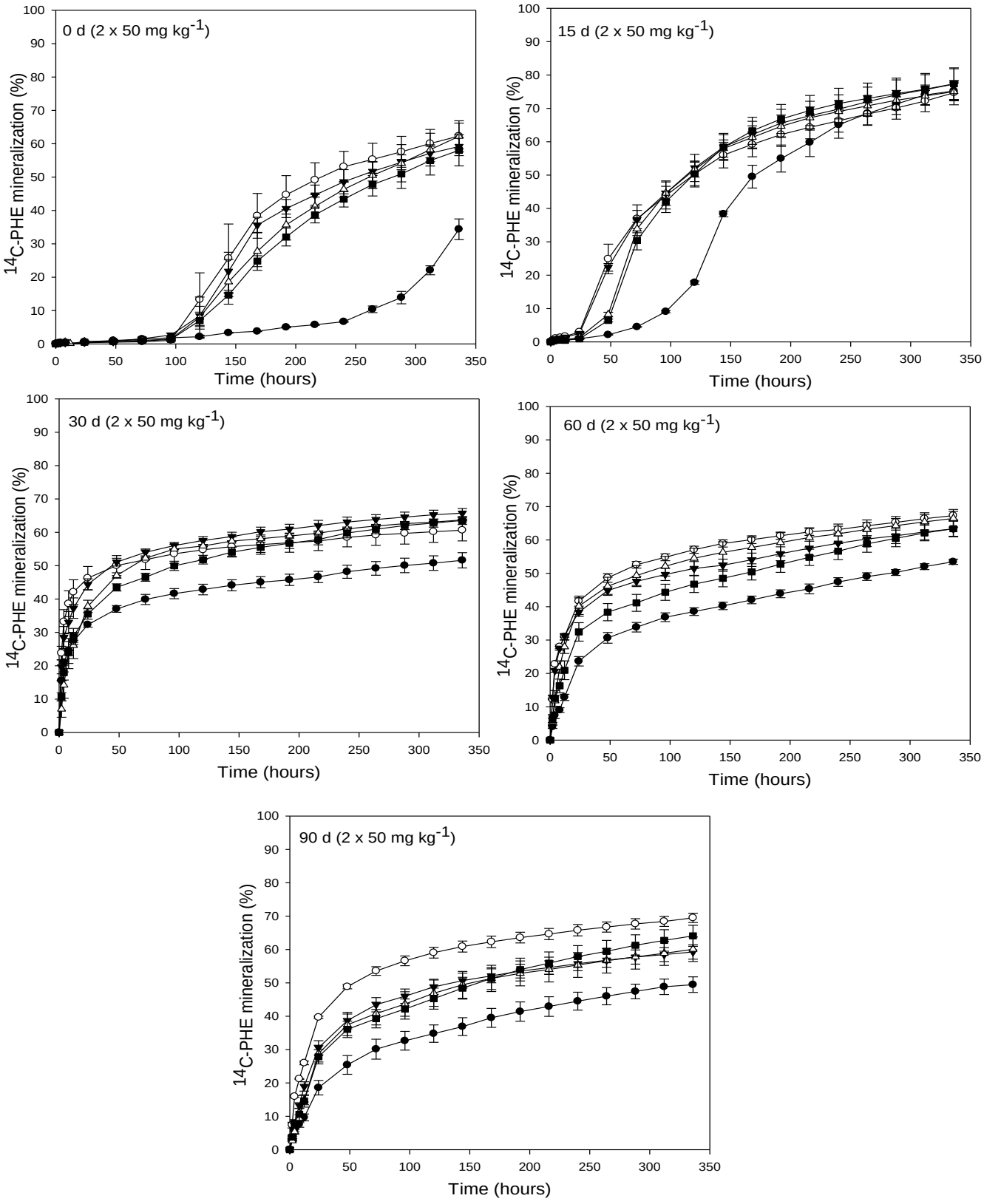

Figure 2: Amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in the soils exposed to 2 x 50 mg kg^{-1} soil of ^{12}C-phenanthrene in the presence of mixtures of anaerobic digestate and wood-ash (AD-WA) (○), anaerobic digestate (AD) (▼), wood-ash (WA) (Δ) and absence of anaerobic digestate (AD) and/or wood-ash (WA) (controls 2) (■), and soils with 1 x 100 mg kg^{-1} of ^{12}C-phenanthrene's exposure in the absence of anaerobic digestate and/or wood-ash (controls 1) (●) at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values are the means (n = 3) ± SEM.

Table 4: The parameter of mineralisation of ^{14}C-phenanthrene in the soils exposed to 2 x 50 mg kg^{-1}soil of ^{12}C-phenanthrene, in the absence and presence of anaerobic digestate (AD) and/or wood-ash (WA) (controls 2), and soils with 1 x 100 mg kg^{-1} of ^{12}C-phenanthrene's exposure in the absence of anaerobic digestate (AD) and wood-ash (WA) (controls 1), at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values in the columns followed by different letters are statistically different (Turkey, LSD; n = 3; *P < 0.05*); also indicated are shorter lag phases (▪) and higher mineralisation (*) which were not statistically significant *(P > 0.05)*; values are the means *(n = 3)* ± SEM.

Soil-PAH contact times (d)	Concentrations of ^{12}C-PAH (mg kg^{-1})	Soil amendments	Lag phases (h)	Maximum rates (% h^{-1})	Cumulative extents (%)
0 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	192.6 ± 4.1 A	0.5 ± 0.1 A	34.4 ± 3.1 A
	2 X 50	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	115.5 ± 5.9 A	0.5 ± 0.1 A	58.0 ± 4.7 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	111.2 ± 8.4 A	0.8 ± 0.2 B	62.3 ± 3.9 A
		soil + $^{12\,\&\,14}$C-PAH + AD	109.7± 8.3 A	0.8 ± 0.2 A*	59.2 ± 2.7 A
		soil + $^{12\,\&\,14}$C-PAH + WA	118.2 ± 9.2 A	0.6 ± 0.1 A*	62.2 ± 4.7 A
15 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	74.4 ± 0.8 A	0.9 ± 0.0 A	75.2 ± 3 A
	2 X 50	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	42.0 ± 1.5 A	1.0 ± 0.1 A*	77.3 ± 4.8 A*
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	26.5 ± 0.7 A	0.9 ± 0.2 A	74.7 ± 3.6 A
		soil + $^{12\,\&\,14}$C-PAH + AD	27.0 ± 0.3 A	0.8 ± 0.0 A	77.2±4.6 A*
		soil + $^{12\,\&\,14}$C-PAH + WA	37.1 ± 1.2 A	1.1 ± 0.1 A*	75.0±2.6 A
30 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	0.6 ± 0.0 A	7.8 ± 0.0 A	51.5 ± 2.4 A
	2 X 50	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	1.0 ± 0.1 A	5.3 ± 0.6 A	63.5 ± 1.2 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.4 ± 0.0 A	11.9 ± 1.0 B	60.6 ± 3.2 A
		soil + $^{12\,\&\,14}$C-PAH + AD	0.5 ± 0.1 A	9.8 ± 1.1 A*	65.7 ± 1.4 A
		soil + $^{12\,\&\,14}$C-PAH + WA	1.8 ± 0.6 B	3.9 ± 1.0 A	63.7 ± 0.2 A
60 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	2.7 ± 0.3 A	2.1 ± 0.2 A	53.5 ± 0.8 A
	2 X 50	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	1.6 ± 0.2 A	3.3 ± 0.6 B	63.4 ± 2.3 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.8 ± 0.0 A	6.1 ± 0.1 B	67.3 ± 1.3 A
		soil + $^{12\,\&\,14}$C-PAH + AD	0.8 ± 0.0 A	6.5 ± 0.3 B	63.3 ± 2.4 A
		soil + $^{12\,\&\,14}$C-PAH + WA	1.9 ± 0.3 A	3.1 ± 0.3 A*	66.4 ± 2.7 A
90 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	3.4 ± 0.3 A	1.6 ± 0.1 A	49.5 ± 2.3 A
	2 X 50	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	2.8 ± 0.4 A▪	1.9 ± 0.2 A*	64.1 ± 3.2 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	1.4 ± 0.2 B	4.5 ± 0.3 B	69.5 ± 1.4 A
		soil + $^{12\,\&\,14}$C-PAH + AD	3.3 ± 0.9 A▪	2.3 ± 0.2 B	59.3 ± 2.2 A
		soil + $^{12\,\&\,14}$C-PAH + WA	4.0 ± 0.6 A	1.6 ± 0.2 A	60.1 ± 3.7 A

3.1.3 Mineralisation of ^{14}C-phenanthrene in the soils exposed to 4 x 25 mg kg^{-1} of ^{12}C-phenanthrene in the presence and absence of renewable bioenergy residues

The mineralisation of ^{14}C-phenanthrene in the soils with previous exposure to 4 x 25 mg kg^{-1} soil of ^{12}C-phenanthrene in the absence and presence of AD and/or WA was studied, at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d (Fig. 3 and Table 5). In the absence of AD and/or WA, soils exposed to 4 x 25 mg kg^{-1} soil concentration of ^{12}C-phenanthrene (controls 2) showed significantly shorter lag phases after 0, 15 and 90 d soil-PAH contact time than the soils exposed to 1 x 100 mg kg^{-1} soil concentration of ^{12}C-phenanthrene (controls 1). Significantly shorter lag phases were also observed after 60 and 90 d with additions of AD, as well as mixtures of AD and WA, compared to the similar unamended soils (controls 2) and controls 1.

There was no significant difference in the rates of mineralisation between the soils exposed to 4 x 25 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 2) compared to the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1). However, additions of mixtures of AD and WA, as well as AD only significantly enhanced the mineralisation rates than the two controls from 0 d to 90 d soil-PAH contact time. The extents of mineralisation were higher ($P < 0.05$) in the absence of AD and/or WA in the soils exposed to 4 x 25 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 2) than the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1). Similarly, additions of AD and WA, as well as their mixtures, significantly increased ($P < 0.05$) the extents of mineralisation than both controls from 30 to 90 d soil-PAH contact time; while after 0 d, mineralisation extents for all amended soils including controls 2 were only significantly higher than the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1).

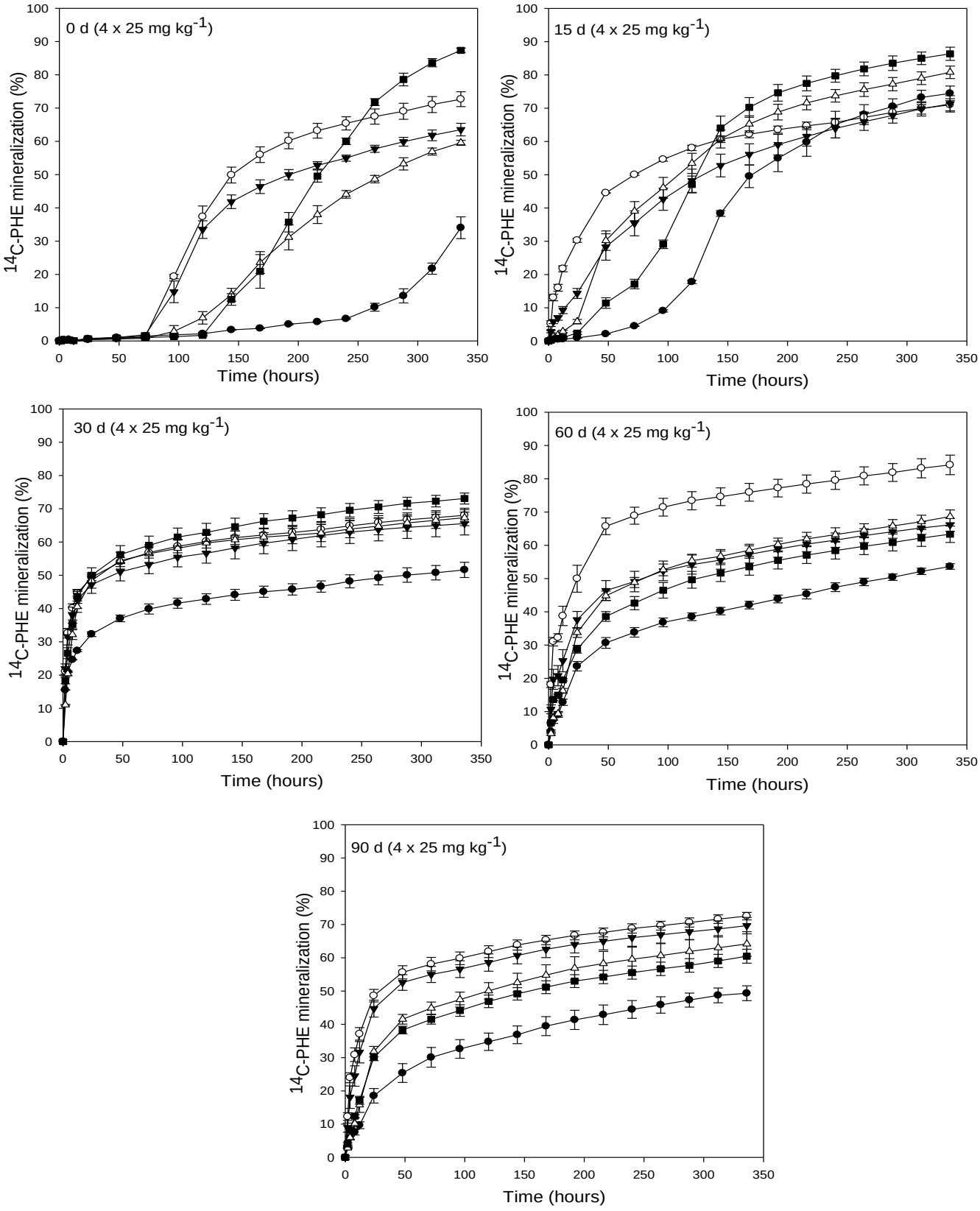

Figure 3: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in the soils exposed to 4 x 25 mg kg^{-1} soil of ^{12}C-phenanthrene in the presence of mixtures of anaerobic digestate and wood-ash (AD-WA) (○), anaerobic digestate (AD) (▼), wood-ash (WA) (Δ) and absence of anaerobic digestate (AD) and/or wood-ash (WA) (controls 2) (■), and soils with 1 x 100 mg kg^{-1} of ^{12}C-phenanthrene's exposure in the absence of anaerobic digestate and/or wood-ash (controls 1) (●) at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values are the means (n = 3) ± SEM.

Table 5: The parameter of mineralisation of ^{14}C-phenanthrene in the soils exposed to 4 x 25 mg kg^{-1}soil of ^{12}C-phenanthrene, in the absence and presence of anaerobic digestate (AD) and/or wood-ash (WA) (controls 2), and soils with 1 x 100 mg kg^{-1} of ^{12}C-phenanthrene's exposure in the absence of anaerobic digestate (AD) and wood-ash (WA) (controls 1), at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values in the columns followed by different letters are statistically different (Turkey, LSD; *n = 3*; *P < 0.05*); also indicated are shorter lag phases (▪) and higher mineralisation (*) which were not statistically significant *(P > 0.05)*; values are the means (n = 3) ± SEM.

Soil-PAH contact times (d)	Concentrations of ^{12}C-PAH (mg kg^{-1})	Soil amendments	Lag phases (h)	Maximum rates (% h^{-1})	Cumulative extents (%)
0 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	192.6 ± 4.1 A	0.5 ± 0.1 A	34 ± 3.3 A
	4 X 25	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	132.8 ± 4.7 A	0.7 ± 0.1 A*	87.4 ± 0.7 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	76.9 ± 0.2 A	0.9 ± 0.0 B	72.7 ± 2.2 B
		soil + $^{12\,\&\,14}$C-PAH + AD	78.5 ± 1.6 A	0.8 ± 0.1 B	63.5 ± 1.9 B
		soil + $^{12\,\&\,14}$C-PAH + WA	109.1 ± 10.0 A	0.4 ± 0.0 A	59.6 ± 0.7 B
15 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	74.4 ± 0.8 A	0.9 ± 0.0 A	74.4 ± 2.2 A
	4 X 25	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	31.5 ± 1.9 A	0.8 ±.0.0 A	86.3 ± 2.0 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	1.9 ± 0.2 A	3.9 ± 0.1 B	71.0 ± 1.8 A
		soil + $^{12\,\&\,14}$C-PAH + AD	3.8 ± 0.7 A	1.5 ± 0.1 B	71.3 ± 2.5 A
		soil + $^{12\,\&\,14}$C-PAH + WA	20.8 ± 2.2 A	1.0 ± 0.1 A*	80.8 ± 1.9 A*
30 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	0.6 ± 0.0 A	7.8 ± 0.0 A	51.6 ± 2.3 A
	4 X 25	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	0.5 ± 0.0 A▪	9.2 ± 0.5 A*	73.1±1.7 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.5 ± 0.0 A▪	10.6 ± 0.7 B	68.0 ±2.1 B
		soil + $^{12\,\&\,14}$C-PAH + AD	0.5 ± 0.0 A▪	10.9 ± 0.8 B	65.5 ± 3.5 B
		soil + $^{12\,\&\,14}$C-PAH + WA	0.9 ± 0.0 A	5.5 ± 0.1 A	67.3 ± 2.4 B
60 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	2.7 ± 0.3 A	2.1 ± 0.2 A	53.6 ± 0.8 A
	4 X 25	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	1.5 ± 0.1 B	3.5 ± 0.1 A*	63.3 ± 2.5 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.6 ± 0.0 B	9.1 ± 0.4 B	84.1 ± 2.9 B
		soil + $^{12\,\&\,14}$C-PAH + AD	1.0 ± 0.1 B	5.4 ± 0.7 B	66.1 ± 1.9 B
		soil + $^{12\,\&\,14}$C-PAH + WA	2.7 ± 0.2 A	2.4 ± 0.1 A*	68.6 ± 2.0 B
90 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	3.4 ± 0.3 A	1.6 ± 0.1 A	49.3 ± 2.2 A
	4 X 25	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	2.5 ± 0.2 A▪	2.1 ± 0.1 A*	60.5 ± 2.1 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.8 ± 0.1 B	6.3 ± 0.4 B	72.5 ± 1.1 B
		soil + $^{12\,\&\,14}$C-PAH + AD	1.2 ± 0.2 B	4.8 ± 0.8 B	69.6 ± 2.5 B
		soil + $^{12\,\&\,14}$C-PAH + WA	3.5 ± 0.3 A	1.7 ± 0.3 A*	64.1 ± 3.7 B

3.1.4 Mineralisation of ^{14}C-phenanthrene in the soils exposed to 2 x 100 mg kg^{-1} of ^{12}C-phenanthrene in the presence and absence of renewable bioenergy residues

The mineralisation of ^{14}C-phenanthrene in the soils exposed to 2 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene in the presence and absence of AD and/or WA was studied at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d (Fig. 4 and Table 6). From 0 d to 30 d, there was no significant difference in the lag phases between the soils exposed to 2 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 2) and the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1). However, after 60 and 90 d, the length of the lag phases in controls 2 was shorter than Control 1. Also, additions of mixtures of AD and WA significantly reduced ($P > 0.05$) the length of the lag phases after 90 d, while shorter lag phases were observed with the mixtures of AD and WA after 60 d, as well AD only incubations after 60 and 90 d, although they were not different ($P > 0.05$) from the two controls.

There was no significant difference in the rates of mineralisation between the soils exposed to 2 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 2) compared to the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1). However, the rates of mineralisation were increased ($P < 0.05$) with additions of mixtures of AD and WA after 60 d and 90 d, and AD after 60 d compared to the controls. After 0, 15 and 90 d soil-PAH contact time, extents of mineralisation were higher ($P < 0.05$) in the absence of AD and/or WA in the soils exposed to 2 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 2) than the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1). On a general note, the extents of mineralisation were enhanced ($P < 0.05$) with additions of mixtures of AD and WA than controls 1 and controls 2 after 0, 30, 60 and 90 d soil-PAH contact time points. Similarly,

higher ($P < 0.05$) extents of mineralisation were observed with additions of AD after 30 and 60 d, as well as WA after 60 d compared to the two sets of controls.

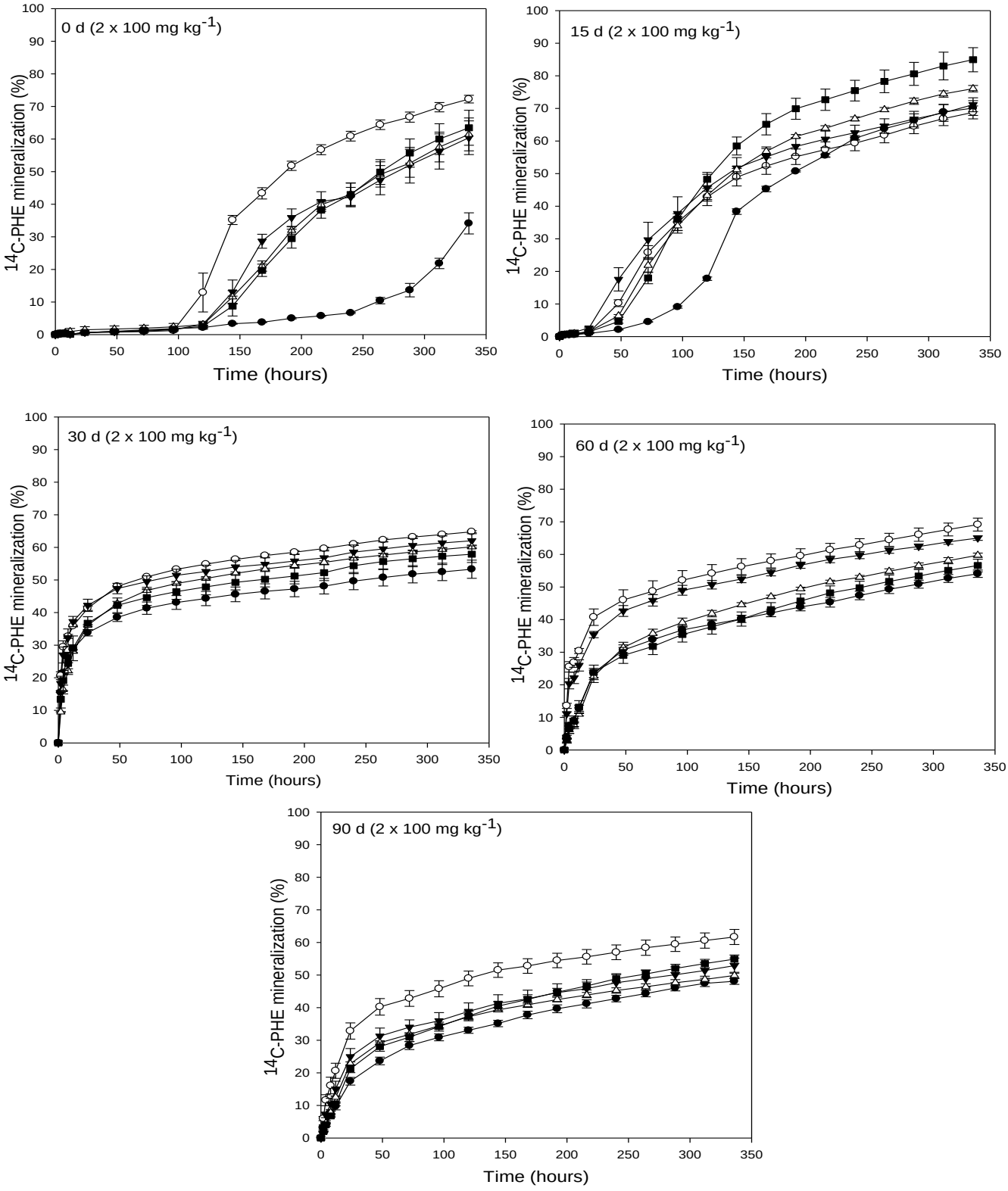

Figure 4: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in the soils exposed to 2 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene in the presence of mixtures of anaerobic digestate and wood-ash (AD-WA) (○), anaerobic digestate (AD) (▼), wood-ash (WA) (∆) and absence of anaerobic digestate (AD) and/or wood-ash (WA) (controls 2) (■), and soils with 1 x 100 mg kg^{-1} of ^{12}C-phenanthrene's exposure in the absence of anaerobic digestate and/or wood-ash (controls 1) (●) at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values are the means (n = 3) ± SEM.

Table 6: The parameter of mineralisation of ^{14}C-phenanthrene in soils exposed to 2 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene, in the absence and presence of anaerobic digestate (AD) and/or wood-ash (WA) (controls 2), and soils with 1 x 100 mg kg^{-1} of ^{12}C-phenanthrene's exposure in the absence of anaerobic digestate (AD) and wood-ash (WA) (controls 1), at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d. Values in the columns followed by different letters are statistically different (Turkey, LSD; n=3; P<0.05); also indicated are shorter lag phases (▪) and higher mineralisation (*) which were not statistically significant (P>0.05); values are the means (n=3) ± SEM.

Soil-PAH contact times (d)	Concentrations of ^{12}C-PAH (mg kg^{-1})	Soil amendments	Lag phases (h)	Maximum rates (% h^{-1})	Cumulative extents (%)
0 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	192.6 ± 4.1 A	0.5 ± 0.1 A	34.1 ± 3.2 A
	2 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	133.1 ± 7.3 A	0.5 ± 0.1 A	63.4 ± 5.4 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	108.3 ± 7.2 A	1.0 ± 0.1 A*	72.2 ± 1.2 B
		soil + $^{12\,\&\,14}$C-PAH + AD	135.2 ± 9.6 A	0.8 ± 0.1 A*	60.4 ± 5.2 B
		soil + $^{12\,\&\,14}$C-PAH + WA	124.8 ± 4.6 A	0.5 ± 0.0 A	61.4 ± 5.1 B
15 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	74.4 ± 0.8 A	0.9 ± 0.0 A	71.3 ± 1.1 A
	2 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	47.5 ± 2.3 A	0.8 ± 0.1 A	84.9 ± 3.7 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	34.3 ± 1.4 A	0.7 ± 0.1 A	68.8 ± 2.0 A
		soil + $^{12\,\&\,14}$C-PAH + AD	28.4 ± 1.4 A	0.6 ± 0.1 A	71.1 ± 2.1 A
		soil + $^{12\,\&\,14}$C-PAH + WA	41.3 ± 1.6 A	0.7 ± 0.1 A	76.0 ± 1.0 A*
30 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	0.6 ± 0.0 A	7.8 ± 0.0 A	53.3 ± 2.8 A
	2 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	0.8 ± 0.2 A	6.7 ± 1.3 A	57.9 ± 2.5 A*
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.5 ± 0.0 A▪	10.3 ± 0.6 A*	64.8 ± 0.3 B
		soil + $^{12\,\&\,14}$C-PAH + AD	0.6 ± 0.1 A	9.1 ± 1.1 A*	62.0 ± 2.2 B
		soil + $^{12\,\&\,14}$C-PAH + WA	1.1 ± 0.0 B	4.7 ± 0.1 A	60.1 ± 0.5 B
60 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	2.7 ± 0.3 A	2.1 ± 0.2 A	54.1 ± 1.1 A
	2 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	3.9 ± 1.3 A	1.9 ± 0.5 A	56.6 ± 1.9 A*
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.7 ± 0.0 A▪	6.8 ± 0.4 B	69.1 ± 2.0 B
		soil + $^{12\,\&\,14}$C-PAH + AD	0.9 ± 0.1 A▪	5.5 ± 0.3 B	65.0 ± 0.7 B
		soil + $^{12\,\&\,14}$C-PAH + WA	3.5 ± 0.6 A	1.7 ± 0.3 A	59.7 ± 0.6 B
90 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	3.4 ± 0.3 A	1.6 ± 0.1 A	47.9 ± 0.7 A
	2 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	5.4 ± 0.6 A	1.1 ± 0.1 A	54.9 ± 1.3 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	1.7 ± 0.2 B	3.1 ± 0.4 B	61.7 ± 2.3 B
		soil + $^{12\,\&\,14}$C-PAH + AD	3.0 ± 0.3 A▪	1.9 ± 0.2 A*	52.9 ± 2.3 A*
		soil + $^{12\,\&\,14}$C-PAH + WA	4.5 ± 0.3 A	1.3 ± 0.1 A	49.9 ± 1.0 A*

3.1.5 Mineralisation of ^{14}C-phenanthrene in the soils exposed to 4 x 100 mg kg^{-1} of ^{12}C-phenanthrene in the presence and absence of renewable bioenergy residues

The mineralisation of ^{14}C-phenanthrene in the soils previously exposed to 4 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene in the absence and presence of AD and/or WA was studied at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d (Fig. 5 and Table 7). In the absence of AD and/or WA, soils exposed to 4 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 2) showed shorter ($P < 0.05$) lag phases after 0 and 15 d than the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1). Additions of AD, WA as well as the mixtures of AD and WA shortened ($P < 0.05$) the lag phases than the two controls after 0 and 15 d; while from 30 d to 90 d, there was no significant difference in the lag phases both in the presence and absence of the AD and/or WA, as well as compared with controls 1. Noticeably, the length of lag phases was generally higher in all amended soils with WA when compared to the other amendments and controls (1 and 2) respectively.

The rates of mineralisation were higher in the absence of AD and/or WA after 30 d in soils exposed to 4 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 2) than soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1). The mineralisation rates were also higher ($P < 0.05$) with the addition of mixtures of AD and WA after 30, 60 and 90 d than all the controls, while AD addition significantly increased the mineralisation rates after 30 and 60 d. The extents of mineralisation were increasingly greater ($P < 0.05$) in soils exposed to 4 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 2) in the absence of AD and/or WA than the soils exposed to 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene (controls 1) after 0 d and with increasing soil-PAH contact time, except after 60 d incubation. Further, WA addition enhanced the extents of mineralisation after 90 d ageing. More so, the increase in soil-PAH

contact time resulted in higher extents of ^{14}C-phenanthrene mineralisation in all the AD and/or WA amended soils from 0 d to 30, such that significant extents of mineralisation were also observed after 90 d compared to the 0 d soil-phenanthrene contact time.

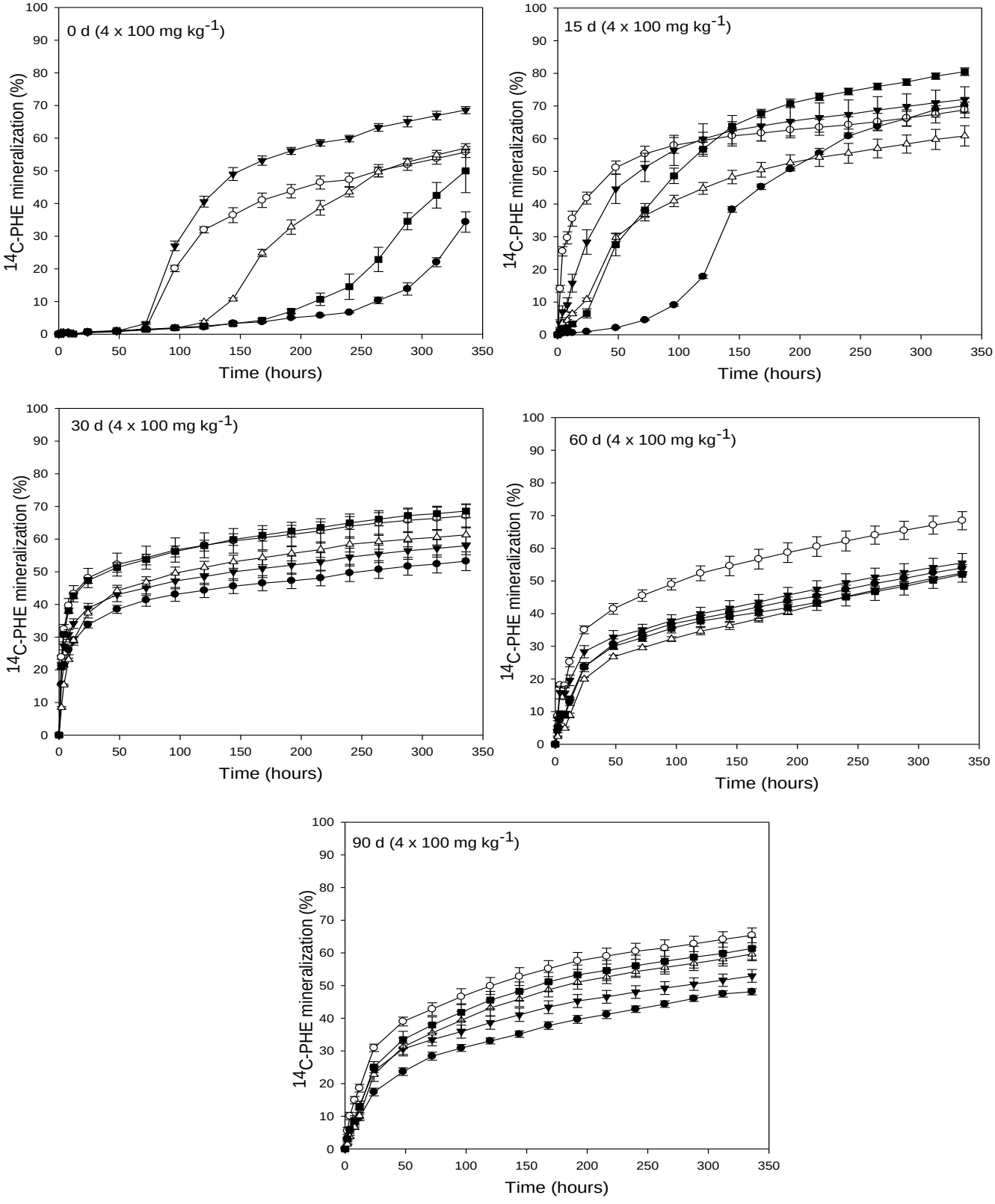

Figure 5: The amounts of $^{14}CO_2$ (%) produced from the mineralisation of ^{14}C-phenanthrene in the soils exposed to 4 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene in the presence of a mixture of anaerobic digestate and wood-ash (AD-WA) (○), anaerobic digestate (AD) (▼), wood-ash (WA) (∆) and absence of anaerobic digestate (AD) and/or wood-ash (WA) (controls 2) (■), and soils with 1 x 100 mg kg^{-1} of ^{12}C-phenanthrene's exposure in the absence of anaerobic digestate and/or wood-ash (controls 1) (●) at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values are the mean (n=3) ± SEM.

Table 7: The parameter of mineralisation of ^{14}C-phenanthrene in soils exposed to 4 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene, in the absence and presence of anaerobic digestate (AD) and/or wood-ash (WA) (controls 2), and soils with 1 x 100 mg kg^{-1} of ^{12}C-phenanthrene's exposure in the absence of anaerobic digestate (AD) and wood-ash (WA) (controls 1), at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d. Values in the columns followed by different letters are statistically different (Turkey, LSD; n = 3; P<0.05); also indicated are shorter lag phases (▪) and higher mineralisation (*) which were not statistically significant (P>0.05); values are the means (n = 3) ± SEM.

Soil-PAH contact times (d)	Concentrations of ^{12}C-PAH (mg kg^{-1})	Soil amendments	Lag phases (h)	Maximum rates (% h^{-1})	Cumulative extents (%)
0 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	192.6 ± 4.1 A	0.5 ± 0.1 A	34.3 ± 3.1 A
	4 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	174.5 ± 3.3 B	0.5 ± 0.1 A	50.0 ± 6.7 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	78.1 ± 1.9 B	0.8 ± 0.0 B	55.8 ± 1.7 A
		soil + $^{12\,\&\,14}$C-PAH + AD	66.1 ± 7.9 B	1.0 ± 0.1 B	68.6 ± 1.1 A
		soil + $^{12\,\&\,14}$C-PAH + WA	124.3 ± 1.6 B	0.6 ± 0.0 A*	57.0 ± 1.3 A
15 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	74.4 ± 0.8 A	0.9±0.0 A	70.1±2.3 A
	4 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	18.7 ± 3.5 B	0.9±0.1 A	80.5±1.2 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.7 ± 0.1 B	7.0±0.5 B	68.8±2.4 A
		soil + $^{12\,\&\,14}$C-PAH + AD	4.1 ± 1.9 B	2.0±0.3 B	72.0±3.9 A*
		soil + $^{12\,\&\,14}$C-PAH + WA	9.1 ± 0.7 B	1.0±0.1 A*	60.8±3.1 A
30 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	0.6 ± 0.0 A	7.8 ± 0.0 A	53.2 ± 2.9 A
	4 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	0.5 ± 0.0 A	10.4 ± 0.2 B	68.6 ± 2.0 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	0.4 ± 0.0 A	11.9 ± 0.3 B	67.1 ± 3.7 A
		soil + $^{12\,\&\,14}$C-PAH + AD	0.5 ± 0.0 A	10.6 ± 0.5 B	57.9 ± 2.7 A*
		soil + $^{12\,\&\,14}$C-PAH + WA	1.2 ± 0.0 B	4.2 ± 0.1 C	61.3 ± 2.4 A*
60 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	2.7 ± 0.3 A	2.1 ± 0.2 A	54.0 ± 1.0 A
	4 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	2.7 ± 0.7 A	2.5 ± 0.2 A*	52.0 ± 2.4 A
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	1.2 ± 0.1 A▪	4.8 ± 0.1 B	68.4 ± 2.8 B
		soil + $^{12\,\&\,14}$C-PAH + AD	1.3 ± 0.1 A▪	4.2 ± 0.5 B	55.5 ± 2.9 A*
		soil + $^{12\,\&\,14}$C-PAH + WA	7.0 ± 1.5 B	1.3 ± 0.1 A	52.4 ± 0.3 A
90 d	1 X 100	Controls 1 (soil + $^{12\,\&\,14}$C-PAH)	3.4 ± 0.3 A	1.6 ± 0.1 A	48.1 ± 0.9 A
	4 X 100	Controls 2 (soil + $^{12\,\&\,14}$C-PAH)	3.4 ± 0.0 A	1.5 ± 0.0 A	61.4 ± 3.4 B
		soil + $^{12\,\&\,14}$C-PAH + AD + WA	1.9 ± 0.3 A▪	2.8 ± 0.5 B	65.3 ± 2.2 B
		soil + $^{12\,\&\,14}$C-PAH + AD	3.9 ± 0.9 A	1.5 ± 0.2 A	52.9 ± 2.0 A*
		soil + $^{12\,\&\,14}$C-PAH + WA	5.9 ± 0.8 B	1.1 ± 0.1 A	59.6 ± 2.1 B

3.2 Soil microbial biomass carbon and nitrogen in the soils

The effect of the addition of anaerobic digestate (AD) and/or wood-ash (WA) on the microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) of the ^{12}C-phenanthrene-exposed soils was determined after 0, 60 and 90 d incubations (20 ± 2 ^{o}C), with chloroform fumigation method (Table 8).

In the soils exposed to a single addition of 1 X 100 mg kg^{-1} of ^{12}C-phenanthrene, there is no significant ($P > 0.05$) effect of the presence of AD and/or WA on the MBC and MBN compared to the similar unamended soils (controls). However, in the soils exposed to 2 X 100 mg kg^{-1} of ^{12}C-phenanthrene, the MBC and MBN were higher than the controls after 90 d in the presence of mixtures of AD and WA as well as AD only. Soil exposure to 4 X 100 mg kg^{-1} of ^{12}C-phenanthrene showed higher MBC after 0 d compared to the controls in the presence of mixtures of AD and WA as well as WA only. A similar observation was made with AD addition after 60 d and 90 d. MBN was observed higher than the controls after 90 d in the presence of the mixtures of AD and WA as well as AD only.

In the soils with additions of 2 X 50 mg kg^{-1} of ^{12}C-phenanthrene, there was no significant ($P > 0.05$) effect with the addition(s) of AD and/or WA on the MBC and MBN compared to the similar unamended soils (controls). However, in the soils exposed to 4 X 25 mg kg^{-1} of ^{12}C-phenanthrene, the MBC increased significantly ($P < 0.05$) than the controls after 0 d, 60 d and 90 d with additions of mixtures of AD and WA, as well as AD only; while the addition of WA increased ($P < 0.05$) the MBC after 0 d and 90 d compared to the controls. MBC was significantly ($P < 0.05$) low in the absence of AD and/or WA compared with the addition of AD and/or WA. MBN was significantly higher than the controls as well as similar phenanthrene-exposed soils only after 0 d. Significantly higher MBN was observed after 60

d both in the presence and absence of AD and/or WA and after 90 d in the presence of the mixtures of AD and WA.

Table 8: Amounts (mg kg^{-1}) of the microbial biomass carbon (C) and nitrogen (N) in the soils with single and multiple additions of ^{12}C-phenanthrene in the presence and absence of anaerobic digestate (AD) and/or wood-ash (WA), and the soils with a single addition of ^{12}C-phenanthrene in the absence of anaerobic digestate (AD) and/or wood-ash (WA) (controls) after 0 d, 60 d and 90 d incubations; values (n = 3) represent the mean ± standard error of the mean (SEM); values in columns followed by different letters are statistically different (Turkey, LSD; *P<0.05*); letter with an asterisk sign (*) indicates higher mineralisation that was not statistically significant (P > 0.05).

Additions of ^{12}C-PAH (mg kg^{-1} soil)	**Soil-amendments**	**0 d Soil-PAH contact time**		**60 d Soil-PAH contact time**		**90 d Soil-PAH contact time**	
		MBC (mg kg^{-1})	**MBN (mg kg^{-1})**	**MBC (mg kg^{-1})**	**MBN (mg kg^{-1})**	**MBC (mg kg^{-1})**	**MBN (mg kg^{-1})**
	Control (soil + $^{12\ \&\ 14}$C-PAH)	45.7 ± 9.2 a	34.4 ± 4.7 a	20.2 ± 6.4 a	83.2 ± 5.5 a	77.7± 11.5 a	26.5 ± 1.6 a
1 x 100	soil + ^{12}C-PAH + AD + WA	43.9 ± 10.8 a	47.6 ± 10.9 a*	57.8 ± 7.4 a*	134.3 ± 2.2 b	37.2 ± 3.5 a	58.3 ± 1.0 b
	soil + ^{12}C-PAH + AD	37.1 ± 4.3 a	21.1 ± 5.0 a	86.8 ± 6.7 b	49.5 ± 9.4 a	165.9 ± 9.6 b	65.5 ± 2.6 b
	soil + ^{12}C-PAH + WA	16.3 ± 4.0 a	12.8 ± 2.4 a	77.6 ± 4.0 b	26.5 ± 4.7 b	54.1 ± 5.3 a	37.1 ± 4.4 a
2 x 50	soil + ^{12}C-PAH + AD + WA	21.6 ± 1.4 a	34.0 ± 3.9 a	32.1 ± 7.1 a*	94.0 ± 20.2 a	56.5 ± 4.5 a	83.4 ± 6.1 a
	soil + ^{12}C-PAH + AD	39.7 ± 13.2 a	18.8 ± 3.1 a	28.3 ± 12.4 a*	33.4 ± 0.4 a	99.5 ± 1.9 a*	46.2 ± 5.7 a
	soil + ^{12}C-PAH + WA	31.8 ± 6.1 a	19.3 ± 5.5 a	20.0 ± 2.1 a	47.1 ± 4.3 a	81.5 ± 7.0 a	38.4 ± 5.6 a
	soil + ^{12}C-PAH	101.4 ± 5.9 b	17.8 ± 0.0 a	20.2 ± 3.5 a	90.5 ± 2.4 a	60.8 ± 2.4 a	65.3 ± 19.0 a
4 x 25	soil + ^{12}C-PAH + AD + WA	670.6 ± 8.1 b	143.5 ± 13.5 b	76.9 ± 13.5 b	91.4 ± 13.8 b	123.4 ± 6.6 b	152.1 ± 17.0 b
	soil + ^{12}C-PAH + AD	571.3 ± 18.3 b	86.6 ± 3.0 a*	115.5 ± 3.4 b	21.1 ± 0.7 a	231.3 ± 8.7 b	50.0 ± 4.5 a
	soil + ^{12}C-PAH + WA	665.1 ± 2.0 b	67.3 ± 2.3 a*	58.4 ± 4.4 a*	10.3 ± 0.5 a	149.0 ± 11.1 b	38.4 ± 0.8 a
	soil + ^{12}C-PAH	86.1 ± 7.8 a	109.0 ± 6.2 b	21.2 ± 2.5 a*	98.3 ± 7.8 b	100.5 ± 6.8 a*	41.1 ± 9.3 a
2 x 100	soil + ^{12}C-PAH+ AD + WA	91.3 ± 6.7 a*	81.0 ± 7.2 b	26.5 ± 3.2 a*	71.9 ± 8.6 a	122.3 ± 5.9 b	716.0 ± 11.7 b
	soil + ^{12}C-PAH + AD	99.3 ± 9.1 a*	46.0 ± 2.2 a*	20.9 ± 2.6 a*	18.1 ± 3.1 b	134.9 ± 8.4 b	679.9 ± 9.6 b
	soil + ^{12}C-PAH + WA	72.9 ± 4.9 a*	26.0 ± 3.8 a	39.8 ± 4.0 a*	12.0 ± 0.5 b	48.5 ± 13.7 a	16.3 ± 1.9 a
	soil + ^{12}C-PAH	114.0 ± 0.6 b	57.8 ± 6.5 a*	50.4 ± 11.1 a*	27.7 ± 3.5 b	82.3 ± 5.8 a	40.1 ± 1.6 a
4 x 100	soil + ^{12}C-PAH + AD + WA	166.4 ± 6.0 b	51.1 ± 8.4 a*	20.2 ± 3.0 a	27.8 ± 5.5 a	71.1 ± 8.2 a	123.0 ± 11.0 b
	soil + ^{12}C-PAH + AD	48.4 ± 6.0 a*	36.8 ± 1.2 a*	95.9 ± 10.1 b	34.4 ± 1.8 a	72.2 ± 2.3 a	532.1 ± 6.4 b
	soil + ^{12}C-PAH + WA	51.0 ± 5.6 b	25.3 ± 3.1 a	58.9 ± 9.0 a*	27.5 ± 2.2 a	43.2 ± 2.3 a	25.8 ± 2.1 a
	soil + ^{12}C-PAH	28.7 ± 9.5 a	17.1 ± 0.2 a	51.7 ± 15.1 b	19.1 ± 1.0 a	74.6 ± 21.3 a	83.1 ± 4.7 a*

3.3 Concentrations of phospholipid fatty acids in the soils

Changes in microbial community structure, as the concentrations of phospholipid fatty acids (PLFAs), of the ^{12}C-phenanthrene-exposed soils with and without the additions of AD and/or WA were determined after 90 d aged incubation at 20 ± 2 ^{o}C (Table 9). The soils with a single exposure of 1 x 100 mg kg^{-1} soil of ^{12}C-phenanthrene and amended with AD, WA and mixtures of AD and WA showed higher total and bacterial PLFAs compared to the similar unamended soils (controls).

There were higher bacterial and total PLFAs in the soils with multiple ^{12}C-phenanthrene-exposures (2 x 50 and 4 x 25 mg kg^{-1} soil) in the absence of AD and/or WA (controls). Also, the soils pre-exposed to 2 x 50 mg kg^{-1} soil showed lower amounts of bacterial and total PLFAs with separate additions of AD and WA compared to the similar unamended soils. However, there were higher total and bacterial PLFAs than the controls with the additions of the mixtures of AD and WA. Furthermore, the total and bacterial PLFAs were lower after 0 d in the soils pre-exposed to 4 x 25 mg kg^{-1} of ^{12}C-phenanthrene and amended with AD and/or WA compared to the controls, but increased with combined addition of AD and WA. However, the bacterial PLFAs were higher than the controls' with the addition of AD.

Similarly, there were higher total and bacterial PLFAs than the controls' in the soils pre-exposed to higher concentrations of ^{12}C-phenanthrene (2 x 100 mg kg^{-1} soil and 4 x 100 mg kg^{-1} soil) in the presence of AD, WA and mixtures of AD and WA. Furthermore, greater concentrations of bacteria to fungi, as well as gram-positive to gram-negative bacteria, were observed in most of the ^{12}C-phenanthrene-exposed soils in the presence of AD, WA as well as the mixtures of AD and WA.

Table 9: The concentrations of the phospholipid fatty acids (PLFA) (nmol g^{-1} dry weight soil) in 90 d aged ^{12}C-phenanthrene-exposed soils in the presence and absence of anaerobic digestate (AD) and/or wood-ash (WA).

Amounts of ^{12}C-PHE added to soils (mg kg^{-1})	Amended soils	Total PLFAs	Total fungal	Total bacteria	Fungal:Bacterial	Total gram +ve	Total gram -ve	Gram +ve : Gram -ve
1 x 100	soil + $^{12\ \&\ 14}$C-PHE (Control)	147.8	11.3	67.6	0.1	39.1	27.7	1.5
1 x 100	soil + 17.31 g AD + 0.94 g WA	253.3	11.3	111.9	0.1	58.2	52.4	1.1
	soil + 17.31 g AD	313.4	22.7	147.2	0.2	76.3	68.6	1.2
	soil + 0.94 g WA	268.4	17.0	132.0	0.1	64.2	65.6	1.0
2 x 50	soil + 17.31 g AD + 0.94 g WA	163.9	13.4	75.4	0.2	39.8	35.0	1.2
	soil + 17.31 g AD	66.7	5.1	29.3	0.1	10.9	18.3	0.8
	soil + 0.94 g WA	91.7	7.9	36.2	0.3	15.9	19.4	0.6
	soil + 12C-PAH	270.1	30.1	133.2	0.2	70.1	60.8	1.2
4 x 25	soil + 17.31 g AD + 0.94 g WA	201.0	21.5	99.0	0.2	40.5	57.0	0.7
	soil + 17.31 g AD	144.2	13.7	74.2	0.1	36.0	37.3	0.8
	soil + 0.94 g WA	95.6	10.4	46.6	0.3	19.6	26.7	0.6
	soil + 12C-PAH	245.5	23.8	109.7	0.2	48.0	60.3	0.8
2 x 100	soil + 17.31 g AD + 0.94 g WA	382.0	248.3	75.6	0.1	27.7	47.4	0.4
	soil + 17.31 g AD	284.7	30.1	124.6	0.2	60.0	63.1	1.0
	soil + 0.94 g WA	205.5	20.7	95.9	0.2	44.0	50.6	0.9
	soil + 12C-PAH	367.4	42.6	193.2	0.2	107.7	82.7	1.3
4 x 100	soil + 17.31 g AD + 0.94 g WA	223.2	18.6	121.1	0.2	49.3	70.7	0.7
	soil + 17.31 g AD	319.7	36.8	174.4	0.2	95.1	77.0	1.2
	soil + 0.94 g WA	343.9	39.6	175.5	0.2	92.9	80.4	1.2
	soil + 12C-PAH	261.9	26.3	131.3	0.2	61.0	68.4	0.9

4. Discussion

4.1 Biodegradation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils in the absence of renewable bioenergy residues

Generally, soil characteristics and microbial populations are known to be important for the promotion of indigenous mineralisation of PAHs (Bento *et al.*, 2005). Microorganisms possess diverse capacities for attacking hydrocarbons and are involved in nutrients cycling, organic matter turnover, as well as stabilization of soil structure (Maletic *et al.*, 2013; Lang *et al.*, 2016; Siles and Margesin, 2018). Also, past studies revealed that soils with high levels of organic matter are known to have a strong affinity for hydrophobic organic compounds, (such as PAHs) (Yang *et al.*, 2010; Okere and Semple 2012), and a larger capacity to sequester organic compounds within their particles (Semple *et al.*, 2003; Stokes *et al.*, 2006; Marini and Frapiccini, 2013). These properties and factors consequently contribute to the persistence of organic compounds in soils.

However, under suitable environmental conditions, microorganisms are known with the ability to mineralize organic contaminants to simple inorganic compounds, such as CO_2 (Semple *et al.,* 2003; Griffiths *et al.*, 2012). Studies further show that prior exposure of soil to organic contaminants or their analogues can positively influence indigenous microbial metabolic adaptation and mineralisation of subsequent organic contaminants, especially since the occurrence of a single contaminant is not common (Reid *et al.*, 2002; Macleod and Semple, 2002; Macleod and Semple 2006; Couling *et al.*, 2010).

In this present study, the influence of soil pre-exposure to ^{12}C-phenanthrene in the absence and presence of AD and/or WA on catabolic evolution of ^{14}C-phenanthrene was

investigated. The results showed higher percentage of catabolically evolved $^{14}CO_2$ from the mineralisation of the freshly added ^{14}C-phenanthrene across all the kinetics of mineralisation. This mineralisation result suggests an occurrence of enhanced indigenous microbial activity, which have a significant implication on the biodegradation of the ^{14}C-phenanthrene. The soil used for the study is rich in organic matter content, which suggests a deeper level of soil-contaminant interaction, in terms of affinity and sequestration of the ^{14}C-phenanthrene within the soil matrix during ageing. Therefore, this mineralisation results showed the biodegradation of the ^{14}C-phenanthrene was increased. This was observed as the shorter lag phases and greater extents of mineralisation in the soils pre-exposed to multiple additions of ^{12}C-phenanthrene (Figs 2 and 3; Tables 4 and 5, controls 2) and higher concentrations of phenanthrene (Figs 4 and 5; Tables 6 and 7, controls 2), amended and unamended with AD and/or WA, compared to the soils with a basic single exposure to ^{12}C-phenanthrene (Table 3, controls 1) in the absence of AD and/or WA. The history and level of the soil exposures to ^{12}C-phenanthrene (an analogue of the ^{14}C-phenanthrene) and the increasing interactions between the soil and the phenanthrene play important roles in the bioavailability and/or bioaccessibility of the ^{14}C-phenanthrene. For instance, from results of, the loss of the ^{14}C-phenenthrene was observed in the soils with multiple additions and additions of higher concentrations of ^{12}C-phenanthrene across all the ageing period (0 d to 90 d) and over all the mineralisation kinetics, compared to the soils with a single exposure to ^{12}C-phenanthrene.

Also, this effect of biostimulation can contribute to the abundance of the indigenous microbial PAH-degraders and/or induction of the relevant microbial PAH-catabolic enzymes, which can significantly contribute to the desorption rates of PAHs from the soil matrixes (Rhodes *et. al.*, 2008). These observations agree with past studies where the persistent exposures of soil to particular organic contaminants or their analogues

enhanced indigenous microbial metabolic adaptation to mineralize the same organic contaminants subsequently added to the soil (Macleod and Semple 2006; Couling *et. al.*, 2010). In previous studies shorter lag phases and higher mineralisation of PAHs have been observed in the soils where the PAH-degraders were previously exposed to the same or analogues of the organic contaminants (Megharaj *et al.*, 2011; Patowary *et al.*, 2016; Truskewycz *et al.*, 2019). For instance, in a study by Macleod and Semple (2002), there was a faster mineralisation of ^{14}C-pyrene in a non-sterile pasture soil previously exposed to ^{12}C-pyrene, compared to a similar soil with no prior exposure to ^{12}C-pyrene, where there was no evidence of mineralisation at any of the soil–pyrene contact time. Similarly, in Macleod and Semple (2006), soils exposed to multiple additions and higher concentrations of ^{12}C-pyrene showed higher levels of mineralisation of freshly added ^{14}C-pyrene. In a study by Couling *et. al.* (2010), both the numbers and concentrations of ^{12}C-pyrene added to soils were reported rapidly and extensively enhanced mineralisation of freshly added ^{14}C-pyrene.

4.2 Biodegradation of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils in the presence of renewable bioenergy residues

Anthropogenic alteration of soil physico-chemical properties (such as pH, moisture content, aeration and availability of nutrients) by organic contaminants can cause adverse environmental conditions that hamper microbial activity, and consequently, biodegradation of PAHs (Watanabe and Baker, 2000; Semple *et al.*, 2001; Rhodes *et al.*, 2008; Haritash and Kaushik, 2009). In this study, the addition of AD and/or WA improved the development of catabolic evolution of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils, compared to the similar unamended soils. The effect of the nutrients addition (as AD and/or WA) was observed with a higher occurrence of biodegradation, which was

predominant with the addition of the mixtures of AD and WA, with reduced ($P < 0.05$) lag phases and increased ($P < 0.05$) mineralisation rates and extents from 0 d to 90 d soil-PAH contact time compared to the similar unamended soils. The microbial adaptation was pronounced as indicated by the shorter lag phases, and the rates and extents of the biodegradation were enhanced as shown by the faster rates and greater extents of the mineralisation in the presence of AD and/or WA. Some of the AD-amended soils showed higher mineralisation rates than WA-amended soils, which may be due to the amount of organic matter and N in AD compared to WA, where N is known to be absent or present in a negligible amount (Perucci *et al.*, 2006; Whelan *et al.*, 2010; Fernández-dayelgado Juárez *et al.*, 2013; García-Sánchez *et al.*, 2015a; García-Sánchez *et al.*, 2015c).

From the results of the present study, it is noticed that the addition of mixtures of AD and WA positively influenced the biodegradation of the ^{14}C-phenanthrene more than their separate additions. For example, the cumulative extents of mineralisation of the ^{14}C-phenanthrene were greater in the presence of AD and/or WA with increasing soil-phenanthrene contact time or interactions compared to the similar soils unamended with AD and/or WA. These findings suggest a further development compared to previous studies where soil pre-exposures to organic contaminants were not accompanied with the addition of organic nutrients or nutrients sources. For example, in a study by Macleod and Semple (2006), soils exposure to multiple additions of ^{12}C-pyrene reduced the lag phases after 8 and 12 weeks; however, in this present study, our results demonstrated enhanced mineralisation from the incubation onset (0 d) and with increasing soil-PAH contact time (0 d – 90 d). The complementary N, P and K elements present in the AD and WA (Bougnom *et al.*, 2010; Bougnom *et al.*, 2012) could have contributed to the effectiveness of their combined use as amendments in contaminated soils. The occurrence of biostimulation in soil following the addition of organic amendments is well documented in

previous studies (Haritash and Kaushik, 2009; Bougnom *et al.*, 2012; Koszel and Lorencowicz, 2015).

Also, the effect of addition(s) of AD and/or WA on the amounts of soil microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) in the ^{12}C-phenanthrene-exposed soils were assessed, as concentrations of total organic C and N (mg kg^{-1} soil) (Joergensen *et al.*, 2011) (Table 8). The MBC and MBN of most of the soils with multiple additions of ^{12}C-phenanthrene and the soils with higher applications of ^{12}C-phenanthrene increased in the presence of the AD and WA, compared to the similar unamended soils, and the soils with a single exposure to ^{12}C-phenanthrene only (Table 8). The effects observed in this study suggest increased growth and degradation activity of the indigenous PAH-degraders and, consequently, enhanced biodegradation of the ^{14}C-phenanthrene as the soil-phenanthrene interactions increased. The soil microbial biomass is known to consist of the living components of the SOM and is actively involved in biogeochemical processes (Ren *et al.*, 2019). Soil microbial biomass is also considered as a sink/source of nutrients (Griffiths *et al.*, 2012), and have implications on the biodegradation of PAHs. In previous studies, soil microbial biomass has been successfully used to indicate the occurrence of soil contamination (Joergensen and Wichern, 2018), and changes in soil microbial biomass have been traced to nutrients availability as well as microbial growth and death processes (Fujita *et al.*, 2019; Ren *et al.*, 2019).

4.3 The concentrations of phospholipid fatty acids in the ^{12}C-phenanthrene-exposed soils in the presence and absence of renewable bioenergy residues

PLFAs are known to be the components of microbial cell membranes and represent the fingerprints of viable soil microbial communities (Hanif *et al.*, 2010; Quideau *et al.*, 2016). In previous studies, PLFA analysis has been used for quantitative assessment of microbial community compositions and the monitoring of soil responses to environmental changes (Kaiser *et al.*, 2010; Buyer and Sasser, 2012), such as availability of nutrients, application of organic amendments and contamination by hydrocarbons (Buyer *et al.*, 2010; Willers *et al.*, 2015; Quideau *et al*., 2016). Also, the composition and function of soil microbial community structures have been employed for bioremediation and biomonitoring (Hanif *et al.*, 2010). In this present study, there were positive changes on the microbial community structure following the addition(s) of AD and/or WA, particularly, the mixtures of AD and WA, as the amendments resulted in higher total and bacterial PLFAs than the controls. There were greater changes compared to the single ^{12}C-phenanthrene-exposed soils unamended with AD and/or WA (controls) (Table 9). The readily microbial utilizable essential nutrients supplied by the AD and/or WA significantly contributed to the catabolic activity and abundance of the indigenous microorganisms, especially the PAH-degraders and, consequently, influenced the mineralisation of ^{14}C-phenanthrene in amended soils. From the results, the soil pre-exposures to ^{12}C-phenanthrene and subsequent amendments with the AD, WA and mixtures of AD and WA led to an increase in the microbial abundance in the soils. The changes in the microbial community structure in the present study agree with a study by Abubaker *et al.* (2013), where they observed significant differences in the bacterial community structure of different soils amended with biogas residues and cattle slurry, compared to the similar unamended soils after 120 d of incubation.

5. Conclusion

This study showed the influence of soil's pre-exposure to phenanthrene and the effect of subsequent amendment with AD and/or WA on the development of microbial degradation of subsequently added fresh ^{14}C-phenanthrene. The results showed that the complementary approach of soil-exposure to organic compounds or their analogues and subsequent amendment with organic nutrients can be a strategy to enhancing indigenous microbial adaptation and overcoming the limitations of a shortage of nutrients supply induced by the presence of organic contaminants in soil. The positive changes in microbial community composition following the addition of AD and/or WA suggested the enhanced biodegradation of the phenanthrene contaminant observed. Also, these findings have positive implications on indigenous bioremediation of PAH-contaminated soils with limited nutrients as a result of the contaminant exposure; the results from this investigation provided insights into a remediation strategy that can significantly enhance biodegradation of PAHs within a short period.

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6.4 Paper 4: The influence of contact time on biodegradation and extraction of ^{14}C-phenanthrene in anaerobic digestate- and wood-ash-amended soils.

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Abstract

The environmental persistence of polycyclic aromatic hydrocarbons (PAHs), and their harmful exposure to humans and other biota, has aroused significant interests in the bioaccessibility and biodegradation of PAHs in soils. This study investigated the influence of contact time on indigenous and inoculum-induced biodegradation and chemical extraction of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with nutrient-rich anaerobic digestate (AD) (a biogas residue) and/or wood-ash (WA) (a biomass combustion residue). In the presence of AD and/or WA (compared to the similar unamended soils): significantly low ^{14}C-phenanthrene activity (dpm) was recovered in most of the soil microcosms, particularly with the mixtures of AD and WA before and after mineralisation, which also declined with increasing soil-PAH contact time; the DCM- and HP-β-CD-extractable ^{14}C-phenanthrene were low and the amounts declined with increasing soil-PAH contact time, while there was a nearly 1:1 relationship between the HP-β-CD-extractable and mineralised ^{14}C-phenanthrene fractions; greater levels of $^{14}CO_2$ (%) was produced from the mineralisation of the ^{14}C-phenanthrene in the inoculated and uninoculated mineralisation (with little difference between them) and the results were predominant with the mixtures of AD and WA while the $^{14}CO_2$ (%) also reduced with increasing soil-PAH contact time; the heterotrophic bacteria were higher at the incubation onset, while the inoculant's number increased at the end of mineralisation; and the levels of mineralisation (%) in some of the uninoculated microcosms suggest the mineralisation was independent of the bacterial density.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic contaminants (HOCs), which can be introduced into the environment through natural activities like thermal geologic production and anthropogenic sources that involve industrial activities (Okere and Semple, 2012; Oyelami *et al.*, 2013; Lang *et al.*, 2016). Many PAHs, especially those with high molecular weight, sorb on to the organic matters and/or clay fractions in the soil, and result in desorption resistant fractions that persist in the natural environments over time because of their hydrophobicity (Reid *et. al.*, 2000; Papadopoulos *et. al.*, 2007; Marini and Frapiccin, 2013). These enhance the resistance of the PAHs to biological, chemical and physical loss processes (Couling, *et. al.*, 2010). The occurrence and accumulation of PAHs in the soil mean that human and other organisms may be exposed to their putative toxic and genotoxic effects (Das and Chandran, 2011). Therefore, the awareness of their environmental persistence and exposure risks arouses significant interests in studies relating to their bioaccessibility and biodegradation in the soil.

The intrinsic catabolic potential of soil microflora has been known as a route of removal of PAHs in the environment (Leys *et. al.*, 2005; Tyagi, *et. al.*, 2011). The catabolism PAHs of has been preferred to physical and chemical methods due to its environmental sustainability and low cost (Alvarez *et. al.*, 2011; Maletić *et. al.*, 2013; Naseri *et. al.*, 2014; Tiwary *et. al.*, 2015; Ning *et. al.*, 2017). However, the natural microbial degradation process often takes longer than the physical and chemical methods (Yu *et al.*, 2005; Lang *et al.*, 2016). Also, the increased interaction of the PAHs with the soil particles over time is known to reduce the bioavailability of the PAHs (a process called ‘ageing') (Doick and Semple, 2003; Doick *et. al.*, 2003; Semple *et. al.*, 2003; Doick *et. al.*, 2005a). In past studies, strategies involving the addition of nutrients and pre-adapted catabolic inocula

with required degradation capability have been explored to enhance microbial degradation of PAHs in the soil (Tyagi *et. al.*, 2011). However, no study has reported the impact of the addition of organic amendment on both indigenous and inoculum-induced biodegradation of PAHs.

Two nutrient-rich bioenergy residues, AD and WA, were added to the soil, in the present study, to enhance the microbial growth and degradation of PAHs. Both AD and WA (fly-ash) are known to contain considerable amounts of major elements (which include calcium (Ca), potassium (K) and sodium (Na)) (García-Sánchez *et. al.*, 2015a). Also, AD is known to have a large amount of organic matter and N contents (Insam *et. al.*, 2015; Tiwary *et. al.*, 2015), while N is mostly or completely absent in WA (Perucci *et. al.*, 2006). Studies documented the positive effects of soil amendment with AD and WA on soil properties (Sharma and Kalra, 2006; Insam *et al.*, 2009; García-Sánchez *et. al.*, 2015a). Also, because of the complementary nutrient elements of the AD and WA (Bougnom *et al.*, 2012), their mixture can be a valuable soil amendment for enhancing biodegradation of PAHs, which is not currently documented in studies.

In this study, the influence of contact time on indigenous and inoculum-induced biodegradation, as well as the chemical extraction of ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed AD- and/or WA-amended soils was investigated. The PAH degrading inoculum employed was *Pseudomonas* species. The mineralisation of the ^{14}C-phenanthrene was assessed by (1) determining the ^{14}C-phenanthrene residues in the soils, before and after mineralisation, by combustion in a sample oxidizer; (2) determining the chemical-extractable ^{14}C-phenanthrene residues in the soils, using dichloromethane (DCM) (for total ^{14}C-phenanthrene residue) and hydroxypropyl-β-cyclodextrin (HP-β-CD) (for comparing the chemical extractability and microbial availability of the ^{14}C-

phenanthrene); (3) catabolic evolution of the ^{14}C-phenanthrene (using a ^{14}C-respirometry system) (Stroud *et. al.*, 2009); and (4) estimating the microbial population size of the soil samples before and after mineralisation, as colony-forming units per gram (CFU g^{-1}) soil, by using the standard aseptic agar-plate technique.

2. Materials and Methods

2.1 Materials

The chemicals and other materials used for this study have been previously listed in Paper 1, Section 2.1 above. Additional materials used in this study include nutrient agar (supplied by Sigma Life Science), ringer's pellets and plate count agar (PCA) powder (supplied by Oxoid). The information about the AD and WA used for this study have been discussed in Paper 1, Section 2.1 above.

2.2 Soil sampling and characteristics

The soil used in this study was collected (5 - 20 cm depth) in Myerscough, UK. The information about the soil and its physico-chemical properties (Table 1) have previously been discussed in Paper 1, Section 2.2 above.

Table 1: Physico-chemical characteristics of Myerscough soil, anaerobic digestate (AD) and wood-ash (WA); measurements were in dry weight (d/w), except those indicated wet weight (w/w); n = 3 ± standard error of the mean (SEM); parameter marked with (*) (Couling, et. al., 2010); <BDL = below the detection 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 (EC) (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 (C)	19.0 ± 1.0	349.0 ± 1.0	13.0 ± 0.1
	Total nitrogen (N)	2.8 ± 0.1	43.5 ± 0.5	0.7 ± 0.1
	Total phosphorus (P)	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		

2.3 Soil spiking and amendments

Soil (2.396 kg w/w, 30 % SMC) was spiked with non-labelled (^{12}C-) phenanthrene (100 mg kg^{-1}_{soil}), using acetone as the carrier solvent. The mixtures were carefully homogenized and left in the fume cupboard for 2 hours (h) to allow the acetone to volatilize (Macleod and Semple, 2000). Afterward, the soil was amended with AD and WA as follow: (1) soil + ^{12}C-phenanthrene + AD, (2) soil + ^{12}C-phenanthrene + WA, (3) soil + ^{12}C-phenanthrene + AD + WA. The control soils were similarly exposed to ^{12}C-phenanthrene without the addition of AD and/or WA.

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 AD- and WA-amended and unamended soils remained in the fume cupboard for 24 hours, before spiking with ^{14}C-phenanthrene (35 kBq kg^{-1}_{soil}) alongside its non-radiolabelled analogue (10 mg kg^{-1}_{soil}), using acetone as the delivery solvent. The spiked soils were left in the fume cupboard for 24 h, to allow the acetone to volatilize before ageing (20 ± 2 ^{o}C) *(n = 3)* in separate labelled and pre-cleaned amber glass jars with loose Teflon–lined screw-caps to (allow ambient oxygen exchange) for 90 days (d).

2.4 The recovery and mineralisation of the ^{14}C-phenanthrene in the soils

2.4.1 Recovery of total ^{14}C-phenanthrene originally applied to soil.

The total activity applied as ^{14}C-phenanthrene to AD- and WA-amended and unamended ^{12}C-phenanthrene-exposed soils was determined by combustion in a sample oxidizer (Packard 307), at increasing soil-PAH contact time 0 d, 15 d, 30 d, 60 d and 90 d. This is to evaluate the concentration of the ^{14}C-activity in the soil samples at each of the above-defined time points after before and after the microbial mineralisation of the ^{14}C-phenanthrene. Each of the soil samples was weighed (ca. 1 g) into separate cellulose combustion cones and combusted for 5 min (using Packard A3070L Sample Oxidizer) with the aid of Combustaid® (200 µl). The evolved $^{14}CO_2$ was trapped with Carbosorb-E® (10 ml) and Permafluor-E® (10 ml) was used as a scintillation cocktail. The trapping efficiency of the sample oxidizer (>96 %) was determined before burning the soil samples. The trapped ^{14}C-activity was quantified with a liquid scintillation analyzer (Canberra Packard Tri-Carb 2250CA) by using standard calibration and quench correction techniques and protocols (Reid *et. al.*, 2004).

2.4.2 Extractability of ^{14}C-phenanthrene from the soils

The first chemical extraction of ^{14}C-phenanthrene from the soil samples was carried out with dichloromethane (DCM). This is an exhaustive method, and it was used to determine the total extractable ^{14}C-phenanthrene concentrations in the AD- and WA-amended and unamended soils (Semple *et. al.*, 2003; Allan *et. al.*, 2007) at increasing soil-PAH contact time 0, 15, 30, 60 and 90 d. Each amended soil (2.5 g w/w) (n = 3) was placed in separate Teflon centrifuge tubes (50-ml capacity), and DCM (30 ml) and Na_2SO_4 granules were added. The tubes were tightly closed with screw-caps and agitated end-to-end (150 rpm) on an orbital shaker (SANYO Gallenkamp) for 22 h at 21 °C. The resultant extracts were centrifuged and the soil pellets were re-washed with fresh DCM solution (30 ml) (as described above). Each supernatant (3 ml) was decanted into separate labelled acetone-cleaned economy vials (20-ml capacity) and 17 ml of Ultima Gold scintillation fluid was added to each. The mixtures were subsequently analysed by liquid scintillation counting (above-described). The resultant pellets (after extraction) 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 (as previously described) to recover the solvent-nonextractable fractions.

The second chemical extraction of ^{14}C-phenanthrene from the soil samples was carried out with hydroxypropyl-β-cyclodextrin (HP-β-CD). This is a non-exhaustive and mild method (Reid *et. al.*, 2000; Doick *et. al.*, 2003), and it was used to estimate the extent of mineralisation (bioaccessibility/bioavailability) of the ^{14}C-phenanthrene in the soils at increasing soil-PAH contact time 0 d, 15 d, 30 d, 60 d and 90 d (Reid *et. al.*, 2000; Doick *et. al.*, 2003). Each amended soil (1.25 g w/w) (n = 3) was placed in separate Teflon centrifuge tubes (50 ml capacity) and 50 mM of HP-β-CD solution (25 ml) was added to

each. The tubes were properly closed with screw-caps and agitated end-to-end (150 rpm) on an orbital shaker (SANYO Gallenkamp) for 22 h at 21 °C (Semple *et al.*, 2006). The mixtures were centrifuged for 30 min at 15 ^{o}C (using Beckman JA 21/2 Centrifuge) and the supernatants were separately decanted in similar pre-cleaned and labelled Teflon centrifuge tubes. The soil pellets were re-suspended in fresh HP-β-CD solution (25 ml) and the above process was repeated. The supernatants (6 ml each) were sampled into separate acetone-rinsed and labelled economy vials (20 ml capacity), and 14 ml of Ultima Gold scintillation fluid was added to each. The mixtures were quantified by liquid scintillation counting (previously described) and the solvent-nonextractable fractions in the soil pellets were recovered by sample oxidation as previously described.

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

The mineralisation of ^{14}C-phenanthrene to $^{14}CO_2$ was performed in modified 250 ml Schott bottles, with Teflon-lined screw-caps incorporated with metal clips (Couling, et. al., 2010) at increasing soil-PAH contact time 0, 15, 30, 60 and 90 d, using a ^{14}C-respirometric system. Soil from each of the soil treatments (13.7 g w/w) was weighed into separate respirometer, and a soil-to-liquid ratio of 1:3 slurry was used to ensure a complete $^{12/14}$C-PAH distribution (Doick and Semple 2003). Each inoculated soil contains 25 ml of autoclaved mineral basal salts (MBS) medium and 5 ml of a bacterial inoculum of ^{14}C-phenanthrene-degrading *Pseudomonas* sp. (~10^7 bacteria kg^{-1}_{soil}) in MBS medium. Each uninoculated soil contains 30 ml of MBS medium. Scintillation vial (7 ml-size) containing 1 M sodium hydroxide (NaOH) (2 ml) was suspended in each respirometer (from the cap metal clip) to trap any catabolically evolved $^{14}CO_2$ during the mineralisation of the ^{14}C-phenanthrene. The MBS medium contains 0.3 g l^{-1} NaCl, 0.6 g l^{-1} $(NH_4)_2SO_4$, 0.6 g l^{-1} KNO_3, 0.25 g l^{-1} KH_2PO_4, 0.75 g l^{-1} K_2HPO_4, 0.15 g l^{-1} $MgSO_4.7H_2O$, and the

following micronutrients: $LiCl(LiBO_2)$ (20 mg l^{-1}), $CaSO_4.5H_2O$ (80 mg l^{-1}), $ZnSO_4.7H_2O$ (100 mg l^{-1}), $Al(SO_4)_3.16H_2O$ (100 mg l^{-1}), $NiCl.6H_2O(CoNO_3)$ (100 mg l^{-1}), $CoSO_4.7H_2O(CoNO_3)$ (100 mg l^{-1}), KBr (30 mg l^{-1}), KI (30 mg l^{-1}), $MnCl_2.2H_2O$ (600 mg l^{-1}), $SnCl_2.2H_2O$ (40 mg l^{-1}) and $FeSO_4.7H_2O$ (300 mg l^{-1}).

The respirometers were incubated (20 ± 2 ^oC) for 14 days on a flat-bed orbital shaker (SANYO Gallenkamp) (100 rpm), to ensure adequate mixing of the slurries. The NaOH-CO_2 traps were removed at 2, 4, 8, 12, 24 h, and henceforth every 24 h for 14 d (336 h) and replaced instantly with the fresh ones, while the collected NaOH-CO_2 traps were instantly mixed with 5 ml of Ultima Gold scintillation fluid. The vials were wiped-cleaned 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 (Canberra Packard Tri-Carb 2300TR) (Reid *et. al.*, 2004).

2.6 Bacterial source, seeding and enumeration

The catabolically active phenanthrene-degrading inoculum used In this study was *Pseudomonas* species (sp.), and the inoculum density was ~10^7 CFUs kg^{-1} soil (Doick *et. al.* 2005a). The inoculant was obtained from a pure strain previously cultured in mineral basal salts (MBS) medium, and subsequently re-cultured in fresh MBS medium with ^{12}C-phenanthrene as the carbon (C) source, on an orbital (100 rpm) shaker. The inoculant was harvested after 4 d (late exponential phase) of incubation by centrifugation (4000 rpm) at 15 ^oC for 30 min. The cell pellets were recovered and re-washed in fresh MBS medium (as described above) to ensure the total removal of any residual phenanthrene (Reid *et. al.*, 2004). Before and after each mineralisation assay, the numbers of culturable

heterotrophic and PAH-degrading bacteria in the AD- and WA-amended and unamended soils were quantified (CFU g^{-1}), using the standard aseptic agar-plate technique.

Soils and slurries (1 ± 0.2 g w/w, each) were extracted before and after each mineralisation with one-quarter strength Ringer's solution (1:10). An aliquot of each soil suspension (1 ml) was ten-fold serially diluted with sterile distilled water. 0.1 ml of each resultant solution was inoculated on separate plate count agar (PCA) (for heterotrophic bacteria), and agar plates impregnated with phenanthrene (0.2 %) (for PAH degrading bacteria) (Oyelami *et. al.*, 2013; Ite *et. al.*, 2015). Plates were incubated (25 ± 1 ^{o}C) and distinct colonies were counted after 48 h for heterotrophic bacteria, and 10 d for the phenanthrene-degrading *Pseudomonads*.

2.7 Statistical Analysis

Following blank-correction, the data were plotted with SigmaPlot 10.0. The differences in the mineralisation of the ^{14}C-phenanthrene in the AD- and WA-amended and unamended soils were evaluated through one-way analysis of variance (ANOVA), at 95 % confidence level ($P < 0.05$), to determine the least significant difference. The comparison of the means within and across the ^{12}C-phenanthrene-exposed soils amended with AD, WA and mixtures of AD and WA were analysed using Turkey and LSD's Post-hoc tests (SPSS). Pearson correlation coefficient (r) was performed to describe the relationship between the microbial numbers and the rates and extents of the ^{14}C-phenanthrene mineralisation; while the value of r is ranked on a scale between +1 and −1.

3. Results

3.1 Recovery of ^{14}C-phenanthrene activity by combustion of soil

The ^{14}C-activity (%) in the ^{12}C-phenanthrene-exposed soils amended and unamended with AD and/or WA were recovered by combustion of the soil samples (sample oxidation) at increasing soil-PAH contact times of 0, 15, 30, 60 and 90 d (Figure 1). The ^{14}C-activity recovered before mineralisation was higher in the absence of AD and/or WA (controls) after 0 d and 15 d, which gradually declined from 30 d to 60 d in the presence of AD and/or WA. However, the recovered ^{14}C-activity after 90 d was higher in the presence of AD and/or WA than the controls. The ^{14}C-activity recovered in inoculated mineralisation containing mixtures of AD and WA were lower ($P < 0.05$) than the controls' after 0 d and 30 d with WA addition. Lower ^{14}C-activity compared to the controls were also recovered in the presence of WA (0 d, 15 d and 90 d), AD (15 d) as well as the mixtures of AD and WA (30 d) but they were not statistically significant. In uninoculated mineralisation, recovered ^{14}C-activity was lower ($P < 0.05$) than the controls in the soils with additions of AD (0 d) as well as WA and the mixtures of AD and WA (60 d). The lower recoveries observed in the soils with the additions of mixtures of AD and WA (0 and 30 d) as well as WA (90 d) were not statistically different ($P > 0.05$) from the controls.

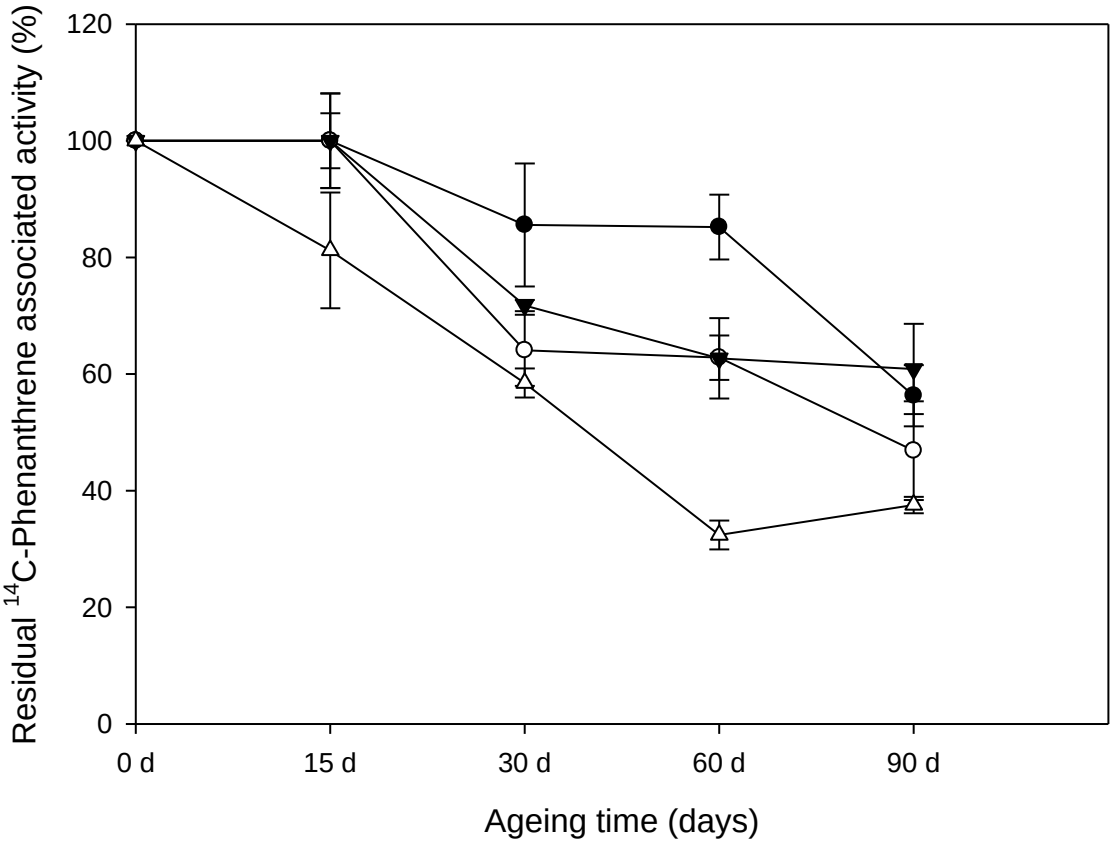

Figure 1: The ^{14}C-phenanthrene's loss curves in ^{12}C-phenanthrene-exposed soils amended with anaerobic digestate (AD) (○), wood-ash (WA) (▼) and mixtures of anaerobic digestate and wood-ash (Δ), as well as unamended soils (controls) (●) over 90 days; values (n = 3) represent the mean ± standard error of the mean (SEM).

3.2 Chemical extraction of ^{14}C-phenanthrene

The DCM- and HP-β-CD-extractable ^{14}C-phenanthrene fractions (%) in the uninoculated ^{12}C-phenanthrene-exposed soils amended and unamended with AD and/or WA were determined (Table 2). The total amounts of the ^{14}C-phenanthrene were extracted with DCM, while the non-extractable fractions were recovered from the soil pellets by combustion in a sample oxidizer (Doick *et. al.*, 2003). Most of the DCM-extractable ^{14}C-phenanthrene fractions were higher from 0 d to 90 d in the ^{12}C-phenanthrene-exposed soils unamended with AD and/or WA (controls) compared to the similar soils with AD and/or WA amendments. Also, the DCM-extractable ^{14}C-phenanthrene fractions were lower ($P < 0.05$) than the controls with the additions of WA after 30 and 60 d as well as AD and the mixtures of AD and WA after 60 d. However, DCM-extractable fractions reduced after 90 d but they were not statistically different ($P > 0.05$) from the controls.

Similarly, the amounts of HP-β-CD-extractable ^{14}C-phenanthrene fractions were lower ($P < 0.05$) than the controls with the additions of mixtures of AD and WA from 0 d to 90, AD after 0 d and 60 d, as well as WA after 0 d, 30 d and 60 d. Generally, it was noted that the HP-β-CD-extractable ^{14}C-phenanthrene fractions were lower ($P < 0.05$) than the controls as the soil-phenanthrene contact time increased.

Table 2: Dichloromethane (DCM)- and Hydroxypropyl-β-cyclodextrin (HP-β-CD)-extractable ^{14}C-phenanthrene residues in ^{12}C-phenanthrene-exposed soils amended and unamended with anaerobic digestate (AD) and/or wood-ash (WA); values in columns followed by different letters are statistically different (Turkey, LSD; $P < 0.05$); values (n = 3) represent the mean ± SEM.

Soil-PHE contact times (d)	Samples	DCM-extractable [$^{9-14}$C]-PHE activity (%)	HP-β-CD-extractable [$^{9-14}$C]-PHE activity (%)
1 d	Controls (soil + ^{12}C- & ^{14}C-PHE)	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 a
15 d	Controls	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 d	Controls	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 a
	Soil + AD + WA	31.7±4.7 a	11.2 ± 16.1 b
60 d	Controls	37.4 ± 3.1 a	8.4 ± 4.5 a
	Soil + AD	22.1 ± 4.8 b	5.4 ± 2.7 a
	Soil + WA	23.8 ± 4.3 b	1.3 ± 1.3 a
	Soil + AD + WA	25.6 ± 4.3 a	4.6 ± 1.1 a
90 d	Controls	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 a

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

The indigenous and inoculum-induced mineralisation of the ^{14}C-phenanthrene in the ^{12}C-phenanthrene-exposed soils amended and unamended with AD and/or WA were studied at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d (Figure 2 and Table 3). The adapted inoculum used in this study was *Pseudomonas* sp. Lag phases were shorter ($P < 0.05$) than the controls after 0 d with the additions of WA and mixtures of AD and WA. Shorter lag phases were also observed with additions of AD after 0 d and 30 d, as well as the mixtures of AD and WA after 60 d and 90 d but they were not statistically different ($P > 0.05$) from the controls.

The rates of mineralisation were higher ($P < 0.05$) than the controls after 0 d with the additions of WA as well as mixtures of AD and WA. The higher rates of mineralisation observed with the additions of AD after 0 d, as well as mixtures of AD and WA after 30 d were not statistically different ($P > 0.05$) from the controls. The extents of mineralisation were greater ($P < 0.05$) than the controls after 60 d with the addition of the mixtures of AD and WA.

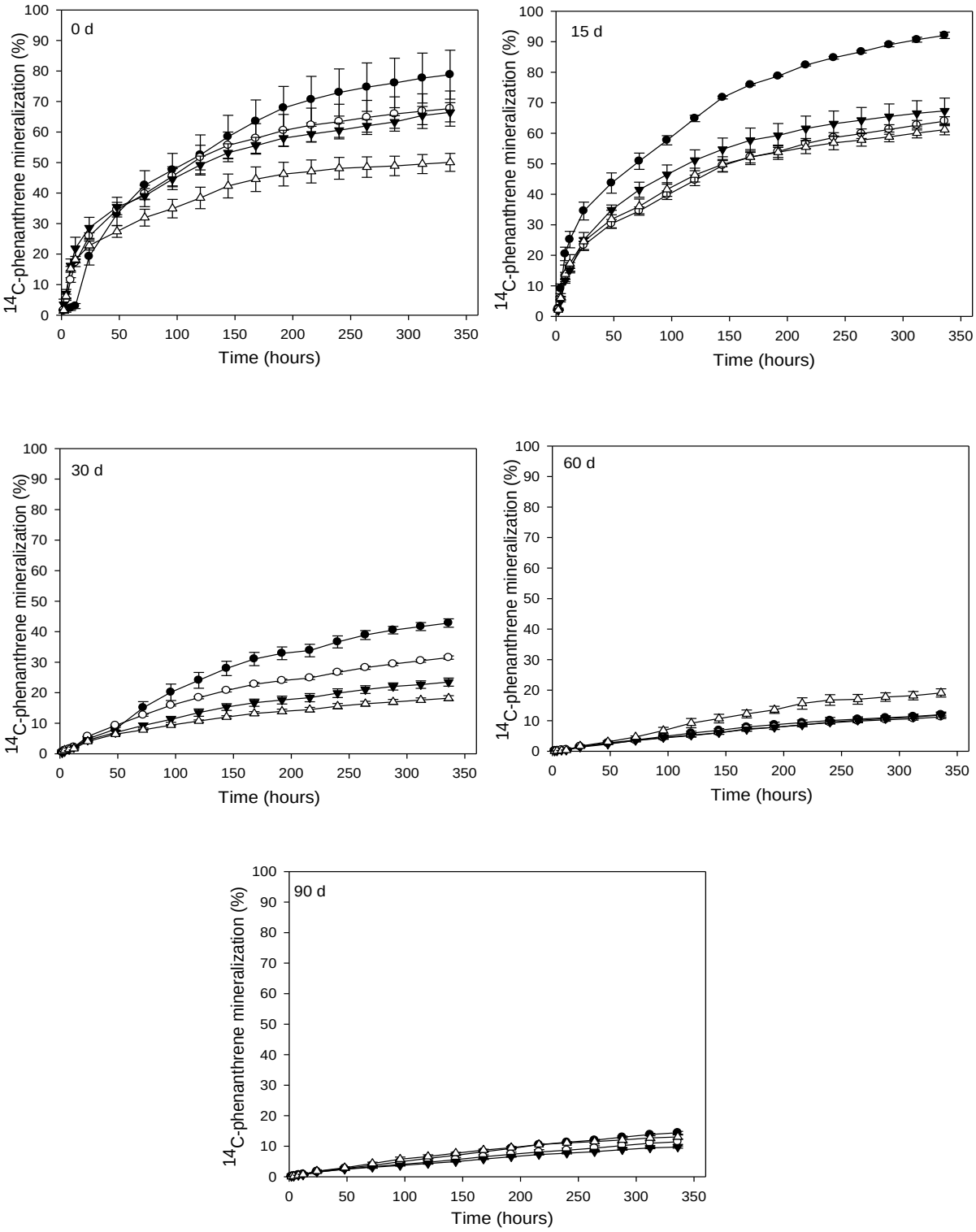

Figure 2: The mineralisation of ^{14}C-phenanthrene (%) in inoculated ^{12}C-phenanthrene-exposed soils amended with anaerobic digestate (AD) (○), wood-ash (WA) (▼) and mixtures of anaerobic digestate and wood-ash (AD-WA) (Δ), as well as unamended soils (controls) (●), at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values (n = 3) represent the mean ± standard error of the mean (SEM).

Table 3: The parameters of mineralisation of ^{14}C-phenanthrene in inoculated ^{12}C-phenanthrene-exposed soils amended with anaerobic digestate (AD) and/or wood-ash (WA), and unamended soils (controls) at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; 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 mineralisation (*) than the controls that are not statistically significant (P > 0.05); values (n = 3) represent the mean ± SEM.

Soil-PAHs contact times (d)	Soil amendments	Lag phase (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
0 d	Control (soil + ^{12}C- & ^{14}C-PAH)	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 d	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 d	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 d	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 d	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 aged ^{14}C-phenanthrene in uninoculated soils

The mineralisation of the ^{14}C-phenanthrene in uninoculated ^{12}C-phenanthrene-exposed soils amended and unamended with AD and/or WA were studied at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d (Figure 3 and Table 4). Lag phases were shorter ($P < 0.05$) than the controls with the additions of AD after 0 d and WA after 15 d. Also, shorter lag phases were observed with the additions of WA after 0 d, AD after 15 d and 30 d, as well as mixtures of AD and WA after 60 d but they were not statistically significant compared to the controls. The rates of mineralisation were higher ($P < 0.05$) than the controls after 15 d with the addition of WA. The higher rates of mineralisation observed with the additions of mixtures of AD and WA after 15 d, as well as AD after 30 d were not statistically significant compared to the controls. The extents of mineralisation were greater ($P < 0.05$) than the controls after 60 d with the addition of mixtures of AD and WA.

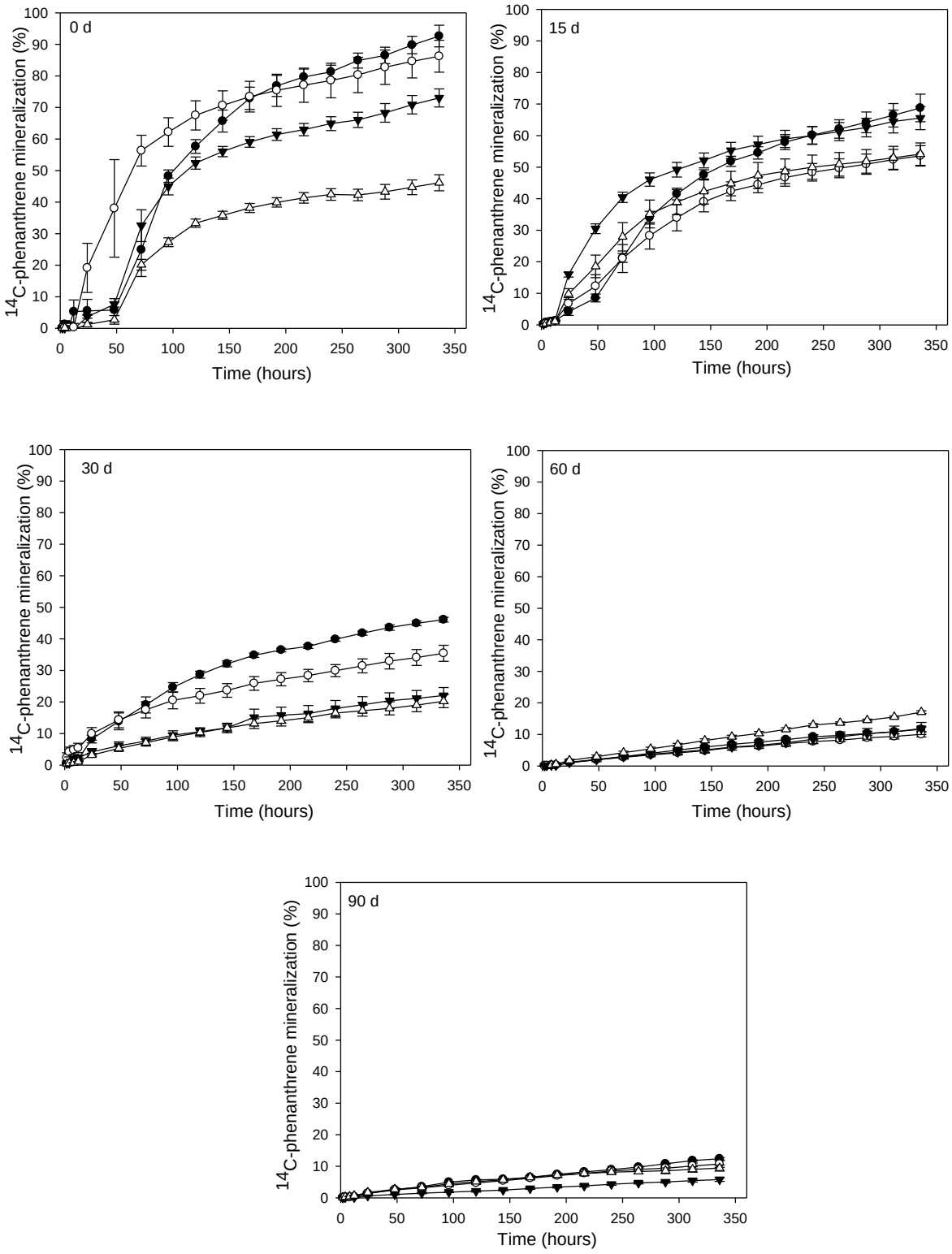

Figure 3: The mineralisation of ^{14}C-phenanthrene (%) in uninoculated ^{12}C-phenanthrene-exposed soils amended with anaerobic digestate (AD) (○), wood-ash (WA) (▼) and mixtures of anaerobic digestate and wood-ash (AD-WA) (Δ), as well as unamended soils (controls) (●), at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; values (n = 3) represent the mean ± SEM.

Table 4: The parameters of mineralisation of ^{14}C-phenanthrene in uninoculated ^{12}C-phenanthrene-exposed soils amended with anaerobic digestate (AD) and/or wood-ash (WA), and unamended soils (controls) at increasing soil-PAH contact times 0, 15, 30, 60 and 90 d; 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 mineralisation (*) 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 d	Control (soil + ^{12}C- &^{14}C-PAH)	37.4 ± 13.9 a	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 ± 3.1 a**+**	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 d	Control	29.3 ± 5.2 a	0.6 ± 0.1 a	68.8 ± 4.4 a
	Soil + AD	24.3 ± 5.5 a**+**	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 ± 1.3 a**+**	0.8 ± 0.2 a*****	54.1 ± 3.6 a
30 d	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 ± 10.9 a	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 d	Control	120.5 ± 10.9 a	0.1 ± 0.0 a	11.6 ± 0.7 a
	Soil + AD	144.6 ± 9.1 a	0.1 ± 0.0 a	10.0 ± 0.8 a
	Soil + WA	145.5 ± 32.8 a	0.1 ± 0.0 a	11.9 ± 1.9 a
	Soil + AD + WA	87.7 ± 8.9 a**+**	0.1 ± 0.0 a	17.1 ± 0.4 b
90 d	Control	85.8 ± 1.6 a	0.1 ± 0.0 a	12.4 ± 0.4 a
	Soil + AD	107.2 ± 10.6 a	0.1 ± 0.0 a	10.7 ± 1.1 a
	Soil + WA	226.6 ± 15.0 b	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 hydroxypropyl-β-cyclodextrin–extractable and mineralised ^{14}C-phenanthrene fractions in the soils

The mineralized and HP-β-CD-extractable ^{14}C-phenanthrene fractions (%) of the ^{12}C-phenanthrene-exposed soils amended and unamended with AD and/or WA were compared at increasing soil-PAH contact time 0 d, 15 d, 30 d, 60 d and 90 d (Table 5; Figure 4). Regression analyses showed that the HP-β-CD-extractable fractions (%) in both inoculated and uninoculated AD- and/or WA-amended and unamended soils positively correlated to the mineralized fractions (%) at each soil-PAH contact time.

In the inoculated ^{12}C-phenanthrene-exposed soils amended with AD and/or WA, the mineralised and HP-β-CD-extractable ^{14}C-phenanthrene fractions (%) were close in terms of 1:1 proportionate relationship, as well as strongly correlated (r^2 = 0.94) after 0 d and 15 d. Similarly, in most of the uninoculated ^{12}C-phenanthrene-exposed soils amended with AD and/or WA, the mineralised and HP-β-CD-extractable ^{14}C-phenanthrene fractions (%) have a proportionately 1:1 relationship, and were positively correlated (r^2 = 0.95) (Figure 4) with increasing soil-PAH interactions.

Table 5: The comparison of the hydroxypropyl-β-cyclodextrin (HP-β-CD)-extractable ^{14}C-phenanthrene (%) and biodegradable (%) ^{14}C-phenanthrene in ^{12}C-phenanthrene-exposed soils amended with AD and/or WA; (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 uninocula-ted soils (%) C	Ratio (A:C)
1 d	Control (soil + ^{12}C- &^{14}C-PAH)	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 d	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 d	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 d	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 d	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

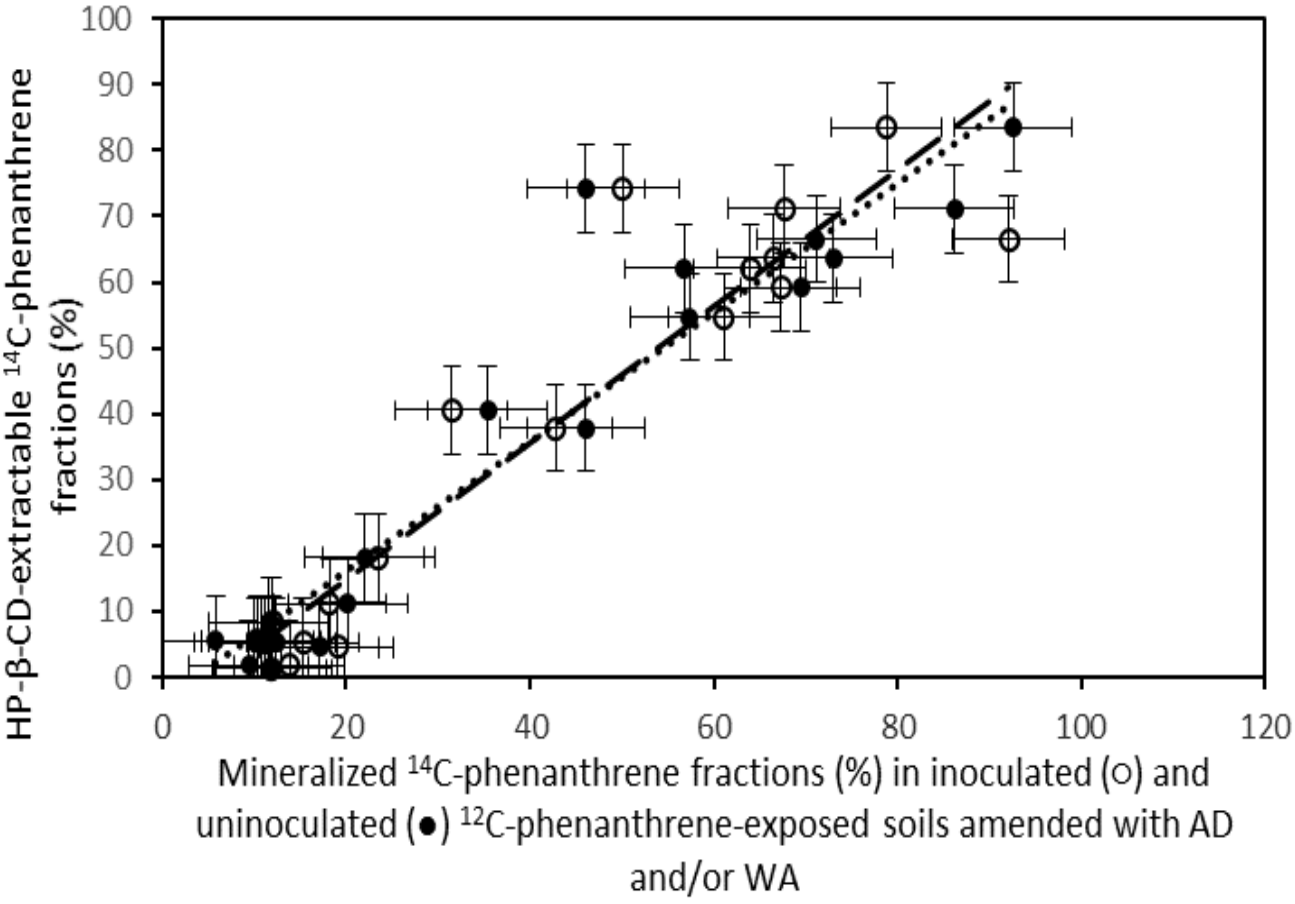

Figure 4: Relationship between hydroxypropyl-β-cyclodextrin (HP-β-CD)-extractable and mineralised fractions (%) in inoculated (o) and uninoculated (●) ^{12}C-phenanthrene-exposed soils amended with anaerobic digestate (AD) and/or wood-ash (WA); in the inoculated soils, R^2 = 0.94 and in the uninoculated soils, R^2 = 0.95.

3.6 Bacteria enumeration before and after mineralisation of ^{14}C-phenanthrene

3.6.1. The of heterotrophic and PAH-degrading Pseudomonas species in the inoculated soils

The numbers of bacteria (CFU g^{-1}) in the uninoculated ^{12}C-phenanthrene-exposed soils, amended with AD and/or WA, were evaluated before and after mineralisation at increasing soil-PAH contact time 0, 15, 30, 60 and 90 d (Table 7). Before mineralisation, the numbers of the heterotrophs (CFUs kg^{-1} soil) increased after 0 d in the presence of AD and/or WA than the similar unamended soils (controls). Also, after 15 d, the bacterial numbers in the WA-amended soils as well as the mixtures of AD and WA were higher than the controls.

After mineralisation, the numbers of the heterotrophs reduced after 0 d soil-PAH contact time in AD- and/or WA-amended soils than the controls. Also, the numbers of the heterotrophs in the soils after mineralisation were lower than the bacterial counts before mineralisation. However, the population size of the PAH-degraders (*Pseudomonas* sp.) increased from the initial ~10^7 inoculum size to ~10^8 density in the AD- and/or WA-amended and unamended soils. After 30 d, the PAH-degraders increased more than the controls in AD-amended soils and the soils amended with the mixtures of AD and WA. After 60 d, the PAH degraders in the soils amended with the mixtures of AD and WA and the controls are not different. However, after 90 d, the numbers of PAH degraders increased more than the controls in the presence of the AD and/or WA.

Table 6: The heterotrophic and PAH-degrading bacterial colony-forming units, per gram soil (CFU g^{-1} soil) in inoculated ^{12}C-phenanthrene-exposed soils amended with anaerobic digestate (AD) and/or wood-ash (WA), at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d; values (n = 3) represent the mean ± SEM.

Soil-PAH contact times (d)	Soil amendment	Bacterial counts (CFU g^{-1} soil)								
		Before mineralisation (Heterotrophs)			After mineralisation (a) Heterotrophs			After mineralisation (b) PAH-degrader		
0 d	Control (soil + ^{12}C- &^{14}C-PAH)	3.3E+06	±	2.4E+05	1.3E+08	±	7.2E+07	6.1E+08	±	1.5E+08
	Soil + AD	2.2E+07	±	2.6E+06	1.2E+07	±	8.1E+06	2.4E+08	±	3.1E+07
	Soil + WA	1.5E+07	±	1.1E+07	1.5E+07	±	8.1E+06	8.0E+08	±	2.4E+08
	Soil + AD + WA	2.5E+07	±	1.7E+07	1.2E+07	±	8.1E+06	5.2E+08	±	2.1E+08
15 d	Control	1.2E+07	±	2.6E+06	1.2E+09	±	1.5E+08	2.2E+09	±	1.5E+09
	Soil + AD	2.5E+07	±	1.4E+07	4.3E+09	±	3.9E+09	7.7E+08	±	1.1E+08
	Soil + WA	8.3E+06	±	3.0E+06	6.6E+08	±	9.3E+07	3.1E+09	±	4.5E+08
	Soil + AD + WA	1.9E+07	±	6.8E+06	5.8E+08	±	3.8E+08	4.6E+08	±	1.9E+08
30 d	Control	6.4E+06	±	2.3E+06	1.7E+09	±	6.1E+07	7.7E+08	±	2.3E+08
	Soil + AD	5.2E+06	±	2.7E+06	8.8E+08	±	3.1E+08	1.1E+09	±	1.8E+08
	Soil + WA	6.2E+06	±	1.6E+06	8.0E+08	±	1.0E+08	6.1E+08	±	6.8E+07
	Soil + AD + WA	6.2E+06	±	3.4E+06	8.5E+08	±	3.6E+08	1.1E+09	±	6.3E+08
60 d	Control	1.7E+07	±	5.1E+06	2.7E+09	±	2.4E+09	1.3E+09	±	4.3E+08
	Soil + AD	4.3E+06	±	4.1E+05	2.6E+08	±	5.6E+07	5.9E+08	±	7.8E+07
	Soil + WA	1.3E+07	±	4.9E+06	7.3E+08	±	1.1E+08	8.6E+08	±	2.2E+08
	Soil + AD + WA	1.0E+07	±	4.4E+06	2.2E+08	±	4.0E+07	1.3E+09	±	5.0E+08
90 d	Control	7.7E+07	±	3.4E+07	5.8E+09	±	3.4E+09	2.5E+08	±	3.8E+07
	Soil + AD	2.0E+07	±	3.1E+06	1.3E+09	±	2.0E+08	3.7E+08	±	1.4E+08
	Soil + WA	4.9E+07	±	9.0E+06	6.5E+08	±	1.5E+08	6.5E+08	±	1.6E+08
	Soil + AD + WA	5.9E+07	±	1.1E+07	9.4E+08	±	4.4E+08	8.0E+08	±	2.4E+08

3.6.2. Numbers of heterotrophic bacteria in the uninoculated soils

The numbers of the heterotrophic bacteria (CFU g^{-1}) in the uninoculated ^{12}C-phenanthrene-exposed soils amended and unamended with AD and/or WA were evaluated before and after mineralisation, at increasing soil-PAH contact time 0 d, 15 d, 30 d, 60 d and 90 d (Table 8). Before mineralisation, the bacterial number increased than the controls after 0 d in the presence AD and/or WA, and after 15 d with the additions of AD as well as mixtures of AD and WA. After mineralisation, the bacterial numbers in the AD- and WA-amended soils increased more than the controls after 30 d; while after 90 d, the bacterial counts were predominantly higher in the presence of the AD and/or WA compared to the controls.

Table 7: The indigenous bacterial colony-forming units, per gram soil *(CFU g^{-1} soil)*, present in uninoculated ^{12}C-phenanthrene-exposed soils amended with anaerobic digestate (AD), wood-ash (WA) and a mixture of anaerobic digestate and wood-ash (AD-WA), at increasing soil-PAH contact times of 0 d, 15 d, 30 d, 60 d and 90 d; values (n = 3) represent the mean ± SEM.

Soil-PAH contact times (d)	Soil amendment	Heterotrophic bacteria (CFU g^{-1} soil)					
		Before mineralisation			After mineralisation		
0 d	Control (soil + ^{12}C-&^{14}C-PAH)	3.3E+06	±	2.4E+05	1.3E+08	±	7.2E+07
	Soil + AD	2.2E+07	±	2.6E+06	1.2E+07	±	8.1E+06
	Soil + WA	1.5E+07	±	1.1E+07	1.5E+07	±	8.1E+06
	Soil + AD + WA	2.5E+07	±	1.7E+07	1.2E+07	±	8.1E+06
15 d	Control	1.2E+07	±	2.6E+06	3.8E+09	±	1.9E+09
	Soil + AD	2.5E+07	±	1.4E+07	5.1E+08	±	1.0E+08
	Soil + WA	8.3E+06	±	3.0E+06	4.7E+08	±	2.6E+08
	Soil + AD + WA	1.9E+07	±	6.8E+06	7.6E+08	±	8.0E+07
30 d	Control	6.4E+06	±	2.3E+06	5.4E+08	±	8.4E+07
	Soil + AD	5.2E+06	±	2.7E+06	3.6E+09	±	2.7E+09
	Soil + WA	6.2E+06	±	1.6E+06	2.3E+09	±	1.5E+09
	Soil + AD + WA	6.2E+06	±	3.4E+06	2.0E+08	±	4.9E+07
60 d	Control	1.7E+07	±	5.1E+06	2.7E+09	±	2.4E+09
	Soil + AD	4.3E+06	±	4.1E+05	2.6E+08	±	5.6E+07
	Soil + WA	1.3E+07	±	4.9E+06	7.3E+08	±	1.1E+08
	Soil + AD + WA	1.0E+07	±	4.4E+06	2.2E+08	±	4.0E+07
90 d	Control	7.7E+07	±	3.4E+07	3.7E+09	±	9.2E+08
	Soil + AD	2.0E+07	±	3.1E+06	1.3E+10	±	5.2E+09
	Soil + WA	4.9E+07	±	9.0E+06	8.5E+09	±	6.6E+08
	Soil + AD + WA	5.9E+07	±	1.1E+07	6.7E+09	±	1.3E+08

4. Discussion

4.1 The impact of soil amendment with renewable bioenergy residues on the extractability of ^{14}C-phenanthrene in soil

Soil characteristics and the prevailing environmental factors are known to contribute significantly to microbial availability and degradation of organic contaminants in soils (Rhodes *et. al.*, 2008; Roberto *et. al.*, 2009; Marini and Frapiccin, 2013). Although the hydrophobic nature of PAHs enhances their persistence in soil (Reid *et. al.*, 2000; Stokes *et. al.*, 2006; Couling, *et. al.*, 2010), their labile fractions are rapidly lost within a short period, and then followed with a gradual loss (Abdel-Shafy and Mansour, 2016). This is known to be due to the increasing PAH interactions with the soil fractions over time (Semple *et. al.*, 2003; Okere and Semple 2012; Riding *et al.*, 2013; Umeh *et. al.*, 2017). In studies, biodegradation has been identified as the major loss process among all the natural physico-chemical and biological processes (adsorption, volatilization, photolysis, leaching and biodegradation) involved in the loss of PAHs in soil (Papadopoulos *et. al.*, 2007).

The presence of PAHs in the soil is known to have a significant effect on the nutrient availability, microbial performance (Margesin and Schinner, 2001; Warr *et al.*, 2013) and, consequently, biodegradation of the PAHs. Therefore, the determination of the phenanthrene's ^{14}C-activity in the soils, in this present study, is significant to assess the impact of the soil amendment with AD and/or WA before and after mineralisation. The findings in this present study suggest that the AD and/or WA supplied essential nutrients required by the microorganisms for growth and/or optimal activity, which consequently, contributed to the biodegradation of the ^{14}C-phenanthrene in the soils. For example,

before and after mineralisation, significantly low phenanthrene's ^{14}C-activities were recovered in most of the inoculated and uninoculated ^{12}C-phenanthrene-exposed soils in the presence of AD and/or WA, particularly the mixtures of AD and WA. It is thought that the nutrients supplied by the AD and/or WA were utilized by the microorganisms for growth and degradation of the ^{14}C-phenanthrene. Also, the ^{14}C-activity (dpm) recovered generally declined with increasing soil-PAH contact time, which defines the impact of ageing and the high organic matter content of the soil used in the current study (Table 1). This observation agrees with past studies where the SOM affects the sorption of PAHs to soil particles and/or sequestration of PAHs within the soil, particularly as the soil-PAH contact interactions increased (Semple *et al.*, 2003; Lima *et al.*, 2005; Stokes *et al.*, 2006; Papadopoulos *et. al.*, 2007; Okere and Semple 2012; Riding *et al.*, 2013; Umeh *et. al.*, 2017).

Similarly, the amounts of ^{14}C-phenanthrene recovered by DCM extraction (Table 2) in ^{12}C-phenanthrene-exposed AD- and/or WA-amended soils were lower compared to the similar unamended soils. The DCM extraction is known to be an exhaustive method that gives the total chemically-extractable PAHs in the soil samples (Semple *et. al.*, 2003; Allan *et. al.*, 2007). However, the organic carbon and the N in the AD as well as other essential nutrients contents in both AD and WA, which are readily utilized by the soil microflora, would have positively influenced or enhanced the catabolic evolution of the ^{14}C-phenanthrene, and consequently, affected the DCM-extractable ^{14}C-phenanthrene. For example, from the results, the DCM-extractable ^{14}C-phenanthrene and their non-extractable residues were lower than the controls' in the presence of AD and/or WA from 0 to 90 d of the soil-phenanthrene interactions. Another reason could have been due to the natural losses and diffusion or occlusion of the phenanthrene fractions within the clay or organic matters of the soil, as the soil-phenanthrene interactions increased (Riding *et*

al., 2013; Umeh *et. al.*, 2017). Consequently, the DCM-extractable ^{14}C-phenanthrene at each time point varied. Generally, there was a gradual decrease in the amounts of the DCM-extractable phenanthrene, as the soil-phenanthrene contact time increased (due to the ageing effect). Similarly, the non-extractable ^{14}C-phenanthrene residues, as well as the total amounts of both extractable and non-extractable ^{14}C-phenanthrene fractions gradually reduced as the soil-phenanthrene contact time increased. Studies where the chemical extractability of organic contaminants in the soil was found to decrease with increasing soil-contaminant interactions are also documented (Reid *et. al.*, 2000; Doick *et. al.*, 2003; Papadopoulos *et. al.*, 2007).

In the HP-β-CD extraction, low amount of ^{14}C-phenanthrene was recovered in most of the ^{12}C-phenanthrene-exposed soils in the presence of AD and/or WA (Table 3). This low recovery may also be related to the amount and sequestration of the organic or mineral matters in the soil, as mentioned earlier, and the impact they have made on indigenous microbial activity and/or mineralisation of the ^{14}C-phenanthrene. Also, the HP-β-CD-extractable ^{14}C-phenanthrene generally declined with increasing soil-PAH contact time, which showed the influence of ageing on the HP-β-CD-extractability of the ^{14}C-phenanthrene. For example, the HP-β-CD non-extractable ^{14}C-phenanthrene residues began to increase after 15 d, and continued with increasing soil-PAH contact time both in the absence (controls) and presence of the AD and/or WA. This result agrees with past studies on HP-β-CD-extraction, where the extractability of organic contaminants decreased as the soil-contaminant interactions increased (Papadopoulos *et. al.*, 2007; Doick *et. al.*, 2005; Rhodes *et. al.*, 2008). In studies, HP-β-CD-extraction has been used to mimic indigenous catabolic degradation of PAHs, or to 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).

However, the length of the soil-PAH contact time has been known to impact on the chemical extractability of PAHs (Macleod and Semple, 2000; Northcott and Jones, 2001; Reid *et. al.*, 2004; Papadopoulos *et. al.*, 2007; Doick *et. al.*, 2005a; Rhodes *et. al.*, 2008). The formation of non-extractable residues with the DCM and HP-β-CD was explained could be due to the consequence of the phenanthrene's slow diffusion and sequestration within the micropores or components of the SOM, as the soil-phenanthrene interactions increased (Northcott and Jones, 2001; Doick *et. al.*, 2003; Doick *et. al.*, 2005b; Okere and Semple, 2012; Riding *et al.*, 2013; Umeh *et. al.*, 2017).

The observations in this study also established the influence of contact time and soil properties on the mineralisation of organic contaminants in soils, as revealed by the loss of the ^{14}C-phenanthrene both in the absence and presence of AD and/or WA. Generally, the chemically-extracted phenanthrene reduced in the presence of AD and/or WA compared to the controls. This shows the biostimulatory influence of the growth- or rate-limiting nutrients supplied by the AD and/or WA, the effect of which, consequently, enhanced the mineralisation of the ^{14}C-phenanthrene. Also, the loss of the ^{14}C-phenanthrene in this study supports the consideration of biodegradation as a major loss process for organic contaminants in soils (Papadopoulos *et. al.*, 2007). Similar findings were presented by Doick *et. al.* (2005b) where soil amendment with transformer oil contributed to an increase in the chemical extractability of PCBs over a period of contact time with the soil amended with transformer oil.

4.2 The impact of soil amendment with renewable bioenergy residues on the mineralisation of ^{14}C-phenanthrene in soil

Microbial degradation of organic contaminants is known to be efficient, environmentally sustainable and less expensive (Das and Chandran, 2011). Therefore, bioremediation technology has been considered suitable for a long-term restoration of land contaminated with petroleum hydrocarbons and their derivatives (Alvarez *et. al.*, 2011). Mills *et. al.* (2003) reported successful biodegradation of PAHs in a petroleum-contaminated wetland by natural attenuation. Although, natural bioremediation approach maintains the soil's ecological characteristics, the required number of the relevant PAH-degrading microflora can be less abundant in a PAH-contaminated soil. Also, the increase in soil carbon (C), 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 the 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); and where biodegradation needs to be improved, indigenous microbial populations have been supported with pre-adapted microbial inoculants that possess the relevant hydrocarbon-degradation potential (Lang *et al.*, 2016).

In this present study, there were low levels of $^{14}CO_2$ (%) from the mineralisation of the ^{14}C-phenanthrene in some of the inoculated and uninoculated mineralisations in the absence of AD and/or WA. This implies there were relevant indigenous PAH degraders in the soils but were less abundant (Li *et al.*, 2009). However, the positive effect of the nutrients (especially N and/or P) supplied from the AD and/or WA enhanced the microbial activity, which led to the greater levels of $^{14}CO_2$ (%) from the mineralisation of the ^{14}C-phenanthrene in both inoculated and uninoculated mineralisation in the presence of AD

and/or WA. This finding agrees with past studies where biodegradation of PAHs was increased following soil amendments with organic nutrients (Hollender *et al.*, 2003; Haritash and Kaushik, 2009; Ruberto *et al.*, 2009; Tyagi et. al., 2011; Scotti *et al.*, 2013, Scotti *et al.*, 2014).

In the inoculum-induced mineralisation, the lag phases were shorter from the incubation onset and this continued with increasing soil-phenanthrene contact time in the presence of AD- and/or WA. This implies the inoculant (*Pseudomonas* sp.) was able to utilize the nutrients supplied by the AD and WA for proliferation and degradation activity. For example, in the inoculated ^{12}C-phenanthrene-exposed soils amended with AD, WA, as well as mixtures of AD and WA, the mineralisation was enhanced at the incubation onset compared to the similar unamended soils. Also, the enhanced mineralisation rates positively correlated to the population size of the PAH-degrading bacteria before (R^2 = 0.58) and after (R^2 = 0.50) the mineralisation. However, in the uninoculated soils amended with WA, no significant effect was observed on the mineralisation at the incubation onset but as the soil-phenanthrene contact time increased. Also, there was no correlation between the WA addition and the microbial population size before (R^2 = -0.8) and after (R^2 = -0.3) mineralisation. In these particular findings, although the indigenous microbial activity was supported by the soil amendments, microorganisms with the required metabolic PAH-degrading enzymes might not be abundant.

Biostimulation has been known to provide suitable nutrients and/or conditions for the soil microflora as well as PAH-degrading inoculants (Tyagi, et. al., 2011). Therefore, in this present study, biostimulation was complemented with microbial inoculation. The positive effect of these applications was observed from the incubation onset and as the soil-phenanthrene contact time increased. This development was predominant in the ^{12}C-

phenanthrene-exposed soils amended with the mixtures of AD and WA, where significantly increased extents of mineralisation were observed compared to the similar unamended soils. Also, inoculation with pre-adapted PAH-degraders shortened the lag times and increased the mineralisation than observed in the uninoculated AD- and/or WA-amended soils. These findings agree with a study by Hamdi *et. al.* (2007), where biostimulation was complemented with bioaugmentation for biodegradation of aged PAHs in soils, which improved biodegradation compared to the similar unamended soils. The microbial enhancement observed in the inoculum-induced mineralisation in this present study agrees with the report of Tyagi, *et. al.* (2011) where microbial inoculation is indicated to be a better bioremediation strategy compared to biostimulation.

However, the length of the soil-PAH contact time has been known to impact on the bioavailability of PAHs (Riding *et al.*, 2013; Umeh *et. al.*, 2017). Therefore, the impact of ageing on the bioaccessibility of the ^{14}C-phenanthrene was observed in both inoculated and uninoculated AD- and WA-amended soils. The levels of $^{14}CO_2$ (%) produced from the mineralisation of the ^{14}C-phenanthrene reduced significantly as its interactions with the soils increased; the lag phases became significantly longer, while the mineralisation rates and extents also declined. The soil used in this present study has a considerable amount of organic matter which could have enhanced the sorption of the ^{14}C-phenanthrene on to the soil materials and/or sequestration within the SOM, as the soil-phenanthrene interactions increased. Consequently, the bioaccessibility of the ^{14}C-phenanthrene gradually declined, as shown by the results, with increasing contact time with the soil. This observation agrees with past studies where the bioaccessibility of PAHs in soils were reduced due to the ageing effect (Semple *et al.*, 2004; Doick *et. al.*, 2005b; Okere and Semple 2012; Riding *et al.*, 2013; Umeh *et. al.*, 2017).

4.3 The correlation of HP-β-CD extractability and PAH mineralisation

The HP-β-CD-extraction of the ^{14}C-phenanthrene was compared with the extent to which the ^{14}C-phenanthrene was biodegraded both in the presence and absence of AD and/or WA. A relationship close to 1:1 is expected between the HP-β-CD-extractable and mineralised ^{14}C-phenanthrene (Reid et. al., 2000); however, in this present study, some of the HP-β-CD-extractable ^{14}C-phenanthrene fractions were relatively greater than the mineralised fractions (Table 6). This could have been due to several reasons. The nutrients supplied from the AD and/or WA would have been utilized by the microorganisms and contributed to the enhancement of the mineralisation of the ^{14}C-phenanthrene. This could have resulted in the gradual loss of the ^{14}C-phenanthrene. Also, the soil used for the study was clayey-loam with a considerable amount of organic matter, which could have either enhanced the sorption of the phenanthrene contaminant to the soil materials or reduced the desorption of the ^{14}C-phenanthrene from the soil matrices (Reid *et. al.*, 2004; Papadopoulos *et. al.*, 2007). The findings and/or observations in this study agree with similar reports of HP-β-CD-extractable fractions of the soil organic contaminants being relatively greater than the mineralised fractions due to their slower desorption rates from the soil (Reid et. al., 2000; Rhodes *et. al.*, 2008). However, the regression analyses of the HP-β-CD-extractable and mineralised fractions in this present study showed positive correlations at each time point, both in inoculated and uninoculated mineralisation. These results agree with documented studies where the HP-β-CD-extractable and bioavailable fractions of PAHs were not in a 1:1 relationship but very close, with a relative decrease in the bioavailable fractions compared to the HP-β-CD-extractable fractions (Reid et. al., 2000; Papadopoulos *et. al.*, 2007; Rhodes *et. al.*, 2008).

4.4 The impact of soil amendment with renewable bioenergy residues on the bioaccessibility of ^{14}C-phenanthrene in soil

The presence of microorganisms with the required metabolic capability to degrade PAHs is known to influence the rate of hydrocarbons biodegradation in soil (Das and Chandran, 2011). Several studies showed the composition of the naturally occurring microbial populations that contribute to the biodegradation of petroleum hydrocarbons and their derivatives in different environments (Kanaly & Harayama, 2000; Haritash and Kaushik, 2009; Alvarez *et. al.*, 2011; Das and Chandran, 2011; Tyagi *et al.,* 2011). Their studies identified bacteria as the most active agents in the degradation of hydrocarbons, and various bacterial strains are known for their catabolic capability as well as metabolic routes required for the degradation of PAHs (Haritash and Kaushik, 2009; Das and Chandran, 2011; Tyagi *et.al.,* 2011). Bacterial species known to degrade PAHs have been isolated from hydrocarbon-contaminated soils or sediments (Haritash and Kaushik, 2009). In this present study, the indigenous microbial components were not analysed but the bacterial population size was determined. Also, a pure strain of pre-adapted phenanthrene-degrading *Pseudomonas* species was employed for the inoculum-induced mineralisation at a population density of $\sim 10^6$ to 10^7 CFUs g^{-1} soil. This step agrees with the report of biodegradation of PAHs with the use of catabolically active bacteria of relatively high inoculum densities, from $\sim 10^6$ to 10^8 cells g^{-1} soil (Doick *et. al.*, 2005a). The inoculant was pre-exposed to phenanthrene, as a sole carbon source, over a period of time to enhance adaption before inoculation into the ^{14}C-phenanthrene-contaminated soils. Pseudomonads are one of the bacterial strains identified to possess the metabolic routes required for the degradation of recalcitrant compounds (Hwang and Cutright, 2002; Tyagi, *et. al.*, 2011). The use of both indigenous and inoculum-derived degradations, in this present study, helped to: evaluate the level of occurrence of indigenous PAH-

degrading microbes in the soil samples; and to appraise the effects of the catabolic inoculum on the degradation of the ^{14}C-phenanthrene, in the presence of the AD and/or WA.

In the present study, the influence of the addition of AD and/or WA on the indigenous and PAH-degrading bacterial population size was evaluated. In both inoculated and uninoculated ^{12}C-phenanthrene-exposed soils amended with AD and/or WA, the numbers of heterotrophic bacteria were higher at the onset of the incubation before mineralisation compared to the end of mineralisation. This could have been due to the effect of the ^{14}C-phenanthrene on the microbial adaptation or growth. As the soil-phenanthrene contact time increased, the bacterial population size increased at the end of mineralisation compared to the start of mineralisation. This observation implies an increase in the proliferation and/or activity of the ^{14}C-phenanthrene-degraders in both indigenous and inocula-induced mineralisation. Soil amendments with the AD and/or WA, which supplied readily available essential nutrients useful for microbial growth and/or activity, as well as maintained favourable soil conditions could have contributed to the abundance of the relevant indigenous phenanthrene-degraders in the soils.

Also, the results of the study of the uninoculated mineralisation show that the levels of mineralisation (%) in some of the soil samples was independent of the density of the microorganisms present in the soils. Some of the uninoculated ^{12}C-phenanthrene-exposed AD- and/or WA-amended soils showed enhanced mineralisation; the results showed the population size of the heterotrophic bacteria at the end of mineralisation was less than those at the start of mineralisation. These imply the some of the soil microflora must have possessed the relevant microbial metabolic pathways or phenanthrene-degrading enzymes (Das and Chandran, 2011) for the degradation of phenanthrene.

However, in the inoculated ^{12}C-phenanthrene-exposed AD- and WA-amended soils, the mineralisation rates at the incubation onset positively correlated to the bacterial densities before (R^2 = 0.6) and after (R^2= 0.5) mineralisation, while there was no linear correlation with the extents of mineralisation. Also, the population size of the inoculant increased after mineralisation compared to the initial inoculum dilution at the start of the mineralisation. This observation supports the mineralisation results, where significantly shorter lag phases and improved mineralisation rates were observed in the inoculated AD- and/or WA-amended soils, at the incubation onset (0 d) compared to the uninoculated AD- and/or WA-amended soils, where there was no significant effect on the lag phases and rates of mineralisation.

7. Conclusion

The ameliorative effect of WA and stimulating effect of the AD on biodegradation of ^{14}C-phenanthrene in soils was investigated. Improved mineralisation of ^{14}C-phenanthrene was observed in the ^{12}C-phenanthrene-exposed soils amended with AD and/or WA, especially from the onset of the soil-contaminant contact time, compared to the similar soils without AD- and/or WA-amendment(s). Higher mineralisation was observed in the inoculated mineralisation compared to the indigenous mineralisation in the presence of AD and/or WA, from the onset of the soil-phenanthrene contact time. Although, the rates and/or extents of mineralisation reduced as the soil-phenanthrene interactions increased over time, higher extents of mineralisation were still observed in both inoculated and uninoculated mineralisation in the presence of the AD and/or WA, especially the mixtures of AD and WA. The presence of relevant hydrocarbon-degrading microorganisms and the sustainability of their optimal growth and activity (with adequate supplies of rate-limiting organic nutrients) can contribute to microbial accessibility and degradation of PAHs. Therefore, this study gives insight into the bioremediation system that can enhance microbial availability and degradation of ageing PAHs in the soil.

8. References

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9. General conclusion

The results of the investigations in this thesis established the positive influence of soil amendment with AD and/or WA on indigenous biodegradation of PAHs. This is due to the organic matter content of the AD, as well as the nutrient contents of both AD and WA. Soil amendment with correct amounts of AD and WA, especially as a mixture, can potentially ameliorate the soil conditions and augment the supply of essential nutrients for optimal microbial activity. The presence of abundant hydrocarbon-degrading microbes, as well as the sustainability of their optimal growth and/or activity, can boost the degradation of PAHs in soil. Therefore, soil amendment with a mixture of AD and WA can contribute to the development of a soil adapted to optimal bioaccessibility and/or biodegradation of PAHs. The thesis presents the insights (obtained from these present empirical studies) that can contribute to the design of sustainable remediation strategies that will enhance the biodegradation of PAHs in PAH-contaminated soils lacking in nutrients.

10. Recommendations for further studies

The positive impact of the AD and/or WA additions to soil on microbial activity has been established, and the positive findings of this thesis on the effects of soil amendments with AD and/or WA on biodegradation of PAHs suggest their addition to the soil to be a suitable option of recovering and retaining their recyclable nutrients, rather than disposal in landfills or dumping sites where they tend to become environmental hazards. Their use as soil amendments can maximise the sustainability of the soil nutrients and microbial activity. However, more practical investigations may be required on the potential amounts of AD and WA that could be applied as soil amendments, especially as a mixture due to their complementary nutrient contents, and the implication on the total available nutrients after amendment. This will prevent excessive addition(s) of AD and/or WA, as well as mitigate hazardous effects of over-application to soil and environmental health (previously mentioned).

Therefore, the following recommendations are suggested to maximize the field applicability of the AD and/or WA to enhance biodegradation of PAHs in the soils lacking in nutrients:

1. The available nutrients in PAH-contaminated soil should be assessed before the addition of AD and/or WA to ascertain the amounts of macro- and micro-nutrients available in the soil. This will assist in knowing the correct amounts of AD and WA that could be added to soil as amendments due to their significant impact on soil microbial activity and community structure. For example, a major constraint to the use of WA for soil amendment was identified in studies to be the uncertainty of its correct application amount (Ferreiro *et al.*, 2011; Fernández-Delgado Juárez *et al.*, 2013). Also, the AD

addition is known to effectively increase the soil available P content and degradable organic matter without increasing the soil organic carbon content (Alburquerque *et. al.*, 2012a).

The entry/occurrence of organic contaminants affects the soil carbon-to-nitrogen-to-phosphorus (C:N:P) ratio which significantly influences the soil microbial growth and activity (Stroud *et al.*, 2007; Silva and Fay, 2012; Zam and Mustafa, 2012; Warr *et al.*, 2013; Naseri *et al.*, 2014). However, soil amendment with the admixture of the nutrient-rich AD and WA can compensate for any shortage(s) in the available microbial growth-limiting nutrients due to their complementary nutrient components (Alburquerque *et al.*, 2012a; García-Sánchez *et al.*, 2015a). The application of the correct amounts of these residues will help the soil quality (and protect the environmental health) (Kuba *et al.*, 2008; Gómez-Brandón *et al.*, 2016). This can enhance the development of an optimal indigenous bioaccessibility or biodegradation of PAHs or lead to the design of a sustainable remediation strategy that will enhance the biodegradation of PAHs in soils lacking in nutrients. This recommendation agrees with studies where soil physico-chemical analysis was carried out before the additions of nutrients sources (Fernández-Delgado Juárez *et al.*, 2013; Koszel and Lorencowicz, 2015; García-Sánchez *et al.*, 2015b; Nabeela *et al.*, 2015).

2. The nutrients composition or contents of every batch of the AD and WA to be applied to soil should be characterised before their soil application to prevent excessive addition of nutrients to the soil. For example, in the European Union, the amount of AD to be applied to soil is defined according to the national legislation that outlines the limits for N and P use per hectare (Tambone *et. al.*, 2010). This recommendation agrees with other studies

where the quality of the ameliorative materials was evaluated before their addition to soils (Gómez-Brandón *et al.*, 2016; García-Sánchez *et al.*, 2015a; Nabeela *et al.*, 2015).

Residues are generated from different sources/feedstocks which make their elemental contents and/or qualities to be significantly different. Also, the characteristics of the AD and WA are known to be affected by the characteristics of their feedstocks and preparation processes. For example, the anaerobic digestion process and type of bioreactor have significant impacts on AD (Tiwary *et. al.*, 2015; Tampio *et. al.*, 2016). Similarly, in the WA, the nature of the parent coal, conditions of the combustion, type of emission control devices, as well as the storage and handling methods have been reported to cause a wide variation in the physico-chemical and mineralogical properties of WA (García-Sanchez *et. al.,* 2015).

Also, this step will help to mitigate the excessive application of AD and WA, due to the quantity and quality of the organic matter in the AD, as well as other impurities (such as heavy metals, organic contaminants) in both AD and WA (Tambone *et. al.,* 2010). They can adversely affect the soil properties and microbial osmotic balance or cause higher environmental emissions, while the accumulated heavy metals can become toxic to the soil microbial activities, population size and community structure (Demeyer et. al., 2001; Perucci *et. al.*, 2006; Ochecova *et. al.*, 2014; Monlau *et. al.*, 2016; Fernández-Bayo *et. al.*, 2017). Similarly, high organic matter content can lead to excess microbial activity as well as immobilization of N (Tambone *et. al.*, 2010) and, consequently, reduce the bioaccessibility of PAHs.

3. For sustainable recycling of the AD and WA, certain quality characteristics which involve biological stability and hygiene were reported must be satisfied (Tambone *et. al.,*

2010). The hygiene quality and stabilization of the AD should be evaluated and ensured/maintained to mitigate the introduction of heavy metals, radioactive materials and unwanted micro-organisms (most importantly, pathogens) into the soil. Similarly, the quality of WA should be ensured to mitigate the introduction of heavy metals and radioactive materials into the soil. The safety and stability of the AD and WA were also considered significant in past studies (Alburquerque *et al.*, 2012a; Pezzolla *et al.*, 2012; Fernández-Delgado Juárez *et al.*, 2013).

The use of AD as a bio-fertiliser is known to be influenced by its compliance with the quality standards regulated by the European and United Kingdom's guidelines (Alburquerque *et. a.*, 2012c). Generally, the legislative trends in wastes management have been known to centre on adding value to the AD and WA through their nutrients' recovery for agricultural and/or ecological improvement (Alburquerque *et. a.*, 2012c). Therefore, soil amendments with AD and WA can remain a nutrients' recovery process instead of a disposal method.

4. Environmental risk assessments should be carried out on the soil and air following the short- and long-term applications of AD and WA to monitor their impact on the soil properties and environment. For example, the microbial responses in the AD- and WA-amended soils should be monitored, and the environmental emissions, such as nitrates and greenhouse gas (GHG) should be evaluated (Demeyer *et al.*, 2001; Pezzolla *et al.*, 2012; Fernández-Delgado Juarez *et al.*, 2013; Monlau *et al.*, 2016).

This insight will help to ensure the best management practices that will maximise a sustainable nutrient supply from the residues while minimising the environmental emission of nitrates. For example, excess nitrates from AD application can cause soil

acidity, erode to surface waters, leach to underground waters or denitrify into gaseous form as GHG (Fernández-Delgado Juarez et al., 2013; Maletić et al., 2013; Naseri et al., 2014; Gómez-Brandón et al., 2016; Ning et al., 2017; Richard et al., 2018). Good management practices were also encouraged to protect the water quality across Europe by preventing the pollution of ground and surface waters with nitrates from agricultural sources (Nicholson et. al., 2017).

Similarly, the excess WA may negatively affect the soil properties such as texture, aeration, water holding capacity and salinity (Perucc*i et al.*, 2008). Also, the excess WA particles may block the soil pores as they swell when in contact with water and cause a decrease in soil aeration and loss of contaminants (Demeyer *et al.*, 2001; Reid *et. al.*, 2004). WA alkalinity may influence the soil electrical conductivity (EC) and soil elements solubility (Demeyer *et al.*, 2001; Bougnom and Insam, 2009; Bougnom *et al.*, 2010), while the heavy metals in WA may accumulate in soil (Ning *et al.*, 2017; Richard *et al.*, 2018) and consequently, inhibit microbial growth and/or activity.

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