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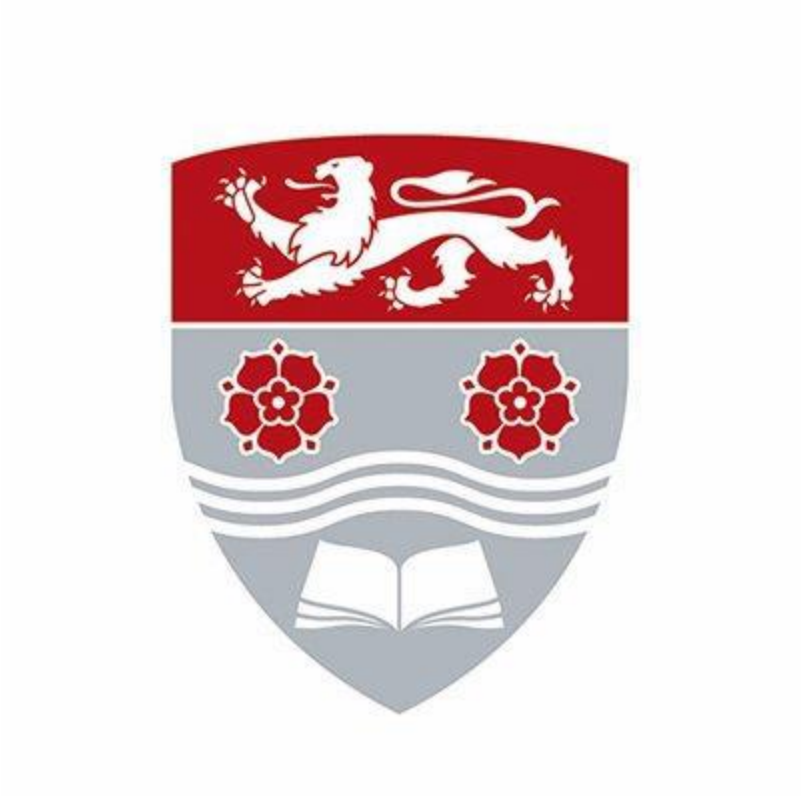

The impact of organic amendments and fungal treatments on the biodegradation of polycyclic aromatic hydrocarbons in soil.

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BSc (Hons), M.Sc

Submitted for the degree of Doctor of Philosophy

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Declaration

I hereby declare that this thesis is my original work and the work has not been submitted elsewhere, in whole or in part, to qualify for any other academic award of a higher degree. All sections of this thesis with joint research/collaborators have been appropriately acknowledged and referenced.

Victor Taghoghor Omoni

Lancaster University, August 2021

Statement of authorship

This thesis has been prepared in a conventional format and contained the following papers:

Chapter 3 has been published as: **Omoni, V.T.**, Lag-Brotons, A.J., and Semple, K.T. (2020). Impact of organic amendments on the development of phenanthrene catabolism in soil. *International Biodeterioration and Biodegradation*, 151:104991. **Mr Omoni** carried out all laboratory work, data analysis, wrote the manuscript and prepared it for submission **(90 %**). Dr Lag-Brotons assisted with the laboratory work and commented on the manuscript (5 %). Prof. Semple (5 %) commented on the manuscript and provided great recommendations.

Chapter 4 has been published as: **Omoni, V.T.**, Baidoo, P.K., Fagbohungbe, M.O., and Semple, K.T. (2020). The impact of enhanced and non-enhanced biochars on the catabolism of ^{14}C-phenanthrene in soil. *Environmental Technology and Innovation*, 20:101146. **Mr Omoni** performed all laboratory work, wrote the manuscript and prepared it for submission (**75 %**). Mr Paul assisted in the laboratory set-up, sample analysis and this research formed part of his MSc dissertation (15 %). Dr Fagbohungbe (5 %) and Prof. Semple (5 %) commented on the manuscript and provided recommendations for improvement.

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Chapter 7 has been submitted as: **Omoni, V.T.**, Nwosu, T.F-X., Bankole, P.O., Onyeri, C.A., Ojo, S.A., and Semple, K.T. (2021). The impact of different nitrogen amendments on the biodegradation of ^{14}C-phenanthrene by endophytic fungal strains in liquid culture. *Biodegradation.* **Mr Omoni** performed all laboratory work, wrote the manuscript and prepared it for submission (**65 %**). Mr Nwosu (15 %) assisted in the laboratory set-up, sample analysis and this research formed part of his MSc dissertation. Dr Bankole (5 %) assisted in the sample analysis (molecular) and commented on the manuscript. Dr Ojo (5 %) and Miss Onyeri (5 %) assisted in the laboratory set-up and sample analyses. Prof. Semple (5 %) commented on the content and provided recommendations.

I hereby consent with the above statements in my thesis:

Prof Kirk T. Semple Mr Victor Taghoghor Omoni

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Abstract

Soil is the major sink for a broad range of potentially toxic hydrophobic organic contaminants (HOCs), including polycyclic aromatic hydrocarbons (PAHs). PAHs are widely distributed affecting the physicochemical and biological properties of soil. These HOCs have significant human and environmental health concerns. The application of mineral-based conventional fertilisation and other chemical treatment methods to stimulate microbial catabolic activities is however costly as well deleterious to soil quality and health. Soil amendment with organic waste materials with a particular interest in the lignocellulosic ones for the remediation of contaminated soil has become a good substrate option for microbial growth and improvement of soil quality due to their lower cost, eco-friendly, sustainable, feasible and practical nature in the environment. Therefore, this thesis aimed to investigate the impact of various organic materials as sources of nutrients and microbial inocula for enhanced PAHs biodegradation in contaminated soils. The study comprised of three sets: the effects of application ratios of different organic materials (spent brewery grains, spent mushroom compost and biochars); fungal treatment and immobilisation study of organic material (in particular brewery spent grains) and nitrogen amendments on the biodegradation of ^{14}C-phenanthrene. Results revealed that the biodegradation of phenanthrene was improved in soils after the addition of organic waste materials. In addition, the results showed that microbial catabolism and biodegradation of phenanthrene can be facilitated by the addition of smaller amounts of organic waste materials into the soil. Furthermore, improving the efficiency of organic waste material by pre-treatment and immobilisation methods using white-rot fungi were observed to promote higher catabolic response as well as influence the physicochemical properties and biological activity of amended soils. This thesis provides insights and evidence supporting the values of organic materials as nutrient supplements; appropriate ratios for soil amendment, and fungal immobilisation and fungal pre-treatment of waste materials as key mechanisms to stimulate and promote the bioactivities and biodegradation of PAHs in contaminated soil.

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List of Abbreviations

ABTS – 2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid

ACP – Acid phosphatase

BRF – Brown-rot fungi

C – Carbon

C:N – Carbon-to-nitrogen

Ca – Calcium

CEC – Cation exchange capacity

CFUs – Colony Forming Units

EbioC – Enhanced biochar

EPA – Environmental protection agency

H_2O_2 – Hydrogen peroxide

HMW – High molecular weight

HOC – Hydrophobic organic contaminants

K – Patassium

Kd – Soil/sediment-water distribution coefficient

Koc –Soil organic carbon partition coefficient

Kow – Octanol-water partition coefficient

Kp – Partition coefficients

LAC – Laccase

LiP – Lignin peroxidase

LMW – Low molecular weight

MnP – Manganese peroxidase

N – Nitrogen

NEbioC – Non-enhanced biochar

OC – Organic carbon

TOC – Total organic carbon

P – Phosphorus

PAHs – Polycyclic aromatic hydrocarbons

SBG – Spent brewery grains

SMC – Spent mushroom compost

SOM – Soil organic matter

SRF – Soft-rot fungi

SSF – Solid state fermentation

WRF – White-rot fungi

Chapter 1

General Introduction and Literature Review

1. Introduction

Rapid growth of the world population, human civilization and modernization, as well as unprecedented increase in industrialization and development, had led to increased production of poorly understood chemical compounds, including the polycyclic aromatic hydrocarbons (PAHs). These had influenced the sustainable quality of life (Li *et al.*, 2018) and consequently gave rise to major environmental challenges such as climate change, loss of biodiversity as well as ecological threats to humans, plants, and animals due to environmental contamination with the organic chemical wastes (Umeh *et al.*, 2017). PAHs are among the major organic environmental contaminants of interest in recent times due to their high exposure risks in different environmental matrices, such as soil, water and air (Adeniji *et al.*, 2019). The understanding of the behaviour, catabolism and challenges of these chemical contaminants will enhance their safe removal and, consequently, minimise their effects in an impacted environment.

PAHs are aromatic hydrocarbons which are mostly colourless, white, or pale-yellow solids consisting of several hundred chemically related compounds. They are class of ubiquitous organic compounds consisting of two or more fused aromatic rings with several structural configurations and varied toxicity in aquatic and terrestrial ecosystems. The PAHs that are mostly encountered in the environment contained the simplest two (naphthalene) to seven (coronene) fused benzene rings; however, PAHs with greater fused rings are also reported (Boehm, 2005; Allison and Burgess, 2015). PAHs are persistent organic compounds and are classified into light molecular weight (LMW) PAHS which contain up to four fused rings, and heavy molecular weight PAHs (HMW) which contain more than four fused rings and are more stable as well as toxic in the environment (Lawal, 2017). The partition behaviour of PAHs

showed that the LMW PAHs may exist mainly in dissolved form whereas the HMW PAHs are predominantly bound to organic-rich surfaces or solids (Chiou *et al.*, 1998; Liu *et al.*, 2017).

Recently, environmental degradation and primarily soil contamination by these organic contaminants has received greater attention due to high variability in the nature and extent of different contaminated soils and the costs of remedial measures (Zabbey *et al.*, 2017). For these chemicals to be environmentally safe, their exposure levels should be as low as reasonably practicable (ALARP) or after a remedial action (clean-up exercise) the exposure levels should be as low as reasonably achievable (ALARA) especially in the contaminated soil (Teaf, 2008; Trellu *et al.*, 2016). These complex safety measures have led researchers to examine the successes and failures of remedial techniques with respect to the residual level of the target chemical contaminants after soil remediation. However, the concentrations of PAH contaminants in soil remain relatively high, even after the sites have been certified remediated (Sam *et al.*, 2017). Feasible techniques including physical, chemical, biological, thermal, and electrical processes have been discussed for the remediation of contaminants in soil (Sakshi *et al.*, 2019; Dadrasnia and Agamuthu, 2013; Kuppusamy *et al.*, 2017). Of the large researches and technologies employed to remediate PAHs-contaminated soils, none has proven 100 percent effective in removal rates and most of the remedial techniques available are yet to be replicated with real-life scenario (field studies) to ascertain their efficiency. Therefore, remediation of PAHs as organic contaminants in the environment is still a global concern.

The fate of PAHs in soil environment is controlled by their physicochemical properties such as hydrophobicity and low aqueous solubility which promotes their persistence nature and sorption unto soil matrices (Patel *et al.*, 2020). Furthermore, most of them persist in the environment due to their slow degradation by natural attenuation or chemical and biological

processes (Trellu *et al.*, 2016). These properties, including the number of fused rings have shown that PAHs are carcinogenic, mutagenic, genotoxic and environmentally toxic; and they can bioaccumulate in the environment such as in the food chains (Al-Hawash *et al.*, 2018). These have posed serious public health concerns and thereby trigger the interest of the scientific and regulatory bodies. About 16 specific PAHs are listed by the United States Environmental Protection Agency (US EPA) among the 126 priority pollutants (Hussain *et al.*, 2018). Therefore, the environmental effects of these organic contaminants outweigh the benefits they provide to humans thereby necessitating their degradation either after being used or when they are present in the environment (Megharaj *et al.*, 2011).

Soil quality is greatly affected by the deposition of PAHs and other hydrophobic organic pollutants which are generated from different routes and activities. PAHs have both natural and anthropogenic sources; however, the latter are more dominant because they are the major routes of PAHs in the environment (Abdel-Shafy and Mansour, 2016; Omoni *et al.*, 2020a). They can occur through three main processes namely, petrogenic, pyrogenic and biogenic (Gennadiev *et al.*, 2015; Kuppusamy *et al.*, 2017). Today, in most parts of the world where oil activities (exploration and production) are predominant such as in the Niger-Delta axis of Nigeria, there are over 2000 estimated land-based crude oil contaminated sites resulting from oil spill (Sam *et al.*, 2017; UNEP, 2011), which has greatly impacted on ecosystem services. Such contaminated sites contain elevated exposure levels of PAHs and related organic contaminants (Obida *et al.*, 2021).

Nutrients provided by waste streams applied to land could offer a sustainable approach to resolving the environmental problems arising from petroleum hydrocarbons contaminated land. The use of organic waste materials (e.g. lignocellulosic waste materials) generated from

the agro-industrial processes, including straw (pea and rice), rice husks, sugarcane bagasse, spent mushroom compost, corn cobs, spent brewery grains are potential sources of organic nutrients for enhancing microbial growth and metabolism in PAH contaminated soils (Brändli *et al.*, 2005; Chiu *et al.*, 2009; García-Delgado *et al.*, 2015; Omoni *et al.*, 2020b). Recently, there has been growing interest in the application of processed organic waste materials such as biochar as an emerging soil amendment material in nutrient-deficient soil (Zhang *et al.*, 2018) and PAH contaminated soil (Kong *et al.*, 2018; Omoni *et al.*, 2020a). Soil amendments with biochar can improve both the physicochemical and biological properties of the soil. These low-cost organic materials are rich sources of macro-and micro-nutrients (Dhaliwal *et al.*, 2019), which when added to soil can provide organic carbon, nitrogen and phosphorus to the soil biota (Larney and Angers, 2012; Chojnacka *et al.*, 2020). A large amount of food wastes is generated annually from different parts of the world industry especially in Africa and China (Li *et al.*, 2019; Omoni *et al.*, 2020b). In Africa, most of these wastes have contributed to massive environmental pollution due to their alternatives uses as well as improper disposal routes such as landfilling, illegal dumping and burning (Omoni *et al.*, 2020b).

Organic materials can be colonised and used by microorganisms as ecological niches in soil, when they are used as microbial-support systems (immobilisation) for contaminant removal in soil. These materials can enhance the survival of fungal mycelia, optimizes enzyme secretion, protect both the fungi and enzymes from harsh environmental conditions associated with organic pollution, and increase fungal viability and activity in the soil (Sari *et al.*, 2014; Dzionek *et al.*, 2016; Andriani and Tachibana, 2016). Another approach to exploring organic waste materials involves the biological pre-treatment with lignin-degraders such as white-rot, soft-rot, and brown-rot fungi and some bacterial species which can delignify and degrade hemicellulose present in the organic materials. This specific function is associated with the

production of extracellular ligninolytic and non-ligninolytic enzymes secreted by the rot fungi (Baldrian, 2006; Abdel-Hamid *et al.*, 2013). The white-rot fungi (WRF) basidiomycetes are more extensively studied because they have a higher potential for delignification and degradation of cellulose and hemicellulose in lignocellulose-containing materials (Isroi *et al.*, 2011; Rouches *et al.*, 2016). The fungal pre-treatment is usually carried out to remove non-metabolisable lignin and provide access to the readily and rapidly metabolizable carbon source for microbial growth and metabolism.

1.1 Polycyclic aromatic hydrocarbons (PAHs): soil contamination and physico-chemical properties

Soil is the major sink for a broad range of hazardous hydrophobic contaminants, especially the polycyclic aromatic hydrocarbons (PAHs), and these contaminants may affect the physicochemical and biological properties as well as qualities of soil (Xiao *et al.* 2014; Sakshi *et al.*, 2019). Soil is a very useful natural resource that requires more attention; it provides the necessary conditions that support life and thus forms a complex habitat for macro, meso and micro-organisms. Soil harbours an estimated one-third of organisms on earth, and is responsible for several biogeochemical cycling and activities, including the functioning of the ecosystem. Anthropogenic sources of PAHs including incomplete combustion of organic materials (wood, coal, oil, garbage, and other organic substances) have been recognised as the larger contributor of PAHs in the environment than natural sources such as forest fires and volcanoes (Abdel-Shafy and Mansour, 2016; Kuppusamy *et al.*, 2017).

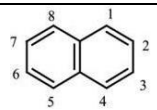
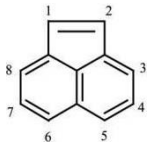
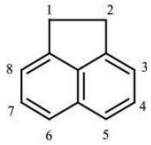
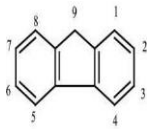
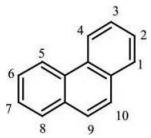
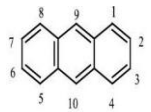
PAHs are class of organic compounds with two or more single or fused aromatic rings consisting of a pair of carbon atoms shared between rings in their molecules (Abdel-Shafy and Mansour, 2015). The structural arrangements of aromatic rings in space can be linear, angular or in clusters (Patel *et al.*, 2020). Linear PAHs are likely more unstable than the clustered PAHs, however both are rapidly attacked and degraded by photooxidation and chemical oxidation. The angular arrangement is thermodynamically the most stable configuration of PAHs, but their bay regions are susceptible to enzymatic attack and thus more degradation than both linear and clustered (Abdel-Shafy and Mansour, 2016; Patel *et al.*, 2020).

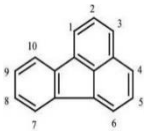
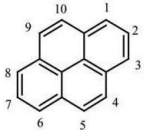
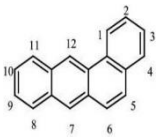
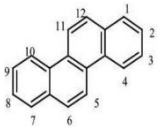
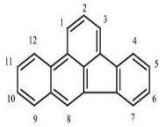
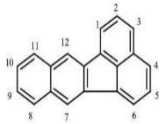
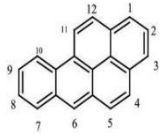
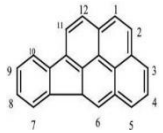
Currently, more than 100 different PAHs have been identified and a few hundred PAH-derivatives are known (Alexander, 1999). Of which 16 PAHs are primarily listed among the 126 priority pollutants by US EPA due to their major concerns in the environment (Hussain *et al.*, 2018), resulting from their potential nature of carcinogenicity, mutability, teratogenicity and toxicity. These priority contaminants were classified based on their highest concentrations, exposure rates, recalcitrance nature and toxicity (Patel *et al.*, 2020). Based on their structural configuration, PAHs are also characterised through their low water solubility, low vapour pressure, and high melting and boiling points (Lee and Vu, 2010). The vapour pressure and aqueous solubility of PAHs tend to decrease with increasing molecular weight, whereas the aqueous solubility reduces with each added fused ring (Adeniji *et al.*, 2017). These properties influence their fate in the environment from their susceptibility to microbial degradation, to a large extent. The chemical structure and physico-chemical properties of the 16 priority PAHs are listed in Table 1. These 16 PAHs are amongst the PAHs present in petroleum hydrocarbons including the petroleum-specific alkylated (PAH1-PAH4) homologues of the five alkylated or targeted PAHs groups: alkylated naphthalene, phenanthrene, dibenzothiophene, fluorene, and chrysene. These PAH compounds are highly hydrophobic, recalcitrant, lipophilic and less

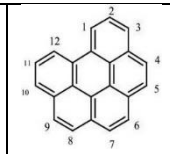
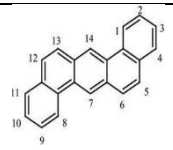
aqueous soluble in the environment, hence they persist and accumulate in the environment thereby refractory to microbial degradation (Cerniglia, 1992). They are mostly found in the environment such as water, air, sediments and soil due to their chemical stability. Most countries have added these toxic pollutants to their environment monitoring programmes.

The partitioning, persistence and accumulation of different PAHs and other hydrophobic contaminants in the soil depends on contaminants physicochemical properties, including their molecular weight, aromaticity, water solubility, phase distribution and partition coefficients (Table 1). The soil natural heterogeneity promotes the complex nature of hydrophobic PAH contaminants based on its physicochemical properties which include polarity of soil matrix (moieties in soil organic matter containing nitrogen and oxygen), soil mineral and organic matrix, texture, degree of structure development, water retention capacity, carbon type and content in soil (Semple *et al.*, 2003; Loganathan, and Lam, 2012). Both properties (soil and PAHs) are determinants for the fate and behaviour (diffusion) of PAHs and soil-PAH interaction. Specifically, other PAH properties such as chemical structure, hydrophobicity and high lipophilicity have further increased the persistence of these contaminants in our environment, rendering them less available for biological uptake and degradation (Wang *et al.*, 2009). Coupled with this, their highly lipophilic nature increases their binding ability and bio-accumulation into fatty tissues of humans and animals on either exposure or consumption and, consequently, their potential to cause a long-term harm (Abdel-Shafy and Mansour, 2016; Kadri *et al.*, 2017).

Table 1. The physical and chemical properties of the 16 polycyclic aromatic hydrocarbons (PAHs) as priority pollutants (IARC, 2010; Gateuille *et al.*, 2012; Ghosal *et al.*, 2016; Hussain *et al.*, 2018; Patel *et al.*, 2020)

Chemical name	Physical appearance	Molecular structure/nomenclature	Molecular weight (g/mole)	Aqueous solubility (mg/L)	Phase distribution	Melting point (^{0}C)	Boiling point (^{0}C)	Vapour pressure (mmHg)	Log Kow	Log Koc	Estimated half-lives (days)
Naphthalene	White, volatile, solid		128.18	31	Gas	80.26	218	0.087	3.29	2.97	5.660
Acenaphthylene	Colourless crystalline solid		152.20	16.1	Gas	92–93	265–275	0.029	4.07	1.40	30.70
Acenaphthene	White-to-beige crystals		154.20	3.8	Gas	95	96	4.47×10^{-3}	3.98	3.66	18.77
Fluorene	Small, white, crystalline plates		166.23	1.9	Gas	116–117	295	3.2×10^{-3}	4.18	3.86	15.14
Phenanthrene	Colourless, monoclinic crystals		178.24	1.1	Particle gas	100	340	6.8×10^{-4}	4.45	4.15	14.97
Anthracene	White crystals or flakes		178.24	0.045	Particle gas	218	340–342	1.75×10^{-6}	4.45	4.15	123.0

Fluoranthene	Colourless solid, often pale yellow		202.26	0.26	Particle gas	110.8	375	5.0×10^{-6}	4.90	4.58	191.4
Pyrene	Flakes/powder; pale yellow or colourless solid		202.26	0.132	Particle gas	156	393–404	2.5×10^{-6}	4.88	4.58	283.4
Benzo[a]anthracene	Colourless-to-greenish-yellow fluorescence		228.30	0.011	Particle	158	438	2.5×10^{-6}	5.61	5.30	343.8
Chrysene	Colourless-to-beige crystals/powder		228.30	0.0015	Particle	254	448	6.4×10^{-9}	5.9	No data	343.8
Benzo[b]fluoranthene	Colourless crystals (needles)		252.32	0.0015	Particle	168.3	No data	5.0×10^{-7}	6.04	5.74	284.7
Benzo[k] fluoranthene	Yellow crystals		252.32	0.0008	Particle	215.7	480	9.59×10^{-11}	6.06	5.74	284.7
Benzo[a]pyrene	Pale yellow needles		252.32	0.0038	Particle	179–179.3	495	5.6×10^{-9}	6.06	6.74	421.6
Indeno[1,2,3-c,d]pyrene	Yellow crystals (needles)		276.34	0.062	Particle	163.6	530	10^{-10}– 10^{-16}	6.58	6.20	349.2

Benzo[g,h,i]perylene	Pale yellow-green crystals		276.34	0.00026	Particle	273	550	1.03×10^{-10}	6.50	6.20	517.1
Dibenzo[a,h]anthracene	Colourless crystalline powder		278.35	0.0005	Particle	262	No data	1.0×10^{-10}	6.84	6.52	511.4

* Kow- Octanol-Water Partition Coefficient; Koc- Soil Sorption Partition Coefficient.

1.2 Sources and pathways of PAHs in soil

1.2.1 Sources of PAHs

PAHs are widely distributed in air as well as terrestrial and aquatic environments (Hussain *et al.*, 2018). In soil, PAHs are primarily produced by three main sources: biogenic (or diagenetic), petrogenic and pyrogenic in the environment (Sakshi *et al.*, 2019; Abdel-Shafy and Mansour, 2016). The distribution and sources of PAHs in the environment are presented in Table 2. Both natural and anthropogenic processes/activities can release PAHs into the soil environment from the three main sources: petrogenic, pyrogenic and biogenic (Gennadiev *et al.*, 2015; Patel *et al.*, 2020). Although some PAHs in the environment are released from natural sources of combustion such as forest fires and volcanoes along with some minor biogenic sources. However, the exposure from anthropogenic sources are the major routes of PAH pollution, and these involve mainly four types which are industrial, mobile, domestic and agricultural emissions (Ravindra *et al.*, 2008; Kuppusamy *et al.*, 2017; Hussain *et al.*, 2018), owing to their high occurrence and toxicity compared to the natural sources of PAHs.

The anthropogenic sources of PAHs can be grouped into pyrogenic and petrogenic sources. Pyrogenic PAHs formed at high temperature (350–1200°C) under low or no oxygen conditions (Abdel-Shafy and Mansour, 2016) followed by a rapid arrangement into non-alkylated PAH structures (Ramesh *et al.*, 2011). Some pyrolytic processes include unintentionally incomplete combustion of fossil fuels and biomass (e.g. petroleum, wood, coal and related products); these include emissions from industrial-related activities such as, coal-fired power plants, burning of organic matter, wood and coal (Ravindra *et al.*, 2008; Szewczyńska *et al.*, 2013).

Pyrogenic PAHs are also emitted into the atmosphere and they are partitioned between the gaseous and particulate forms in the atmosphere thus facilitating their fate, mobility and soil deposition which forms the basis of developing effective remedial technologies (Bouloubassi *et al.*, 2012; Patel *et al.*, 2020). Meanwhile, the petrogenic PAHs are produced from petroleum-related sources, when ancient organic matter is converted to petroleum and coal under low geological temperature (~ 100–150°C) and high pressure over millions of years (Stout *et al.*, 2015). The PAHs produced from petrogenic sources are predominant with alkylated structures. Industrial petrogenic processes are present in petroleum and by-products that are widespread due to storage, transport, uses and leakages of crude oil and its products (refined products) including asphalt, diesel, creosote, gasoline, lubricants and unprocessed coal and crude oil (Alexander, 1999; Baldwin *et al.*, 2020; Patel *et al.*, 2020). Pyrogenic sources are usually composed of LMW PAHs while the HMW PAHs are present in petrogenic sources (Bouloubassi *et al.*, 2012). Natural pyrogenic PAHs are emitted from forest wildfires and volcanic eruptions while the natural petrogenic PAHs are present in oil seeps (natural leaks of crude oil and gas) from oil wells/reservoirs.

The PAHs of the biogenic or diagenetic origin, are synthesized by plants (phytoplankton and algae), animal remains and microorganisms (biogenic) or the process of slow transformation of non-hydrocarbon natural substrates to hydrocarbons in lake sediments (diagenetic) (Readman *et al.*, 2002). Soil bacteria and fungi are known to be significant producers of some volatile hydrocarbons, such as isoprene which was shown to be produced by actinomycetes and by various bacterial species (Ladygina *et al.*, 2006). Evidence of biological formation of naphthalene in termite nests of tropical forest; perylene from pigments of fungi, insects, or marine organism; phenanthrene, methyl-phenanthrene and methyl-anthracene in preindustrial

lake sediments; and phenanthrene homologous (retene and pimanthrene) from pristine soil/sediments confirmed the biogenic sources of PAHs (Wilcke, 2007; Cabrerizo *et al.*, 2011).

Table 2. Sources of PAHs in contaminated soil (Abdel-Shafy and Mansour, 2016; Sakshi *et al.*, 2019)

Sources of PAHs	Origin of PAHs	Activities	Process/route
Pyrogenic	Combustion of wood, petroleum products, coal)	Industrial (anthropogenic)	Wood burning Burning of tires Burning of fossil fuel Burning of tobacco Burning of agricultural waste Combustion of oil, diesel, and coal
		Natural	Volcano eruption Forest fire
Petrogenic	Substances originated from crude oil/	Industrial (anthropogenic)	Oil spill Outlets from oil refinery Petroleum handling facilities like kerosene tank, generating plant, petrol stations, mechanic workshops, leaking pipeline, and airport fuel dump Petroleum oil industries and industrial activities Creosote, asphalt production Used engine oil, jet fuel, kerosene Manufactured gas plants
		Natural	Oil seeps
Biogenic (or diagenetic)		Natural	Surface waxes of leaves and insect cuticles Wood of tropical forests PAH synthesised by termite organisms Bacterial and algal synthesis Pine needles, Decomposition of vegetable litter fall

1.3 Fate and behaviour of PAHs in soil

The fate and behaviour of PAHs in soil is controlled by several intrinsic soil processes (biological, chemical and physical) including the soil porosity, texture, structure, minerals and organic matter (Semple *et al.*, 2003), together with the physicochemical properties of PAHs which include hydrophobicity, polarity, aqueous solubility, lipophilicity and molecular structure (Figure 1). Other factors such as biological diversity and microbial activity, soil-PAH interaction, prevailing environmental conditions/processes (e.g. adsorption, volatilisation, photolysis, leaching) and natural attenuation process (biodegradation) also influence the fate of PAHs and, consequently lead to their persistence in soil (Semple *et al.*, 2007; Riding *et al.*, 2013). PAHs can be lost from soil (possible fates of PAHs) through different viable mechanisms which include microbial breakdown (biodegradation), photo-oxidation, chemical oxidation, volatilization, crop uptake, accumulation in plants and leaching (Reilley *et al.*, 1996; Semple *et al.*, 2003; Haritash and Kaushik, 2009; Hussain *et al.*, 2012). Since microorganisms have the ability to degrade hazardous contaminants, they are involved in remediation and soil self-purification processes (Sakshi *et al.*, 2019). The HMW PAHs are more resistant to microbial degradation than LMW PAHs due to the higher binding affinity of HMW PAHs in soil (Srogi, 2007; García-Alonso *et al.*, 2008). The fate and behaviour of contaminants are the primary aspects during their risk estimation and remediation (Riding *et al.*, 2013).

The fate of PAHs in the environment mostly depends on their molecular weight (Gateuille *et al.*, 2012). The lightest compounds are known to be the most water soluble and the most volatile, whereas the heaviest ones are the most lipophilic. Also, PAHs with low volatility are classified as semi-volatile organic chemicals (Semple *et al.*, 2003; Stogiannidis and Laane, 2015) and this decreases with the increasing number of aromatic rings (Abdel-Shafy and

Mansour, 2016). Therefore, PAHs with less aromatic rings such as naphthalene, anthracene and phenanthrene are more likely to escape into the air from the soil environment. PAHs are generally lipophilic and hydrophobic (Stogiannidis and Laane, 2015) and, hence, may tend to sorb to organic matter than the aqueous phase in the soil.

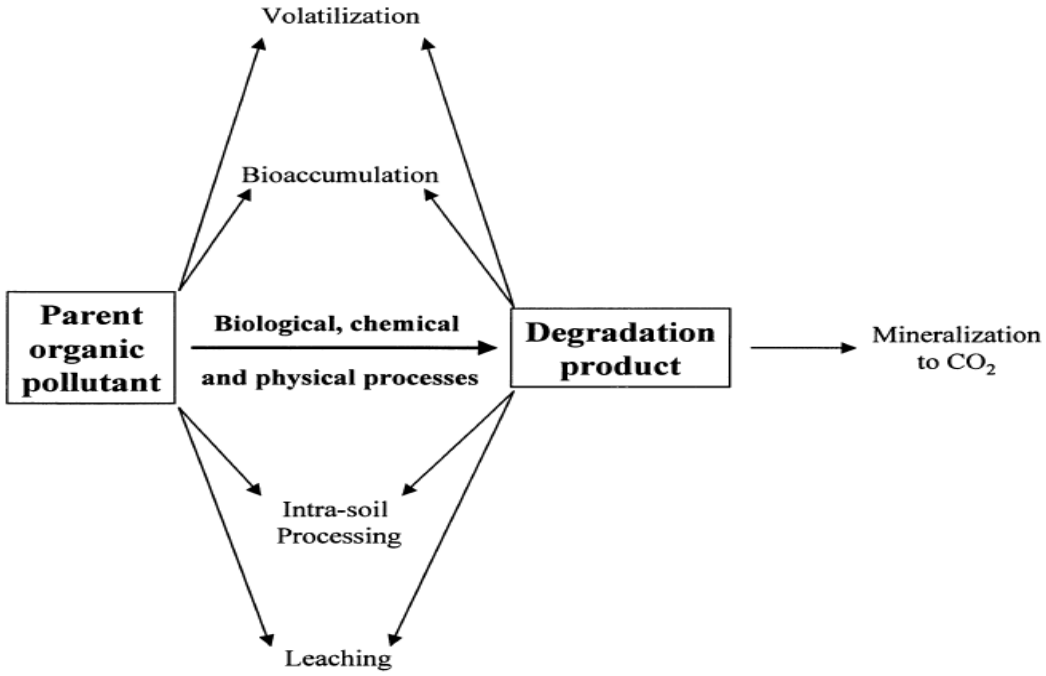


Figure 1. Environmental fate of PAHs in soil (Semple *et al.*, 2001).

PAHs generally have low solubility in water and this decreases with increasing number of aromatic rings (Abdel-Shafy and Mansour, 2016). This implies that LMW PAHs such as naphthalene, anthracene and phenanthrene has a relatively high solubility in water than HMW PAHs, such as benzo[a]anthracene, chrysene, pyrene and benzo[a]pyrene. They may be sorbed to soil organic matter due to their low solubility and high lipophilicity. PAHs are usually present in virtually all hydrocarbons contaminated environment. Petrogenic PAHs are released into the environment through the discharge of petroleum products such as road leaching (Ngabe *et al.*, 2000). Pyrolitic PAHs are mostly emitted to the atmosphere where they partly sorb onto

particles (Gateuille *et al.*, 2012). Some are photo-oxidized; others are transported by wind erosion. The heaviest PAHs mostly sorb onto particles because of their low volatility and may rapidly deposit on soil and vegetation (Smith and Jones, 2000). Once deposited onto soils, PAHs can be sorbed or accumulated within the soil biota or can be sequestered within the soil's matrix into organic matter and tiny complex structures (e.g., glass and rubbery phase) making it difficult for larger sized microorganisms to access (Figure 2) (Semple *et al.*, 2003).

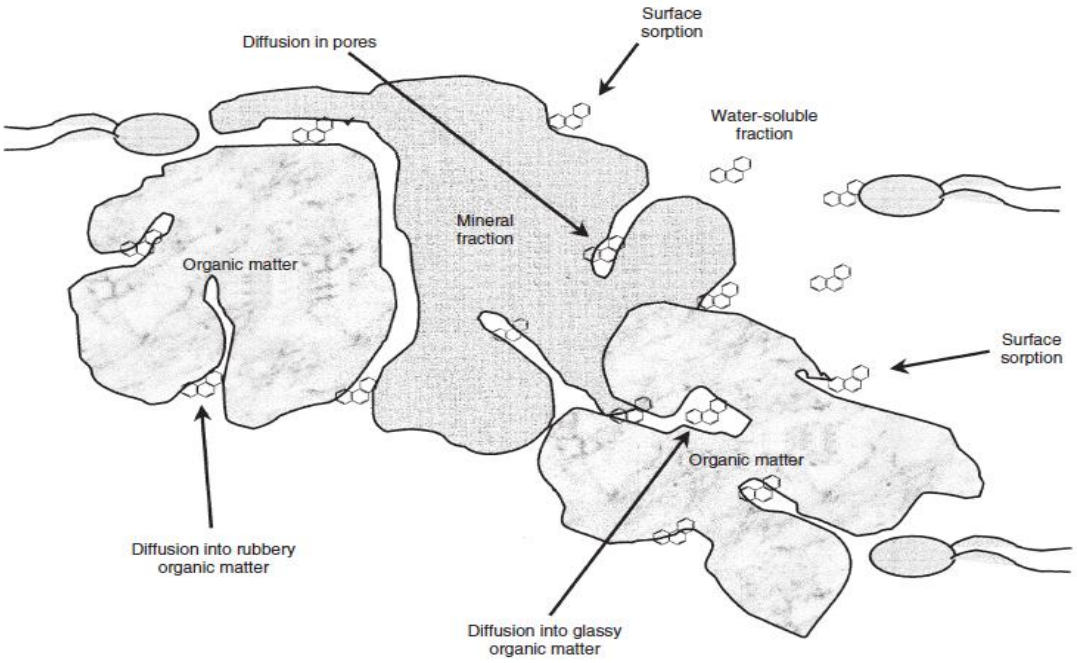


Figure 2. A summarised representation of the physical behaviour of a hydrophobic contaminant within the soil (Semple *et al.*, 2003).

Furthermore, PAH contamination is often spatially heterogeneous because of the great variety of anthropogenic sources. Hence, PAH contamination may display markedly different temporal and spatial distributions. This high degree of spatial and temporal source variability has also complicated our understanding and prediction of the environmental fate of PAHs (Gateuille *et al.*, 2012) and developing strategies for remediation of polluted soils. Basically, the soil

behaves as a dual-function sorbent with organic contaminants by adhering to the (i) surface of mineral matters in the soil by adsorption and (ii) soil organic matter by partition (Moyo *et al.*, 2014). Apparently and to a large extent, the sorption of hydrocarbon contaminants to the soil matrix (both the mineral and organic matters) and the primary constituents of the soil are the key factors that determine the fate, mobility and distribution of a contaminant in the environment (Semple *et al.*, 2003; Luo *et al.*, 2012). Similarly, the fate and availability of PAHs for microbial biotransformation in soil often depends on the absorption to soil solids and desorption to the aqueous phase (Yang *et al.*, 2013).

1.4 Contaminant sorption and transport

1.4.1 Sorption of PAHs in soil

Majorly, aqueous solubility, bioavailability, and environmental behaviour of PAHs in soil ecosystems are significantly influenced by sorption to dissolved organic matter (DOM) and clay minerals. These factors influence the partition of organic pollutants between water and dissolved organic matters and in turn the sequestration of the pollutants in the soil. Soil as a major sink for PAHs is associated with their strong affinity to mineral and organic matters (Okere and Semple, 2012; Xiao *et al.* 2014) and are retained within the narrow width of mesopores and micropores. In aqueous medium (sediments and water), persistence of PAHs is due in part to their strong sorption potential onto settling particles, preserving them for an extended period (Baran *et al.*, 2021). Sorption indicates the partitioning of a solute (ions, molecules, and compounds) between the liquid phase and soil particle interphase.

Following entry of PAHs into the soil environment, the contaminants rapidly bind to the soil mineral and organic matters through physical and chemical processes. Sorption influences the

bioavailability and bioaccessibility of contaminant in soil as well a major factor that determine complete bioremediation of hydrocarbons in soil (Semple *et al.*, 2003; Megharaj *et al.*, 2011). Contaminant properties such as low aqueous solubility and high hydrophobicity facilitate PAHs sorption to soil mineral and organic matters, and this tends to increase with increasing soil ageing (Luo *et al.*, 2012; Ukalska-Jaruga *et al.*, 2020). This facilitates the persistence of PAHs and makes them less bioaccessible in contaminated soils (Riding *et al.* 2013; Rhodes *et al.*, 2012). Sorption to hydrophobic contaminants increases with increasing organic content and dependent on the particle size of the soil.

In particular, as with PAHs, the influencing factor of their sorption to soil matrix are solubility, which is the most determining factor of sorption of such aromatic compounds in soil (Rivas, 2006). Owing to the fact that some contaminants are sorbed to the organic matters in the soil matrix, it reduces the toxicity of the chemical to soil biota; however, the sequestration and ageing of the contaminants will further decrease their bioavailability and biodegradation in the soil. The mechanism of physical sorption of contaminants to organic matters and dissolved organic matters is as a result of the supramolecular structure of the organic matters. This interacts with contaminants through weak van der Waals forces, hydrophobic interactions, hydrogen bonding, entrapment in organic matters, π-π interaction, dipole-dipole interaction, steric effects etc (Pignatello and Xing, 1996; Wang and Zhang, 2014; Kollist-Siigur *et al.*, 2001).

1.4.2 Transport and Partitioning of PAHs in soil

Adsorption/sorption of PAHs to soil organic matter (SOM) occurs by partitioning depending on the physicochemical properties of the specific's compound (Smith *et al.*, 1997). The SOM comprises of two main domains, namely the glassy domain which have non-linear sorption

behaviour and the rubbery domains with linear sorption behaviour of the HOC (Figure 3) (Xia *et al.*, 2007). The latter is associated with faster sorption rates to HOC than the former.

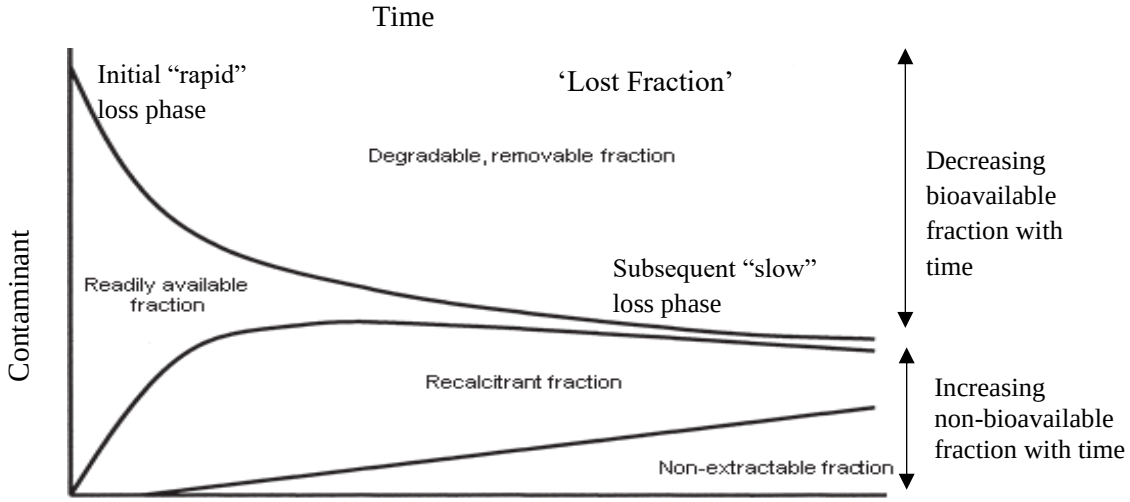


Figure 3. A graphical representation of the influence of contact time on the bioavailability and bioaccessibility of a hydrophobic contaminant in soil (Riding *et al.*, 2013).

Although many soil hydrophobic contaminants show two major biphasic sorption/degradation kinetic phases, the initial phase of rapid sorption onto SOM together with rapid desorption then followed by a period of slow desorption rate. In this phase, the rate of contaminant removal is high however limited by the kinetics of microbial degradation. The second phase involves a gradual sorption or partitioning of the retained PAH fractions within the soil matrix over time and consequently rendering the contaminant less bioaccessible and, thus, reducing the degradation rate by limited mass transfer of the contaminant (Figure 4) (Riding *et al.*, 2013; Barnier *et al.*, 2014; Umeh *et al.*, 2017). Strongly bound PAHs (reversible or desorbable) fractions can be extracted chemically from the soil often referred to as the extractable fraction while some fractions of PAH can be tightly bound to the soil organic matters (irreversible or non-desorbable) and become the non-extractable or recalcitrant to biodegradation in soil (Gao *et al.*, 2009; Riding *et al.*, 2013).

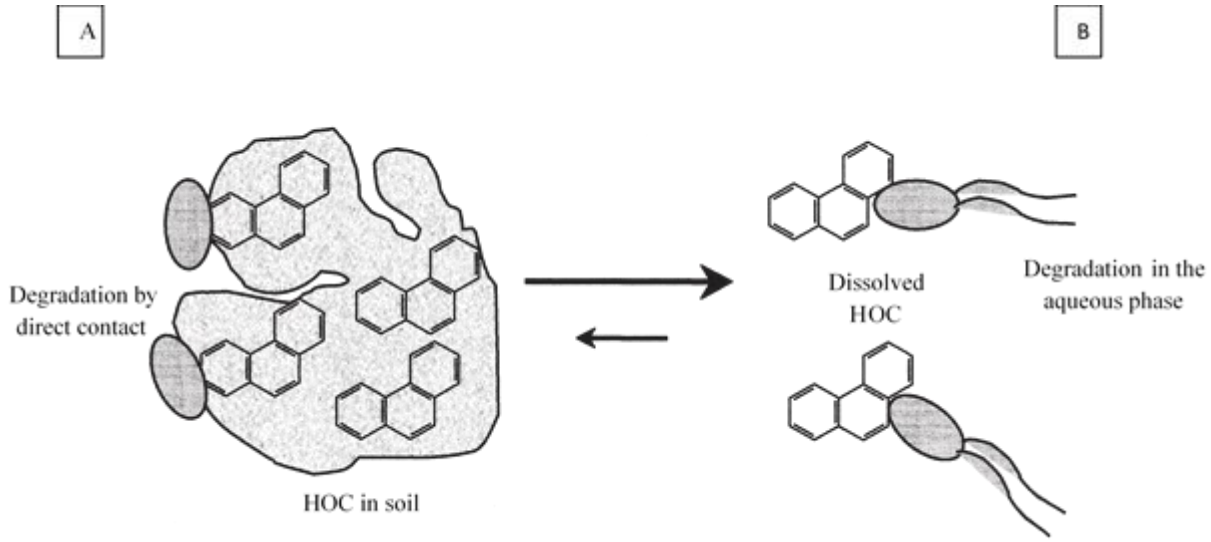


Figure 4. Microbial attack on hydrophobic organic contaminants in soil: A represents direct contact and B represents degradation in the aqueous phase. The direction and size of the arrows represent the interaction (sorption-desorption) between the contaminant and the soil and the pore water (Semple *et al.*, 2003).

Studies have shown that sorption of PAHs to soil is also determined by transport and partitioning of PAHs in the soil and they are related to water solubility, and soil-water partitioning of PAHs such as octanol-water partition coefficient (Kow) and organic carbon partition coefficient (Koc) (Hussain *et al.*, 2018; Yang *et al.*, 2018; Patel *et al.*, 2020). The Kow estimates the potential of PAHs mobility from the water phase to a lipid phase (i.e., the soil-water distribution coefficient) and is related to the solubility of PAHs in water (Abdel-Shafy and Mansour, 2016). The Koc is the potential of PAHs to bind to the soil organic carbon and the degree of PAHs' partitioning to the soil organic carbon (Chiou *et al.*, 1998). As the number of aromatic rings and molecular weights increases, the Kow also increases and the tendency for sorption to soil increases and consequently, the PAHs become more recalcitrant and difficult to biodegradation (Yang *et al.*, 2018). Hence, the distribution and transportation of PAH in soils will differ. Due to the strong relationship between both Kow and Koc with the

soil properties, the prediction of the distribution of a PAH (concentration/sorption) in the soil can be estimated (Abdel-Shafy and Mansour, 2016).

1.5 Biodegradation and bioremediation of PAHs in soil

Biodegradation of PAHs have been extensively studied as a degradation process for contaminated soils. Biodegradation is a technology that uses the science of bioremediation whereby microorganisms are utilized to completely biotransformed a more toxic compound into less toxic compounds with evidence of water, carbon dioxide and inorganic substance (termed as mineralisation). PAH biodegradation can be either aerobic or anaerobic degradation; however, researchers have focused more on both aerobic and anaerobic metabolism for enhanced degradation of PAHs (Carmichael and Pfaender, 1997; Mcnally *et al.*, 1998; Aydin *et al.*, 2017; Dhar *et al.*, 2020). Soil indigenous organisms can efficiently remove hydrocarbon pollutants through biodegradation mechanism (Guarino *et al.*, 2017). This, however, depends on the physiological state of the potential organisms, owing to their ability to resist harsh environmental conditions, as well as, the toxic nature of the pollutants (Dzionek *et al.*, 2016). Several conditions such as the presence of the right organism(s) with the right catabolic enzymes for the target contaminant (specificity for the enzyme active site), accessibility of the target contaminant to the right organism(s) and enzyme action (intracellular or extracellular) on the target contaminant are all required for efficient biodegradation of any contaminant in soil (Alexander, 1999). Furthermore, the ability of a HOCs such as PAHs to be completely mineralised, being used as source of nutrients for carbon and energy, is the most important aspect of microbial transformation in the environment because incomplete mineralisation can produce hazardous metabolites more toxic to the parent compound.

Researchers have extensively studied the mineralisation of PAHs by using ^{14}C-radiolabelled compound (^{14}C-PAHs) (Johnsen *et al.*, 2005; Quilliam *et al.*, 2013; Okere *et al.*, 2017; Umeh *et al.*, 2018; Omoni *et al.*, 2020a). The soil and contaminant properties (e.g. aromatic structure), bioavailability of the contaminant in soil, extent of weathering of the contaminant (sorption and desorption) and microbial activity including the genetic capability of the native populations are important factors that strongly influence any biodegradation process or the rates of biodegradation of PAHs (Jiang *et al.*, 2016). However, a wide variety of autochthonous microorganisms that metabolise both LMW and HMW PAHs are well documented in the literatures, for example, few genera of microorganisms have been reported with capacity to degrade HMW PAHs whereas a large diversity of organisms with capacity to degrade LMW PAHs are reported (Juhasz and Naidu, 2000; Fernández-Luqueño *et al.*, 2011; Ghosal *et al.*, 2016).

Bioremediation is the productive use of biodegradative process to remove or detoxify pollutants concentration to a harmless state which are threat to public health (Thapa *et al.*, 2012) Basically, the goal of bioremediation is to transform organic pollutants into harmless metabolites or mineralize the pollutants into carbon dioxide and water (Alexander, 1999). Before bioremediation can take place, the following must be present: (i) a contaminant; (ii) suitable microorganisms; and (iii) an electron acceptor (Labana *et al.*, 2007; Latinwo and Agarry, 2015)). Bioremediation methods are considered to be more economical, cleaner and safer for treatment of PAHs contaminated sites (Sharma, 2021). The biology depends more often on the intrinsic metabolic efficiency of the actors (i.e., the microbes) to mineralize the pollutant. This green, cost-effective and viable science has proven effective for the remediation of PAHs and other organic contaminants in soils (surface and subsurface), groundwater, freshwater and marine ecosystems than most widely used traditional remediation techniques

such as chemical, physical and thermal methods. The traditional methods only transfer the contaminants to a pristine area without complete mineralisation (Megharaj *et al.*, 2011; Perelo, 2010). The conventional physicochemical treatment techniques commonly used in soil remediation includes soil washing and flushing, soil vapour extraction, solidification, dispersion, stabilization, vitrification, electrokinetic treatment, chemical oxidation or reduction, use of oil booms, excavation and off-site treatment (storage), land-filling, evaporation and incineration (Khan *et al.*, 2004; Gomes, 2012; Agarry and Latinwo, 2015; Koshlaf *et al.*, 2016). Presently, there is still no single bioremediation strategy that serve as a "*'silver bullet*" to restore polluted environments especially due to the nature and type of pollutant that may be present (Azubuike *et al.*, 2016). However, bioremediation is cost-effective and a green technological putative technique used to mitigate recalcitrant hydrocarbons in contaminated soil (Dzionek *et al.*, 2016).

Prior to a bioremediation project in a contaminated environment, identification of the problem, the selection criteria such as nature of the pollutant, degree and depth of pollution, type of environment, location, cost, environmental policies and regulations, duration of pollution and the best choice of remedial action should be considered as they determine the feasibility and success of the remediation (Juwarkar *et al.*, 2010; Azubuike *et al.*, 2016). Site characterization and *ex situ* treatability studies often give a mirror-image of the site before the remediation proceeds. More so, several physical, biochemical, matrix adjustment through provision of readily available nutrients, electron donors (energy sources), electron acceptors, environmental factors (e.g., oxygen, pH, nutrient concentrations, temperature), presence of the right microorganisms or seeding with competent microorganisms are amongst the major factors that speed up biodegradation as well determine the overall success of a remediation process (Labana *et al.*, 2007; Kim *et al.* 2014; Latinwo and Agarry, 2015).

1.5.1 Microbial action and mechanisms of PAHs biodegradation

Microorganisms have played essential roles in soil ecosystem functions, services and processes which include nutrient cycling, degradation of organic matter, stabilisation of soil structure and sustainable soil quality for plant growth (Gougoulias *et al.*, 2014). Most microbial communities, prokaryotes (bacteria and archaea) and eukaryotes (algae, protozoa, fungi and actinomycetes) are highly diverse, synergistic and capable of degrading large numbers of various organic compounds, including PAHs in soil (Ghosal *et al.*, 2016; Lang *et al.*, 2016; El-Sheekh and Mahmoud, 2017; Patel *et al.*, 2019). It has been observed that PAHs degradation in soils is dominated by bacterial and fungal strains (Sakshi and Haritash, 2020). Although bacteria are the most active agents of microbial degradation (Ghosal *et al.*, 2016), fungi and their strong oxidative enzymes are key players in degrading recalcitrant and xenobiotic chemicals such as PAHs (Fernández-Luqueño *et al.*, 2011). Bacterial/archaeal genera have been historically reported for their capacity to degrade PAHs (both LMW and HMW) which includes the species of *Pseudomonas*, *Sphingomonas*, *Sphingobium*, *Norcardia*, *Mycobacterium*, *Alcaligenes*, *Bacillus*, *Rhodococcus* and others (Fernández-Luqueño *et al.*, 2011; Haritash and Kaushik, 2009). The indigenous fungi which belongs to the phylum Ascomycota, with subphylum mucoromycota and basidiomycota are the dominant fungal phylum in hydrocarbons degradation in polluted environments (Bash *et al.*, 2015). Particularly, the white-rot fungi such as *Pleurotus ostreatus*, *Trametes versicolor*, *Bjerkandera adusta*, *Irpex lateus*, and *Phaerochaete chrysosporium* have played key roles in PAH-degradation in different soils (Quintella *et al.*, 2019; Gao *et al.*, 2010; Haritash and Kaushik, 2016).

Microbial degradation has been focused on the assessment of their intrinsic microbial activity and metabolic enzymatic function during PAH degradation (Pozdnyakova, 2012; Kadri *et al.*,

2017; Cao *et al.*, 2020). Researches have acknowledged enough and stimulated microbial population in harsh PAH-contaminated soils but the low response of PAH-degrading microorganisms could reduce PAH catabolism (Omoni *et al.*, 2020a) and their synergistic role with non-PAH degraders is also important in PAH metabolisation (Leahy and Colwell, 1990). The biodegradation of organic contaminants can involve a team of microbial consortium rather than a single organism because the consortia possess broad enzymic versatility for metabolisation of single or complex hydrocarbon mixtures (Olajire and Essien, 2014). The compounds are ultimately catabolized into simpler organic substrates using various pathways and subsequently used by different members of a microbial communities for complete biodegradation (Abbasian *et al.*, 2016). The ability of autochthonous microorganisms to adapt or resist sudden exposure to high concentrations of hydrocarbon pollutants is extremely important in effective microbial degradation. Through horizontal gene transfer some non-degrading microbes develop the intrinsic ability to degrade vast hydrocarbons in a contaminated system (Abbasian *et al.*, 2016). Also, it is well known that microorganisms (both prokaryotes and eukaryotes) can enhance their chances of survival by deploying battery of specific responses and mechanisms which generates genetic and phenotypic diversity to environmental signals such as sudden change in PAHs (Aertsen and Michiels, 2005; Fernández-Luqueño *et al.*, 2011).

1.5.2 *In situ* and *ex situ* bioremediation

Traditional remediation methods for hydrocarbons-contaminated soil, such as soil flushing, chemical and electro-chemical oxidation, electrokinetic separation, soil vapour extraction, solidification/stabilization and thermal treatments, are expensive and not sustainable because the pollutants only reduce in concentrations with incomplete mineralisation in the soil matrix

(Guarino *et al.*, 2017). *In situ* bioremediation is the use of microorganisms to detoxify or immobilise contaminants under natural conditions without any excavation of the medium while *ex situ* bioremediation focuses more on the reduction or elimination of the contaminant (mineralisation) without considering the abiotic factors that interplay in the field and the bio-efficiency of the native microorganisms to degrade the contaminant (Kästner and Miltner, 2016). However, *in situ* bioremediation is often preferred to *ex situ* bioremediation; however, both involved the microbial metabolism of the target contaminants. *In situ* bioremediation are cost-effective, less disturbance to the surrounding soil structure due to non-excavation of the contaminated media, although the process is limited to a particular depth (30-60cm) where microorganisms can attack pollutants within the soil matrix (Vidali, 2001). Bioremedial techniques that involve *ex situ* bioremediation allow modifications of the biological, chemical and physiochemical conditions of the contaminated soil for effective and efficient bioremediation (Azubuike *et al.*, 2016). *Ex situ* bioremediation does not require extensive preliminary study of soil/site contamination; thus, this approach is relatively short, less laborious and less expensive. Similarly, the behaviour and degradation of most organic contaminants in soils have been extensively studied using *ex situ* bioremediation because the process is easier to control and rapid. Furthermore, *in situ* bioremediation relies on the mass flow of limiting nutrients such as nitrogen and phosphorus and electron acceptors, and intrinsic environmental conditions for indigenous microbes for a target contaminant to be biodegraded in soil. Biostimulation and bioaugmentation are widely used bioremediation techniques for contaminated soils.

1.5.2.1 Biostimulation

This is one of the best and cheapest technologies for increasing the efficiency of bioremediation of soil and groundwater contaminated sites. Biostimulation is the addition of organic wastes (organic biostimulants), inorganic nutrients (nitrogen, phosphorus, sulphur, potassium, and carbon sources), oxygen (electron acceptor) and slow/fast releasing fertiliser to change the physico-chemical and biological properties of soil (Sakshi *et al.*, 2019). These nutrients are added to encourage indigenous microbial activities and metabolism of PAHs, as PAH-contaminated soils are naturally low or depleted of available nutrients due to contamination (Fernández-Luqueño *et al.*, 2008; Dzionek *et al.*, 2016). This strategy improves the potential of indigenous microorganisms (microbial biomass and activity) to synthesise the specific degrading enzymes required or capable for PAH biodegradation especially in nutrient-deficient soils. It can be applied in both *in situ* and *ex situ* bioremediation processes. Studies have shown increased or stimulated PAH dissipation after addition of inorganic nutrients to contaminated soils (Carmichael and Pfaender, 1997; Atagana *et al.*, 2003; Sun *et al.*, 2012; Kalantary *et al.*, 2014). In recent times, researchers have incorporated the use of green organic wastes from industry as organic nutrients for enhanced degradation of PAHs in soil (García-Delgado *et al.*, 2015; Lukić *et al.*, 2016; Sayara and Sánchez, 2020; Omoni *et al.*, 2020a) due to their cost-effectiveness, safety, and rich sources of nutrients, enzymes and organic matter compared to inorganic nutrients such as NPK fertiliser (Kästner and Miltner, 2016; Omoni *et al.*, 2020a).

1.5.2.2 Bioaugmentation

Bioaugmentation involves the addition of active microorganisms (autochthonous or allochthonous) that have the capacity to degrade target contaminant by supporting the

autochthonous or native microorganisms in the contaminated medium. These potentially active microorganisms can be supplied through organic substrates or addition of genetically engineered microbes (GEMs) (Pant *et al.*, 2021). However, the latter is restricted and regulated owing to environmental and public health safety. In many cases, the native contaminant degraders in soil may lack the intrinsic ability to degrade the organic compound, or the microbial population may not be sufficient to promote degradation or possible oligotrophic conditions; therefore, microorganisms are added through a process of bioaugmentation (Megharaj *et al.*, 2011; Dzionek *et al.*, 2016). PAH-degrading microorganisms make up only 10% of the total microbial population in soil, which often leads to a delay and eventually takes a longer time in the remediation process than expected (Dzionek *et al.*, 2016). Bioaugmentation has been effectively used for the degradation of PAHs in contaminated soils (Vásquez-Murrieta *et al.*, 2016; Haleyur *et al.*, 2019; Villaverde *et al.*, 2019; Forján *et al.*, 2020) and can be applied *in situ* or *ex situ*. However, the use of PAH-degrading microbes with known exposure history, adaptive status and longer chances of survival are important factors for a successful bioaugmentation process. Single microbial strain or cometabolic microbial consortium of different degrading abilities with genetic traits that provide selective advantages in a given environment can be employed during bioaugmentation process (Megharaj *et al.*, 2011). In theory, the introduced microbial bugs would not only attack the chemical pollutant but also generously transmit the relevant skills to native species (Shekhar, 2012). Although bioaugmentation with a single organism rather than microbial consortium may not be suitable for contaminated field soil with low intrinsic metabolic machinery which may have resulted from sudden changes to stresses and other prevailing physico-chemical factors such as temperature, pH, moisture content, nutrient and organic matter content, initial pollutant concentrations and additional carbon sources (Cycoń *et al.*, 2017).

1.5.3 Some factors that limit degradation of PAHs in soil

PAHs are stable in soil and are resistant to biological and chemical remediation; therefore, they are more persistent in comparison with other recalcitrant compounds. The rates and extents to which PAH degradation can occur in soil are determined by the interplay of several abiotic and biotic factors presented in Figure 5. These abiotic factors, sometimes referred to as environmental factors such as soil and PAH properties will primarily influence the behaviour of microorganisms and their metabolic capabilities for PAH degradation in contaminated soil. Microorganisms are the actors which control and determine the fate of PAH degradation in soil, irrespective of the main environmental drivers in the contaminated soils (Duran and Cravo-Laureau, 2016). However, the microbial transformation and degradation rate can be influenced by manipulating or controlling and optimizing the limiting factors for enhanced PAH biodegradation in soil.

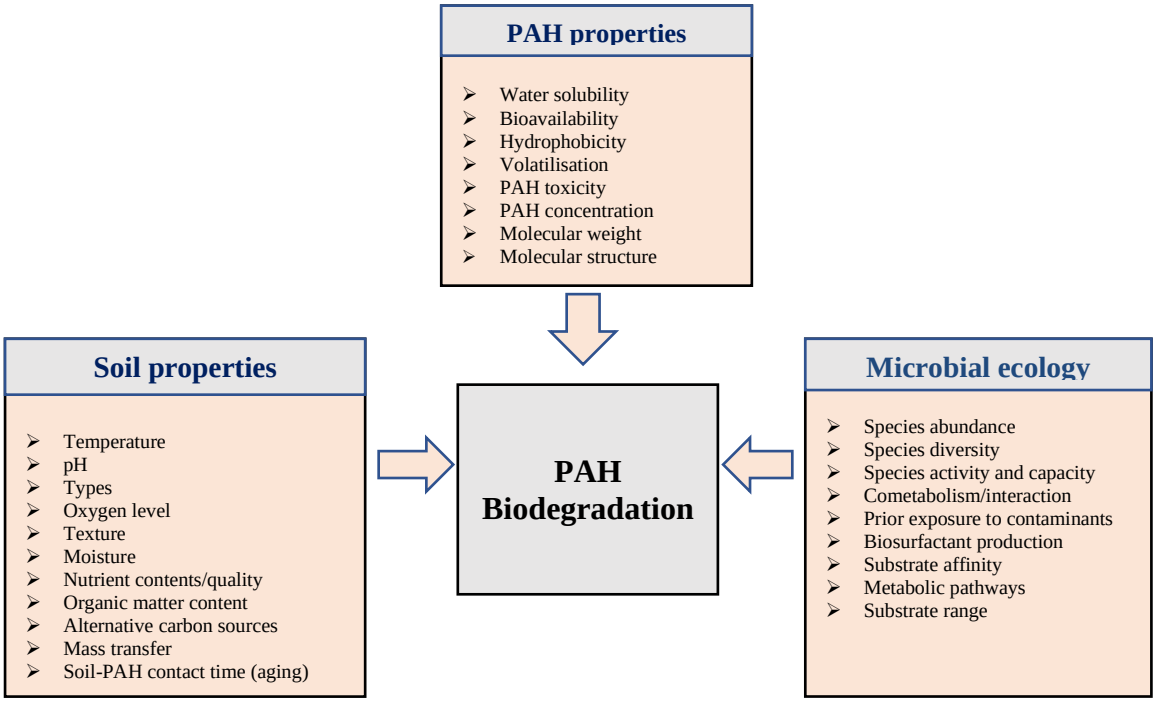


Figure 5. Factors influencing the biodegradation of PAHs in soil.

1.5.3.1 Bioavailability

Optimised bioremediation process of a hydrophobic contaminant depends on two key conditions, namely the bioavailable and bioaccessible fractions of the contaminant for microbial metabolism, uptake and degradation (Semple *et al.*, 2004). Bioavailability represents the fraction of the target chemical in soil that can be taken up or transformed by specific organisms whereas the bioaccessibility is the available fraction of a chemical that is able to move across the cellular surface membrane of an organism in soil and this chemical may be physically removed from the organism or only bioavailable after a period of time (Semple *et al.*, 2001; Semple *et al.*, 2003). The amount of a contaminant in the soil that is available depends on different factors including the physicochemical properties of the specific compound, the soil properties (e.g. soil type) and some environmental conditions (Leahy and Colwell, 1990; Semple *et al.*, 2003; Naidu *et al.*, 2008). For example, bioavailability of a contaminant in the environment can be reduced by its low aqueous solubility and strong interaction with the soil organic matter. The aqueous solubility of PAHs decreases with increasing molecular weights which in turn reduces the bioavailability of the compound. Other properties such as contact time between soil-contaminant interaction (soil ageing) also have a significant role in the bioavailability of PAHs in soil (Alexander, 1999; Luo *et al.*, 2012). The biological processes (activity and metabolism) taking place in the soil systems (Megharaj *et al.*, 2011; Ghosal *et al.*, 2016) are also limiting factors for subsequent and ultimate biodegradation in soil.

1.5.3.2 Temperature

Temperature is a major limiting factor that can determine microbial growth rates and metabolism, organic matter decomposition and changes in soil matrix, as well as the

physicochemical state (fate and behaviour) of the contaminants (Liu *et al.*, 2009; Leahy and Colwell, 1990). Temperature also determines the gas solubility and extent of degradation. However, the effect of temperature will also depend on the type of sorption involved. Temperature can cause a change in PAH solubility governed by aqueous-solubility interactions in soil. As soil temperature increases, the solubility of PAH increases and thus become more bioavailable for microbial metabolism (Hiller *et al.*, 2008). For example, Hiller *et al.* and co-workers reported that the extents of PAHs sorption decreased with increasing temperature from 4 ^{o}C to 27 ^{o}C in contaminated soil/sediment. Soil-water partition coefficient (Kow) can decrease with increasing temperature, which in turn increase the diffusion rate of PAHs through micro-, meso- and nanopores in soil (Haritash and Kaushik, 2009). For most PAH-degrading microorganisms, the ideal temperature for efficient PAH degradation ranges from 20–30 ^{o}C (Simarro *et al.*, 2013; Al-Thukair *et al.*, 2020). The growth rate of microbes increases with temperature but ceases beyond a certain maximum which could slow or halt microbial activities and denature enzymes (Gounot, 1991). At high temperature such as in the tropics, some organic contaminants can be biologically transformed into new compounds which are more toxic than the parent compounds (Ghosal *et al.*, 2016), while at lower temperature in the environment such as the Antarctica, microbial growth rate may be very slow in the rapid uptake and biodegradation of contaminant (Habib *et al.*, 2018).

1.5.3.3 Nutrients availability (Biostabilisation)

Nutrient's availability is by far the most important factor for microbial degradation of PAHs in soils. Major limiting nutrients such as carbon, nitrogen, and phosphorus are basically required by microbes for microbial growth, proliferation and metabolic activity. Most PAH-contaminated soils supplied with nutrients (either organic or inorganic) through several sources

such as direct supplementation/application including fresh and composted organic waste materials (Kästner and Miltner, 2016; Lukić *et al.*, 2016;; Omoni *et al.*, 2020a), inorganic nutrients (Zhu *et al.*, 2016; Nazifa *et al.*, 2018) can enhance PAH biodegradation. Microbial degradation of PAHs is reduced by limited amount of nutrients in the soil. The addition of nutrients to soils (biostimulation) which are poor or deficient in microbial nutrients is often required to stimulate indigenous microbial growth and metabolism and thus facilitate microbial degradation (Vásquez-Murrieta *et al.*, 2016). Also, biostimulation by nutrient addition to soils with high exposure rates of PAHs can lead to the adaptation of selective microbes with the capacity to survive and utilise the target contaminant (Patel *et al.*, 2020).

Carbon-to-nitrogen (C/N) ratios are vital for microbial growth, activity and metabolism where carbon is used as the energy source and nitrogen is used for the protein and nucleic acids synthesis (Mooshammer *et al.*, 2014), which are all efficient for biodegradation of organic compounds in soils (Teng *et al.*, 2010). Most studies have reported the enhanced microbial degradation of PAHs in soil with a C:N ratio of 100:10:1 (Smith *et al.*, 1997; Chen *et al.*, 2008; Teng *et al.*, 2010; Medaura *et al.*, 2021). Although, high nutrients levels can also negatively affect the degradation of PAHs in soils (Chaîneau *et al.*, 2005). For example, soil with high organic matter such as loamy soil may affect the bioavailability of PAHs due to increased sorption to soil (Nam *et al.*, 1998).

1.5.3.4 Soil pH

Soil pH can determine the optimal biodegradation of PAHs; pH values within 6.5–7.5 favour most microbial activity and function. Most natural environments (soil and groundwater) possess often near neutral or slightly alkaline pH range with high buffering capacity (Fu *et al.*,

2016), which is suitable for microbial growth, proliferation and degradation of environmental contaminants. This pH range is maintained by a natural buffering capacity that exists in most fertile native soils due to the presence of carbonates and other minerals (Robinson *et al.*, 2009). However, this buffering capacity is changed over time as a result of acidic by-products of microbial degradation. Majority of bacteria generally exhibit optimum growth at or nearly neutral pH whilst fungi are more resistant to soils with low pH as most soils are acidic throughout the world containing low fungi population when compared to bacterial population. In addition, soil pH can decrease with increase in soil-contaminant contact time, which may be due to increased production of more toxic metabolites from organic contaminant transformation (Ghosal *et al.*, 2016) and/or production of tannic and humic acids during lignin biodegradation (Clemente, 2001). Several studies have revealed that most acidic soils change to neutral after processed hydrocarbons contamination (Osuji and Nwoye, 2007; Trindade *et al.*, 2005; Maddela *et al.*, 2015). Although the effect of extreme pH on the biodegradation process might be variable, but these might have a negative effect by modifying the metabolic activity of the microbial populations capable of degrading the contaminants (Leahy and Colwell, 1990).

1.5.3.5 Moisture content

Soil water content is a critical factor for microbial activities, limiting both soil respiration and enzymatic activities and thus influencing microbial metabolic transformation of hydrophobic contaminants, including PAHs, in soils (Alexander, 1999). Microbial responses to soil moisture also depend on the extent of soil contamination by contaminants. In hydrocarbons contaminated soils, a decrease in soil moisture leads to limited substrates diffusion (e.g. nutrients and oxygen) which results to reduce physiological adaptation of microbes or shift the

soil microbial communities by eliminating specific microbial species which can potentially negatively affect bioremediation process or the microbial attack of organic contaminant (Xu-xiang *et al.*, 2006; Abdel-Shafy and Mansour, 2016). Also, soil water may not be available to microbes as it is absorbed or eventually tied up into organic matter components/matrix of soil. Thus, soil-water interaction can determine bioremediation endpoints. In general, low water content may decrease microbial activity in PAH contaminated soils, while high moisture levels may inhibit hydrocarbons' volatilization and degradation, especially in freshly contaminated soil, and could also create resistance to oxygen transfer to microbes for metabolism (Wu *et al.*, 2017). Normally, in most biodegradation strategy, the moisture content of the environment is either maintained optimally throughout or manipulated to favour degradation of microbes to render a pollutant innocuous.

1.5.3.6 Acclimation, microbial inocula and cometabolism

Acclimation (or adaptation) of the microorganisms to PAHs will lead to selective enrichment and an increase in plasmids (Sakshi and Haritash, 2020). In addition, the pre-exposure and microbial adaptation to PAH contaminant can determine the rates and extents of biodegradation (Patel *et al.*, 2020). Microbial survival rates and uptake of PAH can increase with increasing cells adaptation; however, sufficient microbial population must be present in contaminated soil for significant PAH degradation. The synergistic relationship among different microbial communities in soil helps the microbes to acclimatise and modify the changes thereby creating a suitable environment, resulting in increased microbial growth and survival (Sabra *et al.*, 2010). Many hydrophobic contaminants, including PAHs have been metabolized by synergistic microbial action through a process of cometabolic reaction. Cometabolism is a process of transformation of a non-growth promoting substrates (co-substrates) by microorganisms in the

presence of other transformable compounds or growth-promoting substrates provided as the only sources of carbon and energy. Additionally, these contaminants are fortuitously degraded by enzymes or cofactors produced during microbial metabolism of the growth-promoting compound (Hazen, 2010). Bioenergetically, this form of fortuitous metabolism is non-growth linked, yield organic products that can build up and yet carbon is not incorporated into typical cellular components (ends up as wastes). Often, the enzymes exist in the organism to start the degradation process, but biochemically do not complete the process either provide useful products.

1.5.3.7 Aging/soil-contaminant contact time

The length of time of HOCs in soil (soil-contaminant interaction) can influence their bioavailability/bioaccessibility and consequently determine their microbial biodegradation in contaminated soil (Semple *et al.*, 2007). The aging processes of PAHs are highly related to their sorption and sequestration in soils. PAH aging exhibits a biphasic behaviour in soils: (i) a rapid diffusion of PAH controlled by the pore distribution (meso-and micropore) and organic carbon composition and structures and (ii) a slow diffusion controlled by hard OC and pore distribution (Luo *et al.*, 2012) which can occur in weeks, months and years after contact with soil. Strongly bound PAHs (reversible or desorbable) fractions resulting from soil aging can limit the rates and extents of microbial degradation in soils (Riding *et al.*, 2013), while bonding increases with increasing SOM. Although, some studies have shown better rates of microbial sequestration of PAHs in soils amended with suitable rates of organic waste (Sigmund *et al.*, 2018; Omoni *et al.*, 2020a; Sayara and Sánchez, 2020).

1.6 Organic (lignocellulosic) waste materials in soil bioremediation with PAHs: sustainability and environmental clean-up.

In the past decade, microbial nutrients derived from lignocellulosic biomass (referred to as organic wastes) such as household, agricultural and industrial settings, have offered the most cost-effective, highly practical and eco-friendly method for bioremediation of hydrocarbon polluted and contaminated environment (Nwankwegu *et al.*, 2016). In addition, organic wastes have large stimulating biological, physical and chemical effects when they are amended to soil (Juárez *et al.*, 2013; García-Sánchez *et al.*, 2015). Often, microbial activity is stimulated through the addition of different wastes-derived organics as listed in Table 3. Organic amendments with lignocellulose are governed mainly by two mechanisms viz. adsorption of pollutants by organic matter or their degradation by microbes (Puglisi *et al.*, 2007).

Due to the effects of the lignocellulosic materials, as soil conditioner/improver after addition to soil, researchers have explored them in remediating soils contaminated with PAHs to enhance microbial catabolic and enzymatic activities, which are the key factors that governs and drives the degradation of PAH in soil. In addition, soil management practices such as the application of organic and inorganic amendments may influence the microbial biomass, activity, composition and structure of soil communities (García-Sánchez *et al.*, 2015; Heijboer *et al.*, 2016).

Table 3. Chemical composition of different lignocellulosic materials (Sánchez, 2009; Atila, 2019; Longanesi *et al.*, 2020; Coronado *et al.*, 2020)

Lignocellulosic by-products	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Banana waste	13.2	14.8	14
Barley straw	31–34	24–29	14–15
Bast fibre jute	45–53	18–21	21–26
Coffee pulp	35	46.3	18.8
Corn cobs	35–45	35–45	5–15
Coir pith	29	15	31
Flax straw	29	27	22
Fruit and vegetable wastes	7.2–43.6	4.3–33.5	15.3–69.4
Groundnut shell	36	19	30
Hardwood barks/chips	22–40	20–38	30–55
Pine	46	24	27
Poplar wood	35	17	26
Qat straw	31–37	27–38	16–19
Rye straw	33–35	27–30	16–19
Rice straw	25–35	20–30	10–15
Wheat straw	29–35	26–32	16–21
Saw dust	28–34	17–21	25–32
Softwood barks/chips	45–50	25–35	25–35
Sorted refuse	60	20	20
Spent mushroom compost	5.3–27.0	36.5–51.9	24–36.8
Sewage sludge compost	55.3	41	27.8
Spruce	47	22	29
Spent brewery grains	18.4–37.2	13.8–26.8	9.9–17.1
Sugarcane bagasse	32–44	25–35	19–24
Sweet sorghum bagasse	45	25	18
Wastepaper from chemical pulps	60–70	10–20	5–10

PAHs removal in soil amended with organic waste materials (lignocellulosic biomass) are presented in Table 4. The macro-and micro-nutrients supplied by organic materials can stimulate active and relevant microbial activity and positively influence microbial structure and diversity thereby increasing the microbial degradation of organic contaminants in soil (Koszel and Lorencowicz, 2015; Omoni *et al.*, 2020a,b). The positive effects of organic materials on

the soil physicochemical and biological properties (microbial and enzymatic) are well documented in the literatures (Fernández-Luqueño *et al.*, 2011; García-Delgado *et al.*, 2015). Biomaterials can acts as bulking agents and microbial biomass suppliers when added to soil; thereby enhancing the amounts of soluble carbon, nitrogen and phosphorus, as well as reducing soil sorptive sites and increasing mass transfer and mobility of contaminants to microbial cells for microbial uptake and degradation in the soil matrix (Nwankwegu *et al.*, 2016 Kästner and Miltner, 2016). Gaind *et al.* (2006) reported enhanced biological activity in soil amended with P-enriched organic materials compared to soils with inorganic materials amendments. Therefore, the management of organic waste materials for soil amendment has strengthened their environmental sustainability and performance as well as sources of vital nutrients especially in nutrient-depleted soils (Han *et al.*, 2017).

Table 4. Lignocellulosic wastes used in soil amendment during PAH biodegradation from contaminated soil.

Lignocellulosic by-products	PAH	Percentage degradation/removal	References
Corn cobs	15 EPA PAHs	16% (Total PAHs), 16% (benzo[a]pyrene), 44% (anthracene), decreasing order of % degradation: 3-ring > 4-ring > 5- and 6-ring	Wu *et al.*, 2008
Rice straw	2–6 rings PAHs	30.3%	Huang *et al.*, 2019
	Anthracene, pyrene, benzo[a]pyrene	>96% (anthracene and pyrene), >52–60% (benzo[a]pyrene)	Hamdi *et al.*, 2007
	Phenanthrene	64%	Elyamine and Hu, 2020
Wheat straw	13 PAHs	dibenz[a,h]anthracene (52%), anthracene (46%), Total 13 PAHs (23%)	García-Delgado *et al.*, 2015
Wheat stalk	16 PAHs	LMW (59.3%), HMW (24.3%), Total PAHs (33.3%)	Han *et al.*, 2017
Saw dust	2–6 rings PAHs	66.3%	Huang *et al.*, 2019
Spent mushroom compost	Phenanthrene	35 %	Omoni *et al.*, 2020b
	Napthalene, phenanthrene, benzo[a]pyrene, benzo[ghi]perylene	Napthalene (84%), phenanthrene (59%), benzo[a]anthracene, (68%), benzo[ghi]perylene (68%)	Lau *et al.*, 2003
	Phenanthrene	42.7%	Reid *et al.*, 2002
	16 PAHs	LMW (60.2%), HMW (31.2%), Total PAHs (38.7%)	Han *et al.*, 2017

Sewage sludge compost	Anthracene, pyrene, benzo[a]pyrene	>96% (anthracene and pyrene), >52–60% (benzo[a]pyrene)	Hamdi *et al.*, 2007
Spent brewery grains	Phenanthrene	48.7%	Omoni *et al.*, 2020b
Fruit and vegetable wastes	16 EPA PAHs	49%	Lukić *et al.*, 2016
Food and Kitchen waste	16 EPA PAHs	56%	Lukić *et al.*, 2016
Buffalo manure	16 EPA PAHs	49%	Lukić *et al.*, 2016
Pinewood biochar	13 PAHs	dibenz[a,h]anthracene (59%), fluorene (34%), Total 13 PAHs (14%)	García-Delgado *et al.*, 2015
Enhanced biochar	Phenanthrene	45.6%	Omoni *et al.*, 2020a
Non-enhanced biochar	Phenanthrene	32.6%	Omoni *et al.*, 2020a

1.6.1 Soil amendments with spent brewery grains

Brewery wastes (grains and effluents) are sources of microbial biomass, nitrogen, phosphorus and organic solids suppliers in soils. Bioremediation of PAH-contaminated soil by indigenous biota can be stimulated by the addition of SBG (Contreras-Ramos *et al.*, 2008; Abioye *et al.*, 2010; Omoni *et al.*, 2020b). Very few studies have reported the potential of SBG for soil remediation. In a study carried out by Abioye *et al.* (2010), biostimulation of soil artificially contaminated with used lubricating oil with SBG promoted biodegradation. Brewery wastes (grains and effluents) are sources of microbial biomass, nitrogen, phosphorus and organic solids suppliers in soils. Bioremediation of PAH-contaminated soil by indigenous biota can be stimulated by the addition of SBG (Contreras-Ramos *et al.*, 2008; Abioye *et al.*, 2010; Omoni *et al.*, 2020b). Very few studies have reported the potential of SBG for soil remediation. In a study carried out by Abioye *et al.* (2010), biostimulation of soil artificially contaminated with used lubricating soil with brewery spent grain, banana skin and spent mushroom compost removed total petroleum hydrocarbons by 68.73 %, 62.03 % and 57.01 % in amended soils over 84 d incubation, respectively. They also reported significant increase in hydrocarbons utilising bacteria especially in soil amended with SBG compared to the other amendments. In a recent study by Omoni *et al.* (2020b) found that application of SBG to soil influenced the biodegradation kinetics of phenanthrene in amended soil; their results showed that amended soil with 10% and 20% SBG decreased the lag phases as well as increased the rates and extents of mineralisation. The study also reported significant increases in the microbial numbers (bacteria and fungi) of the amended soils over a 100 d soil-PAH contact time. The authors concluded that large amount of organic materials should not be applied as soil additives during the remediation of PAHs. This is the only study that has reported PAH degradation in soil amended with SBG as at present. However, the appropriate amount of SBG application in

contaminated soil should also be considered and determined because high organic matter in soil may decrease the bioavailability of PAHs to PAH-degraders, hence a reduction on the rates of mineralisation, which can affect the extent of mineralisation over time (Omoni *et al.*, 2020b).

1.6.2 Soil amendments with compost

For remediation of contaminated sites, a more sustainable technique without additional degradation cost, as well as reduction in chemical toxicity or harm before complete biodegradation, is generally needed for remediation purposes. It is possible to use organic wastes as natural carriers for various microbial attachments in hydrocarbon polluted soil for bioremediation as well as an excellent biosorbent for bacteria and fungi attachment; however, depends on their biodegradability and availability for remediation (Dzionek *et al.*, 2016). Compost-mediated bioremediation arising from microbial nutrients enhancement derived from organic wastes by-products produced from household, agricultural and industrial settings, is increasingly considered as one of the most cost effective, eco-friendly and super-enrichment methods for bioremediation of organics in soil (Omoni *et al.*, 2015). This could be a suitable and viable alternative to chemical biostimulants such as surfactants and dispersants used in large-contaminated sites. Stable compost (compost stability) derived from the wastes organic matter (biosolids) and balanced soil:wastes amendments provide more microbial accessibility of the PAH contaminants which in turn promotes the biodegradation of PAHs in contaminated soil-compost mixture (Namkoong *et al.*, 2002; Sayara *et al.*, 2010; Mizwar *et al.*, 2016). Immature and unstable compost can reduce microbial activity and metabolism when added to contaminated soil. Generally, the increase in organic matter in soil as a result of compost addition does not only increased microbial activity but also sorb contaminants present in low concentrations as such reduce the bioavailability for degraders (Kästner and Miltner, 2016).

Compost addition or composting increases bound residue (nonextractable) formation of contaminants in PAHs contaminated soil which stimulates microbial activity and higher efficiency of bioremoval of the contaminants (McFarland and Qiu, 1995; Lukić *et al.*, 2016). Humification of organic matter in soil reduces organic contaminants' bioavailability due to the formation of pollutant-humic acid complexes. Although, dissolved or water-soluble humic and fulvic substances in compost can acts as carriers for PAH compounds thus increasing their solubilisation, bioavailability, desorption of hydrophobic pollutants to microbial cells with insignificant toxicity to soil (Plaza *et al.*, 2009; Smith *et al.*, 1997). Strong binding reactions of PAHs with humic acids in soils amended with fresh compost significantly limit their availability and accessibility to PAH-degrading microorganisms when compared to matured compost (Plaza *et al.*, 2009). Furthermore, soil matrices with high PAHs concentration above acceptable or background levels affect the indigenous degraders due to toxicity, and apparently, higher percentage may be sequestered to soil organic matter which can affect the degradation process. Therefore, such soils can be ameliorated by compost addition by acting as a modifier and creates an even distribution between sorption of organic contaminants and biodegradation.

The physicochemical properties and organic particulate nature of the compost can assist the soil native biota to recolonize the niches of the compost microbes and also desorption of the contaminants from the contaminated soil with subsequent sorption to the biodegradable solid organic matter present in the compost (Kästner and Miltner, 2016). Lignin-degrading fungal colonizers with their exoenzymes can be stimulated by lignocellulosic during composting process and may result in better degradation of HOCs with mass transfer limitations like HMW PAHs (Sakshi and Haritash, 2020). As with industrially available wastes, SMC produced from the growth of mushrooms such as *Pleurotus ostreatus*, *Lentinus edodes* or *Agaricus bisporus*

are excellent additives and extracellular-degrading enzymes (laccase, manganese peroxidase and lignin peroxidase) for recalcitrant organic pollutants such as PAHs, phenols, explosives and pesticides (Haritash and Kaushik, 2009; Kästner and Miltner, 2016). A metabolic memory of highly complex metabolic diversity and high functional redundancy of microorganisms are formed from composting processes and thus acts as super-augmentation with high reinforcement of the complete native microbial activity. On addition to biostimulation process, it can stimulate and increase soil abiotic conditions and intrinsic microbial metabolic processes in soil bioremediation (Kästner and Miltner, 2016)). The biodegradation efficiency of PAHs in amended soils with SMC is listed in Table 4.

1.6.3 Soil amendments with biochar

Biochar (referred to as pyrolyzed agricultural residues) are known to exhibit dual properties, namely sorption and biodegradation of organic contaminants in soil. Two mechanisms (biphasic model) have been proposed for biochar degradation: (i) structural alterations in the form of surface oxidation and (ii) degradation of carbon (C) by microorganisms (Anyika *et al.*, 2015). In soil amendments, biochars can influence not only the physicochemical and biological properties of soil (Zhang *et al.*, 2018; Sadegh-Zadeh *et al.*, 2018) as well as play key roles in the biodegradation of PAHs in soil (Ogbonnaya and Semple, 2013; Han *et al.*, 2017; Cao *et al.*, 2016; Heijboer *et al.*, 2016; Kong *et al.*, 2018; Omoni *et al.*, 2020a).

Biochar is a rich source of soil micro- and macronutrients due to their absorptive capacity in soil (Ayaz *et al.*, 2021). For example, freshly prepared biochar could discharge a significant amount of N ranging from 23 to 635 mg kg^{-1} and P ranging from 46 to 1664 mg kg^{-1} (Jiang *et al.*, 2019). In studies, the addition of various biochar in soil produced from pyrolyzed agro-

residues has proven to play a key role in the biogeochemical cycling of nutrients in soil. For example, sewage sludge biochar contains appreciable amounts of micro- and macronutrients required for soil fertility and in turn can increase the soil microbial activity after amendments (Zielińska *et al.*, 2015). The absorption of PAHs onto biochar has been established in literatures (Quilliam *et al.*, 2013; Moreno Jiménez *et al.*, 2018) and the phenomenon has also been confirmed to reduce the exposure, toxicity and associated risks of PAHs in soil (Chen and Yuan, 2011).

However, Kuśmierz *et al.* (2016) reported that the persistence of PAHs in acidic soil was observed and this led to increased PAH content in soil; however, a rapid loss of 3- to 4-ring PAHs from the biochar-amended soil was also observed. The efficiency of PAHs removal by some pyrolyzed organic waste materials can be found in Table 4. In Singh and Cowie (2014), biochar is known to stimulate native SOC mineralisation in a low-C clayey soil but that this effect decreases with time, possibly due to the depletion of labile SOC from initial positive priming, and/or stabilisation of SOC caused by biochar-induced organo-mineral interactions. Biochar addition to soil can also stimulate the enzyme activities in PAH-contaminated soil (Cao *et al.*, 2016; Oladele, 2019). However, this varies in terms of the different properties of the biochar (feedstock, pH, micro- and macro-nutrients content) as well as the different soil properties (Moreno Jiménez *et al.*, 2018). SOC can stimulate extracellular enzyme activities such as acid phosphatase, ligninolytic enzymes, and phenol oxidase in amended soil. (Brzostek *et al*., 2013). Also, soil amended with biochar has been reported by other researchers caused a decrease in the amount of substrates like OM due to mineralization or sorption, and this affected the secretion of some key enzymes in soil (Mierzwa-Hersztek *et al.*, 2016).

Studies on the effects of different biochars namely walnut shell, corn cob, corn stem and rice straw produced at three different temperature of 250 ^{o}C, 400 ^{o}C and 600^{o}C on the biodegradation of PAHs such as napthalene, phenanthrene, pyrene and chrysene in amended soils, respectively (Zhang *et al.*, 2018). In their study, they observed highest percentage degradation for napthalene (84%) and phenanthrene (50%) in amended soils with corn stem biochar produced at 400 ^{o}C, while the soils amended with walnut shell biochar produced at 400 ^{o}C had pyrene (40%) and chrysene (60%) degradation. In addition, they found that the biodegradation rate of chrysene in biochar-treated soils was generally higher than that of pyrene, which was attributed to the higher accessibility of chrysene to the biochars than pyrene in the biochar-treated soils because of its higher hydrophobicity (logKow) than pyrene. Furthermore, biochar is a potentially diverse niche for microorganisms, thus the application of biochar to soils may assist in the preservation and support of soil biodiversity and biotope for the micro and mesobiota in contaminated soils (Beesley *et al.*, 2011).

1.6.4 Other organic materials in soil amendments

Other organic waste materials that are also rich in nutrients and are used for soil amendments, such as straw, manure, fruit and vegetable wastes, food and kitchen wastes, saw dust, straws, and corn cobs have been reported in PAH biodegradation in soil. Also, the soils with amended with these waste materials have also been found to immobilize PAHs and reduce their negative effects on soil microbial populations and enzyme activities. They stimulate accessory enzymes in soil, including the β-glucosidase, phosphatase, aryl alcohol oxidase, glyoxal oxidase, and dehydrogenase. The management and disposal of these organic materials have become increasingly challenging due to their contribution to massive environmental pollution. Recently, a bioenergy residue referred to as anaerobic digestate produced from the anaerobic

digestion of biodegradable wastes and sewage sludge by microorganisms in the absence of oxygen in a controlled environment has been reported as rich nutrient and as soil conditioners improving the soil physical and chemical properties (Parra-Orobio *et al.*, 2021). In a study by Bianco *et al.* (2020), amendment of marine sediment with four PAH mixtures (anthracene, phenanthrene, pyrene and fluoranthene) with digestate, fresh organic fraction of solid municipal waste, and combination of micro-/macronutrients increased the efficiency of PAH degradation by 55% compared to the unamended control (12%). Ibeto *et al.* (2020) reported that the different anaerobic digestate fractions (whole, solid and liquid) amended to soils enhanced the biodegradation kinetics and soil physiochemical properties such as available N and P in phenanthrene contaminated soil. The biosafety of this organic nutrient is necessary as an agricultural fertiliser.

1.7 Role of lignin-degrading fungi in lignocellulose degradation

There are a large variety of food crops that are produced around the world today which generate enormous agricultural waste materials. This lignocellulosic biomass can serve as low-cost feed stocks for fuel, ethanol and other value-added commodity chemicals (Sharma and Arora, 2015). Most of these agro-residues (lignin-containing raw materials) have poor nutritive value due to the presence of higher lignin content and hemicellulose binding matrix which affects the accessibility of energy-rich polysaccharides (Sharma and Arora, 2015). The combination of hemicellulose and lignin forms a protective barrier around the cellulose (Figure 6), which must be removed before the hydrolysis of cellulose (Andlar *et al.*, 2018). The removal of lignin is important to increase the enzyme access to the hemicellulose and cellulose. Lignin is a very complicated polyphenolic polymer that is highly stable and heterogenous by nature and co-

exists with cellulose in softwoods and hardwoods. These characteristics influence their resistance to intracellular enzyme systems.

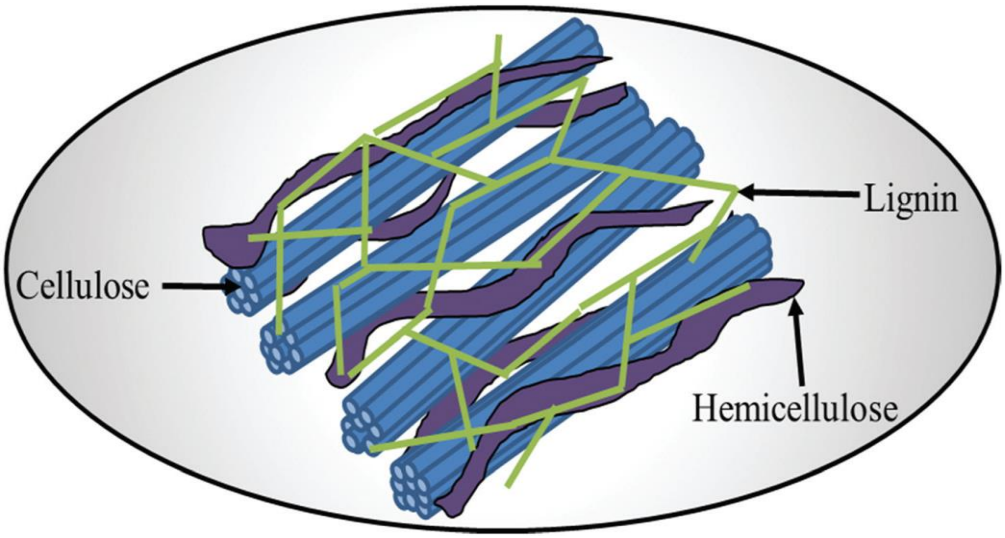


Figure 6. The basic structure of lignocellulosic components in most organic materials (adapted from Meereboer *et al.*, 2020).

Lignocellulosic biomass is composed of two carbohydrate polymers (cellulose and hemicellulose) and a non-carbohydrate phenolic complex heteropolymer (lignin). The degradability and digestibility of the biomass improve their potential; however, this varies with the structure of the lignin content (Janusz *et al.*, 2017). Fungi are better lignin-degraders because of their extracellular enzymatic system and hyphal penetration capacity. The wood-degrading fungi also referred to as lignocellulosic fungi are the predominant fungal species responsible for lignocellulose degradation. These groups of fungi consist of large phylogenetic and phenotypic diversity and required an adequate supply of oxygen for growth and respiration. Lignocellulose-degrading fungi employ a battery of degrading enzymes that are released into the environment to overcome the physical and chemical stability associated with lignocellulose

(Andlar *et al.*, 2018). The intracellular and extracellular systems are produced by the fungi; however, the extracellular enzymes are oxidative enzymes responsible for lignin and polysaccharide degradation. Overall, the mechanisms of wood degradation by these fungi rely on the oxidative enzymes, low pH and the production of organic acids (López *et al.*, 2017). The principal types of lignocellulose degrading fungi with different effects and degradation mechanisms are the white-rot fungi (WRF). Others include the soft-rot fungi (SRF) and brown-rot fungi (BRF). They produced extracellular ligninolytic enzymes system in lignocellulose biomass (Plácido and Capareda, 2015; Falade *et al.*, 2017).

1.7.1 White-rot fungi (Basidiomycetes)

White-rot fungi are heterogeneous group of basidiomycetes fungi making up more than 90% of all wood-rotting fungi (Hatakka and Hamell, 2010). WRFs can degrade all the major lignocellulose constituents (cellulose, hemicellulose and lignin) and are the most efficient lignin degraders in nature. The name was derived due to the white residue or bleached form left after wood degradation. They are well known as producers of lignin modifying enzymes (laccase, lignin peroxidase and manganese peroxidase), hemicellulases and cellulases (Sharma and Arora, 2015). Apart from extracellular ligninolytic enzymes, WRF also possesses intracellular cytochrome P-450 monooxygenase-epoxide hydrolase which synergises with the ligninolytic system in the general degradation mechanism of organic contaminants (Bezalel *et al.*, 1996; Cajthaml *et al.*, 2008). Cytochrome P-450 epoxide hydrolase is an enzymatic system present in all eukaryotic organisms. Ligninolytic enzymes are secreted by WRF during secondary metabolism. Some WRFs such as *Phanerochaete chrysosporium* genes encoding lignin-modifying enzymes are known to be regulated by nutrient limitation (nitrogen, carbon and carbohydrate), oxygen, and metal ions (trace, Mg^{2+} and Ca^{2+}) in culture media (Kumar and

Chandra, 2020), while the ligninolytic enzyme production of *Bjerkandera* sp. and *Pleurotus* sp. can be improved in N-sufficient conditions (Mäkelä *et al.*, 2002; Patrick *et al*., 2011). Some of the WRF capable of selective delignification of lignocellulose biomass include *Trametes versicolor*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Pleurotus eryngii*, *Stropharia coroilla*, *Pycnopus cinnabarinus*, *Dichomitus squalens*, *Coriolus versicolor*, *Cyathus stercoreus* and *Ceriporiopsis subvermispora*. Some white-rot fungi such as *C. subvermispora*, *Phlebia* spp., *Phellinus pini* and *Pleurotus* spp. can remove lignin from wood by preferentially attacking lignin more readily than hemicellulose and cellulose, leaving enriched cellulose. Other white-rot fungi such as *Trametes versicolor*, *Irpex lateus* and *Heterobasidion annosum* degrade the plant cell wall constituents simultaneously (Wong, 2009).

1.7.2 Lignin-degrading fungi and ligninolytic enzymes in soil bioremediation

Fungi require substrates such as cellulose or other carbon sources as a source of energy. Thus, carbon sources, including corn cobs, straw, sawdust, sugarcane bagasse among others (Table 3) can easily be used to enhance fungi degradation of PAHs in contaminated sites. Also, the branching filamentous mode of fungal growth allows for more efficient colonization and exploration in contaminated soil. One distinct advantage of fungi over bacterial systems is that they do not require preconditioning to the particular pollutant. Bacteria usually must be pre-exposed to a pollutant to allow the enzymes that degrade the pollutant to be induced. The pollutant also must be in a significant concentration, otherwise, induction of enzyme synthesis cannot occur; thus, there is a finite level to which bacteria can degrade pollutants (Asamudo *et al.*, 2005). Several species of lignin-degrading fungi such as the WRF have been extensively studied to efficiently degrade and mineralise PAHs especially those of the genera *Trametes*,

Bjerkandera, *Phanerochaete*, *Irpex* and *Pleurotus* as well as the hyphomycetes *Penicillium* and *Aspergillus* (Bezalel *et al.*, 1996; Gao *et al.*, 2010; Pozdnyakova, 2012; Ghosal *et al.*, 2016; Quintella *et al.*, 2019; Cao *et al.*, 2020). However, depending on their ligninolytic enzyme complexes, the degradative function of each strain can potentially differ (Pozdnyakova, 2012; Cao *et al.*, 2020). Most often, the ligninolytic enzymes are secreted and/or stimulated by these groups of fungi in the presence of lignocellulose. Lignin-modifying enzymes are able to delignify lignin-containing substrates through radical reactions and they are known to oxidase a wide range of organic compounds with structural similarity, including soil humic substances and organic contaminants (Hofrichter *et al.*, 1998; Tumeola and Hatakka, 2011). Besides the extracellular degradation pathway, the involvement of the intracellular cytochrome P-450 and the epoxide hydrolase system in the initial step in the pathways of PAH degradation by WRFs have been reported previously (Bezalel *et al.*, 1996; Ichinose *et al.*, 2013).

In a study carried out by Marchand *et al.* (2017), crude oil degradation on contaminated soil seeded with *Rhodococcus* sp., *Trichoderma tomentosum*, and *Fusarium oxysporum*, significantly degraded all four PAH compounds (anthracene, flourene, phenanthrene and pyrene) in a mixture. The authors showed that fungi species effectively remove more than 10%, 13%, 8%, and 17% of anthracene, phenanthrene, fluorene, and pyrene respectively after 49 days (Marchand *et al.*, 2017). Similarly, in a study by Andriani and Tachibana (2016), white-rot fungus *Bjerkandera adusta* SM46 under saline-alkaline stress condition was able to biotransform HMW benzo[a]pyrene as salinity increased to 20 g/l. Emuh (2010) observed in his study, that the crude oil and heavy metals present in contaminated soil are broken down and absorbed by mushroom hypha and mycelia through the secretion of enzymes into environmentally safe levels resulting in increased carbon (IV) oxide, water and biomass production. Zebulun *et al.* (2011) showed enhanced biodegradation during the decontamination

of anthracene-polluted soil through a white-rot fungus (*Pleurotus ostreatus*). The study reported that incubation time, level of contamination and fungal treatment influenced the rate of degradation of all levels of anthracene (76-89%) compared to control soil (33-51%). It was observed that the release of ligninolytic enzymes such as LiP, LAC and MnP by *P. ostreatus* is related to the degradation of anthracene in the polluted soil.

It has also been found that the presence of co-contaminants and mediators can influence PAH biodegradation. In addition, Bhattacharya *et al.* (2014) reported that benzo[*a*]pyrene was significantly degraded by *Pleurotus ostreatus* which was strongly influenced by the presence of heavy metal cations and mediators such as vanillin and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate). This study showed that a 15 mM concentration of copper (Cu) was found to be the best enhancer for benzo[*a*]pyrene degradation (74.2%). The extent of degradation was increased to 83.6 % when 5 mM of vanillin was included in the medium. Moreover, in a study carried out in the Republic of Korea, 150 taxonomically and physiologically diverse white-rot fungi were investigated for their performance on dye decolorization, gallic acid reaction, ligninolytic enzymes, and tolerance to four PAH compounds: phenanthrene, anthracene, fluoranthene, and pyrene. Among these fungi species, six isolates showed the highest (> 90%) tolerance to both individual PAH and mixed PAHs. In their study, *Peniophora incarnata* KUC8836 and *Phlebia brevispora* KUC9033 significantly degraded the four PAHs and can be considered as important fungi species for the degradation of xenobiotic compounds in environmental settings (Lee *et al.*, 2014).

1.8 Immobilisation and biological treatment of lignocellulosic waste materials for PAH biodegradation

Immobilised fungi and their biocatalytic ligninolytic enzyme systems (intracellular and extracellular) have potential applications in the bioremediation of HOCs, including the PAHs (Kumar and Chandra, 2020). It reduces the costs of bioremediation processes and enhances their degradation efficiency. Fungal cell or ligninolytic enzyme immobilisation offers advantages over free cells, and these include protection of mycelial cells against shear force, increase cells/enzymes survival during storage, maintenance of activities as well as stability over varying pH, temperatures and incubation periods by providing a more suitable environment for the fungal cells and its enzymes (Chakraborty *et al.*, 2016). Other advantages include reduction in the risk of genetic mutations, multiple uses of the enzymes, increased tolerance of the cells/enzymes to high pollutant concentration and toxic pollutants and increased resistance to environmental conditions such as the presence of heavy metals (Homaei *et al.*, 2013; Dzionek *et al.*, 2016).

Immobilisation is defined as a technique to limit the mobility of microbial cells or their enzymes with simultaneous preservation of their stability, viability and catalytic potentials (Dzionek *et al.*, 2016). The fungi or their enzymes can be immobilised in a variety of solid support materials such as lignocellulose-containing materials as presented in Table 3 and Table 5. These support materials are biodegradable, hydrophilic, biocompatible, inexpensive, and high surface porosity (Dzionek *et al.*, 2016). The process involves the use of the intrinsic capacity of the microorganisms to form biofilms on the surface of the various materials. It is well known that these lignocellulosic support materials contributed to improved fungal metabolism by inducing their ligninolytic enzyme for the degradation of different organic

compounds in contaminated soils (Ghosal *et al.*, 2016; Kumar and Chandra, 2020). The most extensively studied and most effective group of fungi employed for immobilisation and biological pre-treatment of lignocellulosic materials are the white-rot fungi. Several studies have been carried out to investigate the biodegradation of various organic compounds in the environment by fungi immobilised on lignocellulosic materials (Mohammadi and Nasernejad, 2009; Arum Sari *et al.*, 2014; Kheirkhah *et al.*, 2020) and fungal pretreated lignocellulose materials (Dzul-Puc *et al.*, 2005; Rubilar *et al.*, 2007; Arum Sari *et al.*, 2014). Table 5 outlined some lignocellulosic materials used for PAH mineralisation and the ligninolytics secreted in amended soils. This solid-state fermentation (SSF) promotes the excellent growth of fungi and boosts the enzyme activity by providing nutrients to the fungi (Koyani and Rajput, 2015).

However, several pre-treatment methodologies have been employed to break down the chemical composition and structure of lignocellulosic biomass residues (Baruah *et al.*, 2018). However, biological pre-treatment methods have some advantages over other pre-treatment methods, such as mechanical, chemical and thermal methods which are costly due to high energy input and the formation of toxic inhibitory products such as acetic, furural and phenolic acids, as well as the high solvent cost associated with chemical pre-treatment strategy (Ramarajan and Manohar, 2017). Therefore, the biological pre-treatment processes are more economically viable, superior and eco-friendly compared to the other pre-treatment techniques (Isroi *et al.*, 2011; Wagner *et al.*, 2018). Biological pre-treatment processes represent promising approaches to the removal of lignin from the waste materials while increasing enzymatic hydrolysis of the hemicellulosic and cellulosic contents to monomeric sugars such as xylose, arabinose, mannose, glucose and galactose, the readily metabolizable carbon source for microbial growth and metabolism.

Table 5. PAH degradation in soils amended with lignocellulosic waste materials.

Phylum/type of rot fungus	Lignocellulose support	Type of treatment	Ligninolytic enzyme detected	Source/PAH	Time (days)	Summary (PAH dissipation/efficiency)	References
Trametes versicolor	Wheat straw/Wheat braw	Biological pre-treatment	Lacasse and MnP	4–5 rings	60 d	The extents of degradation were higher for fluoranthene and pyrene higher which ranged from 63–68% and 59–83%, respectively.	Lladó *et al.*, 2013.
Lentinus trigrinus	Wheat straw/Wheat braw	Biological pre-treatment	Lacasse and MnP	4–5 rings	60 d	The extents of degradation were higher for fluoranthene and pyrene higher which ranged from 63–68% and 59–83%, respectively.	Lladó *et al.*, 2013.
Pleurotus ostreatus	Mushroom cultivation substrate	Biological pre-treatment	Not measured	15 EPA PAHs	60-d	32.9% dissipation of the 15 studied PAHs was observed in amended microcosms. Anthracene, benzo(a)pyrene and benzo(a)anthracene are the most degradable PAHs with >60% removal observed.	Li *et al.*, 2012.
	Chopped wheat braw	Immobilisation	Not measured	13 PAHs	60 d	Most effective in degrading 3-ring PAHs (fluorene, phenanthrene and anthracene) and the moderately degradable 4-ring PAH (fluoranthene and pyrene). Members with ≥ 5-rings (benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[a]pyrene were degraded between 32.8–85.2%	Covino *et al.*, 2010.
	Wheat straw	Immobilisation	LiP, MnP	13 PAHs	42 d	73% of the total 13 PAHs was degraded. At 21 d, dibenz[a,h]anthracene (75%) followed by fluorene (70%) showed the greatest	García-Delgado *et al.*, 2015.

						extents of degradation, while 94% and 81% of dibenz[a,h]anthracene and benzo[a]pyrene were removed after 42 d incubation.	
Phanerochaete chrysosporium	Sugarcane bagasse	Biological pre-treatment	Not measured	Benzo(a)pyrene	5 d and 10 d	Benzo(a)pyrene removed 68% at 5 d incubation	Dzul-Pul *et al.*, 2005
	Sugarcane bagasse	Immobilisation	MnP	Anthracene	10 d	84% anthracene was removed from shaken culture compared to free cell (54%) after 7-d incubation	Mohammadi and Nesernejad, 2009
	Pine sawdust	Biological pre-treatment	Not measured	Benzo(a)pyrene	5 d and 10 d	PS removed 74% PAH at 10 d incubation	Dzul-Pul *et al.*, 2005
Phanerochaete velutina	Composted Green wastes	Biological pre-treatment	LAC, MnP	16 EPA PAHs	30 d	Immobilised fungus was able to degrade 96% of 4 rings PAHs and 39% of 5- and 6-ringds PAHs.	Winquist *et al.*, 2014.
Bjerkandera adusta	Rice straw	Immobilisation	LAC, LiP, MnP	Naphthalene, phenanthrene, chrysene, benzo(a)pyrene	30 d	Degradation of LWM-PAHs were greatest: naphthalene (94%), phenanthrene (70%) while chrysene (55%), benzo[a]pyrene (63%) of HMW-PAHs were degraded after 30 d. Mean degradation in the order of naphthalene > phenanthrene > benzo(a)pyrene > chrysene.	Andriani and Tachibana, 2016.
Dichomitus squalens	Chopped wheat braw	Immobilisation	Not measured	13 PAHs	60 d	17.1 % and 61.2% anthracene and phenanthrene were degraded	Covino *et al.*, 2010.
	Ground corncobs	Immobilisation	Not measured	13 PAHs	60 d	15.9 % and 68.1% anthracene and phenanthrene were degraded	Covino *et al.*, 2010.
Penicillium frequentans	Sugarcane bagasse	Biological pre-treatment	Not measured	Phenanthrene	17 d	52% of phenanthrene was removed after 17 d with 20% oxygen	Meléndez-Estrada *et al.* (2006).

Also, apart from lignocellulosic materials, other solid carriers used in immobilisation include both natural (chitosan, alginate, κ-carrageenan, loofah sponge, tezontle) and synthetic (polyvinyl alcohol, polyurethane, palygorskite, montmorillonite, hydromica, porous glass polypropylene, polystyrene porous porcelain) carriers used in bioremediation (Kourkoutas *et al.*, 2004; Dzionek *et al.*, 2016). Different immobilisation techniques are employed for these carriers for the bioremediation of contaminated matrices: adsorption, encapsulation, electrostatic or covalent bonding, flocculation (natural and artificial), and entrapment (Figure 7). Adsorption of microbial cells and enzymes to surface membranes such as lignocellulose materials is the easiest and commonest method used in bioremediation processes owing to its cost-effectiveness, rapid process, simple application, and greener strategy.

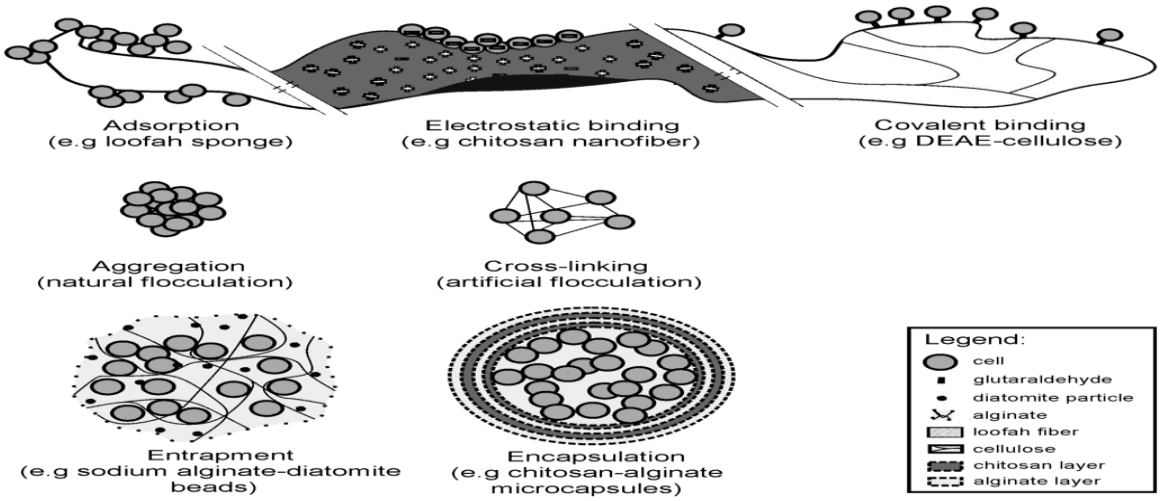


Figure 7. Methods of immobilisation (adapted from Dzionek *et al.*, 2016).

1.9 The aims and objectives of the thesis

The thesis investigated the impact of organic amendments and fungal treatments of organic materials on the biodegradation of phenanthrene in soil and in particular the study of the biological treatment of spent brewery grains. It was hypothesised that the addition of organic materials and fungal treatments of these materials can potentially improve the soil properties by stimulating the physicochemical and biological conditions of soil and consequently, enhance the degradation of PAHs in contaminated soil.

This thesis incorporates available literature, methodological and several scientific/experimental papers which were achieved through the following objectives:

- Review available literatures on the biodegradation of polycyclic aromatic hydrocarbons in soil: fates, treatments and challenges to successful remediation.
- Summarise the effects of organic waste materials and fungal delignification techniques for the enhanced remediation of polycyclic aromatic hydrocarbons in soil.
- Evaluate the impact of various organic waste materials on the development of ^{14}C-phenanthrene catabolism and microbial activities in soil over time under different soil:organic waste regimes using respirometric soil slurry assay.
- Investigate the effect of two biological (fungi) treatment techniques (immobilisation and pre-treatment) for organic waste materials on the physicochemical and biological (enzymes and microbial activities) properties and biodegradability of ^{14}C-phenanthrene in soils over time with different white-rot basidiomycetes.
- Examine the effect of different nitrogen amendments on the mycodegradation of ^{14}C-phenanthrene using several strains of endophytic fungi isolated from the organic waste materials amended soils.

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Chapter 2

Summary of publications

Chapter 3 has been published as: Omoni, V.T., Lag-Brotons, A.J., and Semple, K.T. (2020). Impact of organic amendments on the development of phenanthrene catabolism in soil. *International Biodeterioration and Biodegradation*, 151: 104991.

The influence of increasing waste-to-soil ratios (1:10, 1:5, 1:2, 1:1 & 2:1) on the biodegradation of ^{14}C-phenanthrene from a PAH contaminated soil using two organic materials- spent brewery grains (SBG) and spent mushroom compost (SMC) were examined. Soils amended with smaller amounts (1:10 & 1:5) of the two organic wastes (SBG and SMC) significantly reduced the length of the lag phases, resulted in faster rates and greatest levels of phenanthrene mineralisation in amended soils compared to the larger dose rates (1:1, 1:2 & 2:1) and control soils. Evolution of $^{14}CO_2$ was significantly higher in soil amended with either SBG or SMC throughout this study. Aging the amended soils significantly reduced the lag phases and increased rates and extents of mineralization in the phenanthrene-contaminated soil. The microbial numbers increased in all of the amended soils but phenanthrene-degrading numbers in most amended soils did not correlate with the rates and extents of ^{14}C-phenanthrene mineralization. It was noted that apart from the heterotrophs, phenanthrene- degrading bacteria did not proliferate in the soils containing larger amounts of SBG (2:1); this result was similar to those observed for control soil. Generally, SGB-amended soils showed more efficient biostimulators than SMC as observed in this study. Results from the investigation suggest that a higher degradation of phenanthrene and stimulated microbial activity in contaminated soil might be achieved by amending soil with appropriate and lower amount of organic waste materials and there two organic wastes should be considered as nutrient supplements during bioremediation of contaminated soil.

Chapter 4 has been published as: Omoni, V.T., Baidoo, P.K., Fagbohungbe, M.O., and Semple, K.T. (2020). The impact of enhanced and non-enhanced biochars on the catabolism of ^{14}c-phenanthrene in soil. *Environmental Technology and Innovation*, 20: 101146.

In this study, the effect of different concentrations (0, 0.01, 0.1, 0.2, 0.5 and 1.0%) of the pyrolyzed organic materials- enhanced (EBioC) and non-enhanced biochars (NEbioC) on the catabolism of ^{14}C-phenanthrene in soil was investigated. Higher extents of mineralisation were found in soils amended with biochar amounts 0.01%, 0.1% and 0.2%, the EBioC- as compared to the NEbioC-amended soils. Generally, the smaller amendments (0.1%, 0.2%, 0.01%) in both types of biochar and increasing soil-phenanthrene contact time influenced the lag phase, fastest rates and extents of ^{14}C-phenanthrene mineralisation. The results showed that after 100 d of soil incubation, the addition of 0.5% and 1% EbioC and NEbioC increased the lag phases, reduced the rates and extents of ^{14}C-phenanthrene in amended soil. While soil amendments with both EbioC and NEbioC increased the soil microbial populations; however, it was found that the phenanthrene-degrading microbial populations declined as soil contact time increased especially for larger amounts of both biochar (0.5 % and 1%). In general, soils amended with EbioC showed higher mineralisation and microbial activity than the NEbioC-amended soils. A significant positive correlation was recorded between the phenanthrene degrading fungi with both fastest rates and total extents of ^{14}C-phenanthrene mineralised in 0.2% EbioC-amended soil. For NEbioC-amended to soils, 0.01% and 1.0% amounts showed strong positive correlations between ^{14}C-phenanthrene-degrading fungi and total extents of ^{14}C-phenanthrene mineralisation, respectively. Phenanthrene-degrading bacteria CFUs did not show positive correlation with kinetics of mineralisation. Finding from this study reveals the importance of biochar type and amount that can promote PAH biodegradation. Also, the study suggests that

these two biochar types can employed in contaminated soils to reduce either the risk associated with PAHs or PAH mobility and bioaccessibility.

Chapter 5 has been accepted for publication as: Omoni, V.T., Lag-Brotons, A.J., Ibeto, C.N., and Semple, K.T. (2020). Effects of biological pre-treatment of lignocellulosic waste with white-rot fungi on the stimulation of ^{14}C-phenanthrene catabolism in soils. *International Biodeterioration and Biodegradation. In press.*

In this study, the impact of lignocellulosic material (spent brewery grains) pre-treated with five white-rot fungi (*Phanerochaete chrysosporium, Trametes versicolor*, *Irpex lateus*, *Pleurotus ostreatus*, and *Bjerkandera adusta*) on the catabolic evolution of ^{14}C-phenanthrene was investigated. At the same time, changes in the ligninolytic (laccase, lignin peroxidase and manganese peroxidase), non-ligninolytic (β-glucosidase and phosphatase) enzymatic and microbial activities were also assessed. The results showed higher mineralisation of ^{14}C-phenanthrene, stimulated enzyme activities, and higher microbial population in all amended soils. Results showed enhanced mineralisation of ^{14}C-phenanthrene mineralisation in most amended soils with fungal pre-treated SBG than in the unamended control soils. Generally, shorter lag phases, higher rates and greater extents of mineralisation were found in amended soils with fungal pre-treated SBG with the following trend *T. versicolor* > *B. adusta* > *P. chrysosporium* = *P. ostreatus* > *I. lateus*. More so, the white-rot fungi *T. versicolor* and *B. adusta* are more efficient degraders of the PAH in the soil. In addition, the extents of mineralisation generally reduced as levels of ligninolytic enzyme decreased, while the non-ligninolytic enzymes increased with soil-PAH contact time in all amendment conditions. However, in most cases *T. versicolor* followed by *B. adusta* and *P. chrysosporium* displayed higher levels of soil enzyme activities (ligninolytic and non-ligninolytic) compared to their

counterpart strains and control soil. Overall results suggest biological pre-treatment of waste materials as a promising approach to increase the bioactivities and biodegradation of organic pollutants during *in situ* remediations of contaminated soil.

Chapter 6 has been submitted for publication as: Omoni, V.T., Ibeto, C.N., Lag-Brotons, A.J., Bankole, P.O., and Semple, K.T. (2020). Impact of white-rot fungi immobilised on lignocellulosic waste on the biodegradation of ^{14}C-phenanthrene in contaminated soils. *Science of the Total Environment. Under review*.

Soil amendments with different immobilised white-rot fungi (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Irpex lateus*, *Pleurotus ostreatus*, and *Bjerkandera adusta*) on lignocellulosic waste (spent brewery grains) for the enhanced catabolism of ^{14}C-phenanthrene in contaminated soil was investigated. The soil physiochemical and biological (enzymatic and microbial) properties after SGB-immobilised fungi amendment were also examined. From this study, there was greater reduction in the lag phase as well higher rate of mineralisation in soil amended with *Trametes versicolor* compared to the other fungal soil treatments and unamended (control) soil. However, phenanthrene biodegradation was generally higher in white-rot *Pleurotus ostreatus* and *Phanerochaete chrysosporium* compared to the other soil conditions and control. Data showed the carbon-to nitrogen (C/N) ratio significantly increased in most amendment conditions and at most time points (1 d and 25 d) compared to the unamended (control) soil. SBG-immobilised *P. chrysosporium* increased the soil C/N ratio compared to the other soil treatments. However, significant decrease in the C/N ratios in all amended soils were noticeable from 50 d onwards. Also, soil pH decreased over time in amended soils but remain unchanged with *T. versicolor* amendment. Greatest extent of mineralisation was found at soil C/N ratio of 12.9 and pH 6.3 in amended soil. All amendments

showed higher enzyme activities (ligninolytic and non-ligninolytic enzymes) compared to control soil. The ligninolytic enzyme activities were higher in *P. ostreatus*-amended soil while these enzymes were positively correlated with the extents of $^{14}CO_2$ mineralised in all WRF-amended soils. The manganese peroxidase activities were generally higher in SBG-immobilised WRF-amended soils than the other ligninolytic enzymes examined. However, compared to all the other enzymes assayed, the non-ligninolytic phosphatase activity was significantly higher in all amended soils. Microbial numbers increased in all amended soils while PAH-degrading fungal numbers increased with increased soil-PAH contact time. For *P. chrysosporium, B. adusta* and *T. versicolor*- amended soils, significant positive correlations were observed between the phenanthrene-degrading fungal numbers with fastest rates and with overall extents of ^{14}C-phenanthrene mineralisation. The main findings of this study suggest that the investigated WRFs are good lignocellulosic immobilisers, and this technique can be applied as cheap sustainable remedial approach for the reclamation of hydrophobic hydrocarbon contaminated soil.

Chapter 7 intended for submission as: Omoni, V.T., Nwosu, T.F-X., Bankole, P.O., Onyeri, C.A., Ojo, S.A., and Semple, K.T. (2021). The impact of different nitrogen amendments on the biodegradation of ^{14}C-phenanthrene by endophytic fungal strains in liquid culture. *Submitted to Biodegradation*

In this study, the catabolic evolution (^{14}C-phenanthrene to $^{14}CO_2$) of five selected and screened indigenous endophytic fungal strains: *Fusarium* sp. (KTS01), *Trichoderma harzianum* (LAN03), *Fusarium oxysporum* (KTS02), *Fusarium oxysporum* (LAN04), and *Clonostachys rosea* (KTS05) under different nitrogen (N) amendments were examined. A preliminary experiment was performed to evaluate the abilities of the five fungal species which were

isolated from pristine soil to grow on various concentration of phenanthrene without a source of N as well their ability to effectively grow, colonise and penetrate lignocellulosic biomass (spent brewery grains). The fungal strains were identified using fungi universal specific primers (ITS1 and ITS4). Different carbon/nitrogen ratios (C:N ratios of 10, 20, and 30 to 1) and nitrogen sources (urea, malt extract and ammonium nitrate) in both static and agitated liquid media were used as N amendments for the mineralisation of phenanthrene. The results generally showed that there were variations in the amounts of ^{14}C-phenanthene mineralisation for most of the fungal strains under all N amendments as well in both static and agitated cultures. The results revealed that the identified fungal isolates have between 99.8 to 99.9% match with their close relatives retrieved from NCBI database and are members of endophytic ascomycetes. Generally, in both static and agitated condition significantly shorter lag phases, higher feaster rates and greater extents of mineralisation were found mostly in cultures *Clonostachys rosea* KTS05 and *Fusarium* sp. KTS01 amended with C/N ratio of 10:1 compared to the other fungal strains and C/N ratios. However, *Fusarium* sp. KTS02 showed significantly faster rate than *Fusarium* sp. KTS01 in the agitated culture with same C/N ratio. It was noted that there were faster rates of mineralisation in all static cultures with fungal strain amended with C/N than agitated culture conditions. In contrast, static and agitated culture conditions for most fungal strains resulted in faster rates of mineralisation in urea and malt extract, respectively. The seeded fungal strains *Clonostachys rosea* KTS05 and *Trichoderma harzianum* LAN03 showed greater phenanthrene mineralisation after N source amendments in static and agitated cultures, respectively, particularly with malt extract. The findings suggest that the endophytic fungal strains can be used for organic materials pre-treatment and potential PAH-degrading strains in bioremediation project.

Appendix

The following research was also carried with other co-authors and published as part of the research work developed in the course of my PhD:

Ibeto, C., Omoni, V., Fagbohungbe, M., Semple, K., 2020. Impact of digestate and its fractions on mineralization of ^{14}C-phenanthrene in aged soil. Ecotoxicology and Environmental Safety, 195, 110482.

This research investigated the impact of digestate (by-products or residue from anaerobic digestion of organic materials) and its fractions- whole, solid and liquid digestate on the catabolism of phenanthrene in contaminated soil. Other parameters such as water-soluble nitrogen, phosphorus, total (organic and inorganic) carbon and microbial numbers were also examined in amended soils. The results showed that whole digestate had greater influence on the soil fertility, microbial numbers and the biodegradation kinetics in amended soil compared to the other treatments. In addition, phenanthrene mineralisation was also linked to the amount of available nitrate and phosphate levels in soil. The non-phenanthrene degraders significantly contributed to the catabolic degradation of phenanthrene in amended soil. Findings suggest that the different digestate fractions especially whole digestate should further be exploited as a low cost, and sustainable treatment in soil contaminated with PAHs.

Chapter 3

Impact of Organic Amendments on the Development of Phenanthrene Catabolism in Soil

Impact of Organic Amendments on the Development of Phenanthrene Catabolism in Soil

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3.1 Abstract

This study investigated the impact of spent brewery grains and spent mushroom compost on the development of phenanthrene biodegradation in soil. Two aspects were considered: (i) the influence of increasing waste-to-soil ratios (1:10, 1:5, 1:2, 1:1 & 2:1) and (ii) the impact of soil-PAH contact time (1 – 100 d). Biodegradation was quantified by measuring changes in the lag phase, the fastest rates and extents of mineralization of ^{14}C-phenanthrene, as well as changes in the number of total heterotrophic and phenanthrene degrading bacteria and fungi. The amendment of smaller amounts of the wastes (1:10 & 1:5) resulted in greatest levels of biodegradation. Microbial numbers increased in all of the amended soils but phenanthrene-degrading numbers in most amended soils did not correlate with the rates and extents of ^{14}C-phenanthrene mineralization. This investigation highlighted the value of waste organic materials as nutrient sources to stimulate microbial degradation of contaminants in soil.

Keywords Spent brewery grains, spent mushroom compost**,** phenanthrene, biodegradation, soil

3.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants of major concern in the environment, being potentially harmful to human health due to their carcinogenicity, teratogenicity and mutagenicity potential (European Commission DG Environment, 2013). They are produced primarily through three main processes: biogenic (or diagenetic), petrogenic and pyrogenic (Gennadiev *et al.*, 2015). Both natural and anthropogenic sources can occur through petrogenic and pyrogenic processes.

Pyrogenic sources are formed from incomplete combustion of fossil fuels and biomass (petroleum, wood, coal and related products), which are the major routes of PAHs contamination in the environment compared to the natural (petrogenic) sources (Gennadiev *et al.*, 2015). Thus, making these hydrophobic organic contaminants ubiquitous in the environment (Akpor *et al.*, 2007), and recalcitrant, being composed by two or more fused aromatic rings (Ogbonnaya *et al.*, 2017). Physico-chemical properties of PAHs such as molecular size, solid-liquid partition (Kd) and organic carbon-water partition coefficients (Koc) (Cerniglia, 1993), influence their biodegradability, persistence, and recalcitrance to microbial attack in the environment. In terms of mobility in the environment, properties such as hydrophobicity, lipophilicity, and low water solubility are also important (Semple *et al.*, 2003).

Soils, sediments and other ubiquitous components such as microplastics can act as sinks for PAHs in the environment through sorption to soil mineral and organic fractions (Xiao *et al.*, 2014; Fries and Zarfl, 2012; Lee *et al.*, 2014). This influences the mobility, bioavailability and biodegradation and, as a result, may influence the persistence of these contaminants (Riding *et*

al., 2013). Biodegradation is a major mechanism for the removal of organic contaminants in soils and is dependent on the interaction between hydrocarbonoclastic microorganisms (hydrocarbon degraders) and their surrounding environment; in particular, properties associated with the contaminants (concentration, toxicity, mobility, bioavailability) and nutrient cycling (availability and degrading enzyme presence) (Leahy and Colwell, 1990; Das and Chandran, 2011). Related to the latter aspect, the efficiency of PAH biodegradation may be influenced by low nutrient and organic carbon concentrations in contaminated soil (Zhang *et al.*, 2012). In general terms, the catabolic potential of microorganisms to detoxify and degrade hydrocarbons depends largely on the amount of nutrients available for microbial metabolism (Azubuike *et al.*, 2016). Consequently, ensuring appropriate nutrient supply for microorganism activity (catabolism) has been a recurrent strategy for the remediation of hydrocarbon-polluted sites.

Nutrients provided by waste streams applied to land could offer a sustainable approach to resolving the environmental problems arising from petroleum hydrocarbons contaminated land. The use of organic waste materials (sugarcane bagasse, straw (pea and rice), rice husks, food) have proven to speed up microbial growth and metabolism, thus PAHs biodegradation in contaminated soil (Chiu *et al.*, 2009; García-Delgado *et al.*, 2015). Due to the progressive change in waste streams types and nature throughout time, there is a need to continue exploring their properties, especially those which are relevant for biologically mediated remediation strategies (composition, cost-effectiveness, safety, practicality, sustainability, shorter remediation time).

Today, a huge amount of wastes is generated from the food production industry in the UK (post farm-gate waste is 9.5 million tonnes with commercial and industrial business producing 2.9

million tonnes). In Africa, especially in sub-Saharan Africa, waste generation is estimated to be 62 million tonnes/year; and larger percentage (66-70%) of the total waste generated is organic; however, most countries in Africa have no estimated or documented value for these wastes. This has also contributed to massive environmental pollution due to alternative use as well as improper disposing routes such as landfilling, illegal dumping and burning. For example, in African soils are vulnerable to degradation through environmental conditions (extremes of wetting and drying, leaching, erosion, loss of organic matter) and anthropogenic impacts (fertiliser and pesticide application, land use, deforestation, dumping of wastes and pollution). However, the maintenance of soil health and fertility is crucial to continued sustainable production of food, welfare of families and communities and local and national economies. Some of these rich non-estimated green wastes include spent mushroom compost (SMC) and spent brewery grains (SBG) which could potentially be used for soil bioremediation (Mussatto, 2014). Focusing on the case of SMC and SBG, about 200,000 tonnes of SMC are generated annually in UK (Finney *et al.*, 2009), while in Nigeria more than 750, 000 tonnes of SBG and 3.4 million tonnes in the EU (15% from UK) are produced annually, respectively (Aliyu and Bala, 2011; Olawoye *et al.*, 2017). These organic wastes can potentially improve soil structure, increase soil fertility (directly and indirectly) and stimulate plant growth (Kästner and Miltner, 2016, Sigmund *et al.*, 2018). They also offer rich sources of enzymes, including those involved in lignocellulosic decomposition (Phan and Sabaratnam, 2012), and represent a potential microbial inocula for biodegradation of recalcitrant organic compounds (Leahy and Colwell, 1990). In contaminated soils, these effects result in biodegradation, stimulation and stabilization of contaminated soil matrix, thus promoting soil restoration (Kästner and Miltner, 2016).

To the best of the authors' knowledge, previous studies have not fully investigated the impact of additions of SBG and SMC on biodegradation of PAHs in soil. In this study, the development of phenanthrene (PHE) biodegradation was investigated in soil, which had been amended with two organic wastes (SBG and SMC). Changes in catabolic activity were quantified by measuring the kinetics of ^{14}C-phenanthrene mineralization to $^{14}CO_2$ and changes in microbial numbers over time.

3.3 Materials and Methods

3.3.1 Soil and organic waste collection

Soil (A horizon; 5–20 cm) was collected from a field belonging to Myerscough Agricultural College (Preston, UK). Soil samples were air-dried, homogenized by sieving through a 2mm mesh to remove plant debris, stones, and larger residue fragments and stored at 4^0C in the dark until use. General soil properties are described in Table S1 (Couling *et al.*, 2010). Fresh SBG and spent SMC were collected from Lancaster Brewery (Lancaster, UK) and Drinkwater's Mushrooms Ltd (Galgate, UK), respectively. General properties are described in Table S2. SMCs were pasteurized at 60^0C for 10-12 h prior to sampling. The materials used for SMC preparation were casing soil (peat), mushroom spawn, gypsum, water, and manure. Freshly collected organic wastes were homogenized by mixing and then stored in a sterile air-tight high-density polyethylene bags at a temperature of 4^0C.

3.3.2 Experimental set-up and amendment conditions

Soil (2.1 kg dry weight) was spiked with non-labeled phenanthrene (525 mg kg^{-1} dry weight) as described by Vázquez-Cuevas and Semple (2017). After blending and venting (to remove

acetone), the soils were amended with different amounts of organic wastes (dry weight): 1:10, 1:5, 1:2, 1:1 and 2:1 organic waste-to-soil ratios (in triplicate) and a control soil without amendment. The same water holding (60%) was maintained for all treatments throughout the study (water loss determined gravimetrically). The waste-soil mixtures, including the controls and blank were immediately transferred to amber glass bottles (to prevent photo-oxidation) and incubated in the dark at 21± 1°C over a period of up to 100 days and sampled at 1, 25, 50, 75 and 100 days soil-PAH contact time.

3.3.3 Mineralization of ^{14}C-phenanthrene in soil-waste mixtures

The impact of organic amendments on the catabolic potential of microbial community on ^{14}C-phenanthrene mineralization to $^{14}CO_2$ for each sampling time (1, 25, 50, 75 and 100 days soil-PAH contact time) on the amended and/or aged soil was measured in triplicate using respirometry, which was carried out using modified 250 ml Schott bottles (Reid *et al.*, 2001; Semple *et al.*, 2006). At each time point, 10 ± 0.2 g (dry weight) of the ^{12}C-phenanthrene spiked soil and 30 ml of deionized water was added to a respirometer. A [^{14}C] phenanthrene standard (100 Bq g^{-1} soil) was then added to the respirometer and placed on a flat-bed orbital shaker at 100 rpm and incubated for 14 days at 21 ± 1°C. The $^{14}CO_2$ was trapped in 1 M NaOH (I ml) in a vial suspended from the lid of the respirometer and sampled bihourly for 1 d and then every 24 h for 14 days and measured by liquid scintillation analyzer (LSC, Canberra Packard Tri-Carb2250CA) using standard protocols for counting and automatic quench correction (Semple *et al.*, 2006).

Pristine soil samples spiked with both ^{12}C and ^{14}C-phenanthrene (without organic amendments), and ^{12}C- phenanthrene (without amendment and ^{14}C-labelled compound) were

used as the control and analytical blank, respectively. The catabolic potential of the organic wastes was assessed by measuring the length of the lag phase (the time taken before ^{14}C-phenanthrene mineralization reached 5%), changes in the maximum rate of $^{14}CO_2$ evolution (fastest rate of mineralization resulting from microbial degradation), and the cumulative extent of mineralization of ^{14}C- phenanthrene in the soil samples (Macleod and Semple, 2006).

3.3.4 Enumeration of heterotrophic and phenanthrene-degrading microorganisms

Enumeration of total heterotrophic and phenanthrene-degrading microorganisms was determined by dilution and spread plate method (Okere *et al.*, 2012). For each of the different microcosms, soil microbial numbers were determined before and after each mineralization/respirometry assay. At each time point, 1 g (dry weight equivalent) was taken before the start of the experiment (before CO_2 evolution) and 1 ml of soil slurry after mineralization. Plate count (PCA) and Potato Dextrose (PDA) media were used for enumeration of heterotrophic bacteria and fungi, respectively (in triplicate). For the phenanthrene degraders, bacterial colonies were counted using minimal basal salt (MBS) medium enriched with 50 mg l^{-1} ^{12}C- phenanthrene (carbon source) and supplemented with an antifungal (fungizone) (Okere *et al.*, 2012). Similar microbiological culture medium supplemented with antibacterial (penicillin-streptomycin-glutamate) was used for the enumeration of phenanthrene-degrading fungi (Boochan *et al.*, 2000).

3.3.5 Data analysis

Statistical analyses carried out included paired t-tests, ANOVA ($p < 0.05$) and Pearson's correlation coefficient were performed using the Statistical Package for the Social Sciences

(IBM SPSS Version 23.0). Tukey's post-hoc and Games-Howell test were used to determine significant differences in means of samples within and across groups following the aging effect on ^{14}C-phenanthrene mineralization, lag phase, maximum rate, cumulative extent and microbial numbers at 95% confidence interval ($p < 0.05$) for organic waste-amended soils. Relationships between phenanthrene-degraders versus total extent and fastest rate of mineralization were analyzed using Pearson's product moment correlations. The Pearson's correlation coefficient (r) was ranked on a scale that range between +1 to -1. The value of r is either a perfect positive (+) or negative (-) correlation, when an increase in one variable led to an increase in the other variable (linear relationship) or an increase in one variable causing a decrease in the other variable, respectively. The strength of the relationship is either strong, weak or moderate between the two variables when the absolute values of their relationship approaches +1 or -1. The p-value shows the degree of association between the two variables. Data were plotted using SigmaPlot 10.0 software (Systat Software Inc., USA).

3.4. Results

The development of phenanthrene catabolism was measured in a soil amended with 1:10, 1:5, 1:2, 1:1 and 1:2 SBG:soil and SMC:soil ratios, respectively. Changes in kinetics of ^{14}C-phenathrene biodegradation were measured for 14 days after 1, 25, 50, 75 and 100 days of soil-PAH contact time (Figures 1 and 2). The influence of each organic amendment was assessed by measuring changes in the lag phases, rates and extents of ^{14}C-phenanthrene mineralization to $^{14}CO_2$ (Tables 1 and 2). Changes in the total heterotrophic and phenanthrene degrading bacteria and fungi were also measured over the course of the incubation.

3.4.1 Influence of spent brewery grains on mineralization of ^{14}C-phenanthrene in soils

The length of the lag phase (days) was measured in the SBG-amended soil incubations. Throughout the investigation, the lag phases were significantly shorter ($p < 0.05$) in the soils amended with lower amounts of SBG (1:10 and 1:5). In particular, the shortest lag phases were observed for 1:5 SBG:soil ratio at 100 d of soil-PAH contact time, while the longest lag phases were observed in soils amended with 2:1 SBG:soil ratio ($p < 0.05$), which did not reach 5%. Soil incubations amended with SBG (1:10) were found to have significantly shorter lag phases ($p < 0.05$) than 1:5 SBG:soil incubations after 1 d and 25 d aging (Table 1 and Figure 1). There were no significant differences ($p > 0.05$) between the control and 1:2 SBG:soil incubations, after 1 d soil-PAH contact time and no measurable lag phases were observed for 1:1 and 2:1 SBG:soil incubations. Noticeably, soil-PAH contact time reduced the lag phases in all of the amended conditions and control incubations except 1:1 and 2:1 SBG:soil ratios; an effect which is reflected especially in the lag phases after 100 days ($p < 0.05$), compared to previous time points. With respect to the amount of SBG added to the soil, lower organic waste additions resulted in shorter lag-phases (1:10>1:5>1:2>1:1 > 2:1),although the SBG:soil ratios (1:2, 1:1 and 2:1) showed significantly reduced lag phases compared with unamended control, at most time points during the incubation period.

The influence of increasing amounts of SBG on the fastest rates of ^{14}C-phenanthrene mineralization was also measured at each time point over the 100 d incubation (Table 1 and Figure 1). The fastest rates were observed for soils amended with 1:10 and 1:5 waste:soil incubations, while the lowest were in the 2:1 SBG:soil incubations. The rates of $^{14}CO_2$ mineralized in the 1:10 and 1:5 SBG:soil incubations did not significantly differ ($p > 0.05$).

After 100 d soil-PAH contact time, 1:1 and 2:1 SBG:soil incubations had no effect on rates of ^{14}C-phenanthrene mineralized when compared to un-amended soil.

The influence of increasing amounts of SBG on the extents of ^{14}C-phenanthrene mineralization was measured at each time point over the 100 d incubation. As with the changes in lag phase and fastest rate measurements, the soil-PAH contact time and the amount of SBG had an influence on the amount of ^{14}C-phenanthrene mineralized to $^{14}CO_2$ (Figure 1 and Table 1). At 1 and 25 d soil:PAH contact time, treatments containing organic materials were found with significantly higher ($p < 0.05$) extents of mineralization in the following trend 1:10=1:5>1:2=1:1>2:1. The total extents of $^{14}CO_2$ mineralized were also significantly higher ($p < 0.05$) after 50 d contact time for 1:1 and 1:2 SGB amendments to soil compared to 25d with nearly 120% and 278% increases, respectively, in $^{14}CO_2$ mineralized in amended soils (Table 1). Furthermore, extents of mineralization peaked at 50 d soil-PAH contact time in most amended soils. This treatment period was found significantly higher ($p < 0.05$) in total extents of mineralization compared to the other time points.

The 1:2 SBG:soil amendment showed the highest extent of mineralization (48.7%) after 50 d soil incubation, but this extent of mineralization to $^{14}CO_2$ was not sustained following significant ($p < 0.05$) decreases by 50% and 41% after 75 d and 100 d soil contact time, respectively. However, the lower amounts of SBG amended to soil (1:10 and 1:5) after 100 d incubation, showed significantly higher extents of mineralization by nearly 19% and 13%, respectively, as compared to results observed at 75 d of soil-PAH contact time. The $^{14}CO_2$ produced in 2:1 SBG-amended soil was significantly lower ($p < 0.05$) than any other treatments and control soil at each sampling point during the study.

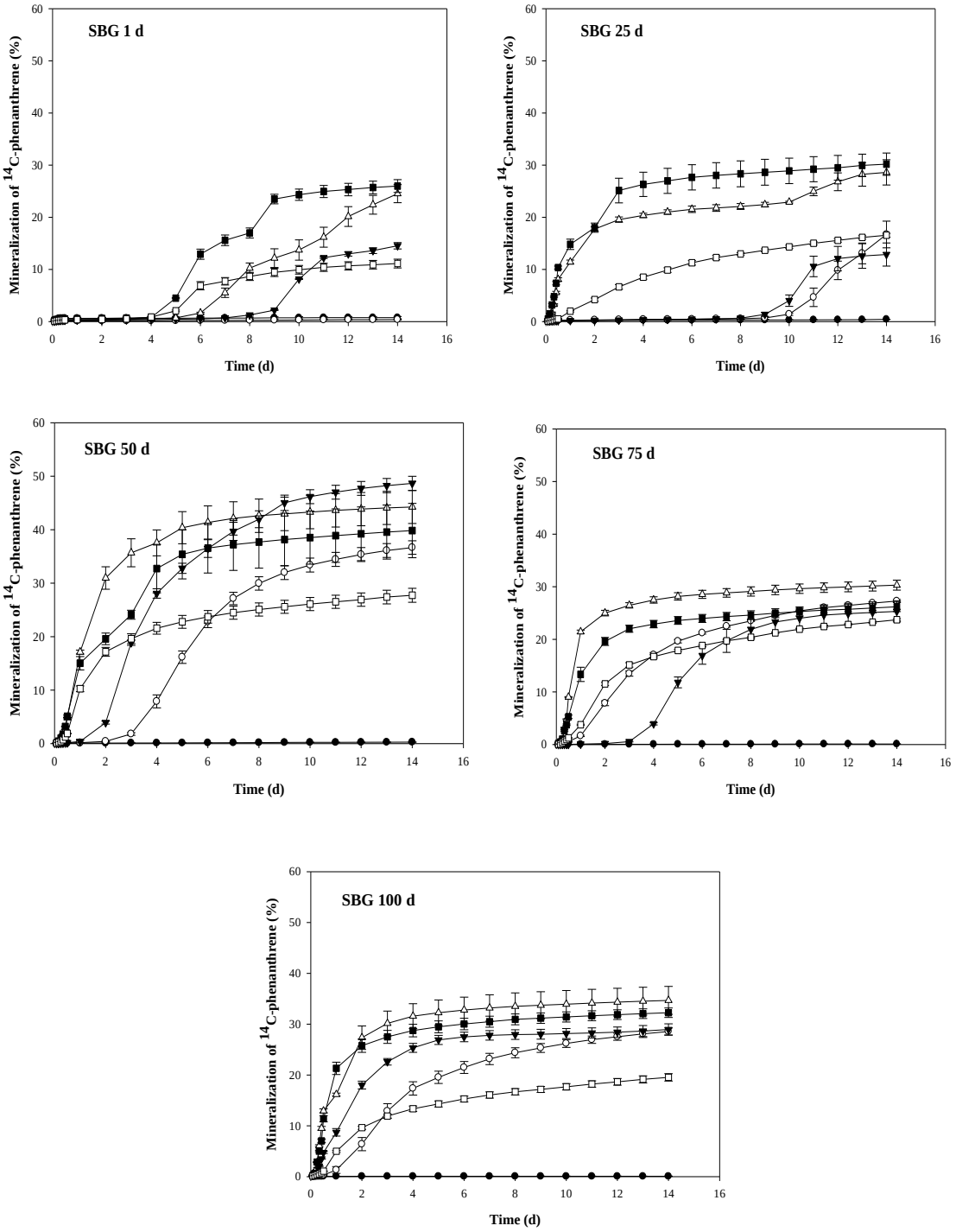

Figure 1. Development of the catabolism of ^{14}C-phenanthrene to $^{14}CO_2$ in soils amended with spent brewery grains: control (unamended)(□), 1:10 (■), 1:5 (△), 1:2 (▼), 1:1 (○) and 2:1 (●) after 1, 25, 50, 75, and 100d soil-phenanthrene contact time. Values of standard error of mean (SEM) are triplicate samples (n = 3).

Table 1. Catabolic profile of ^{14}C-labelled phenanthrene in soil amended with different proportions of spent brewery grains after 14 days respirometric assay. Values are mean ± standard error (n = 3).

Contact time (days)	Amendment (%)	Lag phase ($^{14}CO_2$ ≥5%) d^{-1}	Fastest rate (% $^{14}CO_2$ h^{-1})	Cumulative Extent (%)
1	0	^{a1}9.04 ± 0.08	^{a1}0.20 ± 0.04	^{b1}11.13 ± 1.06
	1:10	^{c1}5.06 ± 0.01	^{a1}0.36 ± 0.03	^{c1}25.99 ± 1.23
	1:5	^{b1}6.91 ± 0.17	^{a1}0.23 ± 0.04	^{c1}24.54 ± 1.73
	1:2	^{a1}9.47 ± 0.01	^{a1}0.25 ± 0.00	^{b1}14.53 ± 0.61
	1:1	N/L*	^{a1}0.02 ± 0.00	^{a1}0.39 ± 0.06
	2:1	N/L*	^{a1}0.23 ± 0.08	^{a1}0.76 ± 0.21
25	0	^{b2}2.34 ± 0.14	^{b1}0.13 ± 0.00	^{b2}16.55 ± 0.32
	1:10	^{d3}0.34 ± 0.00	^{d3}1.50 ± 0.02	^{c1}30.18 ± 2.13
	1:5	^{c2}0.40 ± 0.01	^{d2}1.34 ± 0.08	^{c1}28.60 ± 2.42
	1:2	^{a1}10.16 ± 0.23	^{ac1}0.27 ± 0.05	^{b1}12.86 ± 2.22
	1:1	^{a1}11.05 ± 0.35	^{c2}0.22 ± 0.00	^{b2}16.70 ± 2.56
	2:1	N/L*	^{a1}0.04 ± 0.00	^{a3,4}0.43 ± 0.02
50	0	^{c4}0.69 ± 0.01	^{c3}0.70 ± 0.04	^{b4}27.74 ± 1.27
	1:10	^{d2}0.50 ± 0.01	^{cd2}0.96 ± 0.06	^{c1}39.84 ± 2.08
	1:5	^{d1,3}0.48 ± 0.03	^{d2}1.10 ± 0.02	^{c2}44.25 ± 3.08
	1:2	^{b3}2.05 ± 0.02	^{c3}0.62 ± 0.01	^{c3}48.66 ± 1.32
	1:1	^{a2}3.56 ± 0.11	^{b4}0.35 ± 0.00	^{b3}36.66 ± 2.26
	2:1	N/L*	^{a1}0.01 ± 0.00	^{a2,4}0.28 ± 0.02
75	0	^{c3}1.16 ± 0.01	^{b2}0.32 ± 0.01	^{b3}23.71 ± 0.03
	1:10	^{d2}0.49 ± 0.02	^{d2}0.85 ± 0.06	^{bc1}26.18 ± 0.91
	1:5	^{d2}0.42 ± 0.00	^{cd3}2.11 ± 0.04	^{c1}30.30 ± 0.95
	1:2	^{a2}4.14 ± 0.03	^{b2}0.35 ± 0.04	^{b2}25.31 ± 2.00
	1:1	^{b2}1.55 ± 0.05	^{b3}0.27 ± 0.01	^{bc4}27.28 ± 0.29
	2:1	N/L*	^{a1}0.01 ± 0.00	^{a1,2,4}0.10 ± 0.04
100	0	^{a4}1.01 ± 0.03	^{b2}0.33 ± 0.01	^{b2}19.54 ± 0.46
	1:10	^{b3}0.33 ± 0.03	^{d3}2.18 ± 0.13	^{c2}32.26 ± 0.93
	1:5	^{b3}0.31 ± 0.00	^{d4}1.79 ± 0.09	^{c1,2}34.68 ± 2.75
	1:2	^{ab4}0.69 ± 0.14	^{c3}0.78 ± 0.05	^{c2}28.96 ± 1.10
	1:1	^{ab2}1.78 ± 0.20	^{a3}0.27 ± 0.01	^{c4}28.56 ± 0.71
	2:1	N/L*	^{a1}0.02 ± 0.01	^{a1,4}0.00 ± 0.02

* N/L- No lag phase (Mineralization did not reach or exceed 5%).
* Same letters indicate no statistical differences ($p > 0.05$) in amendment levels within each aging time while different letters indicate significant differences ($p < 0.05$) across amendment levels within each aging time.
* Same numbers indicate no statistical differences ($p < 0.05$) in aged amended soils across the four sampling points while different numbers indicate significant differences in aged amended soils across the four sampling points (1d to 100d).

3.4.2 Enumeration of culturable bacterial and fungal heterotrophs and phenanthrene degraders in SBG-amended soils

The colony forming units (CFUs g^{-1} soil dw) of heterotrophic and phenanthrene degrading bacteria were measured for all treatment conditions and control in SGB amended soils over a 100 d incubation (Table 2). CFUs were also measured at the end of the 14-d respirometric incubations and are presented in supplementary material (Table S3). The total heterotrophs and phenanthrene degraders in amended soils increased significantly ($p < 0.05$) based on the amounts of SBG applied to the soil (Table 2). Compared with the controls, significantly higher numbers ($p < 0.05$) of heterotrophic and phenanthrene-degrading CFUs were observed in the soils amended with SBG. In addition, the smaller amounts of SBG added to the soil (1:10 and 1:5) consistently showed higher CFUs (heterotrophs and phenanthrene-degraders) as compared to other amendment conditions. Furthermore, apart from the heterotrophs, phenanthrene degrading bacteria did not proliferate in the soils containing larger amounts of SBG (2:1); this was also observed in the control soil.

Phenanthrene-degrading bacterial numbers did not statistically correlate with fastest rate of ^{14}C-phenanthrene mineralized in all amended soils throughout this study (1 d to 100 d) (Figure S1), irrespective of the high numbers of phenanthrene-degraders observed in SBG amended soils. Correlations between phenanthrene degraders and total extents of ^{14}C-phenanthrene mineralization showed a significantly weak but negative correlation ($r^2 = 0.40$, $p = 0.02$) with bacterial numbers in 1:5 SBG:soil. Also, the extent of ^{14}C-phenanthrene mineralized for higher dose of SBG added to soils (1:2 and 2:1) showed strong negative and strong positive relationships ($r^2 = 0.50$, $p = 0.003$ and $r^2 = 0.61$, $p = 0.001$) with phenanthrene-degrading bacterial numbers in soils, respectively.

The addition of organic amendment at different ratios resulted in a significant increase ($p < 0.05$) in both heterotrophic and phenanthrene-degrading fungal numbers (Table 2). Overall, both fungal numbers were significantly higher than control before mineralization and after amendment of soils with SBG, although 2:1 SBG application to soil showed a lower heterotrophic fungal number compared to other treatments. Noticeably, 1:2 and 1:1 SBG:soil conditions showed significantly higher fungal numbers (heterotrophs and phenanthrene-degraders) over time (1 d to 100 d) compared to other amendments during the investigation. Similar trends were observed for the 1:5 SBG:soil incubations, but did not increase consistently throughout the 100 d incubation. Additionally, all SBG:soil conditions (except 2:1) showed high fungal proliferation from 75 d to 100 d soil-PAH contact time. After 100 d soil-PAH contact time, the 1:5 SBG:soil incubations displayed the highest CFUs for heterotrophic fungi; while the highest CFUs for phenanthrene-degrading fungi were observed in the 1:2 and 1:1 SBG:soil incubations after 25 d soil-PAH contact time.

As with bacterial numbers, the phenanthrene-degrading fungal numbers for most SBG:soil treatment ratios have no positive relationships with the rates and extents of ^{14}C-phenanthrene mineralization during the study period (Figure S2), except for 1:5 and 1:2 SBG:soil incubations in which the overall extents of mineralization significantly correlated with phenanthrene-degrading fungal numbers. However, weak positive ($r^2 = 0.30$, $p = 0.05$) and strong negative ($r^2 = 0.55$, $p = 0.003$) correlations were found in the 1:5 and 1:2 SBG:soil conditions, respectively.

Table 2. Autochthonous heterotrophic and phenanthrene-degrading microorganisms present before 14 days mineralization of ^{14}C-phenanthrene in spent brewery grains amended soil. Values are mean ± standard error (n = 3).

		Bacteria CFU x 10^8 g^{-1} soil dw		Fungi CFU x 10^6 g^{-1} soil dw	
Contact time (days)	Amendment (%)	Heterotrophs	PHE-degraders	Heterotrophs	PHE-degraders
	0	^{a2}11.6 ± 0.49	^{a4}2.91 ± 0.03	^{a2}0.23 ± 0.00	^{a1}0.03 ± 0.00
	1:10	^{cd4}17.1 ± 0.38	^{c3}15.9 ± 0.34	^{c1}5.71 ± 0.54	^{c2}3.16 ± 0.45
	1:5	^{b4}13.8 ± 0.41	^{c3}13.8 ± 0.41	^{b1}4.44 ± 0.34	^{d2}3.72 ± 0.06
1	1:2	^{a3}9.37 ± 0.48	^{c3}5.02 ± 0.45	^{d1,2}9.52 ± 0.32	^{e2}4.52 ± 0.65
	1:1	^{bc2}15.9 ± 0.18	^{ab3}2.91 ± 0.29	^{f1}11.2 ± 0.12	^{c2}3.11 ± 0.33
	2:1	^{de5}19.0 ± 1.04	^{ab3}2.45 ± 1.23	^{e5}10.2 ± 0.20	^{b1}2.33 ± 0.31
	0	^{a2}0.40 ± 0.03	^{b5}4.05 ± 0.20	^{a2}0.30 ± 0.01	^{a1}0.02 ± 0.00
	1:10	^{f3}13.7 ± 0.17	^{e3}14.1 ± 0.21	^{b2,4}7.76 ± 0.80	^{b2}3.16 ± 0.40
	1:5	^{e3}11.9 ± 0.15	^{d3}12.8 ± 0.26	^{b2}8.23 ± 0.84	^{b5}5.74 ± 0.90
25	1:2	^{b4}6.20 ± 0.19	^{c3}6.79 ± 0.17	^{c1}12.0 ± 0.77	^{c3}9.92 ± 0.70
	1:1	^{d1}10.4 ± 0.15	^{f4}16.0 ± 0.29	^{c2}14.0 ± 1.02	^{c3}9.07 ± 0.74
	2:1	^{c4}8.40 ± 0.08	^{a1}0.00 ± 0.00	^{a4}2.36 ± 0.10	^{b2}5.32 ± 0.34
	0	^{a2}0.05 ± 0.04	^{a2}4.18 ± 0.19	^{a2}0.05 ± 0.00	^{a2}0.05 ± 0.00
	1:10	^{b5}19.0 ± 0.76	^{a2}0.41 ± 0.08	^{c2}7.22 ± 0.77	^{b2}0.24 ± 0.01
	1:5	^{b5}18.3 ± 0.68	^{b2}1.97 ± 0.22	^{b1}4.09 ± 0.40	^{c1}0.41 ± 0.02
50	1:2	^{b5}22.2 ±1.06	^{b2}1.67 ± 0.09	^{e1,2}14.0 ± 0.94	^{c1}0.41 ± 0.05
	1:1	^{b2}21.1 ± 4.96	^{b2}1.46 ± 0.18	^{d1}10.2 ± 0.70	^{d1}0.61 ± 0.03
	2:1	^{a1,2}0.14 ± 0.01	^{a3}0.45 ± 0.05	^{a3}0.99 ± 0.08	^{b1}0.26 ± 0.01
	0	^{a2}0.09 ± 0.03	^{a1}0.02 ± 0.00	^{a4}1.09 ± 0.03	^{a2}0.05 ± 0.00
	1:10	^{b2}0.92 ± 0.02	^{b2}0.36 ± 0.01	^{b4}10.6 ± 0.30	^{a1}0.85 ± 0.04
	1:5	^{c2}2.01 ± 0.07	^{c2}0.77 ± 0.03	^{bc3}12.1 ± 0.37	^{bc3}4.73 ± 0.48
75	1:2	^{d4}1.48 ± 0.04	^{d4}1.14 ± 0.05	^{bc1,2}14.7 ± 0.34	^{d3}7.64 ± 0.70
	1:1	^{e2}2.97 ± 0.09	^{c2}0.87 ± 0.07	^{c2}16.6 ± 0.41	^{cd3}6.37 ± 0.37
	2:1	^{a2,3}2.73 ± 0.03	^{a2}0.04 ± 0.00	^{a2}1.00 ± 0.92	^{b2}3.88 ± 0.18
	0	^{a1}0.05 ± 0.00	^{a3}0.11 ± 0.00	^{a3}0.49 ± 0.02	^{a1}0.03 ± 0.00
	1:10	^{b1,2}0.92 ± 0.00	^{a1}0.15 ± 0.00	^{c5}10.3 ± 0.59	^{b2}3.76 ± 0.18
	1:5	^{d1}2.01 ± 0.11	^{b2}1.32 ± 0.04	^{f4}17.9 ± 0.18	^{c4}6.24 ± 0.18
100	1:2	^{c1}1.48 ± 0.07	^{c1,2}1.45 ± 0.04	^{e2}16.5 ± 0.24	^{c3}6.88 ± 0.30
	1:1	^{e1}2.97 ± 0.16	^{a1}0.11 ± 0.00	^{d2}15.0 ± 0.15	^{c1}7.34 ± 0.53
	2:1	^{e3}2.73 ± 0.06	^{a2}0.07 ± 0.00	^{b2}1.98 ± 0.08	^{a1}0.21 ± 0.01

* Same letters indicate no statistical differences ($p > 0.05$) in amendment levels within each aging time while different letters indicate significant differences ($p < 0.05$) across amendment levels within each aging time

* Same numbers indicate no statistical differences ($p > 0.05$) in aged amended soils across the four sampling points while different numbers indicate significant differences ($p < 0.05$) in aged amended soils across the four sampling points (1d to 100d).

3.4.3 Influence of spent mushroom compost on mineralization of ^{14}C-phenanthrene in soils

In soils amended with SMC, the length of lag phases were also measured at each time point over the 100 d incubation. It was observed that the lag phases generally decreased in all of the treatments with increased soil-PAH contact time (Figure 2 and Table 3). After 1 day of soil incubation, the lag phase observed for control soils were generally not significantly different ($p > 0.05$) from any of the SMC:soil conditions. However, significant reductions ($p < 0.05$) in the lag phases, after 25 days soil-PAH contact time, were observed for all SBG:soil conditions except 2:1 as compared to 1 day contact time. These observed reductions were not significantly different from the other contact points onwards (50 to 100 days). Data showed that the 2:1 SMC:soil incubation displayed the longest lag phases ($p > 0.05$) compared to the other conditions and control throughout the study period. Soil amended with SBG (1:10) also showed significantly shorter lag phase than other amendment conditions and control at 75 days period ($p < 0.05$).

The impact of soil-PAH contact time and the amount of SMC on the fastest rate of ^{14}C-phenanthrene mineralization in soil were studied over 100 d. For all amendment conditions, increases in soil-PAH contact time resulted in faster rates within each SMC-soil treatment, ranging from 0.40% to a maximum of 2.0%. Similarly, the greatest change was observed from 1 d (0.40 – 0.60%) to 100 d (0.80 – 1.90%) soil-PAH contact time: the 1:5 SMC-soil amendment displayed the fastest rate of $^{14}CO_2$ mineralized (2.37% $^{14}CO_2$ h^{-1}) after 100 d soil-PAH contact time. For control soils, the fastest rates of mineralization were not significantly lower ($p > 0.05$) when compared to all SMC amended soils except for 1:5 SMC:soil condition, which had significantly faster rates at 1 and 25 d soil-PAH contact time (both $p < 0.05$).

The influence of increasing amounts of SMC in soil on the extents of ^{14}C-phenanthrene mineralization was also determined over a 100 d period. From this study, the lower SBG:soil ratios (1:10 and 1:5) exhibited significantly greater ($p < 0.05$) extents of ^{14}C-phenanthrene mineralized (50 and 100 d) compared to all other treatment (Table 3). Following 1 d and 25 d of soil-PAH contact time, there were no significant differences in the overall extents in all SMC-amended soils ($p > 0.05$) when compared to their non-equivalent amendment conditions and control soils. Higher extent of mineralization was observed for all amendment conditions, except for the largest dose rate (2:1) from 50 to 100 d soil-PAH contact time. In particular, the lower doses (1:5 and 1:10) displayed higher extents of mineralization (50 to 100 d) in comparison with the other conditions. Furthermore, soils amended with SBG showed the highest extents of mineralization after 75 d but this was not statistically different from 50 d soil-PAH contact time.

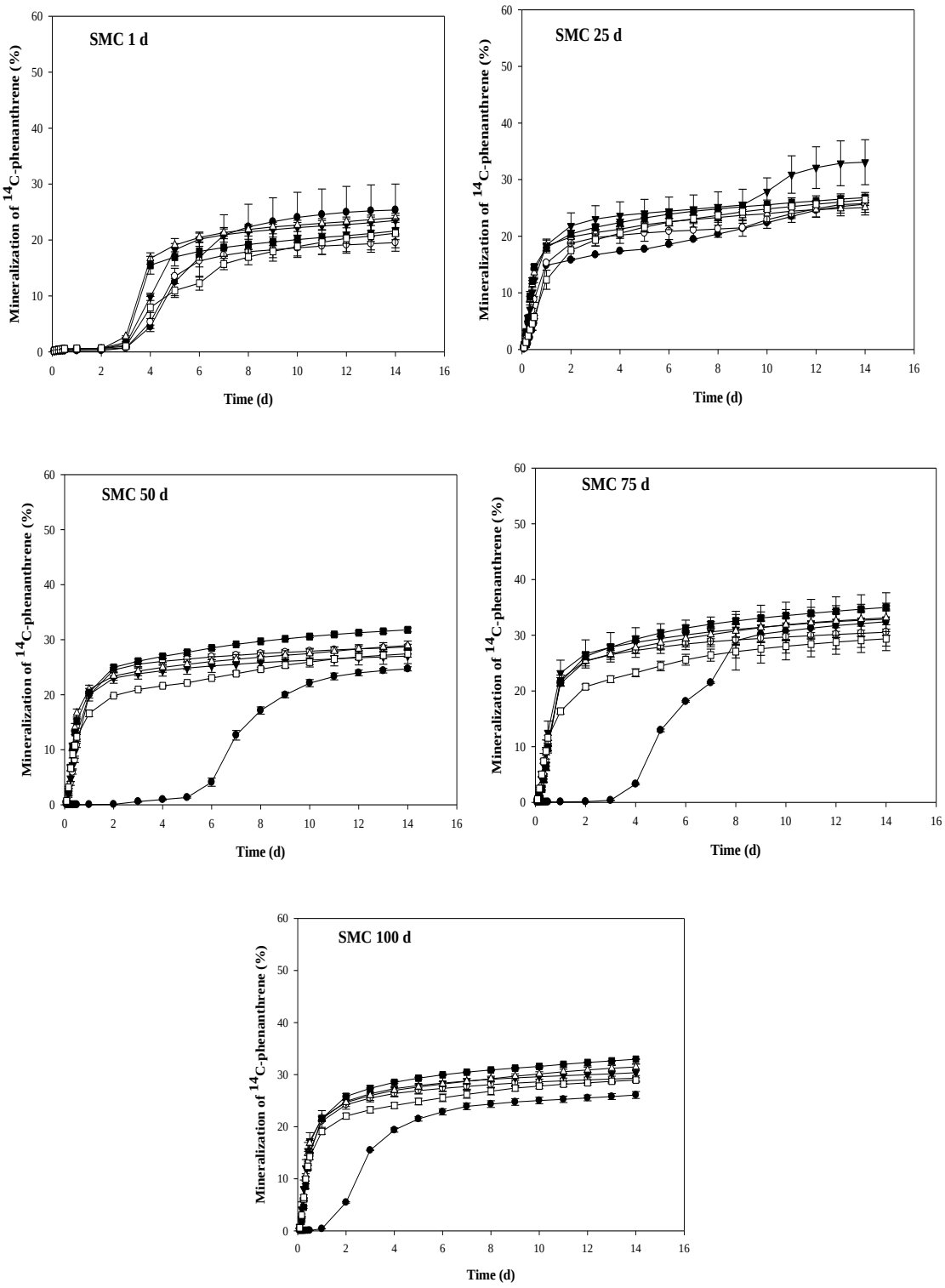

Figure 2. Development of the catabolism of ^{14}C-phenanthrene to $^{14}CO_2$ in soils amended with spent mushroom compost: control (unamended)(□), 1:10 (■), 1:5 (△), 1:2 (▼), 1:1 (○) and 2:1 (●) after 1, 25, 50, 75, and 100d soil-phenanthrene contact time. Values of standard error of mean (SEM) are triplicate samples (n = 3).

Table 3. Catabolic profile of ^{14}C-labelled phenanthrene in soil amended with different proportions of spent mushroom compost after 14 days respirometric assay. Values are mean ± standard error (n = 3).

Contact time (days)	Amendment (%)	Lag phase ($^{14}CO_2$ ≥5%) d^{-1}	Fastest rate (% $^{14}CO_2$ h^{-1})	Cumulative Extent (%)
1	0	^{a1}3.71 ± 0.26	^{a1}0.34 ± 0.00	^{a1}21.22 ± 2.13
	1:10	^{a1}3.25 ± 0.06	^{ab1}0.57 ± 0.00	^{a1}21.61 ± 1.39
	1:5	^{a1}3.50 ± 0.35	^{b1}0.58 ± 0.00	^{a1}23.97 ± 0.49
	1:2	^{a1}3.43 ± 0.06	^{ab1}0.41 ± 0.00	^{a1}23.48 ± 1.32
	1:1	^{a1}3.93 ± 0.21	^{ab1}0.39 ± 0.00	^{a1}19.54 ± 1.48
	2:1	^{a2}4.09 ± 0.05	^{ab1}0.38 ± 0.00	^{a1}25.37 ± 4.57
25	0	^{a2} 0.45 ± 0.02	^{a1}0.66 ± 0.05	^{a1}26.46 ± 0.50
	1:10	^{c3} 0.23 ± 0.02	^{b2}1.94 ± 0.17	^{a1}26.81 ± 0.91
	1:5	^{c3,4}0.24 ± 0.01	^{b2}1.72 ± 0.04	^{a1,2}25.07 ± 1.31
	1:2	^{c2} 0.27 ± 0.02	^{b2}1.64 ± 0.04	^{a1}33.08 ± 3.97
	1:1	^{b2} 0.35 ± 0.01	^{a2}1.14 ± 0.11	^{a2}25.75 ± 1.52
	2:1	^{a4} 0.52 ± 0.00	^{a2}0.86 ± 0.00	^{a1}25.66 ± 0.23
50	0	^{b3} 0.21 ± 0.01	^{bc2} 1.75 ± 0.14	^{ab1} 27.44 ± 0.47
	1:10	^{b3} 0.22 ± 0.01	^{c2} 2.02 ± 0.09	^{c1}31.79 ± 0.47
	1:5	^{b4} 0.21 ± 0.01	^{c3}2.06 ± 0.10	^{bc2,3}28.94 ± 0.83
	1:2	^{b2}0.26 ± 0.02	^{bc2}1.67 ± 0.18	^{ab1}27.02 ± 1.37
	1:1	$^{b2.3}$0.30 ± 0.01	^{b2,3}1.36 ± 0.03	^{bc2}28.69 ± 0.49
	2:1	^{a1} 6.08 ± 0.11	^{a1} 0.36 ± 0.04	^{a1}24.70 ± 0.37
75	0	^{a3}0.25 ± 0.02	^{b2}1.68 ± 0.10	^{a1}29.36 ± 1.55
	1:10	^{b2}0.37 ± 0.02	^{b2}1.62 ± 0.05	^{a2}34.99 ± 0.29
	1:5	^{a2}0.37 ± 0.01	^{b2}1.74 ± 0.03	^{a3}33.14 ± 1.26
	1:2	^{a2}0.30 ± 0.04	^{b2}1.86 ± 0.20	^{a1}32.87 ± 2.89
	1:1	^{a2}0.33 ± 0.00	^{b3}1.61 ± 0.08	^{a2}30.55 ± 0.54
	2:1	^{a3}3.41 ± 1.61	^{a1}0.51 ± 0.12	^{a1}32.43 ± 5.19
100	0	^{b2}0.22 ± 0.01	^{bc3}1.83 ± 0.11	^{ab1}28.96 ± 0.62
	1:10	^{b2,3}0.26 ± 0.01	^{c2}2.03 ± 0.14	^{c1}32.97 ± 0.08
	1:5	^{b4}0.23 ± 0.00	^{b4}2.37 ± 0.04	^{bc3}31.47 ± 0.57
	1:2	^{b2}0.36 ± 0.19	^{b2}2.28 ± 0.25	^{bc1}30.36 ± 1.68
	1:1	^{b3}0.23 ± 0.01	^{b4}1.99 ± 0.05	^{abc2}29.30 ± 0.53
	2:1	^{a3}1.91 ± 0.02	^{ac1}0.42 ± 0.00	^{a1}26.09 ± 0.67

* N/L indicates no lag phase (mineralization did not reach or exceed 5%)

* Same letters indicate no statistical differences ($p > 0.05$) in amendment levels within each aging time while different letters indicate significant differences ($p < 0.05$) across amendment levels within each aging time

* Same numbers indicate no statistical differences ($p > 0.05$) in aged amended soils across the four sampling points while different numbers indicate significant differences ($p < 0.05$) in aged amended soils across the four sampling points (1d to 100d).

3.4.4 Enumeration of culturable bacterial and fungal heterotrophs and phenanthrene degraders in SMC-amended soils

Bacterial numbers (both heterotrophs and phenanthrene-degraders) in the SMC amended soils were significantly higher ($p < 0.05$) than control soil before mineralization throughout the course of the study. In comparison to the bacterial numbers after mineralization, most of the amended conditions were not higher than the non-amended soil except for 1d and 50 d for heterotrophic bacteria (Table 4). CFUs after mineralization is presented in the supplementary material (Table S4). The 1:1 and 2:1 SMC-soil conditions displayed higher bacterial and fungal numbers (both heterotrophs and phenanthrene-degraders) before mineralization throughout the 100 d incubation ($p < 0.05$). The growth of bacteria in amended soils were significantly influenced depending on the SMC dosage application. After 1 d aging, higher organic materials added to soils resulted in greater increases in the bacterial numbers in the following order: 2:1>1:1>1:2:1:5>1:10). Similarly, heterotrophs and phenanthrene-degraders showed higher CFUs in soil amended with higher dose of SBG in rest of the study periods (25 to 100 d).

Relationships between rates and extents of ^{14}C-phenanthrene mineralized with phenanthrene-degrading bacterial numbers for all amended soils over the 100 incubation were explored (Table 4 and Figure S3). The 1:1 SMC-soil incubation showed a strong negative relationship for both fastest rates ($r^2 = 0.76$, $p = 0.000$) and overall extents ($r^2 = 0.69$, $p = 0.000$) with phenanthrene-degrading bacteria, whilst a weak but significant positive correlation was found between extent and phenanthrene-degrading numbers in 1:10 SMC:soil incubation ($r^2 = 0.30$, $p = 0.04$). In addition, both 1:5 and 2:1 SMC:soil incubations displayed weak positive ($r^2 =$

0.30, p = 0.05) and weak negative (r^2 = 0.31, p = 0.03) correlations between the rate of mineralization and phenanthrene-degraders, respectively.

Fungal numbers for SMC amended soils did not show significant differences from control, apart from 1:5 and 1:2 amended soils before (Table 4) and after mineralization (Figure S6). In most cases, the control soil showed a significant heterotrophic and phenanthrene-degrading numbers ($p < 0.05$) than all treatment soils, especially at 50 d and 25d, respectively. After 1 d incubation, the CFUs (heterotrophic and phenanthrene-degrading fungi) from unamended soil were not significantly different ($p > 0.05$) from the rest of the treatments except for 1:5 SMC amended to soils. In contrast to 75 d aging, all amended soils showed significantly higher fungal numbers ($p < 0.05$) compared to 1 d aging. However, results from this study revealed there were no significant relationships ($r^2 < 0.2$; $p > 0.05$) found between rates and extents of ^{14}C-phenanthrene mineralization with phenanthrene-degrading fungal numbers in virtually all amended soils throughout the study.

Table 4. Autochthonous heterotrophic and phenanthrene-degrading microorganisms present before 14 days mineralization of ^{14}C-phenanthrene in spent mushroom compost amended soil. Values are mean ± standard error (n = 3).

		Bacteria CFU x 10^8 g^{-1} soil dw		Fungi CFU x 10^6 g^{-1} soil dw	
Contact time (days)	Amendment (%)	Heterotrophs	PHE-degraders	Heterotrophs	PHE-degraders
1	0	^{a2}0.07 ± 0.00	^{a3}0.14 ± 0.01	^{c1}0.18 ± 0.01	^{c2}0.13 ± 0.00
	1:10	^{b1}0.42 ± 0.02	^{b1}0.14 ± 0.00	^{b1}0.16 ± 0.00	^{d1}0.16 ± 0.00
	1:5	^{c1}0.67 ± 0.02	^{c1}0.23 ± 0.00	^{b1}0.15 ± 0.00	^{c2}0.12 ± 0.00
	1:2	^{d1}0.98 ± 0.01	^{d1,2}1.51 ± 0.02	^{b1}0.15 ± 0.00	^{b2}0.10 ± 0.00
	1:1	^{e1}1.05 ± 0.01	^{e2}2.15 ± 0.03	^{a1}0.06 ± 0.00	^{c1}0.12 ± 0.00
	2:1	^{f1}1.46 ± 0.01	^{f4}2.60 ± 0.01	^{b2}0.51 ± 0.00	^{a1}0.02 ± 0.00
25	0	^{a1}0.03 ± 0.00	^{a3}0.16 ± 0.03	^{a2}0.32 ± 0.06	^{a3}0.29 ± 0.05
	1:10	^{a4}7.02 ± 0.31	^{b4}0.74 ± 0.05	^{c2}0.92 ± 0.10	^{b2}1.59 ± 0.31
	1:5	^{c2}14.3 ± 0.35	^{c5}1.36 ± 0.04	^{bc2}0.69 ± 0.02	^{b2}1.67 ± 0.16
	1:2	^{c4}13.9 ± 0.80	^{c3}1.42 ± 0.14	^{ab3}0.60 ± 0.08	^{b2}2.31 ± 0.40
	1:1	^{b2}8.58 ± 0.42	^{c2}1.36 ± 0.12	^{ab2}0.60 ± 0.08	^{b2}1.84 ± 0.14
	2:1	^{b3}7.07 ± 0.73	^{d3}2.37 ± 0.15	^{a1}0.27 ± 0.02	^{b2}1.60 ± 0.02
50	0	^{a3}0.21 ± 0.01	^{a1}0.01 ± 0.00	^{c1}0.16 ± 0.00	^{a1}0.04 ± 0.01
	1:10	^{a3}1.23 ± 0.04	^{b2}0.43 ± 0.02	^{c1}0.15 ± 0.00	^{a1}0.04 ± 0.00
	1:5	^{a1}3.24 ± 0.31	^{b2}0.44 ± 0.02	^{bc1}0.14 ± 0.00	^{a1}0.03 ± 0.00
	1:2	^{a3}4.84 ± 0.44	^{c1}0.71 ± 0.05	^{a1}0.09 ± 0.00	^{b1}0.09 ± 0.00
	1:1	^{b3}18.2 ± 1.23	^{c1}0.68 ± 0.02	^{b2}0.12 ± 0.00	^{b1}0.08 ± 0.00
	2:1	^{c4}36.4 ± 2.24	^{d2}0.88 ± 0.04	^{bca1}0.14 ± 0.00	^{a1}0.04 ± 0.00
75	0	^{a4}0.45 ± 0.08	^{a2}0.03 ± 0.00	^{a2}0.20 ± 0.00	^{a1}0.03 ± 0.00
	1:10	^{bc3}1.38 ± 0.21	^{b3}0.76 ± 0.02	^{a2}0.42 ± 0.02	^{ab1}0.14 ± 0.00
	1:5	^{b1}1.25 ± 0.28	^{b3}0.76 ± 0.02	^{a2}0.60 ± 0.76	^{ab1}0.60 ± 0.76
	1:2	^{b2}1.31 ± 0.19	^{d1}1.34 ± 0.02	^{a2}13.2 ± 0.76	^{ab1}1.35 ± 0.14
	1:1	^{c1}2.09 ± 0.41	^{c1}1.00 ± 0.09	^{a2}8.14 ± 0.40	^{ab1}1.29 ± 0.11
	2:1	^{d3}5.60 ± 0.27	^{de2}1.17 ± 0.06	^{b4}6.71 ± 0.70	^{c1}2.25 ± 0.14
100	0	^{a2}0.07 ± 0.00	^{a3}0.08 ± 0.00	^{a1}0.00 ± 0.00	^{a1}0.02 ± 0.00
	1:10	^{b2,3}0.71 ± 0.02	^{b2}0.40 ± 0.03	^{b2}0.24 ± 0.00	^{a1}0.03 ± 0.00
	1:5	^{c1}1.01 ± 0.03	^{d4}0.93 ± 0.04	^{b2}0.31 ± 0.02	^{b1}0.04 ± 0.00
	1:2	^{d2,3}1.82 ± 0.04	^{e1}1.32 ± 0.09	^{b2}0.23 ± 0.02	^{c1}0.05 ± 0.00
	1:1	^{d1}1.71 ± 0.05	^{c1}0.56 ± 0.05	^{a1}0.08 ± 0.00	^{a1}0.03 ± 0.00
	2:1	^{e2}3.05 ± 0.07	^{bc1}0.43 ± 0.05	^{c3}1.09 ± 0.05	^{a1}0.03 ± 0.00

* Same letters indicate no statistical differences ($p > 0.05$) in amendment levels within each aging time while different letters indicate significant differences ($p < 0.05$) across amendment levels within each aging time

* Same numbers indicate no statistical differences ($p > 0.05$) in aged amended soils across the four sampling points while different numbers indicate significant differences ($p < 0.05$) in aged amended soils across the four sampling points (1d to 100d).

3.5 Discussion

3.5.1 Organic amendment ratios on ^{14}C-phenanthrene mineralization

The addition of nitrogen-rich nutrients (biostimulation) and potentially viable microbes (bioaugmentation) through organic materials application to soils are two effective approaches for the bioremediation of PAH-contaminated soils (Wang *et al.*, 2016). The results in the present study showed that contact time influenced the development of catabolic activity as defined by decreases in the length of the lag phases and increases in the rates and extents of mineralization of ^{14}C-phenanthrene in amended soils. This agrees with findings reported from previous studies **(**Abioye *et al.*, 2012; Adam *et al.*, 2015), indicating an organic waste stimulatory effect on the biodegradation of phenanthrene. Generally, the addition of SBG and SMC stimulated phenanthrene catabolism in soils, especially with lower amendment ratios (1:5 and 1:10). Application of higher mix ratios (especially 2:1 organic material to soil) of both amendment types largely reduced the extent of mineralization. Das *et al.* (2011) also noted that due to the very high content of organic materials, microbes may metabolize the readily degradable substrate as carbon source, rather than the target PAH and this was further reflected in the microbial population after mineralization. The microcosm for the higher amendment (2:1) could have limited oxygen transport for microbial activity, hence a reduced mineralization, due to the nature of water saturated bulky material formed after amendment. In addition, this could be linked to high sorptive capacity of organic materials for PAH and subsequent decrease in bioavailability of PAH for microbial degradation (Rhodes *et al.*, 2008). More so, the degree of contaminant sorption and their rapidly/slowly desorbing fractions in amended soils are important factors that determine the extent of microbial sequestration and transformation (Rhodes *et al.*, 2012). The decrease in the catabolic response by the higher

amendment (2:1) agrees with previous studies on organic additives addition to PAHs contaminated soils (Namkoong *et al.*, 2002; Semboung Lang *et al.*, 2016).

In this study, soils amended with SBG and SMC (1:10 and 1:5) showed significantly shorter lag phases over time, while the lag phase of organic waste-soil mixture (2:1) was immeasurable compared to other amendment conditions. This clearly indicated that appropriate amounts of amendments could significantly influence microbial metabolism of the target contaminant (Schaefer and Juliane, 2007). In this study, the lower organic waste-soil mixtures indicated stimulated microbial action as a result of adaptation and bioavailability of PAH fraction as observed in the lag phase compared to the higher ones. Our results found that after 100 d soil incubation, both SGB and SMC showed a further reduction in lag phases with a substantive extent of $^{14}CO_2$ mineralized indicating stimulatory effects of the supplements and acclimatization of the degrading microbial populations. Several previous studies have demonstrated that the extent of PAHs-association with soil matrices could potentially facilitate microbial adaptation and subsequently reduce the lag phase for microbial degradation (Oyelami *et al.*, 2015; Okere *et al.*, 2017). In this current study, the fastest rates of mineralization revealed a similar pattern as observed for lag phases with increasing soil-PAH contact period. However, the fastest rates of mineralization remained relatively constant in the SBG-amended soils compared to the SMC-amended soils. Therefore, this may be attributed to the higher available nutrient, and low total organic carbon (TOC) initially present in SMC slurry system. Soil fertility and species richness have been reported as driving force for ^{14}C-phenanthrene degradation (Oyelami *et al.*, 2012). Okere *et al.* (2017) suggested, however, that a higher TOC in soils may decrease the bioavailability of ^{14}C-phenanthrene to PAH-degraders, hence a reduction on the rate of mineralization. Higher rates of ^{14}C-phenanthrene mineralization in amended soils (1:10 and 1:5) of both SMC and SBG in this study indicated the potential

influence of low organic amendments resulting from optimal waste ratios for microbial community uptake and degradation (Namkoong *et al.*, 2002). Abioye *et al.* (2012) and Sigmund *et al.* (2018) also demonstrated that the addition of smaller amounts of organic amendments to soil (1:10) largely increased degradation rates, despite the sequestration of the chemicals in the soil.

The extents of ^{14}C-phenanthrene mineralization in waste-amended soils depended on the amount of organic material added to the soil. Extents of ^{14}C-phenanthrene mineralization were greater at most time points for both SBG- and SMC-amended soils as the application rates decreased (2:1 < 1:1 < 1:2 < 1:5 < 1:10). In addition, we also found that the lower mix ratios (1:10 and 1:5) consistently displayed higher extents of mineralization than the other amendment conditions in almost all time points. This may be attributed to the potential of the organic wastes to reduce or speed up the desorption process of phenanthrene and/or the likely effect of aging on the bioavailability of PAHs in soil matrices (Semple *et al.*, 2007). It is important to emphasize that sorption of contaminants to soil is a slow and reversible process (Hatzinger and Alexander, 1995).

3.5.2 Influence of microbial population on ^{14}C-phenanthrene catabolism in soils

The data from this study showed that the addition of exogenous organic materials to soil increase the microbial populations with soil microbial response (both heterotrophs and degraders). The potential of organic amendments to stimulate microbial numbers in soil have been reported in previous studies (Semple *et al.*, 2001; Agarry and Latinwo, 2015), as well as their catabolic response in PAH-contaminated soil (Zhang *et al.*, 2012; Sigmund *et al.*, 2018).

The present study suggested that the rich ingredients provided by both amendment types increased microbial proliferation and activity. However, the numbers of phenanthrene-degraders were generally low compared to their counterparts (heterotrophs) in amended soils and this may have affected mineralization end-points in this study. Thus, organic supplements could stimulate the microbial populations (heterotrophs and PAH-degraders) but the low response of PAHs-degrading microorganisms could reduce PAH catabolism in soil (Carmichael and Pfaender, 1997). The results from ^{14}C-phenanthrene mineralization (both rates and extents) reported here were not influenced by the number of phenanthrene-degraders, except the mix ratio of 1:5 for SBG and SMC-amended soils. Also, the CFUs (phenanthrene-degrading fungi and bacteria) present in the 1:1 and 2:1 waste-soil conditions; however, had an influence on the rates and extents of mineralization. Hydrocarbonoclastic microorganisms are the key bio-actors on the biodegradation of pollutants in the environments, but their synergistic role with non-PAH degraders may play an important role in PAH metabolization in contaminated soils (Leahy, 1990).

3.6 Conclusion

The present study indicates that organic amendment (SBG & SMC) addition to soil can influence the ^{14}C-mineralization of phenanthrene over time. Mineralization of phenanthrene varies for different amendment ratios to soil as well as the length of soil incubation. We found that the lower mix ratios (1:5 and 1:10) of both amendment types can provide the most optimal conditions and could effectively enhance ^{14}C-phenanthrene mineralization and microbial numbers over time compared to other mix ratios. Aging the soil significantly reduced the lag phases and increased rates and extents of mineralization in phenanthrene-contaminated soil.

These two organic wastes should be considered as nutrient supplements during bioremediation of contaminated soil.

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3.8 Supplementary Data

Table S1. Physicochemical properties of Myerscough soil used in the experiment (Data adapted from Couling *et al.*, 2010). Values are mean ± SEM (n = 3)

Soil properties		Parameter Values
pH (in dH_2O)		6.50 ± 0.08
Moisture content (%)		21.07 ± 2.78
Microbial heterotrophic numbers (CFU g^{-1})		$2.17 \times 10^5 \pm 1.67 \times 10^4$
Elemental analysis	Total extractable carbon	1.80% ± 0.03
	Total extractable nitrogen	0.14% ± 0.01
	Total extractable organic carbon	1.60% ± 0.07
	Soil organic matter	2.70% ± 0.04
Soil particle size	Clay	19.5% ± 0.70
	Silt	20.0% ± 0.87
	Sand – Total	60.4% ± 1.20
	Coarse sand	0.12% ± 0.01
	Medium sand	6.90% ± 0.10
	Fine sand	53.3% ± 0.60
	Surface texture: clay loam	

Table S2. Physicochemical and microbial characteristics (mean ± SEM) of spent brewery grains (SBG) and spent mushroom compost (SMC) used in the experiment.

Parameters measured	SBG	SMC
pH	5.00 ± 0.00	7.18 ± 0.01
EC (mS)/25^{0}C	2.24 ± 0.00	6.92 ± 0.02
Dry matter (%)	18.0 ± 0.58	21.0 ± 0.94
Extractable N (NH_4-N) (mg/kg **dw**)	112.0 ± 15.0	2258.4 ± 28.2
Extractable N (NO_3-N) (mg/kg **dw**)	**bdl**	1.50 ± 0.16
Extractable P (mg/kg **dw)**	762.7 ± 10.4	249.5 ± 6.33
TOC (mg/kg)	382.0 ± 3.41	46.3 ± 0.33
IC (mg/kg)	3.70 ± 0.12	42.3 ± 0.33
TC (%)	47.4 ± 0.05	34.0 ± 0.01
TN (%)	3.13 ± 0.02	1.80 ± 0.02
TP (%)	0.41 ± 0.03	0.09 ± 0.02
C:N	15.1 ± 0.12	18.9 ± 0.20
THBC (CFU x 10^8 g^{-1} dw)	10.5 ± 1.33	0.31 ± 0.24
TFC (CFU x 10^6 g^{-1} dw)	4.45 ± 0.07	0.15 ± 0.04

*****bdl = below detection limit; dw = dry weight; EC= Electrical conductivity; TOC= Total organic carbon; IC= Iorganic carbon; TC= Total carbon; TN= total nitrogen; TP= Total phosphorus; THBC= Total heterotrophic bacterial count, TFC= Total fungal count

Table S3. Autochthonous heterotrophic and phenanthrene-degrading bacteria after 14 days mineralization of ^{14}C-phenanthrene in spent brewery grains amended soil. Values are mean ± standard error (n = 3)

		Heterotrophs CFU x 10^8 g^{-1} soil dw	Phenanthrene degraders CFU x 10^8 g^{-1} soil dw
Ageing days	Amendment (%)	After Mineralization	After Mineralization
	0	6.60 ± 0.10	13.42 ± 0.09
	1:10	11.2 ± 0.26	42.6 ± 0.11
	1:5	9.47 ± 0.30	39.1 ± 0.35
1	1:2	2.20 ± 0.22	15.8 ± 0.76
	1:1	3.17 ± 0.24	0.00 ± 0.00
	2:1	15.2 ± 0.18	0.00 ± 0.00
	0	2.10 ± 0.00	0.66 ± 0.01
	1:10	48.4 ± 0.79	43.4 ± 0.71
	1:5	42.2 ± 0.61	54.2 ± 0.30
25	1:2	46.2 ± 0.61	27.4 ± 3.26
	1:1	15.9 ± 0.61	11.5 ± 0.46
	2:1	0.00 ± 0.00	0.02 ± 0.00
	0	3.18 ± 0.02	9.47 ± 0.11
	1:10	6.77 ± 2.93	1.63 ± 0.13
	1:5	1.09 ± 0.14	3.45 ± 0.20
50	1:2	1.19 ± 0.14	3.17 ± 0.11
	1:1	0.99 ± 0.12	2.17 ± 0.09
	2:1	0.19 ± 0.00	0.98 ± 0.03
	0	0.22 ± 0.03	0.22 ± 0.09
	1:10	0.03 ± 0.00	0.92 ± 0.06
	1:5	4.31 ± 0.17	1.47 ± 0.10
75	1:2	0.99 ± 0.11	1.46 ± 0.11
	1:1	4.13 ± 0.03	2.47 ± 0.13
	2:1	0.19 ± 0.00	1.06 ± 0.03
	0	0.19 ± 0.00	0.07 ± 0.03
	1:10	4.31 ± 0.10	0.28 ± 0.00
	1:5	2.27 ± 0.06	0.24 ± 0.01
100	1:2	0.99 ± 0.03	0.18 ± 0.01
	1:1	4.13 ± 0.09	0.15 ± 0.00
	2:1	0.19 ± 0.00	0.00 ± 0.00

Table S4. Autochthonous heterotrophic and phenanthrene-degrading bacteria present after 14 days mineralization of ^{14}C-phenanthrene in spent mushroom compost-amended soil. Values are mean ± standard error (n = 3)

		Heterotrophs CFU x 10^8 g^{-1} soil dw	Phenanthrene degraders CFU x 10^8 g^{-1} soil dw
Ageing days	Amendment (%)	After Mineralization	After Mineralization
1	0	0.10 ± 0.01	0.27 ± 0.02
	1:10	0.83 ± 0.04	2.65 ± 0.04
	1:5	1.54 ± 0.03	2.29 ± 0.04
	1:2	1.99 ± 0.03	3.02 ± 0.04
	1:1	2.09 ± 0.03	3.98 ± 0.06
	2:1	0.90 ± 0.01	5.73 ± 0.04
25	0	0.35 ± 0.00	0.94 ± 0.01
	1:10	0.99 ± 0.14	2.18 ± 0.11
	1:5	1.22 ± 0.08	4.78 ± 0.74
	1:2	1.02 ± 0.18	1.59 ± 0.06
	1:1	1.03 ± 0.20	2.89 ± 0.13
	2:1	2.65 ± 0.36	3.03 ± 0.37
50	0	0.24 ± 0.03	1.69 ± 0.10
	1:10	1.34 ± 0.11	1.52 ± 0.15
	1:5	1.77 ± 0.04	1.10 ± 0.23
	1:2	2.21 ± 0.19	2.37 ± 0.06
	1:1	1.80 ± 0.04	1.46 ± 0.12
	2:1	8.89 ± 0.17	2.83 ± 0.44
75	0	0.37 ± 0.00	0.00 ± 0.00
	1:10	0.30 ± 0.02	0.89 ± 0.07
	1:5	0.37 ± 0.02	0.86 ± 0.08
	1:2	0.40 ± 0.04	1.59 ± 0.14
	1:1	0.32 ± 0.03	1.09 ± 0.30
	2:1	2.11 ± 0.18	1.10 ± 0.09
100	0	0.18 ± 0.06	0.09 ± 0.02
	1:10	0.90 ± 0.01	0.71 ± 0.03
	1:5	0.83 ± 0.04	0.54 ± 0.01
	1:2	0.72 ± 0.02	1.36 ± 0.01
	1:2	0.36 ± 0.05	1.21 ± 0.20
	2:1	1.43 ± 0.06	1.13 ± 0.10

Table S5. Autochthonous heterotrophic and phenanthrene-degrading fungi present after 14 days mineralization of ^{14}C-phenanthrene in spent brewery grains amended soil. Values are mean ± standard error (n = 3)

		Heterotrophic fungi CFU x 10^6 g^{-1} soil dw	Phen. degrading fungi CFU x 10^6 g^{-1} soil dw
Ageing (days)	Amendment (%)	After Mineralization	After Mineralization
1	0	0.08 ± 0.02	0.40 ± 0.01
	1:10	0.30 ± 0.03	3.39 ± 0.06
	1:5	0.60 ± 0.02	4.01 ± 0.08
	1:2	1.22 ± 0.03	3.33 ± 0.03
	1:1	1.64 ± 0.01	0.10 ± 0.02
	2:1	1.03 ± 0.01	0.03 ± 0.00
25	0	0.06 ± 0.00	0.07 ± 0.01
	1:10	0.57 ± 0.03	0.73 ± 0.06
	1:5	0.78 ± 0.09	1.00 ± 0.07
	1:2	1.35 ± 0.08	1.33 ± 0.11
	1:1	1.82 ± 0.12	0.82 ± 0.05
	2:1	2.13 ± 0.09	0.06 ± 0.00
50	0	0.73 ± 0.07	0.89 ± 0.20
	1:10	4.65 ± 0.27	16.6 ± 1.53
	1:5	5.35 ± 0.20	20.5 ± 1.66
	1:2	14.7 ± 1.36	9.90 ± 0.61
	1:1	20.9 ± 0.93	16.5 ± 2.11
	2:1	0.50 ± 0.05	1.27 ± 0.11
75	0	1.19 ± 0.03	0.54 ± 0.02
	1:10	2.17 ± 0.07	1.31 ± 0.04
	1:5	17.3 ± 0.46	1.09 ± 0.05
	1:2	17.7 ± 0.44	2.21 ± 0.09
	1:1	17.9 ± 0.93	2.77 ± 0.10
	2:1	42.3 ± 1.46	4.12 ± 0.08
100	0	1.10 ± 0.03	0.20 ± 0.00
	1:10	1.05 ± 0.06	0.28 ± 0.00
	1:5	1.42 ± 0.05	0.16 ± 0.00
	1:2	1.63 ± 0.04	0.18 ± 0.00
	1:1	2.70 ± 0.05	0.24 ± 0.00
	2:1	2.90 ± 0.10	0.06 ± 0.00

Table S6. Autochthonous heterotrophic and phenanthrene-degrading fungi present after 14 days mineralization of ^{14}C-phenanthrene in spent mushroom compost-amended soil. Values are mean ± standard error (n = 3)

		Heterotrophic fungi CFU x 10^6 g^{-1} soil dw	Phen. degrading fungi CFU x 10^6 g^{-1} soil dw
Ageing (days)	Amendment (%)	After Mineralization	After Mineralization
1	0	0.22 ± 0.05	0.47 ± 0.12
	1:10	0.28 ± 0.00	0.21 ± 0.00
	1:5	0.23 ± 0.00	0.34 ± 0.00
	1:2	0.40 ± 0.00	0.53 ± 0.00
	1:1	0.35 ± 0.00	0.44 ± 0.00
	2:1	0.37 ± 0.00	0.37 ± 0.00
25	0	0.08 ± 0.00	1.17 ± 0.99
	1:10	0.68 ± 0.06	0.48 ± 0.02
	1:5	1.21 ± 0.24	0.62 ± 0.06
	1:2	0.53 ± 0.07	1.67 ± 0.12
	1:1	0.55 ± 0.08	0.87 ± 0.10
	2:1	1.47 ± 0.29	1.07 ± 0.09
50	0	0.35 ± 0.02	0.23 ± 0.02
	1:10	0.18 ± 0.00	0.09 ± 0.00
	1:5	0.08 ± 0.00	0.20 ± 0.00
	1:2	0.14 ± 0.00	0.24 ± 0.00
	1:1	0.22 ± 0.00	0.17 ± 0.00
	2:1	0.14 ± 0.00	0.18 ± 0.00
75	0	0.09 ± 0.01	0.07 ± 0.20
	1:10	0.35 ± 0.02	1.11 ± 0.01
	1:5	0.60 ± 0.76	0.60 ± 0.76
	1:2	0.43 ± 0.07	0.66 ± 0.03
	1:1	0.43 ± 0.08	1.21 ± 0.06
	2:1	1.11 ± 0.15	1.27 ± 0.15
100	0	0.05 ± 0.00	0.07 ± 0.00
	1:10	0.00 ± 0.00	0.03 ± 0.00
	1:5	0.11 ± 0.01	0.09 ± 0.00
	1:2	0.09 ± 0.00	0.19 ± 0.00
	1:1	0.10 ± 0.00	0.12 ± 0.00
	2:1	0.81 ± 0.00	0.10 ± 0.00

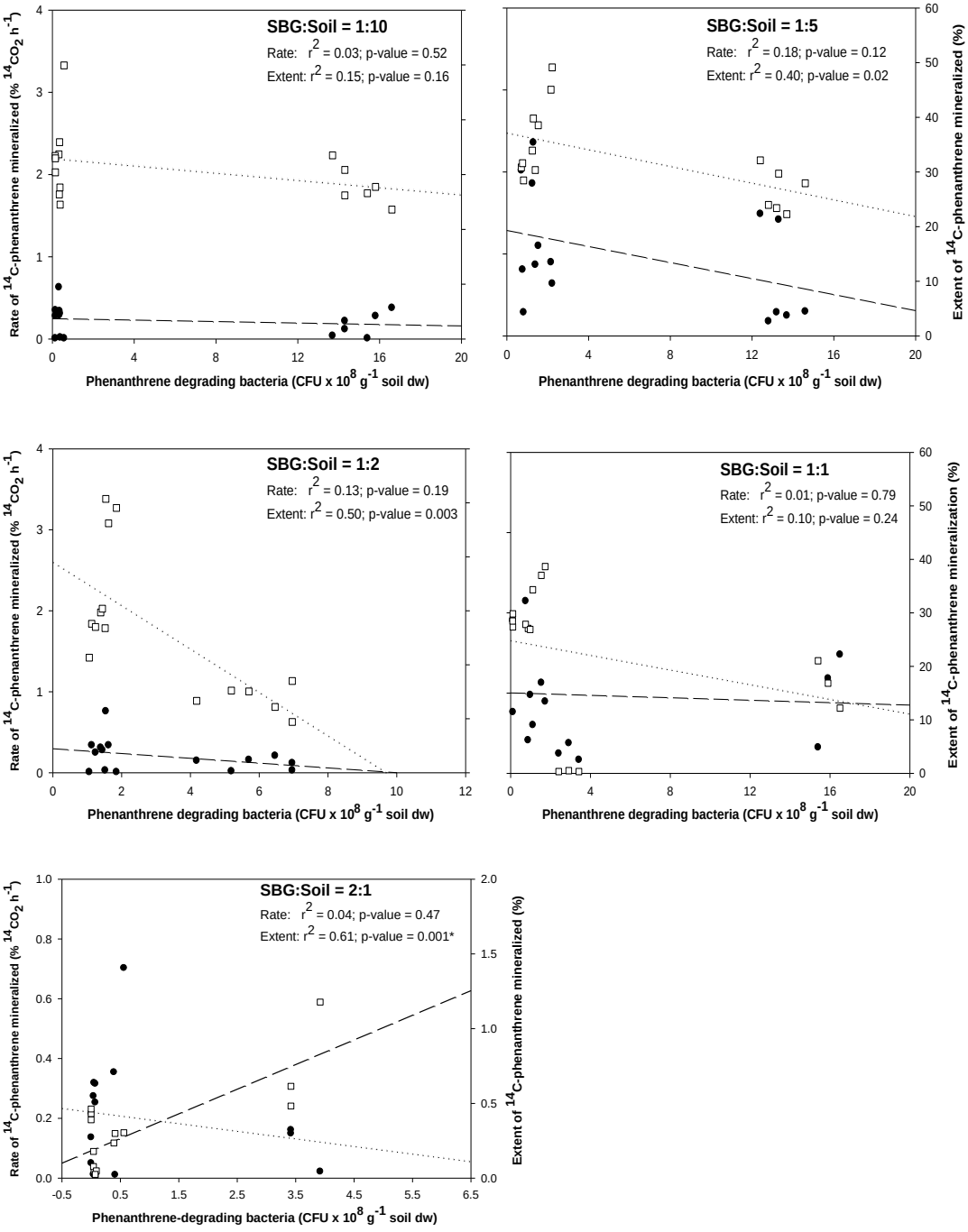

Figure S1. Relationship between both fastest rate and total extent of ^{14}C-phenanthrene mineralization with phenanthrene-degrading bacteria amended with SBG:soil at 1:10, 1:5, 1:2, 1:1 and 2:1 after soil-phenanthrene contact time (1 – 100d)

Keys: *Rate of ^{14}C-phenanthrene mineralized vs phenanthrene-degrading bacteria in amended soil (▫ ·····)

*Extent of ^{14}C-phenanthrene mineralized vs phenanthrene-degrading bacteria in amended soil (● ——).

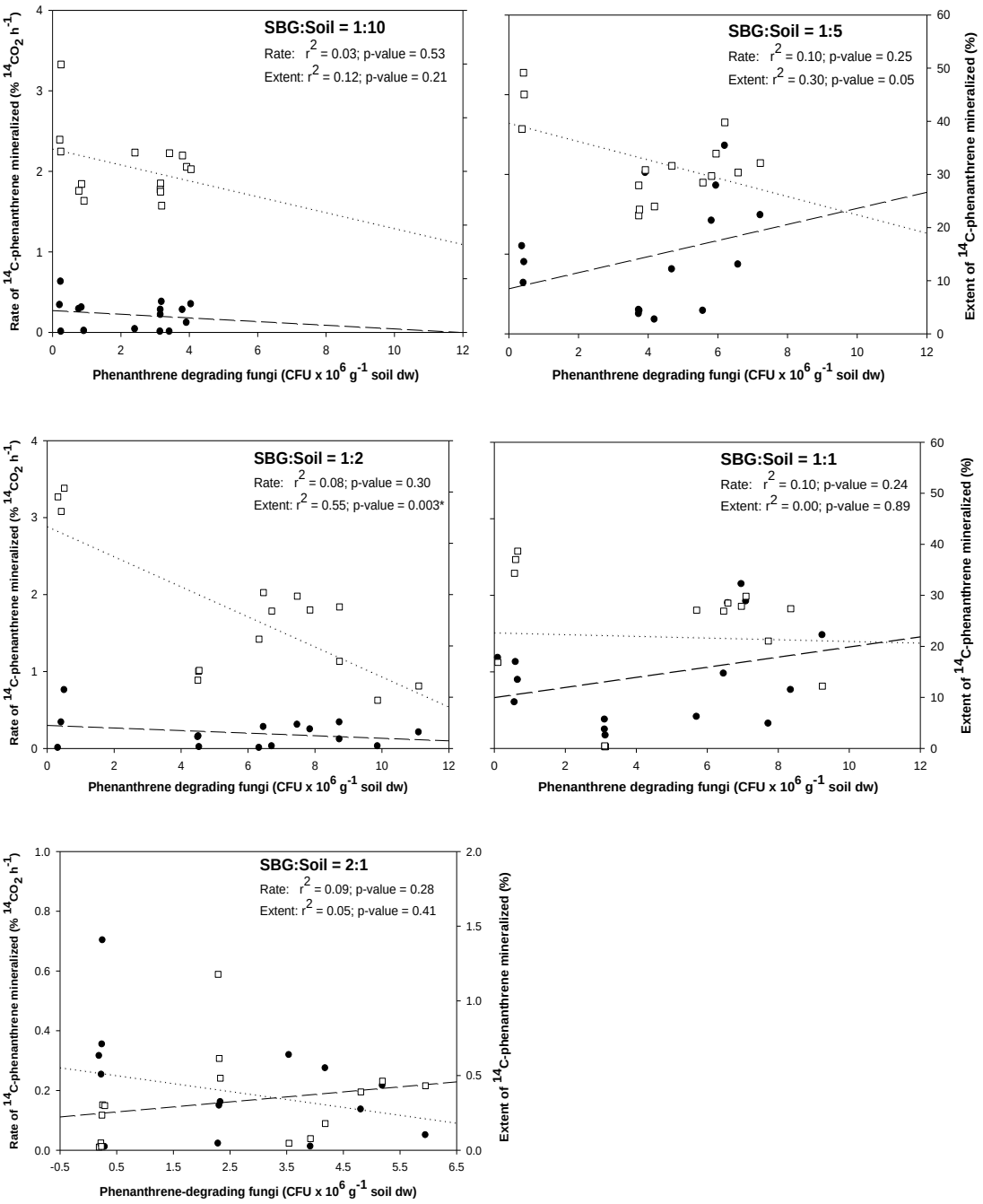

Figure S2. Relationship between both fastest rate and total extent of ^{14}C-phenanthrene mineralization with phenanthrene-degrading fungi amended with SBG:soil at 1:10, 1:5, 1:2, 1:1 and 2:1 after soil-phenanthrene contact time (1 – 100d)

Keys:

*Rate of ^{14}C-phenanthrene mineralized vs phenanthrene-degrading bacteria in amended soil (▭ ┈┈)

*Extent of ^{14}C-phenanthrene mineralized vs phenanthrene-degrading bacteria in amended soil (● — —).

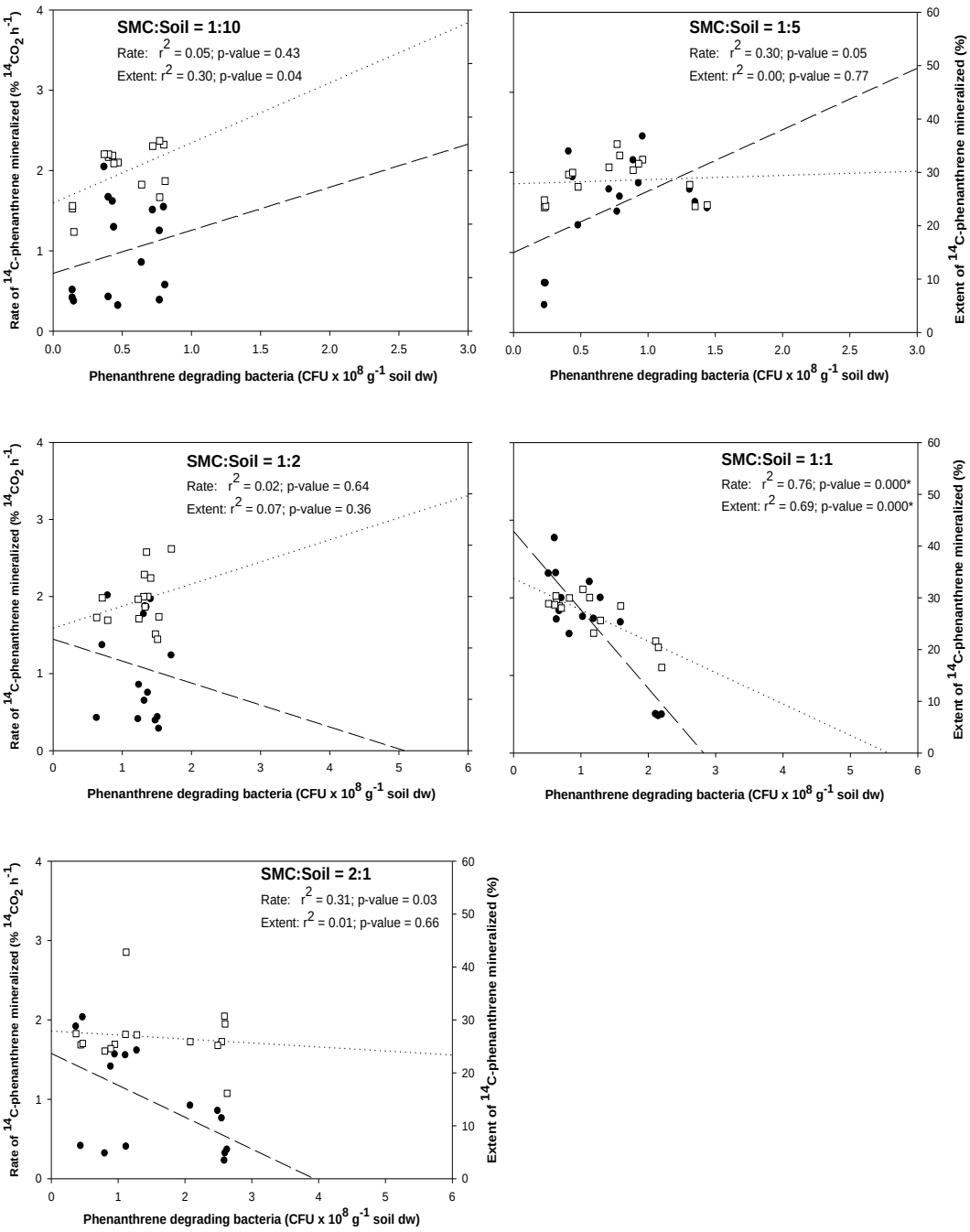

Figure S3. Relationship between both fastest rate and total extent of ^{14}C-phenanthrene mineralization with phenanthrene-degrading bacteria amended with SMC:soil at 1:10, 1:5, 1:2, 1:1 and 2:1 after soil-phenanthrene contact time (1 – 100d)

Keys: *Rate of ^{14}C-phenanthrene mineralized vs phenanthrene-degrading bacteria in amended soil (.....)

*Extent of ^{14}C-phenanthrene mineralized vs phenanthrene-degrading bacteria in amended soil (——)

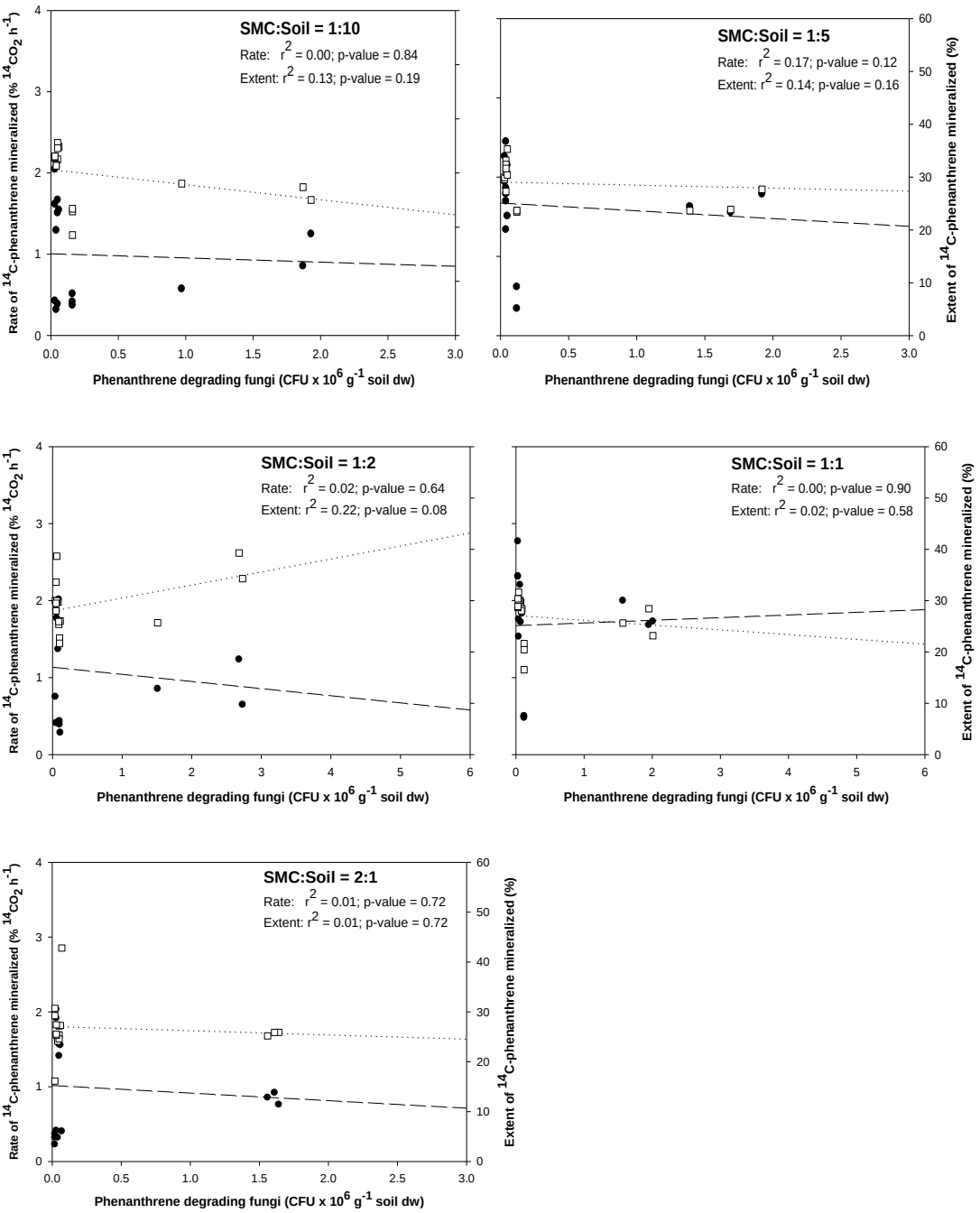

Figure S4. Relationship between both fastest rate and total extent of ^{14}C-phenanthrene mineralization with phenanthrene-degrading fungi amended with SMC:soil at 1:10, 1:5, 1:2, 1:1 and 2:1 after soil-phenanthrene contact time (1 – 100d)

Keys:

*Rate of ^{14}C-phenanthrene mineralized vs phenanthrene-degrading bacteria in amended soil (▭ ·····)

*Extent of ^{14}C-phenanthrene mineralized vs phenanthrene-degrading bacteria in amended soil (▬ — —)

Chapter 4

The Impact of Enhanced and Non-Enhanced Biochars on the Catabolism of ^{14}C-Phenanthrene in Soil

The Impact of Enhanced and Non-Enhanced Biochars on the Catabolism of ^{14}C-Phenanthrene in Soil

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4.1 Abstract

Biochar is a by-product from the pyrolysis of biomass and has a great potential in soil amendment due to its carbon and nutrient-rich properties. The aim of this study was to investigate the impact of increasing amounts (0, 0.01, 0.1, 0.2, 0.5 and 1.0%) of two types of biochar (so-called enhanced and non-enhanced) to soil on the biodegradation of ^{14}C-phenanthrene. Enhanced biochar contains inoculants which are designed to potentially stimulate microbial activity and promote biological function in soil. After 100 d of incubation, the addition of 0.5% and 1% enhanced (EbioC) and non-enhanced biochars (NEbioC) led to longer lag phases, reduced rates and extents of ^{14}C-phenanthrene in amended soil. However, in soils amended with 0.01%, 0.1% and 0.2% amendments, extents of mineralisation of ^{14}C-phenanthrene increased and were found to be higher in the EBioC- as compared to the NEbioC-amended soils. Increasing soil-phenanthrene contact time also increased ^{14}C-phenanthrene mineralisation in soil which had received smaller amounts of EBioC. Application of both EbioC and NEbioC also enriched the soil microbial populations during the incubation. However, it was found that phenanthrene-degrading microbial populations declined as soil contact time increased; this was particularly true for soils receiving larger amounts due to reduction in the mobile/bioaccessible fraction of the phenanthrene in soil. The findings revealed the importance of the type and amount of biochar that may be added to soil to stimulate or enhance organic contaminant biodegradation.

Keywords — Enhanced biochar; non-enhanced biochar; phenanthrene; mineralization; soil-PAH contact time

4.2 Introduction

The degree and impact of polycyclic aromatic hydrocarbons (PAHs) in soil pollution resulting from oil spills, especially from production and exploration activities (Obida *et al.*, 2018), and other anthropogenic sources (Simon and Sobieraj, 2006); pose great threats to human health and the environment. These organic contaminants are known to be carcinogenic and mutagenic in nature (Dong *et al.*, 2012). Microbial degradation is one of the most important mechanisms for removing these contaminants from soil, but the rate and extent of removal is dependent on the chemical and physical properties of the contaminant (Semple et al,. 2007; Simon and Sobieraj, 2006; Xie *et al.*, 2015) as well the soil properties which include nutrient availability, temperature, moisture, presence and activity of the target degrading microorgansims, bioavailable fraction of the contaminant to the degrading microbes, and heterogeneity of the soils (organic matter and mineral fractions) and their associated pore structures (Okere and Semple, 2012; Riding *et al.*, 2013; Semple *et al.*, 2013; Umeh *et al.*, 2017). The heterogenous nature of organic matter and mineral fractions in soil, in part, determines the sorption-desorption mechanisms of contaminants within the soil matrix (Umeh *et al.*, 2017). These properties have contributed to a large extent to the hydrophobicity, lipophilicity, solubility, soil-water partition coefficient (Kd), persistence, mass transfer, mobility, bioaccessibility and biodegradation of the organic contaminants in soil (Abdel-Shafy and Mansour, 2016; Ghosal *et al.*, 2016). In the case of PAHs, increases in the number of fused benzene rings will also increase the persistence as higher molecular weight PAHs are less biodegradable in soil (Couling *et al.*, 2010; Abdel-Shafy and Mansour, 2016).

The application of microbial degradation for the clean up contaminated soils is called bioremediation, which depends on the intrinsic role of microbes and their metabolic enzymes

to metabolize chemicals down to less toxic metabolites or to CO_2. However, using biodegradation as a tool to remediate contaminated soils is often too slow to reduce associated risk to acceptable levels; therefore, interventions are often required to speed up the process (Xu *et al.*, 2018). Recent reports show that several studies have been carried out to enhance the remediation of contaminated soil but present several challenges, including low nutrients and contaminant bioavailability, a reduction in soil microbial activity and a low degradative potential by the indigenous microbes involved in the biodegradation process (Bisht *et al.*, 2015; Zhang *et al.*, 201 6; Kong *et al.*, 2018). It is therefore imperative that the use of nutritional and biological enrichments to contaminated soils should be done through economically viable and environmentally sustainable approaches.

One approach to enhancing bioremediation involves the addition of biomass-derived materials to contaminated soil. Organic materials, such as biochars, may offer a low cost, carbon- and nutrient-rich biomass amendment for stimulating and/or enhancing biodegradation of PAHs in contaminated soil. Biochars are pyrolytic products from organic feedstocks under zero or low-oxygen concentrations at different temperatures of 250^{o}C –1000^{o}C (Kuśmierz *et al.*, 2016; Kumar *et al.*, 2018). Studies have shown that these carbonaceous and sorbent materials improved soil physicochemical properties (structure, stability, pH, water holding capacity, nutrients, carbon energy (Anyika *et al.*, 2015); adsorb and retain nitrogen form (NH_4^+) in soil (Gai *et al.*, 2014), while stimulating microbial activity, growth and composition (Galitskaya *et al.*, 2016), as well as changes in the microbial community structure in soil (Zhang *et al.*, 2018). Additionally, biochar not only influences the oxygen level in soil but also supports aerobic and anaerobic biodegradation (Anyika *et al.*, 2015), especially when used at low dose in biochar-treated soil. Apart from improving microbial activity, the sorptive properties of biochar gives an additional benefit of trapping contaminant in soil.

The sorptive properties of biochars have been widely studied, however few studies have also reported their biodegradative potential for PAHs in soils, owing to their stimulation for substrate bioavailability for microbial degradation through the formation of microhabitat (bacteria and fungi) for actively growing autochthonous soil microflora through electrostatic attraction and attachment to biochar's porous surfaces (Anyika *et al.*, 2015; Ogbonnaya *et al.*, 2016; Zhang *et al.*, 2018). For example, a recent study showed that microbe-biochar interaction enhanced mass transfer of PAHs (making the contaminant more bioavailable) to immobilized cells, thus resulting to higher PAH degradation when compared to un-inoculated biochar in a sorbent-amended system (Xiong *et al.*, 2017). Although the claims of the effectiveness of using biochar to improve bioremediation, its impact on soil bioremediation, depends on the concentration of the contaminant, soil conditions, active sites, types and properties of the biochar (Jones *et al.*, 2012; Yuan *et al.*, 2019). Biochars have been widely studied as a soil conditioner and immobilized carriers particularly for the biodegradation and management of soil remediation. However, the effects of biochar types (enhanced biochar and non-enhanced biochar) and application rate to soil for optimum PAHs metabolism have not been studied extensively.

The main objectives of this research study were (i) to investigate the impact of biochars (enhanced and non-enhanced) on the catabolic potential in soil on ^{14}C-phenanthrene mineralisation (a model PAH compound) over time; (ii) to determine the effects of increasing amounts of both biochars on the mineralisation of ^{14}C-phenanthrene in soil over time, and (iii) to estimate changes in microbial numbers in biochar-amended and phenanthrene-spiked soil.

4.3 Materials and Methods

4.3.1 Soil and Biochar

The surface agricultural soil (5–20 cm depth) used for this study was collected from Myerscough Agricultural College, Preston, United Kingdom. After transferring to the laboratory, soil samples were air-dried, homogenized, sieved through a 2mm mesh and thereafter, stored in the dark until use. Information on the soil characteristics are presented in Table S1 (Couling *et al.*, 2010). Processed biochars, microbially enhanced (EbioC) and non-enhanced (NEbioC), were collected from a biochar processing plant, Lancaster, UK. Biochars were produced from combined lignocellulosic feedstocks (virgin wood and agricultural residues) by slow pyrolysis at a high temperature of 500^{o}C for 4 h under a low oxygen atmosphere in a muffle furnace. Some information on the physical and chemical properties of the biochars are presented in Table 1. After production, some pyrolyzed biochars were cultured (enriched) with mixed microbial inoculants: the arbuscular mycorrhizal fungi *such as Glomus* spp (> 450 propagules/g), *Ascophyllum nodosum* and *Trichoderma* spp (>1x10^{9} CFU/g), as well as wormcasts. Biological amendments were prepared and immobilized (physically attached) onto the biochar surface by spraying onto the material, thereby producing the enhanced biochar. Both biochars were specifically produced for agricultural purposes as soil conditioners to provide required nutrients and to improve soil biological function for plant growth.

Table 1. Physical and chemical properties of the biochars used in the experiment

Parameters analysed		Enhanced Biochar	Non-enhanced biochar	Instruments used and methodologies described
pH		8.6	9.0	pH meter (Jenway model 3504 Bench combined) (Li *et al.*, 2013)
EC (µS/cm)		1136	498	Conductivity meter (Jenway model 3504 Bench combined), (Li *et al.*, 2013)
TC (%)		50.4	78.2	Schimadzu TOC-L analyser (Siudek *et al.*, 2015)
Total N (dry wt) (% dry)		1.15	1.55	Elemental analyser (Vario EL III CHNOS, Hanau, Germany), (Wilke, 2010)
NH_4-N (%)		0.12	0.032	Autoanalyzer model 3HR (AAR 3HR), (Haney *et al.*, 2008).
Ash (%)		35.6	14	Fisher Isotemp 650 Model 58 Programmable Muffle Furnace (Domingues *et al.*, 2017).
Total solids (%)		85.1	66.6	Thermophilic oven (Genlab, UK), (Marmiroli *et al.*,2018)
Total P (%)		0.10	0.045	Spectrophometer (Limwikran *et al.*, 2019)
Mineral components (wt %)				Inductively coupled plasma spectrometry (ICS–MS, PerkinElmer NexION 2000 (Limwikran *et al.*, 2019)
	Total Ca	5.85	5.17	
	Total Mg	0.23	0.17	
	Total K	0.72	0.56	
	Total Na	0.12	0.015	
	Total B	0.002	0.002	
	Total Fe	0.27	0.012	
	Total Mn	0.009	0.002	
	Total Al	0.19	0.015	
	Total S	0.14	0.041	
	Total Mo	-	-	
	Total Cl	0.084	0.019	
	Total Cu	-	-	
	Total Zn	0.002	-	

* EC = electrical conductivity, TC = Total carbon, NH_4-N (ammonium-nitrogen), P = phosphorus, Ca =calcium, Mg = magnesium, K = potassium, Na = sodium, B = boron, Fe = iron, Mn = manganese, Al = aluminium, S = sulphur, Mo = molybdenum, Cl = chlorine, Cu = copper, Zn = zinc

4.3.2 Biochar doses and soil amendment conditions

EbioC and NEbioC were amended to soil at different amounts: 0.0%, 0.01%, 0.1%, 0.2%, 0.5% and 1% (dry weight basis) per total mass of soil. The above doses were amended into the soil to evaluate the potential of each rate to stimulate microbial activities and optimal phenanthrene biodegradation. Soil moisture content (25% on dry matter basis) and pH (7.4 – 7.5) after soil-biochar amendments were monitored and maintained throughout the study period.

4.3.3 Soil spiking and Incubation conditions

Sieved and homogenized soil was spiked with ^{12}C-phenanthrene (100 mg/kg) to give a final concentration of 240 mg/kg; and soil spiking was done according to 'Bolus methodology' (Doick *et al.*, 2003). Briefly, the whole soil sample was divided into four portions; one proportion (approx. 525 g) was spiked with ^{12}C-phenanthrene using acetone as the carrier solvent. This was closely followed by blending the remaining three equal parts of the soil, and afterward, allow to vent for 4hrs (in a fume cupboard) to evaporate acetone (Lee *et al.*, 2003). This was done after rehydration with sterile water based on the water holding capacity (WHC) of soil (55 wt%), while maintaining 25% MC, however. The soil was then amended with different amounts of EbioC and NEbioC: 0.0%, 0.01%, 0.1%, 0.2%, 0.5% and 1% (w/w). All biochar-amended soil samples were prevented from photo-oxidation by storage in air-tight separate amber glass bottles and further incubated in the dark at 21 ± 1°C for 0, 25, 50, 75 and 100 days. Triplicate mixtures were placed in an air-tight amber glass bottle for each condition and allowed to weather in the dark at 21 ± 2°C for 1, 25, 50, 75 and 100 days. Control (non-amended) soils were also incubated alongside with amended soils.

4.3.4 Mineralisation assay in biochar-amended soils

Respirometry assay was carried out to determine the catabolic evolution (^{14}C-phenanthrene to $^{14}CO_2$) from both biochars-amended soil according to standard methods (Reid *et al.*, 2001; Semple *et al.*, 2006). Briefly, the mineralisation of ^{14}C-phenanthrene was monitored in soil microcosms by weighing 10 ± 0.1 g (dry weight) from soil-biochar mixtures into 30 ml of sterile distilled water in 250 ml Scott bottles (Teflon-lined screw cap). Thereafter, the slurry was spiked with ^{14}C-radiolabelled phenanthrene standard (0.5 kBq per respirometer bottle) and incubated at 100 rpm on a flat-bed orbital shaker at 21 ± 1°C. Additionally, each bottle contained a CO_2 trap (7 ml vial) with 1 M NaOH and monitored bihourly for 1 d and then regularly for 24 hrs during an 18 d incubation period. Sampling was done at every time point (1, 25, 50, 75 and 100 d) for biochar-amended soils. Catabolic response in biochar-treated soils were assessed at each sampling period through the lag phases (when mineralisation reached 5%), the fastest rates of $^{14}CO_2$ evolved (expressed also as the maximum rate of $^{14}CO_2$ production) within 1 d in amended soils, and the total extents of ^{14}C-phenanthrene mineralisation after 18 d (Macleod and Semple, 2006).

4.3.5 Microbial enumeration analyses

Spread plate method was used to enumerate the total heterotrophs and phenanthrene degraders in biochar-amended soil (Okere and Semple, 2012; Anyanwu and Semple, 2016). A 10-fold serial dilution was done by weighing 1 ± 0.01 g of biochar-amended soil in 9 ml of Ringer's solution (¼ strength). An aliquot was further transferred into appropriate plates. The plate count agar (PCA) supplemented with an antimicrobial agent (streptomycin-penicillin-glutamic, 8 µl/ml) and, amphotericin-B (5 µl/ml) added to potato dextrose agar (PDA) were used for

enumeration of heterotrophic bacteria and fungi, respectively. Phenanthrene degraders (bacteria and fungi) were counted in minimal basal salt (MBS) media (Vázquez-Cuevas *et al.*, 2018) incorporated with 0.05 mg/ml of ^{12}C-phenanthrene (as sole carbon source) and supplemented with appropriate antimicrobials (bacteria and fungi). Microbial numbers were quantified and presented in CFUs/g_{dw} soil.

4.3.6 Statistical analysis

The biochar-amended experiments in this study were carried out in triplicates. The data were analysed on Statistical Package for the Social Sciences (IBM SPSS Version 23.0). All amended soils were analysed using one-way analysis of variance (ANOVA) at 95% confidence level ($p < 0.05$) to determine the least significant difference (LSD). To test for the effects of both biochars and amounts in phenanthrene-spiked soils, comparison of means within and across time points were analysed using Tukey's Post-Hoc and Games-Howell test to ascertain differences in biochar-treated soils. Pearson product-moment correlation coefficient (r) was performed to describe the relationship between microbial numbers with the rate and extent of ^{14}C-phenanthrene mineralised in biochar-treated soils. The value of r is ranked on a scale between +1 and –1 as clearly described elsewhere (Omoni *et al.*, 2020).

4.4 Results

4.4.1 Mineralisation of ^{14}C-phenanthrene in biochar-amended soils

The impacts of both biochars (EbioC and NEbioC) on the mineralisation of ^{14}C-phenanthrene in soil were studied with varied amounts of biochar (0.0%, 0.01%, 0.1%, 0.2%, 0.5% and 1%)

during 18 d soil respirometry after 1, 25, 50, 75 and 100 d of soil-PAH contact time (Figure 1 and 2; Table 2 and 3). The lag phase represents the time taken to reach 5% mineralisation. The lag phases behaviour in the two treatment soils, EbioC and NEbioC were not obviously different from the non-treated soils in most time points throughout the study. Soil amended with EbioC produced shorter lag periods when compared to their counterpart soil (NEbioC). The shortest lag phase of 0.46 ± 0.00 d and 0.63 ± 0.00 d were achieved in the 0.1% EbioC and NEbioC-amended soils, respectively. On the other hand, the longest lag phase of 8.37 ± 0.01 d and 8.86 ± 0.03 d were achieved in the 0.01% EbioC and NEbioC-amended soils, respectively. However, after 1 d soil-PAH contact time, the biochar types showed significantly longer lag phases in all amended soils. The larger application doses (0.5 and 1.0%) displayed shorter lag phases compared to other treatments and control (0%). This trend was not consistent following further soil ageing, for example, the application doses (0.5 and 1.0%) for both EbioC and NEbioC greatly extended the length of the lag phase (50 > 75 > 100 d) ($p < 0.01$). However, in comparison to 1 d soil-PAH contact time, biochar application doses (0.01, 0.1, 0.2, 0.1 & 1.0%) after 25 d ageing period, displayed reductions in lag phases with these orders of magnitude starting from the lowest to highest amount of EbioC (16 > 15 > 14 > 13 > 7-fold) and NEbioC (12 > 11 > 10 > 9 > 8-fold) (Table 2 and 3). Generally, EbioC (0.1 & 0.2%) consistently reduced the lag phases ($p < 0.001$) compared to other biochar dosages and unamended soil (control) throughout the study period; while soil amended with the NEbioC did not display any similar trend in amended soils (Table 2). Noticeably, the lag phases in the non-amended (control) soil were significantly shorter ($p < 0.05$) than those soils receiving 0.5 and 1.0% amendments for the NEbioC.

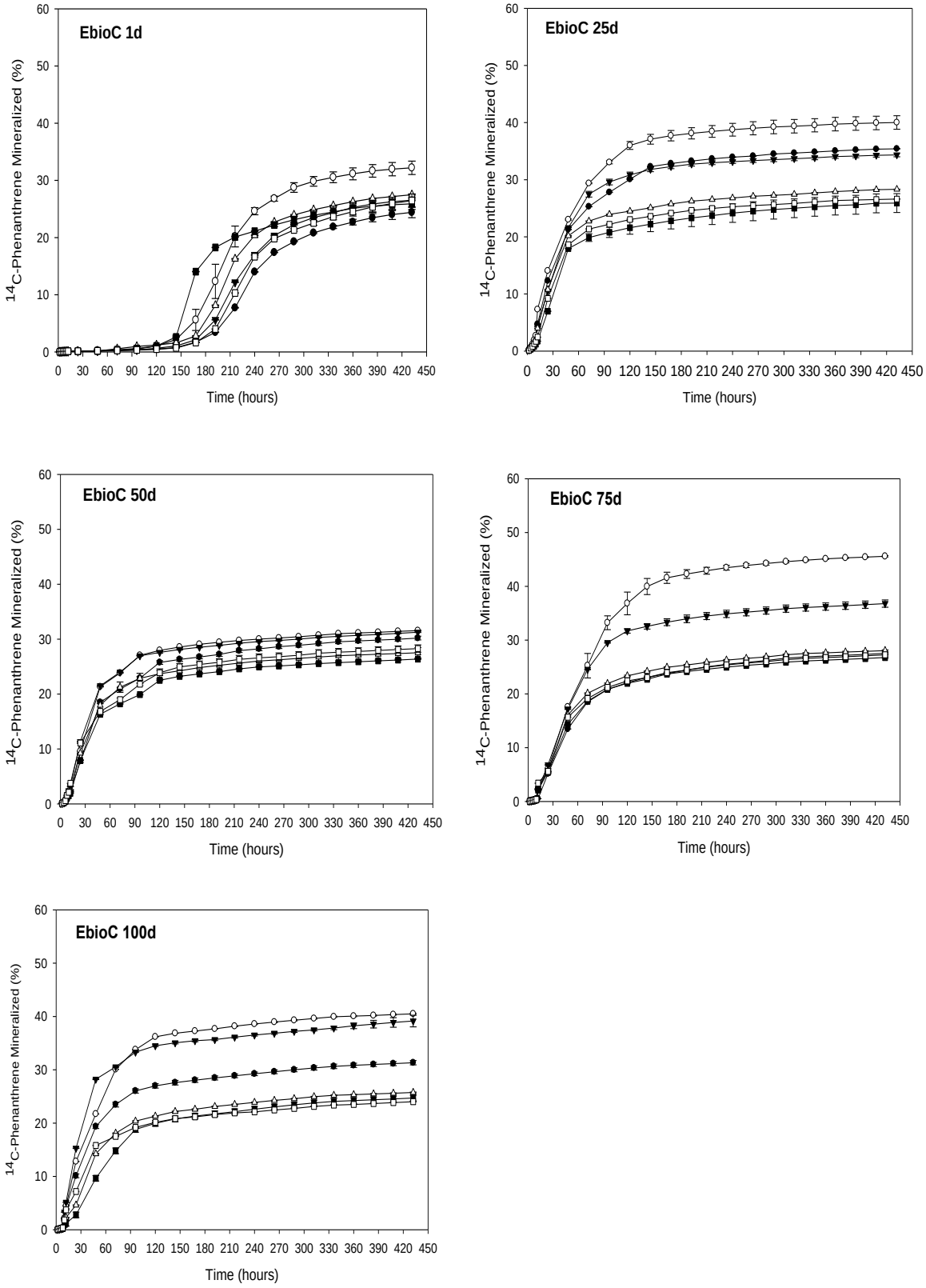

Figure 1. Evolution of $^{14}CO_2$ from the catabolism of ^{14}C-phenanthrene in soil (100mg/kg) amended with enhanced biochar at 0.01% (●), 0.10% (○), 0.20% (▼), 0.50% (Δ), 1% (■) and Control (□) after 1, 25, 75 and 100d soil-phenanthrene contact time. Standard error of mineralisation (SEM) are represented as triplicate samples (n = 3).

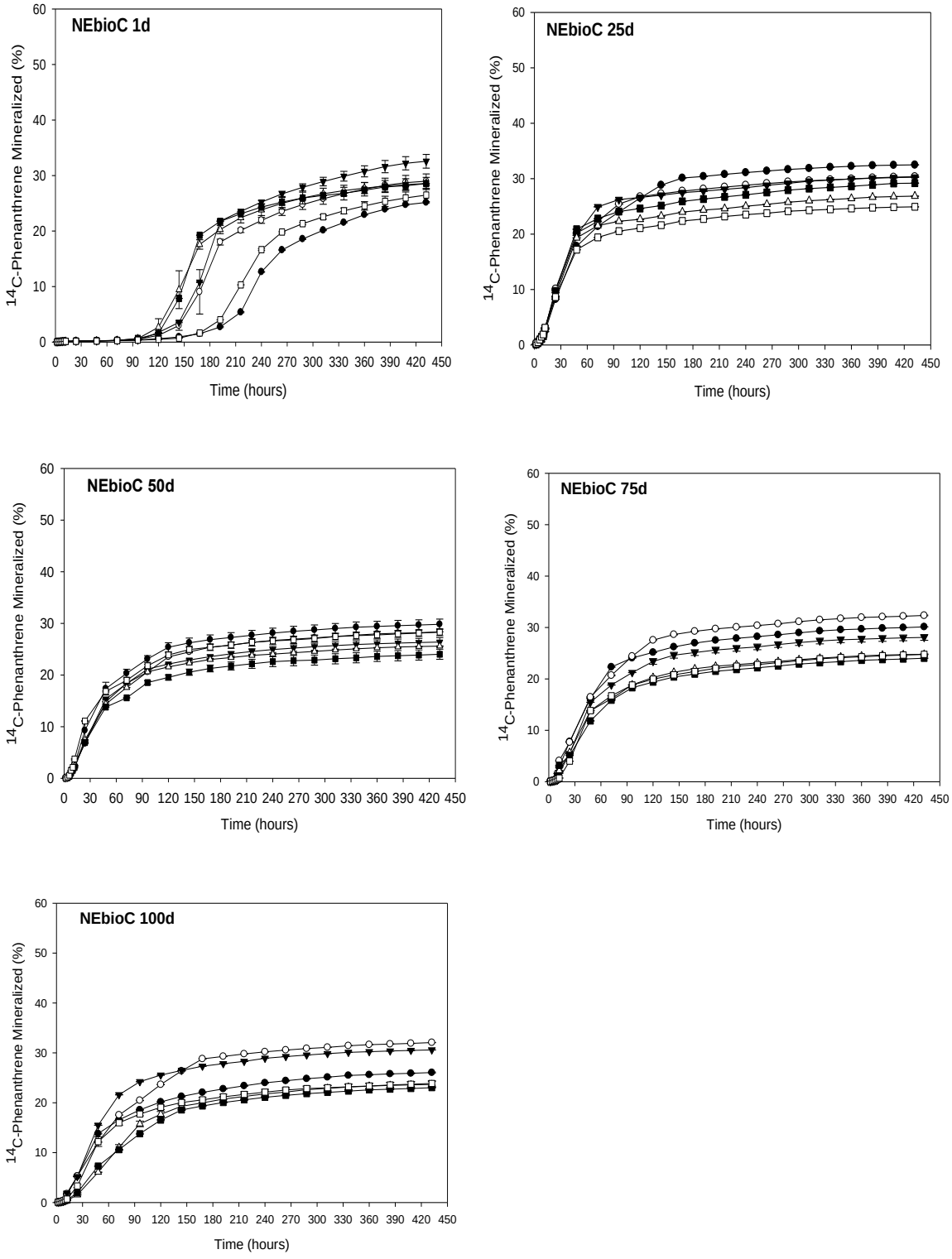

Figure 2. Evolution of $^{14}CO_2$ from the catabolism of ^{14}C-phenanthrene in soil (100mg/kg) amended with non-enhanced biochar at 0.01% (●), 0.10% (○), 0.20% (▼), 0.50% (Δ), 1% (■) and Control (□) after 1, 25, 75 and 100d soil-phenanthrene contact time. Standard error of mineralisation (SEM) are represented as triplicate samples (n = 3)

As observed for the lag phases, the addition of EbioC resulted in faster rates of ^{14}C-phenanthrene mineralisation than those observed in the NEbioC incubations (Tables 2 and 3). In addition, the fastest rates of mineralisation in both EbioC and NEbioC-amended soils were generally higher in the soils that had received the smaller amounts of biochar (0.1, 0.2 & 0.01%) and in more aged soils (1 d to 100 d)**.** However, in 1 d aged soils containing 1% EbioC biochar, there was a significantly increase in the fastest rates of mineralisation compared to other soil amendments and control (Table 2). However, there were no significant changes ($p < 0.05$) in the 1% EbioC biochar from this point onward throughout the incubation. There were significantly faster rates of mineralisation ($p < 0.001$) observed in 0.1% EbioC- (2.29%/d) and NEbioC- (1.24%/d) amended soils compared to the other soil conditions (Table 2 and 3). Further, in soils with no biochar amendment, significantly faster rates of mineralisation were found at most time points compared to the NEbioC-amended soils.

As soil contact time increased in the biochar-amended soils, there were commensurate increases in the fastest rates of ^{14}C-phenanthrene mineralisation, especially in soils receiving smaller amounts (0.01, 0.1 & 0.2%) of biochar. Interestingly, the results for EbioC-amended soils showed that the lower amounts of biochar (0.01, 0.1 & 0.2%) resulted in increases the fastest rates of $^{14}CO_2$ mineralised by 80.7%, 83.4% and 79.4%, after 25 d, respectively, compared to 1-day soil-PAH contact time ($p < 0.001$). Similarly, the results also showed significant increases in fastest rates for 0.01, 0.1 and 0.2% biochar after 75-d (64.2, 21.1 and 38.8%) and 100 d (76.1, 68.1 and 82.2%) incubations when compared to 1-d soil incubation. Correspondingly, the fastest rates of mineralisation were found at 75 d for all NEbioC-amended soils; showing higher rates of 75.6% and 58.1% increases for 0.01 and 0.1% amendments, respectively, compared to 1-d incubation. However, in this investigation, when compared to both biochar-treated soils, the non-amended (0%) soil consistently showed increases in the

fastest rates of mineralisation ($p < 0.001$) as soil-phenanthrene contact time increased: 51.8%, 67.1%, 82.1% and 71.3% after 1, 25, 50 and 100 d, respectively.

The effects of the catabolic potential of both EbioC and NEbioC and their respective dosage application to phenanthrene-spiked soil on the total extent of ^{14}C-phenanthrene mineralisation were also monitored (Figures 1 and 2; Tables 2 and 3). Results indicate that soils amended with 0.1% and 0.2% amounts of biochar showed the highest extent of ^{14}C-phenanthrene mineralisation (45.6% and 32.57%), while the lowest were recorded for 1.0% (24.71% and 23.01%) with EbioC and NEbioC ($p < 0.001$), respectively. At the first time point (1 d), EbioC (0.1%) generally showed considerably higher extents of mineralisation within and across treatments and control ($p < 0.001$) and over the rest of the incubation period. A similar trend was observed for soils with NEbioC amendments, but these were not significantly different from 0.01% biochar amendment ($p > 0.05$). Furthermore, after 75 and 100 d soil-PAH contact time, the total extents of mineralisation significantly increased in the 0.1% (29.3 and 20.5%) and 0.2% (27.9 and 32.26%) EbioC-amended to soils respectively, when compared to 1 d aging period. Greater amounts of biochar amendment (0.5 and 1.0%) did not significantly increase ($p < 0.05$) the total extents of ^{14}C-phenanthrene mineralisation throughout the study for either the EbioC or the NEbioC soil amendments. In general, the presence of the NEbioC-biochar in PAH-spiked soil did not influence the total extents of mineralisation positively compared to EbioC; the exception being in the soils receiving smaller biochar amendments (0.01 and 0.1%).

Table 2. Catabolic and autochthonous microbial number profile (CFU/g) of ^{14}C-phenanthrene mineralisation in soil amended with varying amounts of enhanced biochar. Values are mean ± standard error (n = 3).

Soil-PAH aging (d)	Amended amounts	Lag phase (d)	Fastest rates (/d)	Total Extent (%)	Bacteria		Fungi	
					Total Heterotrophs	PAH degraders	Total Heterotrophs	PAH degraders
					CFU x 10^7 g^{-1} soil dw		CFU x 10^4 g^{-1} soil dw	
1	0	8.16 ± 0.00	0.27 ± 0.00	26.47 ± 0.16	1.66 ± 0.03	0.93 ± 0.02	4.33 ± 0.12	2.13 ± 0.09
	0.01	8.37 ± 0.01	0.26 ± 0.00	24.40 ± 0.94	0.97 ± 0.02	0.83 ± 0.01	2.30 ± 0.12	3.07 ± 0.03
	0.1	7.22 ± 0.01	0.38 ± 0.00	32.20 ± 1.17	1.72 ± 0.03	0.94 ± 0.02	1.87 ± 0.09	3.23 ± 0.12
	0.2	7.82 ± 0.00	0.27 ± 0.00	26.52 ± 0.08	1.43 ± 0.02	0.87 ± 0.02	1.97 ± 0.09	2.23 ± 0.03
	0.5	7.41 ± 0.01	0.34 ± 0.00	27.50 ± 0.08	1.23 ± 0.03	0.59 ± 0.01	2.67 ± 0.12	2.27 ± 0.03
	1	6.21 ± 0.00	0.48 ± 0.00	25.91 ± 0.99	1.17 ± 0.02	1.09 ± 0.01	3.27 ± 0.15	2.93 ± 0.03
25	0	0.69 ± 0.00	0.56 ± 0.00	26.61 ± 0.03	7.17 ± 0.15	0.98 ± 0.01	4.77 ± 0.07	7.40 ± 0.10
	0.01	0.52 ± 0.00	1.35 ± 0.00	35.42 ± 0.05	12.4 ± 0.27	1.20 ± 0.01	2.40 ± 0.06	3.33 ± 0.12
	0.1	0.46 ± 0.00	2.29 ± 0.00	40.20 ± 1.18	19.0 ± 0.35	1.47 ± 0.02	2.40 ± 0.10	2.33 ± 0.07
	0.2	0.55 ± 0.00	1.31 ± 0.00	34.34 ± 0.50	14.3 ± 0.21	1.23 ± 0.01	2.37 ± 0.03	3.47 ± 0.09
	0.5	0.57 ± 0.00	1.15 ± 0.00	28.33 ± 0.02	14.9 ± 0.31	0.77 ± 0.01	5.17 ± 0.09	4.17 ± 0.09
	1	0.81 ± 0.00	0.46 ± 0.00	25.91 ± 1.65	14.6 ± 0.35	1.29 ± 0.02	4.07 ± 0.03	6.10 ± 0.06
50	0	0.59 ± 0.00	0.82 ± 0.00	28.29 ± 0.09	18.1 ± 0.15	2.95 ± 0.03	10.3 ± 0.22	6.12 ± 0.26
	0.01	0.75 ± 0.01	0.47 ± 0.01	30.20 ± 0.34	75.1 ± 0.42	28.2 ± 0.93	3.54 ± 0.19	4.09 ± 0.17
	0.1	0.67 ± 0.00	0.58 ± 0.00	31.56 ± 0.08	38.8 ± 0.42	31.0 ± 0.44	4.22 ± 0.26	3.80 ± 0.15
	0.2	0.61 ± 0.00	0.68 ± 0.02	31.26 ± 0.05	83.1 ± 1.12	23.4 ± 0.42	3.16 ± 0.15	3.04 ± 0.13
	0.5	0.70 ± 0.00	0.58 ± 0.00	27.53 ± 0.94	73.0 ± 0.84	3.44 ± 0.02	5.15 ± 0.30	4.39 ± 0.11
	1	0.76 ± 0.00	0.50 ± 0.00	26.38 ± 0.67	61.6 ± 1.12	3.64 ± 0.02	4.47 ± 0.33	3.63 ± 0.04
75	0	0.87 ± 0.00	1.51 ± 0.00	27.26 ± 0.06	15.4 ± 0.08	2.34 ± 0.02	3.38 ± 0.04	2.93 ± 0.07
	0.01	0.98 ± 0.00	0.39 ± 0.00	27.55 ± 0.04	56.1 ± 1.52	3.61 ± 0.01	5.06 ± 0.07	3.37 ± 0.07
	0.1	0.86 ± 0.00	0.94 ± 0.02	45.56 ± 0.16	88.2 ± 1.84	15.5 ± 0.22	6.14 ± 0.11	3.10 ± 0.12
	0.2	0.80 ± 0.00	0.93 ± 0.03	36.79 ± 0.67	53.2 ± 1.93	11.5 ± 0.23	5.27 ± 0.15	2.33 ± 0.07
	0.5	0.90 ± 0.01	0.90 ± 0.00	28.05 ± 0.05	42.2 ± 1.52	3.33 ± 0.02	4.18 ± 0.15	1.97 ± 0.09
	1	0.93 ± 0.01	0.92 ± 0.03	26.76 ± 0.03	43.9 ± 2.95	2.35 ± 0.01	4.09 ± 0.15	2.57 ± 0.12
100	0	0.69 ± 0.04	0.94 ± 0.00	24.02 ± 0.04	1.57 ± 0.02	2.94 ± 0.03	0.35 ± 0.00	2.91 ± 0.15
	0.01	0.53 ± 0.01	1.09 ± 0.04	31.30 ± 0.42	28.7 ± 0.19	20.8 ± 0.30	8.23 ± 0.15	3.21 ± 0.21
	0.1	0.52 ± 0.00	1.19 ± 0.04	40.50 ± 1.09	29.7 ± 0.37	22.8 ± 0.34	14.1 ± 0.13	2.74 ± 0.04
	0.2	0.49 ± 0.00	1.52 ± 0.02	39.15 ± 0.11	18.8 ± 0.15	29.4 ± 0.32	9.37 ± 0.26	3.54 ± 0.13
	0.5	1.08 ± 0.01	0.93 ± 0.00	25.75 ± 0.07	17.7 ± 0.11	3.16 ± 0.01	3.00 ± 0.21	2.83 ± 0.15
	1	1.27 ± 0.01	0.49 ± 0.00	24.71 ± 0.53	17.2 ± 0.38	18.9 ± 0.11	3.42 ± 0.26	1.94 ± 0.04

Table 3. Catabolic and autochthonous microbial number profile (CFU/g) of ^{14}C-phenanthrene mineralisation in soil amended with varying amounts of non-enhanced biochar. Values are mean ± standard error (n = 3).

					Bacteria		Fungi	
Soil-PAH aging (d)	Amended amounts	Lag phase (d)	Fastest rates (/d)	Total Extent (%)	Total Heterotrophs	PAH degraders	Total Heterotrophs	PAH degraders
					CFU x 10^7 g^{-1} soil dw		CFU x 10^4 g^{-1} soil dw	
1	0	8.16 ± 0.00	0.27 ± 0.00	26.47 ± 0.13	0.66 ± 0.01	0.93 ± 0.01	4.33 ± 0.12	2.13 ± 0.09
	0.01	8.86 ± 0.04	0.30 ± 0.00	25.15 ± 0.09	1.10 ± 0.01	0.92 ± 0.01	2.30 ± 0.11	3.30 ± 0.11
	0.1	7.02 ± 0.06	0.52 ± 0.01	28.98 ± 1.31	1.86 ± 0.02	1.01 ± 0.00	1.93 ± 0.03	3.10 ± 0.06
	0.2	6.20 ± 0.01	0.46 ± 0.00	32.57 ± 1.24	1.43 ± 0.00	0.88 ± 0.03	2.10 ± 0.06	2.53 ± 0.03
	0.5	5.80 ± 0.00	0.44 ± 0.00	28.57 ± 1.10	1.29 ± 0.02	0.56 ± 0.00	2.67 ± 0.12	2.03 ± 0.03
	1	5.55 ± 0.01	0.47 ± 0.00	28.54 ± 0.02	2.06 ± 0.03	1.15 ± 0.02	3.37 ± 0.09	4.17 ± 0.09
25	0	0.69 ± 0.00	0.56 ± 0.00	26.61 ± 0.03	7.17 ± 0.09	0.98 ± 0.01	4.77 ± 0.07	7.40 ± 0.10
	0.01	0.72 ± 0.00	0.49 ± 0.00	32.49 ± 0.02	9.47 ± 0.09	1.19 ± 0.01	2.40 ± 0.06	5.23 ± 0.09
	0.1	0.65 ± 0.00	0.60 ± 0.01	30.36 ± 0.06	12.0 ± 0.03	1.33 ± 0.02	2.33 ± 0.03	7.00 ± 0.06
	0.2	0.65 ± 0.00	0.54 ± 0.00	30.27 ± 0.05	6.53 ± 0.09	1.07 ± 0.01	2.23 ± 0.07	7.94 ± 0.03
	0.5	0.68 ± 0.00	0.57 ± 0.00	26.84 ± 0.01	9.87 ± 0.07	1.06 ± 0.01	4.70 ± 0.06	5.47 ± 0.03
	1	0.65 ± 0.00	0.71 ± 0.02	29.19 ± 0.04	8.84 ± 0.07	1.28 ± 0.01	4.83 ± 0.09	7.30 ± 0.12
50	0	0.59 ± 0.00	0.82 ± 0.00	28.29 ± 0.67	18.1 ± 0.17	2.95 ± 0.03	10.3 ± 0.22	6.12 ± 0.26
	0.01	0.78 ± 0.00	0.46 ± 0.02	29.83 ± 1.00	37.4 ± 0.26	20.9 ± 0.28	5.06 ± 0.13	3.88 ± 0.18
	0.1	0.79 ± 0.00	0.39 ± 0.00	28.37 ± 0.04	33.3 ± 0.26	17.0 ± 0.50	4.26 ± 0.15	4.01 ± 0.11
	0.2	0.79 ± 0.00	0.39 ± 0.00	26.38 ± 0.85	45.0 ± 0.11	16.9 ± 0.38	7.22 ± 0.34	4.85 ± 0.11
	0.5	0.76 ± 0.00	0.42 ± 0.00	25.60 ± 0.04	41.2 ± 0.11	3.12 ± 0.11	3.97 ± 0.11	4.77 ± 0.04
	1	0.80 ± 0.00	0.40 ± 0.00	24.12 ± 0.98	25.9 ± 0.30	3.38 ± 0.11	3.97 ± 0.08	3.12 ± 0.18
75	0	0.87 ± 0.00	1.51 ± 0.03	27.26 ± 0.06	15.4 ± 0.08	2.34 ± 0.02	3.38 ± 0.04	2.93 ± 0.07
	0.01	0.72 ± 0.00	1.23 ± 0.03	30.11 ± 0.02	46.0 ± 2.57	0.34 ± 0.02	2.95 ± 0.04	3.00 ± 0.06
	0.1	0.63 ± 0.00	1.24 ± 0.00	32.35 ± 0.07	66.7 ± 1.84	1.11 ± 0.02	4.73 ± 0.11	3.43 ± 0.09
	0.2	0.94 ± 0.00	0.66 ± 0.00	27.51 ± 0.03	43.5 ± 0.42	0.37 ± 0.03	2.83 ± 0.04	2.83 ± 0.07
	0.5	0.90 ± 0.01	0.90 ± 0.00	24.79 ± 0.05	47.3 ± 1.84	0.22 ± 0.03	2.70 ± 0.11	2.67 ± 0.12
	1	0.97 ± 0.01	1.05 ± 0.03	24.02 ± 0.03	38.8 ± 0.42	0.22 ± 0.04	2.78 ± 0.15	2.27 ± 0.03
100	0	0.69 ± 0.04	0.94 ± 0.02	24.02 ± 0.04	1.57 ± 0.02	2.94 ± 0.03	0.35 ± 0.00	2.91 ± 0.15
	0.01	0.97 ± 0.01	0.59 ± 0.00	26.05 ± 0.10	12.4 ± 0.15	2.65 ± 0.35	9.32 ± 0.24	2.70 ± 0.17
	0.1	0.96 ± 0.00	0.69 ± 0.03	32.06 ± 0.03	10.4 ± 0.19	4.73 ± 0.32	11.9 ± 0.28	3.71 ± 0.11
	0.2	0.97 ± 0.00	0.79 ± 0.00	30.59 ± 0.01	10.7 ± 0.37	7.34 ± 0.15	17.5 ± 0.22	2.36 ± 0.08
	0.5	1.76 ± 0.01	0.22 ± 0.01	23.67 ± 0.57	14.6 ± 0.38	2.04 ± 0.06	16.6 ± 0.26	2.24 ± 0.15
	1	1.57 ± 0.01	0.22 ± 0.00	23.01 ± 0.10	13.7 ± 0.15	1.96 ± 0.06	28.4 ± 0.26	3.08 ± 0.08

4.4.2 Microbial enumeration in biochar-amended soil

4.4.2.1 Bacterial numbers

Soil amendment with biochar (EbioC and NEbioC) significantly increased the total heterotrophic bacterial number at all time points ($p < 0.05$ *and 0.001*), as compared to control (Tables 2 and 3). Further, EbioC-amended soils consistently displayed similar patterns that could be represented in the order of increasing heterotrophic bacterial number (50 > 75 > 100 > 25 > 1) ($p < 0.001$) compared to NEbioC-amended soil. The heterotrophic bacterial numbers were also significantly influenced by 0.1% biochar addition than all other soil amendment conditions (EbioC and NEbioC), including the unamended soil ($p < 0.05$).

Similarly, the addition of both biochars to phenanthrene-spiked soil statistically increased the number of phenanthrene degrading bacteria (CFUs g^{-1} soil) in all soil conditions compared to control (unamended) throughout the study (Tables 2 and 3). Increasing the soil contact time with PAH also significantly influenced ($p < 0.05$) the CFUs in EbioC and NEbioC-amended soils; although these numbers were not consistent in NEbioC-amended soil. The number of phenanthrene-degrading bacteria for both biochar types in amended soils with lower amounts (0.01, 0.1 and 0.2%) presented much higher CFUs as compared to the higher biochar amended soils (0.5 and 1.0%) and control soil ($p > 0.05$). Comparatively, after 1 day, EbioC (0.1 and 0.2%) behaved differently in soil with 32 and 26-fold and 32, 16 and 19-fold increase in phenanthrene-degrading bacterial numbers at 50-day, respectively, when compared to other amendment conditions and incubation period. Similarly, the numbers of phenanthrene degraders in the NEbioC-amended soils resulted in 32, 16 and 19-fold increase in amended soil (0.01, 0.1 and 0.2%), respectively. Furthermore, as mentioned earlier, most contact points did not significantly stimulate increases in the numbers of phenanthrene degraders ($p > 0.05$) when

amended with higher amounts of biochar (0.5 and 1.0%) in both soil amendments (EbioC and NEbioC).

From the data, the number of phenanthrene degraders showed a moderately positive linear correlation ($r = 0.55$, $p < 0.05$) and moderately negative correlation ($r = -0.63$, $p < 0.05$) with total extent of ^{14}C-phenanthrene mineralisation for 0.2% and 1.0% of EbioC-amended soils, respectively. Also, a negative correlation for 0.1% ($r = -0.54$, $p < 0.05$) and 0.2% ($r = -0.64$, $p < 0.05$) between phenanthrene-degraders and total extent of mineralisation for NEbioC were observed, for the respective application doses (Figure S1). Phenanthrene-degraders displayed a significantly strong but negative correlation ($r = -0.72$, $p < 0.01$) with fastest rates of ^{14}C-phenanthrene mineralised in 1.0% NEbioC-amended soil; whilst the number of ^{14}C-phenanthrene degraders observed in 0.1% and 0.5% showed a significant, but negative correlations ($r = -0.52$, $p < 0.05$ and $r = -0.66$, $p < 0.05$) with fastest rates of mineralisation, respectively (Figure S3).

4.4.2.2 Fungal numbers

Overall, the numbers of phenanthrene-degrading fungi were inconsistent within and across biochar-amended soils over time (Tables 2 and 3). The total fungal numbers (TFCs) were significantly higher in the control soils compared to soils amended with biochar at 50 d of exposure. However, this trend changed as both biochar types added to soils significantly resulted in higher TFC after a longer contact (75 to 100 days), resulting in a significant decreasing order of magnitude (1.0% < 0.5% < 0.2% < 0.1% < 0.01%). Phenanthrene-degrading fungi recorded the highest CFUs (7.97×10^4 g^{-1} soil dw) in 0.2% NEbioC-amendment after 25 d of incubation**.** However, significant numbers of phenanthrene degrading

fungi were also recorded in soils amended with biochar (0.01% and 0.1%) compared to other amendment conditions. A significant positive correlation was recorded between the phenanthrene degraders with both fastest rates ($r = 0.80, p < 0.000$) and total extents ($r = 0.52, p < 0.05$) of ^{14}C-phenanthrene mineralised in 0.2% EbioC-amended soil, while 0.1% negatively correlated with both fastest rates ($r = 0.82, p < 0.000$) and total extents ($r = 0.60, p < 0.05$) of mineralisation in amended soils (Figure S2). Finally, NEbioC-amended soils with 0.01% and 1.0% amounts of biochar showed strong positive correlations ($r = 0.72, p < 0.01$ and $r = 0.83, p < 0.01$) between ^{14}C-phenanthrene-degraders and total extents of ^{14}C-phenanthrene mineralisation, respectively (Figure S4).

4.5 Discussion

4.5.1 Effects of enhanced and non-enhanced biochar on ^{14}C-phenanthrene mineralisation

Addition of EbioC to PAH contaminated soil increased microbial activity and greatly impacted on ^{14}C-phenanthrene mineralisation when compared to NEbioC-amended soils, which was even more evident following increases in soil-phenanthrene contact time. This may be due to more bioaccessible fractions of the sorbed-PAH substrate on the immobilised biochar surface for biodegradation (Uyttebroek *et al.*, 2006; Zhang *et al.*, 2018), thus resulting in an increase in soil metabolic activity. Further, Xiong *et al.* (2017) showed that biochar incorporated into soil provided a protective niche allowing for higher metabolic activities and increases in the concentration gradients between sorbed-PAH on biochar surfaces and contact with microbial cell surfaces over shorter distances. Similarly, Galitskaya *et al.* (2016) also observed higher PAH fluxes and stimulated biodegradation in amended soil with biochar. Mass transfer of

PAHs to degrading cells in a biochar-amended soil could be facilitated by dissolved and solid sorbing matrices for PAH degradation (Xiong *et al.*, 2017).

The addition of external nutrient supplies and organic materials to soil would influence the indices of quantifying biodegradation, such as lag phases, fastest rates and overall extents of organic contaminant biodegradation (Jablonowski *et al.*, 2013; Oyelami *et al.*, 2013; Ogbonnaya *et al.*, 2016). In this current study, the immobilised inoculum on the EbioC could have provided an additional support for the indigenous microbial population than NEbioC which led to shorter lag phases, increases in fastest rates and extents of ^{14}C-phenanthrene mineralisation in amended soil. The biological carrier materials (EbioC) can accelerate nutrient uptake and release, improved oxygen and water holding capacity and support metabolic activities and processes in soil rhizosphere (Yakhin *et al.*, 2017; Drobek *et al.*, 2019), thereby indicating their efficacies in nutrient stimulatory action in a poor contaminated or polluted agricultural soil.

Analysis of our results showed shorter lag phases, faster rates and greater extents of mineralisation in the lower biochar doses (0.1% > 0.2% > 0.01%) of both biochar type in amended soils; however, this was more pronounced in EbioC -amended soils. Although, both biochar-amended soils showed significantly extended lag phases after 1 d soil incubation, increasing soil-PAH contact time (25 d onward), the lower biochar amendments (0.1% > 0.2% > 0.01%) impacted on the lag phases, rates and extents of phenanthrene biodegradation in soil. This can be attributed to the fraction of the phenanthrene that is rapidly desorbable or present in the aqueous biochar amended soil phase, which is accessible to microbial cells and their catabolic apparatus (Ogbonnaya *et al.*, 2014; Rhodes *et al.*, 2010).

The fastest rates of ^{14}C-phenanthrene mineralisation depended on the amounts of biochar-amended to soil. Smaller amounts of biochar increased the rates of mineralisation in comparison to the larger amendments over time in both biochar types. Similarly, Ogbonnaya *et al.* (2014) also reported reductions in rates of phenanthrene catabolism in soils which had received large amounts (1%) of wood-derived biochar. It has been reported that properties such as porosity, contaminant concentration and physico-chemical properties, organic matter content, cation exchange capacity, soil contact time, soil properties, microbial activity, diversity and dynamics may all influence rates of mineralisation of organic contaminants in soil (Semple *et al.*, 2003; Ogbonnaya *et al.*, 2013). It is worthy of note that faster rates of mineralisation were measured in soils receiving the EbioC-amendment compared to the NEbioC-amended soils. This clearly indicates the potential of the inoculants in stimulating microbial activity and contaminant catabolism in soil, in particular.

The application of larger amounts (1.0% > 0.5%) of both biochar types reduced ^{14}C-mineralisation in soil. Even though biochar has been reported to have intrinsic ability to biodegrade organic contaminants (Anyika *et al.*, 2015), the sorptive properties of black carbon, including biochars, can reduce mass transfer and bioaccessibility of PAHs to soil microorganisms (Rhodes *et al.*, 2008; 2012; Anyika *et al.*, 2015; Ogbonnaya *et al.*, 2016). This further suggests the likely effect that is seen in this investigation occurs in soils with higher biochar amendments. Biochar type (quantity and quality), dose, and application conditions have also been reported previously as some determining factors which govern the degree of sequestration of PAHs in biochar-amended soil (Sopeña, *et al.*, 2012; Galitskaya *et al.*, 2016; Xiong *et al.*, 2017). For example, in this current study, biochar was prepared at a higher temperature (~500 ^{o}C), which could have increase aromaticity, owing to associated higher surface area and lower cation-exchange capacity (Anyika *et al.*, 2015), suggesting a greater

sorptive effect of PAHs to surfaces as observed for larger biochar amendments reported here (0.5 and 1.0%).

4.5.2 Influence of microbial numbers on the mineralisation of ^{14}C-phenanthrene in biochar-amended soil

Soil amended with biochar can influence the abundance, diversity, and distribution of soil microbial communities, owing to changes in their biological, physical and chemical properties which might also lead to an increase/decrease in soil microbial biomass, the minerals content and organics in soil (Awad *et al.*, 2018). The data obtained in this study revealed stimulated microbial numbers (both bacteria and fungi) in EbioC-amended soils over time. Particularly, it was also found that the lower amendments (0.01, 0.1 & 0.2%) of EbioC added to soil increase the bacterial and fungal CFUs (heterotrophs and phenanthrene-degraders); whereas the 0.5% and 1.0% biochar amendments resulted in lower CFUs especially from 50 d to 100 d in NEbioC-amended soils. The numbers measured in the lower amendments are likely be due to increasing metabolic rates, as seen in the fastest rates of ^{14}C-phenanthrene mineralisation, bioavailable nutrients, lower microbial community disruption and lower water vapour isotherms as a result of reduced soil organic matter (Peake *et al.*, 2014). Soil minerals and organic matter can sorb and bind to biochar surfaces, thus creating more and potentially stronger sorption sites in the biochar for organic contaminants (Semple *et al.*, 2007; Ogbonnaya and Semple, 2013). It may be hypothesised that the phenanthrene may have been more strongly sorbed to the soil-biochar complex in the soils which received larger amendments of 0.5 and 1.0%. This may have caused a slower or irreversible desorption of the PAH or rapidly depleted desorbable fractions of the contaminant over time (50 -100 d) for microbial uptake and biodegradation (Rhodes *et al.*, 2008; 2012). For example, Rhodes *et al.* (2012) noticed a 7.8-

fold decrease in rapidly ($\%F_{rap}$) and a corresponding increase in slowly ($\%F_{slow}$) desorbing fractions of phenanthrene in soils receiving larger amount of black carbon (a form of pyrolyzed carbon) and with increase soil contact time (1 d to 100 d). Further, Rhodes *et al.* (2012) found that there were 50% reductions in the rates of ^{14}C-phenanthrene mineralised with increasing amount of black carbon from 0 to 5% after 20 d soil-PAH contact time. Therefore, it can be suggested that the greater amounts of biochar increased the number of microporous sites for contaminant sorption in biochar-amended soils. Generally, the biochar increased the total numbers of heterotrophs (bacteria and fungi) but caused a decrease in phenanthrene degraders, suggesting reduced bioaccessibility/desorbable (biodegradable) fractions of the PAH studied, which is important for the growth, proliferation and contaminant uptake and metabolism by the PAH-degraders in soil.

It has been concluded that the biodegradation of PAHs in contaminated soil occurs through the actions of microbial consortia rather than by a single microbial population, although this depends on their catabolic potential and enzymes for substrates sequestration (Oyelami *et al.*, 2013; Gupta *et al.*, 2016). In this present study, significant negative correlations were observed between phenanthrene degraders and mineralisation (rates and extents) for most biochar amendments to the soil. This may be as a result of either the microbes are not synthesizing the required enzymes for the target contaminant (however, this was not measured) or they were partially or fortuitously metabolizing the phenanthrene in the presence of non-target carbon substrate (in this case, the biochar) (Namkoong *et al.*, 2002; Das *et al.*, 2011). However, phenanthrene-degrading microorganisms quantified in 0.2% of EbioC and NEbioC positively correlated with rates and extents of mineralisation, respectively; while 0.01% and 1.0% doses also showed a positive correlation between phenanthrene-degrading fungi and rates of mineralisation for NEbioC-amended soils only. Such correlations could explain the effects and

behaviour of different amounts of biochar in the amended soils (Khorram *et al.*, 2016). Therefore, the presence of PAH degrading populations may not be too adequate to indicate the degradation of a contaminant in soil but their high CFUs could contribute to the extent of PAH degradation and provide also useful information on the degradability of the contaminant by indigenous microorganisms in soils.

4.6 Conclusions

The overall findings in this study show that EbioC amendments stimulated the mineralisation of ^{14}C-phenanthrene in soils to greater levels than the NEbioC amendments. Biochar application and soil-contaminant contact time influenced the lag phase, rates and extents of ^{14}C-phenanthrene mineralised, which were particularly evident in the lower amendments (0.1%, 0.2%, 0.01%) in both types of biochar. Enhanced and non-enhanced wood-derived biochars influenced the microbial numbers and catabolic activity in phenanthrene contaminated soil. Phenanthrene-degrading microbial populations markedly reduce with increased biochar-soil contact time and in soils receiving higher amounts of biochar. The finding reported here show that smaller amounts of biochar caused increases in fastest rates and extents of ^{14}C-phenanthrene mineralisation, especially with the enhanced biochar. The reduced rates and extents of ^{14}C-phenanthrene mineralisation found in the soil receiving larger amounts (0.5 and 1.0%) of biochar are likely to be attributed to increase in the number of sites for contaminant sorption in soil-biochar complexes, thus reducing the bioavailability/bioaccessibility of the target contaminant. Therefore, larger amounts of biochar may well inhibit the removal of organic contaminants from the soil. However, both situations have the potential to reduce the

risk associated with PAHs, either through loss by stimulating biodegradation or by reducing the PAH mobility and/or bioaccessibility in soil.

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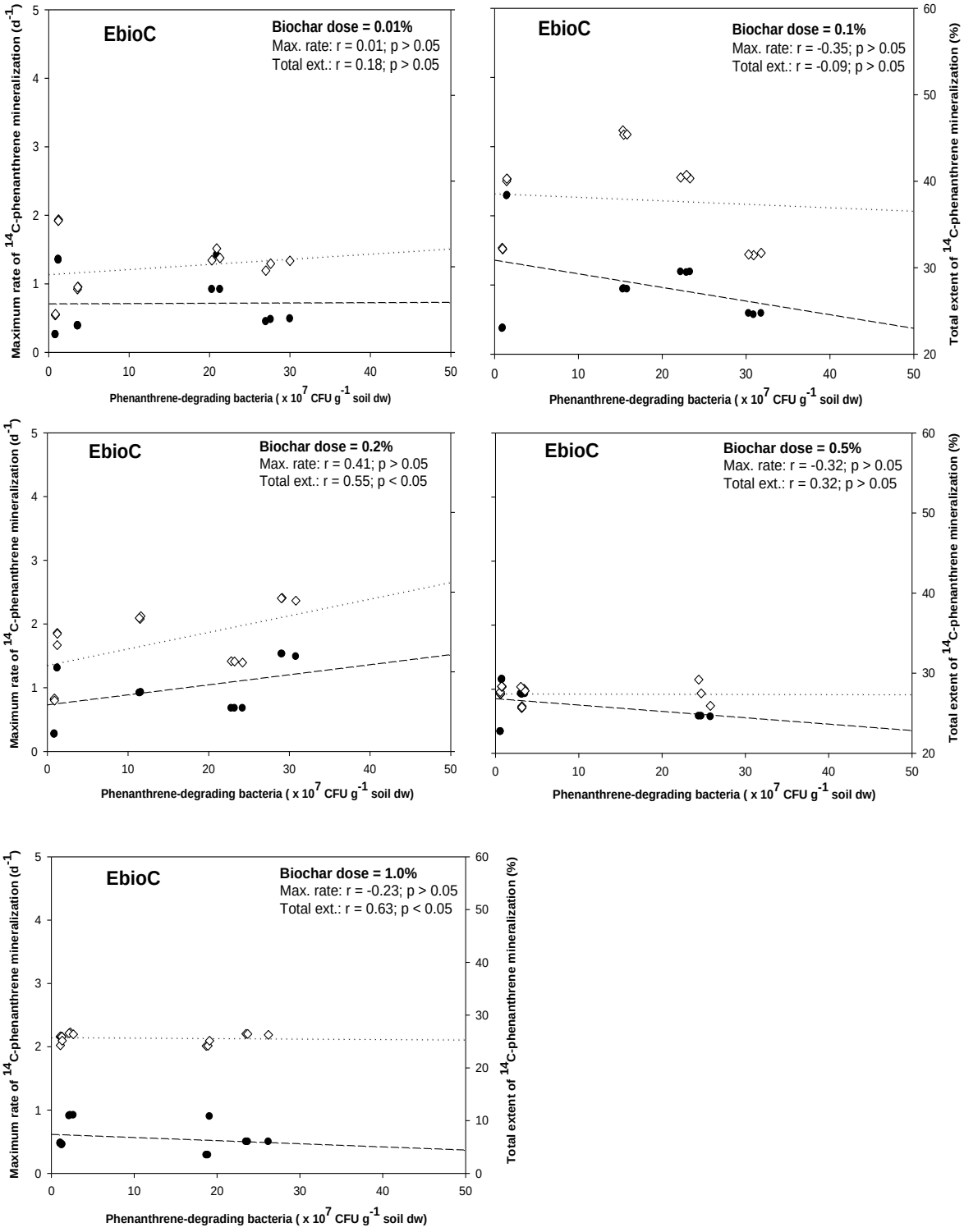
4.8 Supplementary Data

Table S1. Physicochemical properties of Myerscough soil used in the experiment (Couling *et al.*, 2010). Values are mean ± SEM (n = 3)**.**

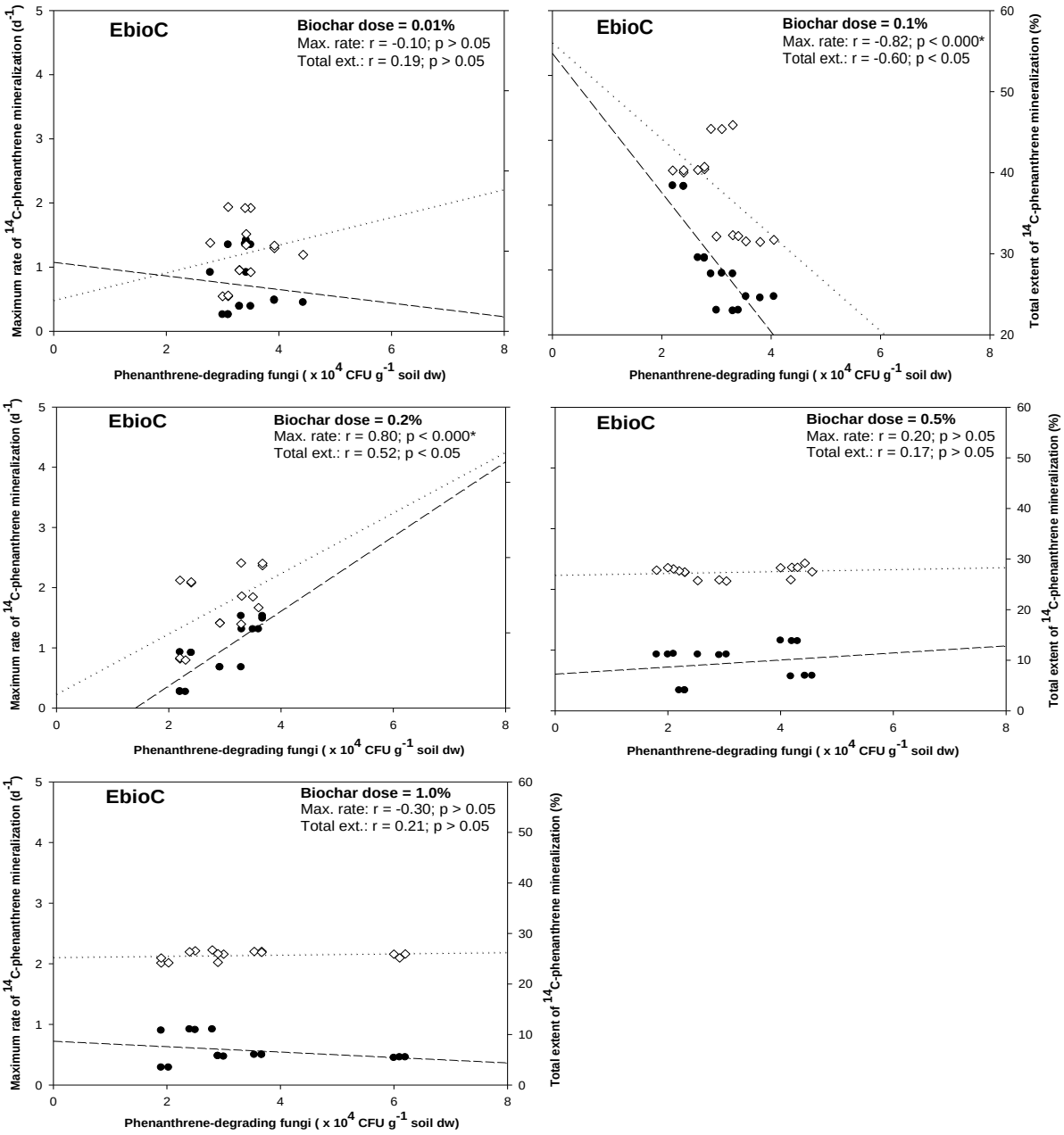
Soil properties		**Parameter Values**
pH (in dH_2O)		6.50 ± 0.08
Moisture content (%)		21.07 ± 2.78
Microbial heterotrophic numbers (CFU g^{-1})		$2.17 \times 10^5 \pm 1.67 \times 10^4$
Elemental analysis	Total extractable carbon	1.80% ± 0.03
	Total extractable nitrogen	0.14% ± 0.01
	Total extractable organic carbon	1.60% ± 0.07
	Soil organic matter	2.70% ± 0.04
Soil particle size	Clay	19.5% ± 0.70
	Silt	20.0% ± 0.87
	Sand – Total	60.4% ± 1.20
	Coarse sand	0.12% ± 0.01
	Medium sand	6.90% ± 0.10
	Fine sand	53.3% ± 0.60
	Surface texture: clay loam	



*Maximum rate of ^{14}C-phenanthrene mineralization with phenanthrene-degrading bacteria in amended soil (●— —)

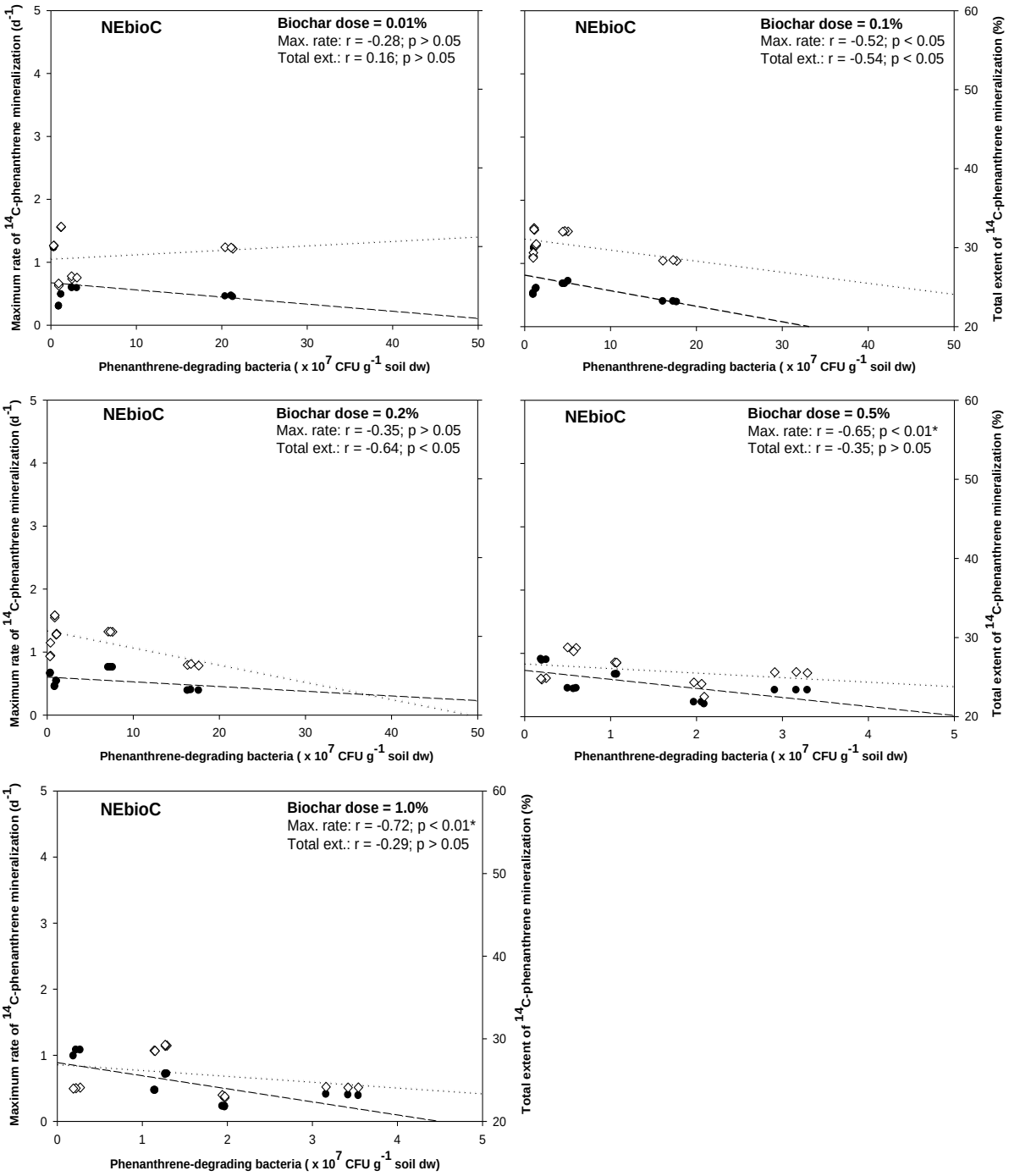
*Total extent of ^{14}C-phenanthrene mineralization with phenanthrene-degrading bacteria in amended soil (◇ ·····)

Figure S1. Pearson correlation in soil between maximum (or fastest rate), total extent of ^{14}C-phenanthrene mineralised versus phenanthrene-degrading bacteria after EbioC-amendments (1 – 100d)



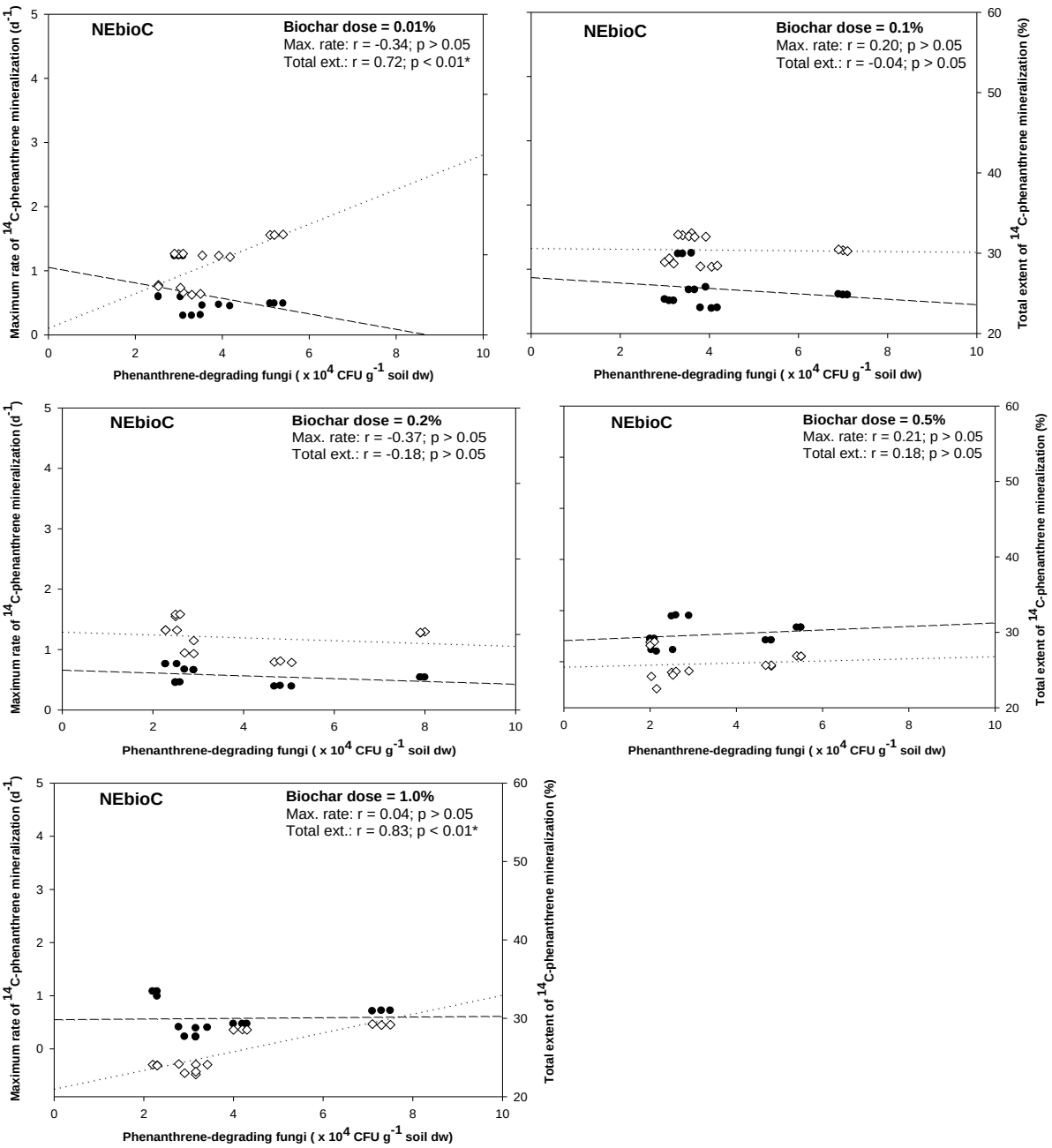
*Maximum rate of ^{14}C-phenanthrene mineralization with phenanthrene-degrading fungi in amended soil (◆ — —)
*Total extent of ^{14}C-phenanthrene mineralization with phenanthrene-degrading fungi in amended soil (◇ ·····)

Figure S2. Pearson correlation in soil between maximum (or fastest rate), total extent of ^{14}C-phenanthrene mineralised versus phenanthrene-degrading fungi after EbioC-amendments (1 – 100d)



*Maximum rate of ^{14}C-phenanthrene mineralization with phenanthrene-degrading bacteria in amended soil (◆ — —)
*Total extent of ^{14}C-phenanthrene mineralization with phenanthrene-degrading bacteria in amended soil (◇ ▪▪▪▪▪▪)

Figure S3. Pearson correlation in soil between maximum (or fastest rate), total extent of ^{14}C-phenanthrene mineralised versus phenanthrene-degrading bacteria after NEbioC-amendments (1 – 100d)



*Maximum rate of ^{14}C-phenanthrene mineralization with phenanthrene-degrading fungi in amended soil (— —)

*Total extent of ^{14}C-phenanthrene mineralization with phenanthrene-degrading fungi in amended soil (⋯⋯)

Figure S4. Pearson correlation in soil between maximum (or fastest rate), total extent of ^{14}C-phenanthrene mineralised versus phenanthrene-degrading fungi after NEbioC-amendments (1 – 100d)

Chapter 5

Effects of biological pre-treatment of lignocellulosic waste with white-rot fungi on the stimulation of ^{14}C-phenanthrene catabolism in soils.

Effects of biological pre-treatment of lignocellulosic waste with white-rot fungi on the stimulation of ^{14}C-phenanthrene catabolism in soils.

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5.1 Abstract

The enhancement of phenanthrene catabolism in soils amended with lignocellulosic waste material (spent brewery grains) was investigated. The soils were pre-treated with five white-rot fungi (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Irpex lateus*, *Pleurotus ostreatus*, and *Bjerkandera adusta*). The changes in the kinetics of ^{14}C-phenanthrene mineralisation (lag phases, the fastest rates and the overall extents) were measured in the inoculated, PAH-amended soils over time (1–100 d). Changes in the ligninolytic (laccase, lignin peroxidase and manganese peroxidase) and non-ligninolytic (β-glucosidase and phosphatase) enzymatic activities were also assessed. Overall results revealed that the amendment of fungal pre-treated SBG influenced the kinetics of mineralisation of ^{14}C-phenanthrene as well as the enzymatic activities in soils. Soil inoculated with fungal pre-treated SBG caused reductions in lag phases as well as higher rates and extents of ^{14}C-phenanthrene mineralisation in the following trend *T. versicolor* > *B. adusta* > *P. chrysosporium* = *P. ostreatus* > *I. lateus*. Furthermore, the extents of mineralisation generally reduced as levels of ligninolytic enzyme decreased, while the non-ligninolytic enzymes increased with soil-PAH contact time in all amendment conditions. These findings provided an insight on the potential of biological pre-treatment of waste materials for enhanced carbon, energy and nutrients on the bioactivities and biodegradation of organic pollutants which may be applicable during *in situ* remediations of contaminated soil.

Keywords: Lignocellulose, white-rot fungi, phenanthrene, biological pre-treatment, soil, pre-treated SBG

5.2 Introduction

Hydrophobic organic contaminants (HOCs), such as the polycyclic aromatic hydrocarbons (PAHs), have been widely studied due to their persistence in the environment and the potential risks they pose to human and environmental health (Wilson and Jones, 1993; Idowu *et al.*, 2019). In addition, factors such as contaminant mobility (controlled by their hydrophobicity, lipophilicity, and water solubility), and bioavailability/bioaccessibility may also contribute to their persistence and susceptibility to biodegradation in soil (Semple *et al.*, 2007; Riding *et al.*, 2013). Numerous studies have shown the efficacy of biodegradation in the removal of HOCs from contaminated soils (e.g. Zhang *et al.*, 2006; Peng *et al.*, 2008; Ghosal *et al.*, 2016); this typically depends on the biological and enzymatic activities of inherent microbiota (Pozdnyakova *et al.*, 2012; Ghosal *et al.*, 2016; Ibeto *et al.*, 2020). The metabolic function of microflora and associated enzymes in nutrient-depleted soils accompanied with low contaminant availability can be reduced or remain inactive, resulting in the persistence of the organic contaminant and further affecting the soil ecosystem's health (Breedveld and Sparrevik, 2000; Al-Hawash *et al.*, 2018). Therefore, to stimulate soil microbial and enzymatic activities, including soil fertility and health, the use of organic amendment using lignocellulosic waste materials, is considered a potential approach for bioremediation and nutrient management strategy (soil organic matter and fertility) for PAH contaminated soils.

Lignocellulosic waste materials, especially those generated from the agro-industrial processes, such as rice straw, sugarcane bagasse, corn cobs, spent brewery grains are potential sources of organic nutrients for microbial growth and metabolism in PAH contaminated soils (Brändli *et al.*, 2005; Ren *et al.*, 2018; Omoni *et al.*, 2020a). Soil amended with lignocellulosic materials can provide organic carbon, nitrogen and phosphorus to the soil biota (Larney and Angers,

2012; Chojnacka *et al.*, 2020). These materials can also be colonised and used by microorganisms as ecological niches in soil, especially when they are used as microbial-support systems for contaminant removal, thereby protecting the microbes against environmental stresses associated with organic pollution (Sari *et al.*, 2014; Andriani and Tachibana, 2016).

Lignocellulosic biomass is composed of two carbohydrate polymers (cellulose and hemicellulose) and a non-carbohydrate phenolic complex heteropolymer (lignin); the degradability and digestibility of the biomass vary with the structure of the lignin content (Janusz *et al.*, 2017). Several pre-treatment methodologies have been employed to break down the chemical composition and structure of lignocellulosic biomass residues (Baruah *et al.*, 2018). However, biological pre-treatment methods have some advantages over other pre-treatment methods, such as mechanical methods which are costly due to high energy input and the formation of toxic inhibitory products such as acetic, furural and phenolic acids, as well as high solvent cost associated with chemical pre-treatment strategy (Ramarajan and Manohar, 2017). Therefore, the biological pre-treatment processes are more economically viable, superior and eco-friendly compared to the other pre-treatment techniques (Isroi *et al.*, 2011; Wagner *et al.*, 2018). Biological pre-treatment processes represent promising approaches to the removal of lignin from the waste materials, while increasing enzymatic hydrolysis of the hemicellulosic and cellulosic contents to monomeric sugars such as xylose, arabinose, mannose, glucose and galactose, the readily metabolizable carbon source for microbial growth and metabolism. The white, soft and brown fungi and some bacterial species can delignify and degrade hemicellulose from the lignocellulose. Specifically, the white-rot fungi (WRF), the basidiomycetes, have been extensively studied because of their potential for higher delignification of the biomass as well as simultaneous degradation of cellulose and hemicellulose (Wan and Li, 2010; Isroi *et al.*, 2011; Rouches *et al.*, 2016). This is mainly due

to their production of extracellular ligninolytic and non-ligninolytic enzymes (Baldrian, 2006; Abdel-Hamid *et al.*, 2013), making them potentially viable candidates for biological pre-treatment of lignocellulosic wastes.

The extracellular ligninolytic enzymes involved in lignin degradation include the peroxidases (lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), dye-decolorizing peroxidase (DyP)) and the laccases (Abdel-Hamid *et al.*, 2013), which can effectively metabolize lignin in a variety of different types of lignocellulose biomass (Isroi *et al.*, 2011). Depolymerisation of the lignin molecules by these enzymes results in materials that are more available for microbial attack, and as sources of nutrients to other soil biota (Saritha *et al.*, 2012; Gao, *et al.*, 2010). However, the lignin-degrading enzymes must be active and stable in the organic substrate (lignocellulose) as well as in the contaminated soil (Lang *et al.*, 1998) for effective biodegradation. Other enzymes, such as the extracellular hydrolases (β-glucosidases and phosphatases) are also secreted by WRF and are known as bio-indicators of soil health, function and quality (Adetunji *et al.*, 2017). These enzymes are involved in biogeochemical cycling of carbon (β-glucosidases) and phosphorus (phosphatases) owing to enzymatic break down of organic matter and nutrient mineralisation in soil (Wali *et al.*, 2020). Previous studies have reported the stimulation of these enzymes by lignocellulosic biomass amendment, including the improvement of soil quality, mainly in PAH-contaminated soils (Tejada *et al.*, 2008; Anza *et al.*, 2019). Very few studies have investigated the addition of organic waste in PAH-contaminated soil with enhanced biodegradation using lignocellulosic material-immobilized WRF (Mohammadi and Nasernejad, 2009; Ros *et al.*, 2010; Lukić *et al.*, 2016), but very few investigations have been reported on PAH degradation in contaminated soils after amendment with biologically pre-treated lignocellulosic waste materials, such as spent brewery grains (SBGs).

For this study, it was hypothesised that the addition of fungal pre-treated SBG would (i) enhance the kinetics of mineralisation of ^{14}C-phenanthrene in soil over time; (ii) stimulate the biological activity (microbial and enzymatic) in PAH-amended soil over time; (iii) develop favourable carbon to nitrogen ratio and pH in the soil to support effective mineralisation of ^{14}C-phenanthrene in soils, and (iv) the fungal pre-treatment would release and increase the accessibility of available sugar monomers and other nutrients from the SBG thereby supporting microbial growth and activity. To address these hypotheses, the aim of this study was to investigate the influence of the addition of fungal pre-treated SBG on the mineralisation of ^{14}C-phenanthrene (a model PAH) in soil and the impact on soil physio-chemical properties and biological activity over time. This was achieved by sampling at different time points (1, 25, 50, 75 and 100 d) to assess the kinetics of mineralisation of ^{14}C-phenanthrene (lag phases, fastest rates and extents) in the soil incubations. Five different lignin-degrading white-rot fungal inocula were studied: *Irpex lateus*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Trametes versicolor* and *Bjerkandera adusta* (Leonowicz *et al.*, 1999; Hatakka and Hammel, 2010; Madadi and Abbas, 2017).

5.3 Materials and methods

5.3.1 Chemicals and other materials

Phenanthrene (^{12}C, 98%), sodium hydroxide, 2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ρ–nitrophenyl–β–D-glucopyranoside (PNG), ρ–nitrophenyl phosphate (PNP), and 3,4-dimethoxylbenzyl alcohol (veratryl alcohol) were purchased from Sigma-Aldrich, UK. [9-^{14}C] Phenanthrene (> 96%, 55.7 mCi mmol^{-1}) was acquired from American Radiolabeled Chemicals, USA. All reagents and salts for buffer solutions, phenol red, microbiological media

(plate count agar and potato-dextrose agar), recipes for minimal basal salt (MBS) solution and antimicrobial agents (Amphotericin-B and Penicillin-Streptomycin-Glutamine) were obtained from Fisher Scientific, UK.

5.3.2 Ligninolytic fungal strains and culture conditions

Irpex lateus (CCBAS 196), *Phanerochaete chrysosporium* (CCBAS 570), *Pleurotus ostreatus* (CCBAS 473), *Trametes versicolor* (CCBAS 614), and *Bjerkandera adusta* (CCBAS 232) were obtained from the Culture Collection of Basidiomycetes (CCBAS), Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic. These strains were selected based on their abilities to delignify lignocellulosic biomass (Abdel-Hamid *et al.*, 2013; Janusz *et al.*, 2017). The fungal cultures were maintained on potato dextrose agar (PDA) slants (pH 5.0) at 4^0C and routinely sub-cultured every 20 days. Four actively growing mycelial plugs (0.5cm diameter) excised from one-week grown PDA agar plates were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (PDB, pH 5.0); thereafter the cultures were blended to form homogenized mycelial mats under sterile conditions for 60 seconds using a high speed Ultra-Turrax homogenizer (10,000 rpm). The blended mycelial suspensions were incubated at room temperature under a rotary shaker at 150 rpm in the dark. After 4 days of rotary incubation, 5% of mycelial pellets were further transferred to 100 ml Erlenmeyer flasks with 50 ml PDB and incubated under the same conditions as mentioned previously. Fungal pellets were harvested using a TX-40R Sorvall centrifuge (Thermo Fisher Scientific, UK) for 10 mins (3500 x *g*, 4^0C), the supernatants decarded and washed thrice with sterile distilled water (autoclaved, 121^0C). Then, the dry weights (dw) of fungal biomass were measured using the oven-dried method at 60^0C until constant weight.

5.3.3 Biological pre-treatment of spent brewery grains with white-rot fungi

Spent brewery grains (SBG) was used as the lignocellulosic waste material for this study. Fresh SBG was collected from Lancaster Brewery in UK, having an initial moisture content of 81% and was dried to 60% using a low-heat oven for 6 h at 60^0C before solid-state fermentation. Other properties of SBG are described in Table S1 (Omoni *et al.*, 2020a). SBG (250 g ; dry matter = 40%) was transferred in sterile 1 l glass bottles and was aerated with sterile moist air then aseptically seeded with a four day old homogenised mycelial pellet (fungal strains designated, A – E) to a final dry weight of 2.5 g (0.01 g/g SBG). These flasks were incubated for 10 days (21 ± 1^0C) under static conditions (solid-state fermentation) for complete fungal growth, colonisation and penetration of the SBG (referred to as fungal pre-treatment).

5.3.4 Soil preparation, spiking and amendments with biologically pre-treated spent brewery grains

Samples of pristine agricultural soil (Dystric Cambisol) with a 2.7% organic matter was collected from a pasture field (5 – 20 cm depth) in Myerscough Agricultural College, Preston, UK. Soil microbial and physicochemical properties have been described in Couling *et al.*, 2010 (Table S2). The soil was air-dried, thoroughly homogenised and sieved with 2mm mesh. The sieved soil was rehydrated to 60% water holding capacity (WHC) with deionized water and soil was spiked with ^{12}C-phenanthrene according to the method as previously described (Doick *et al.*, 2003). Briefly, soil sample was spiked with ^{12}C-phenanthrene with acetone as carrier solvent to a final concentration of 100 mg/kg (dry wt), following bolus methodology and venting for 3 hr in the fume hood. Subsequently, the ^{12}C-phenanthrene spiked soil was amended with 20% of SBG. The amount of SBG added to phenanthrene spiked soil was based

on our previous study (Omoni *et al.*, 2020a). These mixtures were mixed with a stainless-steel spoon for homogeneous distribution in the soil. Soils without phenanthrene and fungal treated SBG serve as blanks. Controls included phenanthrene but lacked pre-treated SBG and abiotic controls (autoclaved-sterilized soils) to ensure that the biocatalytic activity observed in the controls was provided only by soil enzyme. The same soil moisture conditions (20%) after addition of fungal pre-treated SBG were maintained in all soil microcosms throughout the study. Soils with fungal pre-treated SBG were transferred into sterile amber bottles and incubated in the dark at 21± 1°C (n = 3) with sampling period for 1, 25, 50, 75 and 100 d soil-PAH contact time.

5.3.5 Influence of biological pre-treatment of spent brewery grains on the biodegradation of ^{14}C-phenanthrene in soil

Mineralisation of [9-^{14}C] phenanthrene to $^{14}CO_2$ was monitored in SBG amended soils after 1, 25, 50, 75 and 100 days soil-PAH contact time using respirometry assays, in a modified 250 ml Schott bottles (Teflon-lined screw cap) as developed and described by Reid *et al.* (2001) and Semple *et al.* (2006). The respirometry assays were prepared with soil-pre-treated SBG mixtures (10 ± 0.2 g, dw) with 30 ml of deionized water and [^{14}C] phenanthrene standard (98.2 Bq g^{-1} soil) per respirometer in 1:3 soil/water slurry (n = 3). Respirometers were shaken alongside with controls and blanks on a flat-bed orbital shaker (100 rpm) and incubated in the dark at 21 ± 1°C for 14 days with $^{14}CO_2$ traps (1 ml of 1 M NaOH). After the addition of a liquid scintillation cocktail (6 ml), ^{14}C-activity on samples were measured daily by a liquid scintillation counter (LSC) for 10 mins using standard protocols for counting and automatic quench correction (Reid *et al.*, 2001; Macleod and Semple, 2006).

5.3.6. Soil physico-chemical analyses

The influence of the pre-treated SBG by the five selected basidiomycetes fungi on the carbon to nitrogen ratio (C/N) and pH level in amended soils were determined according to the method described by Wilke, 2010 and Larsson *et al.*, 2018, respectively.

5.3.7. Enzyme assays

Soil enzyme activities were monitored by UV- visible spectrophotometry (Japan corporation, Japan) for ligninolytic enzymes- laccase (LAC), peroxidases: manganese peroxidase (MnP) and lignin peroxidase (LiP), as well non-ligninolytic enzymes- β-glucosidase (βGA) and acid phosphatase activities (ACP). LAC enzyme activity was assayed by 0.5 mM ABTS in 1 mM sodium acetate buffer (pH 3.0) as substrate at 420 nm (Bourbonnais and Paice, 1990 - modified). The LiP activity was determined by increasing absorbance at 310 nm, which resulted from the oxidation of 0.2mM veratryl alcohol to veratryl aldehyde in 25 mM sodium tartrate buffer, pH 3.5 (Tien and Kirk, 1988). The MnP activity in soils was monitored as previously described (Chan-Cupul *et al.*, 2016) by the oxidation of 0.01% (wt/vol) phenol-red as substrate in sodium succinate buffer (20 mM, pH 4.5) in a reaction mixture also containing 0.1 mM $MnSO_4$, 25 mM lactate, 0.1 % (wt/vol) bovine serum albumin and 0.1 mM H_2O_2. The reaction was stopped at 30^0C by the addition of 2N NaOH and absorbance measured at 610 nm. β-glucosidase activity was measured using ρ–nitrophenyl–β–D-glucopyranoside (PNG) as substrate (Eivazi and Tabatabai, 1988). Phosphatase activity was determined using ρ–nitrophenyl phosphate (PNP) solution as substrate using the method described by (Tabatabai and Bremner, 1969). All enzyme measurements per sample were done in replicates (n = 3).

5.3.8. Microbial cell numbers

The indigenous microbial population (total heterotrophs and phenanthrene-degraders) was quantified through the cell numbers in fungal pre-treated SBG-amended soils at each ageing period (1, 25, 50, 75, and 100 d) using the spread plate technique as described previously (Okere *et al.*, 2012; Omoni *et al.*, 2020a). Microbial numbers were assessed as colony-forming units per grams soil dry weight ($CFUs/g_{dw}$ soil, n =3).

5.3.9 Statistical analysis

The data were subjected to parametric paired student's t-tests and one-way ANOVA (phenanthrene spiked soil and contact time as factors) using the Statistical Package for the Social Sciences (IBM SPSS Version 25.0) followed by Tukey's post-hoc and Games-Howell tests to compare significant differences in means of samples within and across groups at 95% confidence level ($p < 0.05$). SigmaPlot 10.0 software (Systat Software Inc., USA) was used for the graphical representations of data. Pearson's correlation was used to determine and interpret the relationships between the kinetics of ^{14}C-phenanthrene mineralisation and soil biological activities in organic waste-amended soils (Omoni *et al.*, 2020b). The Pearson's correlation coefficient (r) was ranked based on the linear association between variables on a scale that range between +1 and −1. The strength of the relationship between the two variables is either strong, weak or moderate as their absolute values approaches +1 and −1.

5.4 Results

5.4.1 Fungal pre-treated spent brewery grains on the mineralisation of ^{14}C-phenanthrene in soils

An assessment of the biological pre-treatments on the biodegradation of phenanthrene in soils was determined through biodegradation parameters such as the lag phase, fastest rate and cumulative extent of ^{14}C-phenanthrene mineralisation (Figure 1 and Table 1). In soil treatments, the results revealed significantly shorter ($p < 0.05$) lag phases for PAH amended soils. The longest and shortest lag phases were found for *T. versicolor* (3.21 ± 0.29, 1 d) and *I. lateus* (0.11 ± 0.02, 75 d), respectively in amended soils (Table 1). Compared to the non-amended soil, significantly shortened lag phases were observed in amended soils throughout the study period; in particular, contact points (1d– 50 days) showed considerably reduced lag phases ($p < 0.001$). Treatment with pre-treated SBG of *P. chrysosporium* and *B. adusta* in amended soils, although not significantly different, showed also reduced lag phases when compared to the other soil conditions at 1 d incubation period. Generally, the lag phases in all soil conditions were statistically similar ($p > 0.05$) for most contact points but showed an average lag phase reduction of 66.8, 90.7, 90.4 and 87.8% after 25, 50, 75 and 100 d, respectively, compared to 1 d soil incubation. The reduction in the lag phase was predominant in soils amended with *T. versicolor* (96 %), closely followed by *P. ostreatus* (95.4 %) and *I. lateus* (95.4 %) after 75 d soil aging. Although the *B. adusta* and *P. chrysosporium* also showed significant reductions of 89.6 and 83.2 % in the lag phases after 50 d soil-PAH contact time, respectively. After 100 d of soil incubation, *T. versicolor* and *I. lateus*-amended soils were observed to have significantly shortened the lag phases when compared to other amended and control soils ($p < 0.05$).

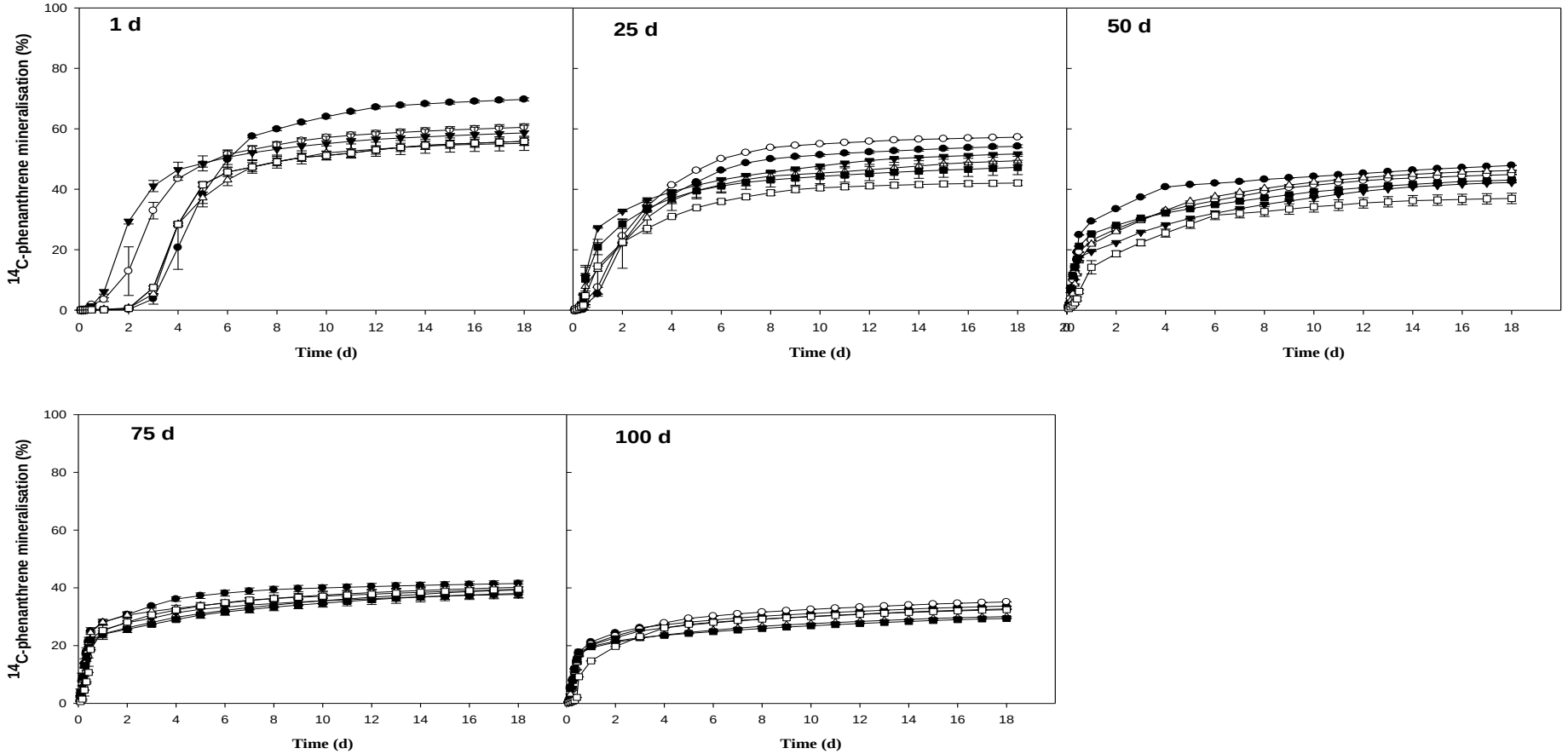

Figure 1. Development of ^{14}C-phenanthrene catabolism in soils amended with fungal pre-treated spent brewery grains after 1, 25, 50, 75, and 100d soil-phenanthrene contact time. Fungal pre-treatment: (●) *T. versicolor*, (○) *B. adusta*, (▼) *P. chrysosporium*, (△) *P. ostreatus*, (■) *I. lateus,* and control (unamended)(□). Values are mean ± SE (n = 3).

Table 1. Kinetics of ^{14}C-phenanthrene in soil amended with fungal pre-treated spent brewery grains of five genera of ligninolytic fungi after 18 days respirometric assay. Values are mean ± standard error (n = 3).

Contact time (d)	Ligninolytic fungi treatment	Lag phase (d)	Fastest rate (%$^{14}CO_2$ d^{-1})	Cumulative Extent (%)	Increase in extent relative to control (%)
1	*T. versicolor*	3.21 ± 0.29	0.95 ± 0.03	69.73 ± 0.49	42.3
	B. adusta	1.73 ± 0.32	1.04 ± 0.03	60.51 ± 1.16	33.5
	P. chrysosporium	0.89 ± 0.08	0.97 ± 0.01	58.66 ± 2.49	31.4
	P. ostreatus	2.92 ± 0.03	0.96 ± 0.03	55.20 ± 2.34	27.1
	I. lateus	2.65 ± 0.04	0.89 ± 0.03	55.85 ± 1.36	27.9
	Control	6.12 ± 0.39	0.79 ± 0.01	40.27 ± 0.75	0.00
25	*T. versicolor*	0.95 ± 0.02	0.83 ± 0.00	54.24 ± 0.48	22.4
	B. adusta	0.73 ± 0.01	0.73 ± 0.02	57.26 ± 0.10	26.5
	P. chrysosporium	0.42 ± 0.02	3.24 ± 0.00	51.73 ± 0.20	18.6
	P. ostreatus	0.89 ± 0.34	2.74 ± 2.09	49.47 ± 1.45	14.9
	I. lateus	0.44 ± 0.03	3.19 ± 1.39	47.25 ± 2.38	10.9
	Control	2.56 ± 0.34	0.70 ± 0.01	42.10 ± 0.61	0.00
50	*T. versicolor*	0.21 ± 0.02	4.52 ± 0.03	47.90 ± 0.01	22.8
	B. adusta	0.18 ± 0.00	3.11 ± 0.04	44.90 ± 0.10	17.6
	P. chrysosporium	0.15 ± 0.00	3.04 ± 0.01	42.26 ± 0.05	12.5
	P. ostreatus	0.23 ± 0.01	2.17 ± 0.03	46.22 ± 0.13	20.0
	I. lateus	0.13 ± 0.00	2.83 ± 0.02	43.08 ± 0.08	14.2
	Control	0.45 ± 0.04	1.22 ± 0.03	36.98 ± 2.98	0.00
75	*T. versicolor*	0.13 ± 0.03	3.41 ± 0.05	41.50 ± 1.09	4.75
	B. adusta	0.27 ± 0.08	3.01 ± 0.07	39.28 ± 2.84	– 0.64
	P. chrysosporium	0.18 ± 0.05	2.86 ± 0.03	37.73 ± 1.10	– 4.80
	P. ostreatus	0.12 ± 0.00	2.91 ± 0.04	40.17 ± 1.10	1.59
	I. lateus	0.11 ± 0.02	2.70 ± 0.01	38.07 ± 0.32	– 3.84
	Control	0.26 ± 0.05	3.96 ± 0.02	39.53 ± 1.01	0.00
100	*T. versicolor*	0.16 ± 0.02	2.56 ± 0.04	33.72 ± 0.03	3.77
	B. adusta	0.26 ± 0.01	2.28 ± 0.01	35.16 ± 0.04	7.71
	P. chrysosporium	0.24 ± 0.00	2.17 ± 0.00	32.73 ± 0.01	0.86
	P. ostreatus	0.23 ± 0.03	2.27 ± 0.00	30.07 ± 0.11	– 7.91
	I. lateus	0.16 ± 0.00	2.21 ± 0.02	29.41 ± 0.04	– 10.3
	Control	0.45 ± 0.03	3.58 ± 0.01	32.45 ± 0.01	0.00

The effect of the fungal pretreatment of waste materials on the fastest rates of ^{14}C-phenanthrene mineralisation (the highest % $^{14}CO_2$ per day) in amended soils was also measured at 1, 25, 50, 75 and 100 d (Figure 1 and Table 1). At 1 d soil-PAH contact time, all soil conditions resulted in significantly faster rates ($p < 0.05$) than the control soils with exception of soil amended with *I. lateus*-pretreated SBG, which was statistically similar to the control soils (Table 1). No significant increases in the fastest rates of mineralisation were observed after 25 d between soil conditions and control ($p > 0.05$), except for soil amended with *P. chrysosporium*-pretreated SBG which showed a significant increase in the fastest rate of mineralisation ($p > 0.001$). However, after 50 d of soil incubation, there were significant increases in the rates of mineralisation ($p < 0.05$) in all amendment conditions compared to the unamended soil. Furthermore, the data showed signficant increases in fastest rates in all pre-treated SBG-amended soils after 50 d (2.83–4.52 % d^{-1}) and 75 d (2.70–3.41 % d^{-1}) with an average increase of 226 and 209%, respectively, compared to 1-d incubation. Notably, amongst the fungal pre-treated SBG investigated, *T. versicolor* showed significantly faster ($p < 0.001$) rates of 4.52, 3.41 and 2.56% d^{-1} in amended soils after 50, 75 and 100 d incubation, respectively. However, in our investigation, when compared to all fungal pre-treated SBG-amended soils, the non-amended soil showed significant increases ($p < 0.01$) in the rates of mineralisation as soil-PAH contact time increased after 75 (24.8%) and 100 d (35.8%), respectively.

The cumulative extents of ^{14}C-phenanthrene mineralisation in soils after amendments with the fungal pre-treated SBG were also assessed (Table 1 and Figure 1). Significant extents of phenanthrene mineralisation ($p < 0.05$) were observed for all soil conditions at each contact point during the investigation (1 d–100 d). The highest extents of ^{14}C-phenanthrene mineralisation were observed at 1 d (69.7 %) soil-PAH contact time, for example, at 1 d incubation, there were higher extents of phenanthrene mineralisation (55.2 – 69.7%) in all

soils' amendments compared to other contact points (25–100 d); the highest being for soil condition with *T. versicolor* (69.7 %, 1d) while the fungus *I. lateus* (29.4%, 100 d) had the least extent of phenanthrene mineralised in amended soils. Similar results were observed at longer incubation periods (25 d and 50 d) where the extents of mineralisation were also significantly higher ($p < 0.05$) in all amended conditions compared to the control soil (Table 1). Notably, at the end of the incubation period (100 d), soil amended with pre-treated SBG of *B. adusta*, followed by *T. versicolor*, showed significant increases ($p < 0.05$) in ^{14}C-phenanthrene mineralisation compared to the control soils and other soil conditions. Generally, the extent of ^{14}C-phenanthrene mineralisation for the five studied fungi in amended soils could be presented in the following order relative to control (*T. versicolor* > *B. adusta* > *P. ostreatus* > *P. chrysosporium* > *I. lateus*).

5.4.2 Soil physicochemical properties on ^{14}C-phenanthrene catabolism in soils

Soil pH in most of the amended conditions were not significantly different ($p > 0.05$) over time except for 25 d and 50 d, which showed significantly higher pH values ($p < 0.05$) in all amended soils investigated (Figure S1). Higher extents of mineralisation (≥ 50%) were generally found between slightly acidic (6.3) and neutral pH (7.2). The extents of ^{14}C-phenanthrene mineralisation were positively correlated ($p < 0.05$) with soil pH in most fungal pre-treated SBG-amended soils (Figure S5). In the case of soil C/N ratio, similar results were observed in all amended soils over a 100 d period (Figure S2). After 1 d soil-PAH contact time, pre-treated SBG in amended soil significantly influenced the C/N ratio ($p < 0.05$) compared to control soils. However, soil pH significantly increased ($p < 0.05$) in amended soils (*T. versicolor*, *B. adusta* and *P. ostreatus*) at 1–50 d soil incubations and then significantly reduced ($p < 0.05$) in all amended conditions with increases in contact time (75–100 d). Similar increases in pH was also observed for amended soil (1 d and 50 d) with *P. chrysosporium*-pretreated SBG. Higher

C/N ratios were observed for *P. chrysosporium* followed by *P. ostreatus* throughout the study period. The correlation analysis showed significantly positive correlations of pre-treated SBG of *T. versicolor*, *B. adusta* and *P. chrysosporium* with the overall extents of mineralisation in amended soils (Figure S5).

5.4.3 Ligninolytic enzyme activities on ^{14}C-phenanthrene catabolism in soils

Changes in LAC activity were monitored over a 100-d soil-PAH contact time (Figure 2 a). LAC activity levels were significantly higher ($p < 0.05$) at all amended soils in most contact points, especially at 1 d and 25 d, compared with the control (non-amended) soils. However, LAC activity were significantly reduced in all amended soil conditions ($p < 0.05$) from this contact points onwards (25–100 d). Furthermore, the LAC activity was highest (2.31 U g^{-1}, 1 d) in *T. versicolor*-amended soil. Results also showed that the *T. versicolor* soil condition displayed higher LAC activity closely followed by *P. ostreatus* and *P. chrysosporium* throughout the study period. After 100 d soil-PAH contact time, pre-treated SBG-amended soils showed no detectable LAC activity.

The LiP enzyme activity in amended soils was also assayed over time (1d–100 d) (Figure 2 b). Significantly higher LiP activity was detected in all the amendments after 1 d incubation compared to the control soil. However, low activity levels were recorded with longer incubation period (25–50 d) and these observed reductions were significantly higher than the controls (Figure 2, $p < 0.05$). Noticeably, there were no measurable LiP activity after 75 d and 100 d aging (Figure 2 b). The highest LiP enzyme level was observed at *P. chrysosporium* soil condition (3.50 U g^{-1}, 1 d), closely followed by *B. adusta* (2.62 U g^{-1}, 1 d) and *I. lateus* (1.42 U g^{-1}, 1 d). Overall, soils amended with both *P. chrysosporium* and *B. adusta* displayed

significantly higher LiP enzyme levels in comparison with other treatment conditions, while the unamended soil showed no detectable LiP activity throughout the study period (Figure 2 b).

MnP activity was detected for all amendment conditions in almost all time points (Fig 2 c). Compared to the other ligninolytic enzymes, MnP enzyme was best stimulated in fungal pre-treated SBG-amended soils throughout the investigation. MnP activity was also measurable in unamended soil, although these levels were significantly lower ($p < 0.05$) compared to all of the amended soils throughout the incubation period. After 1 d soil-PAH contact time, the fungal pre-treated SBG soil incubations showed significant effects on the MnP enzyme activity ($p < 0.001$). On the other hand, all amendment conditions showed significantly reduced MnP activity after 25 d soil-PAH contact time apart from *T. versicolor*-amended soil, which showed significantly higher MnP. In addition, all amendment conditions showed significantly reduced MnPs ($p < 0.05$) with extended soil-PAH contact time (50–100 d). The highest level of MnP activity was observed in soil amended with *B. adusta* (6.24 U g^{-1}) followed by *T. versicolor* (5.88 U g^{-1}) and *I. lateus* (4.26 U g^{-1}). Data revealed that soil conditions (*B. adusta* and *T. versicolor*) generally displayed higher levels of MnP in comparison to the other treatments and control throughout the study period, while *T. versicolor*-amended soil displayed the highest MnP activity (0.61 U g^{-1}) after 100 d soil-PAH contact time. Strong positive correlations ($p < 0.001$) were observed in all amended soils between the ligninolytic enzyme activities (LAC, LiP and MnP) and the lag phases as well as with the overall extents of ^{14}C-phenanthrene mineralisation, respectively; while the fastest rates of mineralisation negatively correlated with all ligninolytic enzymes studied in all of the amended soils (Table S4). Similarly, there were observed positive correlations among the three ligninolytic enzymes (LAC, LiP and MnP) in PAH contaminated soils (Table S4).

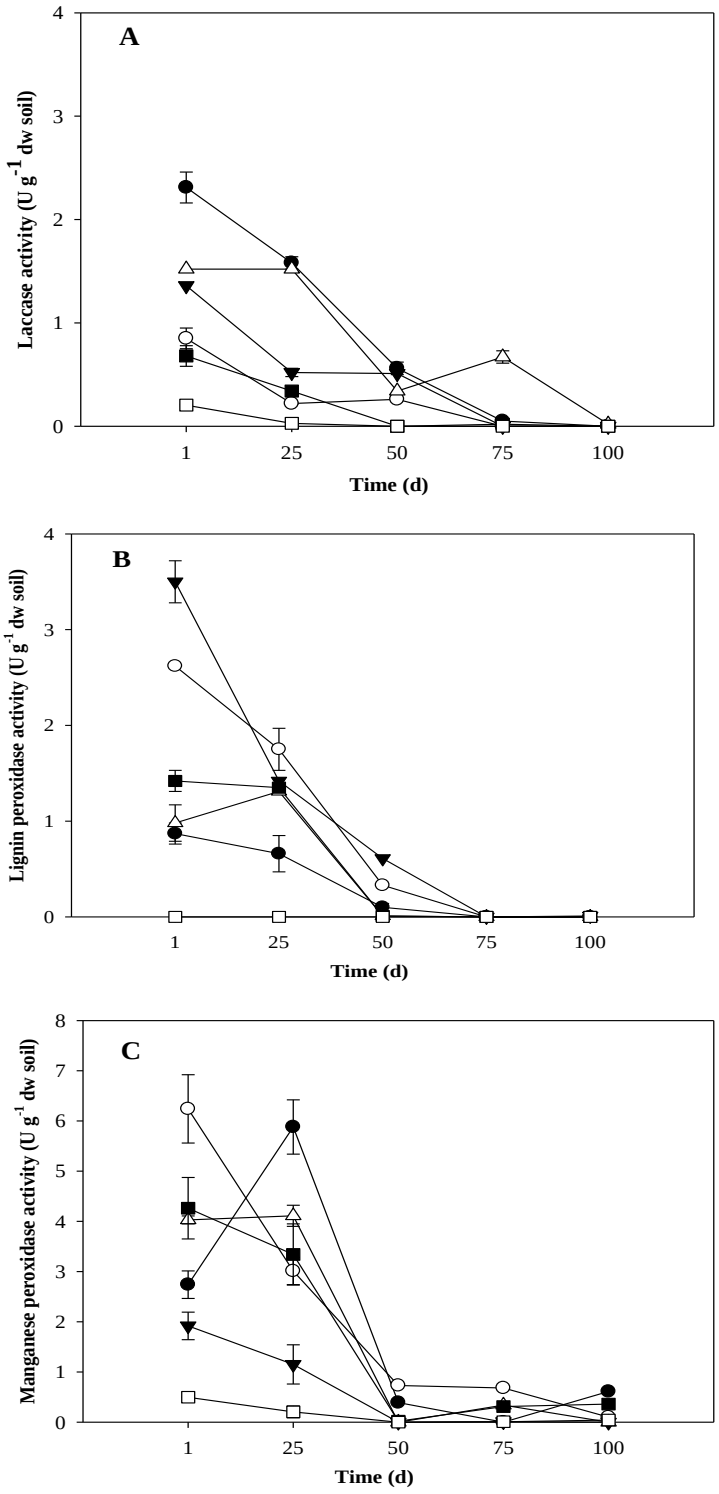

Figure 2. Ligninolytic enzyme activities in soils amended with fungal pre-treated spent brewery grains after 1, 25, 50, 75, and 100d soil-phenanthrene contact time. A = Laccase (LAC) activity in amended soils; B = Lignin peroxidase activity (LiP) in amended soils; C = Manganese peroxidase (MnP) activity in amended soils. Fungal pre-treatment: (●) *T. versicolor*, (○) *B. adusta*, (▼) *P. chrysosporium*, (△) *P. ostreatus*, (■) *I. lateus*, and control (unamended)(□). Values are mean ± SE (n = 3).

5.4.4 Non-ligninolytic enzyme activities on the catabolism of ^{14}C-phenanthrene in soils

Soil βGA activity were significantly increased ($p < 0.01$) by all fungal pre-treated SBG' amendments over time (1 d–100 d) compared to the control soil (Figure 3). Furthermore, the βGA activity were increased by an average of 1.73- and 2.08-fold after 25 d and 50 d, when compared to 1 d soil-PAH contact time, respectively. The highest and lowest βGA activity levels of 12.6 U g^{-1} and 2.60 U g^{-1} in amended soils were measured for *T. versicolor* and *P. chrysosporium* after 50 d and 100 d, respectively. Generally, the βGA enzymes were stimulated in the presence of pre-treated SBG in amended soils with these orders of magnitude (*T. versicolor* > *P. chrysospsorium* > *P. ostreatus* > *B. adusta* > *I. lateus*). The βGA activity was strongly correlated with the fastest rates of mineralisation ($p < 0.001$) for most fungal pre-treated SBG amended to soil however no correlation existed with the lag phases and overall extents of ^{14}C-phenanthrene mineralisation to $^{14}CO_2$ (Table S4).

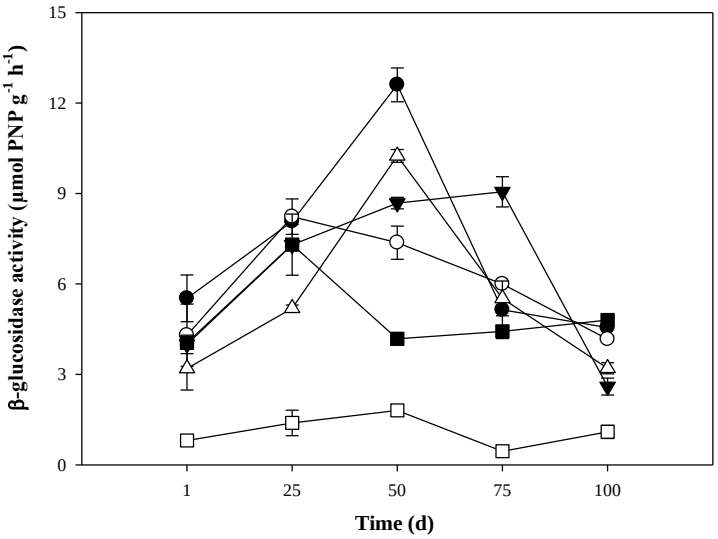

Figure 3. Level of β-glucosidase (µmol PNG g^{-1} h^{-1}) activity in amended soils with fungal pre-treated spent brewery grains after 1, 25, 50, 75, and 100d soil-phenanthrene contact time. Fungal pre-treatment: (●) *T. versicolor*, (○) *B. adusta*, (▼) *P. chrysosporium*, (△) *P. ostreatus*, (■) *I. lateus,* and control (unamended)(□). Values are mean ± SE (n = 3).

The ACP activity in amended soils were markedly influenced within and across all time points and were observed to be significantly higher than the control soils throughout the study period (Figure 4). In comparison to all the other soil enzymes assayed, ACP were greatly stimulated by the addition of pre-treated SBG of *P. chrysosporium* showed significant ACP levels ($p < 0.05$) at 1 d soil-PAH contact time compared to other amendment conditions and control soils. After 25 d onwards, ACP activities were significantly increased in all amended soils except for *P. chrysosporium*-amended soil, which showed significantly reduced ACP activity ($p < 0.05$) with increasing soil-PAH contact time. However, after 50 d incubation, the ACP enzymes were not significantly influenced ($p > 0.05$) except for *T. versicolor*-amended soil, which exhibited a 1.25-fold increase in enzyme activity compared to 25 d incubation. Maximum ACP activity was detected for *B. adusta* (25.2 U g^{-1}, 25 d) followed by *T. versicolor* (19.2 U g^{-1}, 50 d) in amended soils, while the least ACP activity was found in amended soil with *I. lateus* (2.54 U g^{-1}, 75 d). Furthermore, ACP activities were significantly stimulated ($p < 0.05$) at the end of the incubation period (100 d) compared to 50 d and 75 d. Soil ACP activity showed positive correlations with fastest rates in *P. chrysosporium* and *P. ostreatus*-amended soils. Significant positive correlations were also found between ACP enzyme activity and lag phases ($p < 0.01$) and extents of mineralisation ($p < 0.001$), respectively, in *P. chrysosporium* only (Table S4).

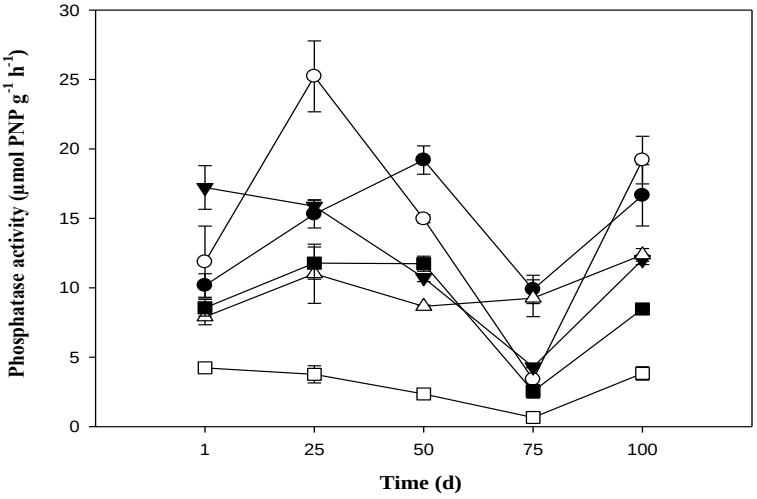

Figure 4. Level of phosphatase (µmol PNP g^{-1} h^{-1}) activity in amended soils with fungal pre-treated spent brewery grains after 1, 25, 50, 75, and 100d soil-phenanthrene contact time. Fungal pre-treatment: (●) *T. versicolor*, (○) *B. adusta*, (▼) *P. chrysosporium*, (△) *P. ostreatus*, (■) *I. lateus,* and control (unamended)(□). Values are mean ± SE (n = 3).

5.4.5 Microbial quantification in fungal pre-treated SBG-amended soils

The CFUs of the microbial populations (bacteria and fungi) were significantly higher ($p < 0.01$) than the control (non-amended) soils throughout the study (1d– 100d) (Table S3). Both heterotrophic and phenanthrene-degrading microbial CFUs were significantly higher ($p < 0.05$) at all time points, while the phenanthrene-degrading bacterial numbers in amended soils increased significantly ($p < 0.05$) from 1 d to up to 50 d soil-PAH contact time. In addition, soil amended with pre-treated SBG (*T. versicolor*, 25 d, 50 d & 100 d) closely followed by *P. ostreatus* (75 d) showed higher phenanthrene-degrading bacterial CFUs as compared to other treatment conditions. In comparison to the phenanthrene-degrading fungal numbers, a significantly higher CFU was observed in *P. chrysosporium* (1 d, 75–100 d) followed by *T. versicolor* (25–50 d) as compared to other soil conditions (Table S3).

Relationships between the soil microbial numbers (bacteria and fungi) and the kinetics of ^{14}C-phenanthrene mineralisation over the 100 d period were examined (Figure S3 and S4). Significant positive relationship ($p < 0.05$) existed between the number of phenanthrene-degrading bacteria and fastest rate of mineralisation in *T. versicolor*-amended soil only. The soil C/N ratios in amended soils with pre-treated SBG (*B. adusta*, *P. chrysosporium* and *T. versicolor*) were significantly correlated ($p < 0.05$) with phenanthrene-degrading fungal numbers. Also, we observed significant positive correlations between the phenanthrene-degrading fungal numbers with fastest rates ($p < 0.05$) and strong positive correlations with overall extents ($p < 0.05$) of ^{14}C-phenanthrene mineralisation in amended soil (*P. chrysosporium, B. adusta* and *T. versicolor*) (Figure S3–S4).

5.5 Discussion

5.5.1 Influence of fungal pre-treated spent brewery grains on the mineralisation of ^{14}C-phenanthrene in soils

In soils, amendment with organic waste materials, in particular the lignocellulosic ones, played important roles in PAH biodegradation in contaminated soil (Novotny *et al.*, 2000; Winquist *et al.*, 2014; Omoni *et al.*, 2020a). However, fungal pre-treatment of lignocellulosic material, such as SBG, increased the accessibility of available sugar monomers and other nutrients for microbial growth and activity. In this study, we found that all fungal pre-treated SBG resulted in shorter lag phases with increases in the rates and extents of mineralisation of ^{14}C-phenanthrene in amended soils; however, the fungal strains showed different PAH biodegradation kinetics within and at each of the sampling time points.

This study showed that the addition of pre-treated SBG of *T. versicolor* to soil greatly reduced the lag phase of ^{14}C-phenanthrene mineralisation, while soil amended with the fungus *I. lateus* was the least influenced. Although there were variations in the lag phase in amended soils, greatest reductions were observed after 75 d and 100 d soil incubations, indicating that increases in soil-PAH contact time led to increase microbial adaptation and activities which might be due to increase in available microbial nutrients and higher released of sugars (glucose) in pre-treated SBG-amended soils. Also, this reduced lag phases may be attributed to the increased survival rates of the fungal mycelia in pre-treated SBG (Czaplicki *et al.*, 2018) and the extent of PAHs-interaction with soil matrices (Wang *et al.*, 2012; Oyelami *et al.*, 2015). In particular, the significantly shorter lag phases displayed by *T. versicolor* compared to other amendment conditions suggests a higher metabolism of SBG as carbon and energy source for PAH dissipation (Han *et al.*, 2017); faster adaptation to phenanthrene and possible synergistic interactions with soil indigenous microorganisms (Kästner and Miltner, 2016; Omoni *et al.*, 2020a). However, the overall findings suggest that all fungal pre-treated SBG led to increased microbial adaptation to the target contaminant and consequently the reduction in the lag phases in amended soils. Omoni *et al.* (2020b) observed that the lag phases were longer in soil amended with SBG without pre-treatment at same phenanthrene concentration when compared to the present work. The authors also reported the shortest and longest lag phases for 20% SBG were 0.21 d and 3.50 d as compared to our study (0.11 d and 3.21 d) in amended soils after SBG pre-treatment, respectively.

The fastest rates of ^{14}C-phenanthrene mineralised to $^{14}CO_2$ depended on the fungal strain employed in the pre-treatment of SBG amended to soil in the present study. This may be associated with the initial preferential attack by fungi for lignin to enriched cellulose, fungal interaction with soil matrix and other soil microbes, and mycelial tolerance to PAH toxicity

(Ghosal *et al.*, 2016; Akhtar and Mannan, 2020). The data showed that all amended soils had significantly faster rates of mineralisation at both 1 and 50 d incubations, indicating the potential of the fungal inoculants for induction of PAH catabolism (Andriani and Tachibana, 2016). However, the rates of mineralisation were negatively affected with extended soil-PAH interaction (75 d and 100 d). This has resulted from increased sorption of PAH into soil matrices and possibly less PAH partitioning, and decrease in the bioavailability of rapidly desorbable PAH fraction to microbial cells in amended soils (Semple *et al.*, 2003; Cui *et al.*, 2013). Consequently, this may hinder the migration capacity of enzyme to reach contaminant sorption site within soil pore for PAH biodegradation. In addition, contaminant concentration and properties, soil properties and contact time, and microbial dynamics may also be attributed (Omoni, *et al.*, 2020b). It was also observed that SBG-associated *T. versicolor* had significantly faster rates of mineralisation in amended soils compared to the other amendment conditions after 50 d and 75 d of soil incubations, which is evident in the strong positive correlations that existed between phenanthrene-degraders (bacteria and fungi) and rates of mineralisation in *T. versicolor*-amended soil. This clearly indicated that this fungus (*T. versicolor*) has shown the greatest ability to improve the rate of PAH mineralisation in amended soil.

Many white-rot fungi are mycoremediators for PAH metabolism in impacted PAH contaminated soils and have the catabolic function to transform toxic organic chemicals to less toxic compounds or CO_2 (Field *et al.*, 1992; Novotný *et al.*, 1999). In this study, we observed significantly higher extents of mineralisation for all fungal pre-treated SBG amended soils in most contact points. Soils amended with pre-treated SGB facilitated the amounts of ^{14}C-phenanthrene mineralised in the present study. Here, it can be hypothesised that there was a (i) higher transport of phenanthrene contaminant by cytoplasmic streaming to the mycelial network (fungal pipelines), and (ii) higher diffusion of phenanthrene to cells in amended soils

(Furuno *et al.*, 2012). It should be noted that greater extents of mineralisation were measured at most time points but in all cases the results depend on the fungal strain used in the pre-treatment of SBG in amended soil. In particular, the pre-treated SBG of *T. versicolor* and *B. adusta* were the most efficient fungal strains with higher extents of mineralisation in amended soils compared to the other amendment conditions in almost all time points. The higher mineralisation of ^{14}C-phenenthrene by both fungi (*B. adusta* and *T. versicolor*) can also be attributed to the high secretion of ligninolytic enzymes in amended soils, especially the MnPs (Lladó *et al.*, 2013; Andriani and Tachibana, 2016). Particularly, previous studies have revealed that *T. versicolor* has the capacity to synthesize extracellular LiP, MnP and laccase (Schlosser *et al.*, 1997; Hossain and Anantharaman, 2006), which also can degrade PAHs (Bamforth and Singleton, 2005). Consequently, it is suggested that these two fungal strains in amended soils possess higher capacity for PAH biodegradation (Peng *et al.*, 2008). Furthermore, we propose that the pre-treatment step with fungal strains improved (by 8–21%) the efficiency of ^{14}C-phenanthrene mineralisation in amended soils when compared to our previous study without fungal pre-treatment (Omoni *et al.*, 2021b). Thus, it may also be hypothesised that all fungal strains used in the pre-treatment of SBG had positive influence on the microbial activities (bacteria and fungi) in amended soils. This is further confirmed by the positive correlations observed in most amended soils between the phenanthrene-degraders (especially fungi) and mineralisation (rates and extents).

5.5.2 The influence of ligninolytic enzymes on ^{14}C-phenanthrene catabolism in soils

Previous studies have showed that the degradation of PAHs in contaminated soils by white-rot fungi depends on ligninolytic enzymes secretion in soil (Pozdnyakova, 2012; Kadri *et al.*, 2017). Soil amended with fungal pre-treated SBG increased all ligninolytic enzyme activities

in this study. However, the fungal strains behaved differently in the secretion of ligninolytic enzymes in amended soils, indicating differences in their enzyme capacity, release and complexity in soil. Fungal pre-treated SBG of *T. versicolor* followed by *P. chrysosporium* in amended soils showed higher levels of LAC than the other amended conditions, indicating the potential of these fungi as proficient LAC inducers in contaminated soil. Similar soil-based studies have reported increases in LAC activity of *T. versicolor* (Lang *et al.*, 1998; Novotný *et al.*, 1999). However, few studies have reported very low levels of LAC activity with *P. chrysosporium* in soil spiked with organic chemicals (Fragoeiro and Magan, 2008; Yu *et al.*, 2011). This work clearly shows that the LAC production by *P. chrysosporium* can be induced in soil amended with fungal pre-treated SBG. On the other hand, the results showed that LiP activities were greatly influenced by *P. chrysosporium* followed by *B. adusta* compared to other fungal strains in amended soils. It has been shown that these two white-rot fungi, particularly *P. chrysosporium,* are key producers of LiPs in the biodegradation of PAHs in lignocellulose-amended soils (Andriani and Tachibana, 2016; Kadri *et al.*, 2017), thereby indicating that the two fungal strains are more efficient LiP producers in PAH contaminated soil in the present study. MnP was the most secreted in amended soils in the present study. This observation of higher levels of MnP enzymes is consistent with the findings of Novotný *et al.* (2004) and Pozdnyakova (2012), who reported significant role of MnPs in the degradation of recalcitrant compounds in soil by similar fungal strains in our study. Although, other fungal enzymes, including cytochrome P450 monooxygenase are also involved in PAH degradation (Durairaj *et al.*, 2016). In our experiments the high levels of MnPs in amended soils, with both *B. adusta* and *T. versicolor* pre-treated SBG, indicated the higher potential of these fungi for the stimulation of MnPs (Lladó *et al.*, 2013; Andriani and Tachibana, 2016).

5.5.3 The influence of non-ligninolytic enzymes on the catabolism of ^{14}C-phenanthrene in soils

Both βGA and ACP activities significantly improved in all PAH-spiked soils following fungal pre-treated SBG amendments. Previous research has also demonstrated increases in these hydrolytic enzymes (βGA and ACP) resulting from the degradation of PAH in contaminated soil (Adetunji *et al.*, 2017; Košnář *et al.*, 2019; Lipińska *et al.*, 2019). In addition, the high production of βGA and ACP observed in the amended soils, which are potential bio-indicator systems of soil quality and health of a degraded and contaminated soils (Dindar *et al.*, 2015; Chang *et al.*, 2017), suggested increased carbon and energy (organic matter and nutrients), and a subsequent increased in microbial growth and activities in all amended soils (especially *T. versicolor* and *B. adusta*). We also found that both βGA and ACP were detected in amended soils as the overall extents of ^{14}C-phenanthrene mineralised decreased over time. As a consequence, these enzymes were not affected by either PAH toxicity or increases in soil-PAH contact time. Although the potential roles of both enzymes (βGA and ACP) in PAH degradation is still unknown; their presence in soil after an organic amendment is very helpful not only for soil remediation but also in soil biology.

5.6 Conclusions

This investigation demonstrated that the biological pre-treatment of lignocellulosic waste material such as the spent brewery grains, has the potential to be used as a cost-effective and sustainable remedial method for enhancing the biodegradation of organic contaminants in soil. Assessment of the soil conditions showed that the fungal pre-treated SBG influenced the mineralisation of ^{14}C-phenanthrene, stimulate the enzyme activities, and microbial population

in all amended soils; however, these primarily depended on the fungal strain used for the pre-treatment of SBG before soil amendment. At most of the sampling time points, reductions in lag phases, faster rates and greater extents of ^{14}C-phenanthrene mineralisation were found in all fungal pre-treated SBG-amended soils (especially *T. versicolor* and *B. adusta*). This study showed that *T. versicolor* and *B. adusta* are more efficient degraders of the PAH in the soil. However, in most cases *T. versicolor* followed by *B. adusta* and *P. chrysosporium* displayed higher levels of both soil enzyme activities investigated (ligninolytic and non-ligninolytic). Overall, the ligninolytic enzymes generally decreased, while non-ligninolytic enzymes increased as the extent of mineralisation diminished in all amended soils over time. The study demonstrated that fungal pre-treatment of organic waste materials provide a promising approach for *in situ* and enhanced bioremediations of organic contaminants in soil.

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5.8 Supplementary Data

Table S1. Physicochemical and microbial characteristics (mean ± SEM) of spent brewery grains (SBG) used in the experiment. Data adapted from Omoni *et al.*, 2010b.

Parameters measured	SBG
pH	5.00 ± 0.00
EC (mS)/25^{0}C	2.24 ± 0.00
Dry matter (%)	18.0 ± 0.58
Extractable N (NH_4-N) (mg/kg **dw**)	112.0 ± 15.0
Extractable N (NO_3-N) (mg/kg **dw**)	**bdl**
Extractable P (mg/kg **dw)**	762.7 ± 10.4
TOC (mg/kg)	382.0 ± 3.41
IC (mg/kg)	3.70 ± 0.12
TC (%)	47.4 ± 0.05
TN (%)	3.13 ± 0.02
TP (%)	0.41 ± 0.03
C:N	15.1 ± 0.12
THBC (CFU x 10^8 g^{-1} dw)	10.5 ± 1.33
TFC (CFU x 10^6 g^{-1} dw)	4.45 ± 0.07

*****bdl = below detection limit; dw = dry weight; EC= Electrical conductivity; TOC= Total organic carbon; IC= Iorganic carbon; TC= Total carbon; TN= total nitrogen; TP= Total phosphorus; THBC**=** Total heterotrophic bacterial count, TFC**=** Total fungal count

Table S2. Physicochemical properties of Myerscough soil used in the experiment (Data adapted from Couling *et al.*, 2010). Values are mean ± SEM (n=3)

Soil properties		Parameter Values
pH (in dH_2O)		6.50 ± 0.08
Moisture content (%)		21.07 ± 2.78
Microbial heterotrophic numbers (CFU g^{-1})		$2.17 \times 10^5 \pm 1.67 \times 10^4$
Elemental analysis	Total extractable carbon	1.80% ± 0.03
	Total extractable nitrogen	0.14% ± 0.01
	Total extractable organic carbon	1.60% ± 0.07
	Soil organic matter	2.70% ± 0.04
Soil particle size	Clay	19.5% ± 0.70
	Silt	20.0% ± 0.87
	Sand – Total	60.4% ± 1.20
	Coarse sand	0.12% ± 0.01
	Medium sand	6.90% ± 0.10
	Fine sand	53.3% ± 0.60
	Surface texture: clay loam	

Table S3. Microbial quantification in amended soils with biopretreated SBG after soil-phenanthrene contact time (1, 25, 50, 75 and 100 d). Values are mean ± standard error (n = 3).

Contact time (d)	Ligninolytic fungi	Bacteria (CFU x 10^9 g^{-1} soil dw)		Fungi (CFU x 10^7 g^{-1} soil dw)	
		Heterotrophs	PAH-degraders	Heterotrophs	PAH-degraders
1	*T. versicolor*	23.1 ± 0.15	0.10 ± 0.00	8.76 ± 0.15	0.12 ± 0.00
	B. adusta	22.4 ± 0.10	0.12 ± 0.00	12.8 ± 0.09	0.15 ± 0.00
	P. chrysosporium	23.0 ± 0.03	0.09 ± 0.00	12.0 ± 0.14	0.93 ± 0.01
	P. ostreatus	21.8 ± 0.12	0.08 ± 0.00	8.29 ± 0.15	0.06 ± 0.00
	I. lateus	22.3 ± 0.09	0.07 ± 0.00	11.5 ± 0.12	0.12 ± 0.00
	Control	0.03 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.05 ± 0.00
25	*T. versicolor*	31.6 ± 0.20	0.15 ± 0.00	9.79 ± 0.10	0.22 ± 0.00
	B. adusta	34.1 ± 0.24	0.13 ± 0.00	3.26 ± 0.15	0.14 ± 0.00
	P. chrysosporium	32.5 ± 0.15	0.08 ± 0.00	2.30 ± 0.06	0.05 ± 0.00
	P. ostreatus	31.3 ± 0.12	0.13 ± 0.00	5.69 ± 0.15	0.22 ± 0.00
	I. lateus	22.1 ± 0.35	0.09 ± 0.00	3.80 ± 0.11	0.11 ± 0.00
	Control	1.27 ± 0.02	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
50	*T. versicolor*	1.20 ± 0.00	0.34 ± 0.00	3.63 ± 0.12	0.07 ± 0.00
	B. adusta	0.49 ± 0.00	0.29 ± 0.00	1.17 ± 0.03	0.05 ± 0.00
	P. chrysosporium	0.33 ± 0.00	0.28 ± 0.00	1.07 ± 0.03	0.02 ± 0.00
	P. ostreatus	0.55 ± 0.00	0.33 ± 0.00	2.33 ± 0.09	0.06 ± 0.00
	I. lateus	0.46 ± 0.00	0.20 ± 0.00	1.33 ± 0.12	0.04 ± 0.00
	Control	0.04 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
75	*T. versicolor*	0.38 ± 0.01	0.11 ± 0.00	0.32 ± 0.01	0.05 ± 0.00
	B. adusta	0.23 ± 0.01	0.14 ± 0.00	0.26 ± 0.01	0.07 ± 0.00
	P. chrysosporium	0.19 ± 0.00	0.10 ± 0.00	0.41 ± 0.01	0.09 ± 0.00
	P. ostreatus	0.27 ± 0.01	0.15 ± 0.00	0.51 ± 0.01	0.04 ± 0.00
	I. lateus	0.14 ± 0.00	0.09 ± 0.00	0.52 ± 0.02	0.07 ± 0.00
	Control	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.00
100	*T. versicolor*	0.21 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
	B. adusta	0.33 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.12 ± 0.00
	P. chrysosporium	0.17 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.16 ± 0.00
	P. ostreatus	0.23 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.11 ± 0.00
	I. lateus	0.26 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.14 ± 0.00
	Control	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00

Table S4. Pearson's correlation between the kinetics of ^{14}C-phenanthrene mineralisation and enzyme activities in amended soils.

Trametes versicolor								
	Lag phase	Fastest rate	Cumulative extent	LAC	LiP	MnP	βGA	ACP
Lag phase	1.00							
Fastest rate	**–0.68****	1.00						
Cumulative extent	**0.90*****	**–0.58***	1.00					
LAC	**0.83*****	**–0.75****	**0.96*****	1.00				
LiP	**0.76*****	**–0.79*****	**0.88*****	**0.95*****	1.00			
MnP	0.37	**–0.81*****	**0.54***	**0.70****	**0.70****	1.00		
βGA	–0.05	**0.46***	0.10	0.06	–0.03	0.03	1.00	
ACP	–0.15	0.37	–0.35	–0.28	–0.27	–0.05	**0.56***	00
Bjerkandera adusta								
Lag phase	1.00							
Fastest rate	**0.70****	1.00						
Cumulative extent	**–0.80*****	**–0.79*****	1.00					
LAC	**0.90*****	**–0.53***	**0.79*****	1.00				
LiP	**0.87*****	**–0.85*****	**0.93*****	**0.84*****	1.00			
MnP	**0.87*****	**–0.75*****	**0.87*****	**0.89*****	**0.93*****	1.00		
βGA	–0.39	–0.02	0.18	–0.30	0.02	–0.16	1.00	
ACP	–0.07	**–0.54***	0.24	–0.08	0.22	0.06	0.35	00
Phanerochaete chrysosporium								
Lag phase	1.00							
Fastest rate	**–0.78*****	1.00						
Cumulative extent	**0.79*****	–0.41	1.00					
LAC	**0.87*****	**0.64****	**0.91*****	1.00				
LiP	**0.92*****	**0.69****	**0.93*****	**0.97*****	1.00			
MnP	**0.85*****	**0.56***	**0.90*****	**0.83*****	**0.92*****	1.00		
βGA	**–0.45***	**0.72*****	–0.05	–0.22	–0.31	– 0.29	1.00	
ACP	**0.68****	**0.45***	**0.73*****	**0.72*****	**0.77*****	**0.79*****	**–0.53***	00

Pleurotus ostreatus								
	Lag phase	Fastest rate	Cumulative extent	LAC	LiP	MnP	βGA	ACP
Lag phase	1.00							
Fastest rate	**–0.52***	1.00						
Cumulative extent	**0.68****	–0.14	1.00					
LAC	**0.72*****	–0.14	**0.83*****	1.00				
LiP	**0.65****	–0.12	**0.66****	**0.91*****	1.00			
MnP	**0.75*****	–0.12	**0.76*****	**0.95*****	**0.96*****	1.00		
βGA	**–0.44***	0.11	0.13	–0.28	–0.36	–0.40	1.00	
ACP	–0.42	**0.68****	**–0.48***	–0.27	–0.02	–0.11	–0.28	00
Irpex lateus								
Lag phase	1.00							
Fastest rate	**–0.61****	1.00						
Cumulative extent	**0.77*****	–0.29	1.00					
LAC	**0.89*****	–0.43	**0.84*****	1.00				
LiP	**0.72*****	–0.23	**0.79*****	**0.86*****	1.00			
MnP	**0.78*****	**–0.48***	**0.79*****	**0.93*****	**0.90*****	1.00		
βGA	–0.23	**0.61****	0.09	0.11	0.41	0.21	1.00	
ACP	0.06	–0.03	0.27	0.22	0.34	0.31	0.26	00

* Values with bold letters showed significant relationship. Significant relationships are shown by * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$). LAC = laccase enzyme; LiP = Lignin peroxidase enzyme; MnP = Manganese peroxidase enzyme; βGA = β-glucosidase; ACP = Acid phosphatase.

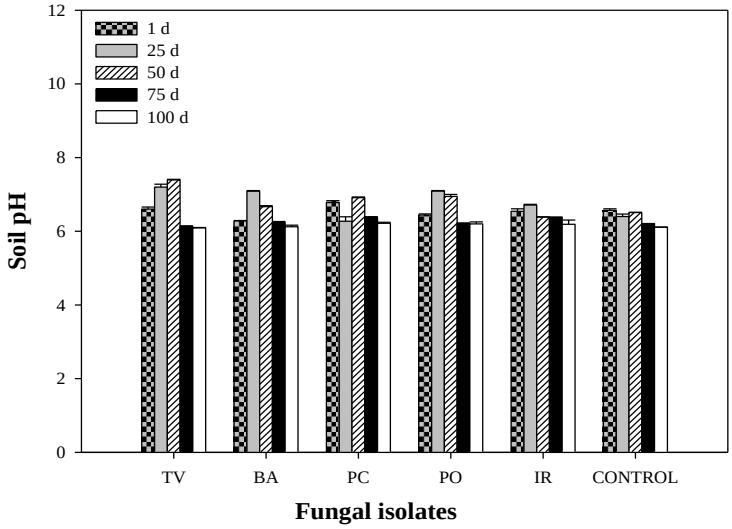

Figure S1. Changes in pH value in amended soils with **spawn** spent brewery grains of *T. versicolor* (TV), *B. adusta* (BA), *P. chrysosporium* (PC), *P. ostreatus* (PO) and *I. lateus* (IR) after 1, 25, 50, 75, and 100d soil-phenanthrene contact time. Values are mean ± SE (n = 3).

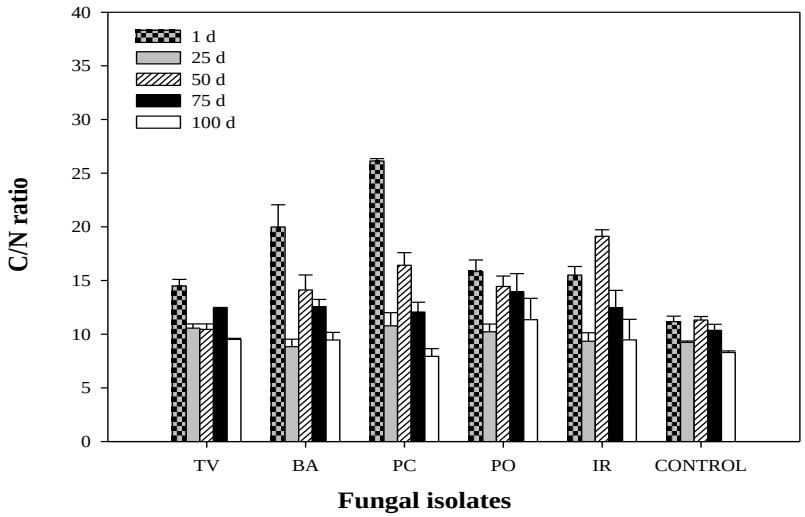

Figure S2. Changes in C/N ratio in amended soils with spawn spent brewery grains of *T. versicolor* (TV), *B. adusta* (BA), *P. chrysosporium* (PC), *P. ostreatus* (PO) and *I. lateus* (IR) after 1, 25, 50, 75, and 100d soil-phenanthrene contact time. Values are mean ± SE (n = 3).

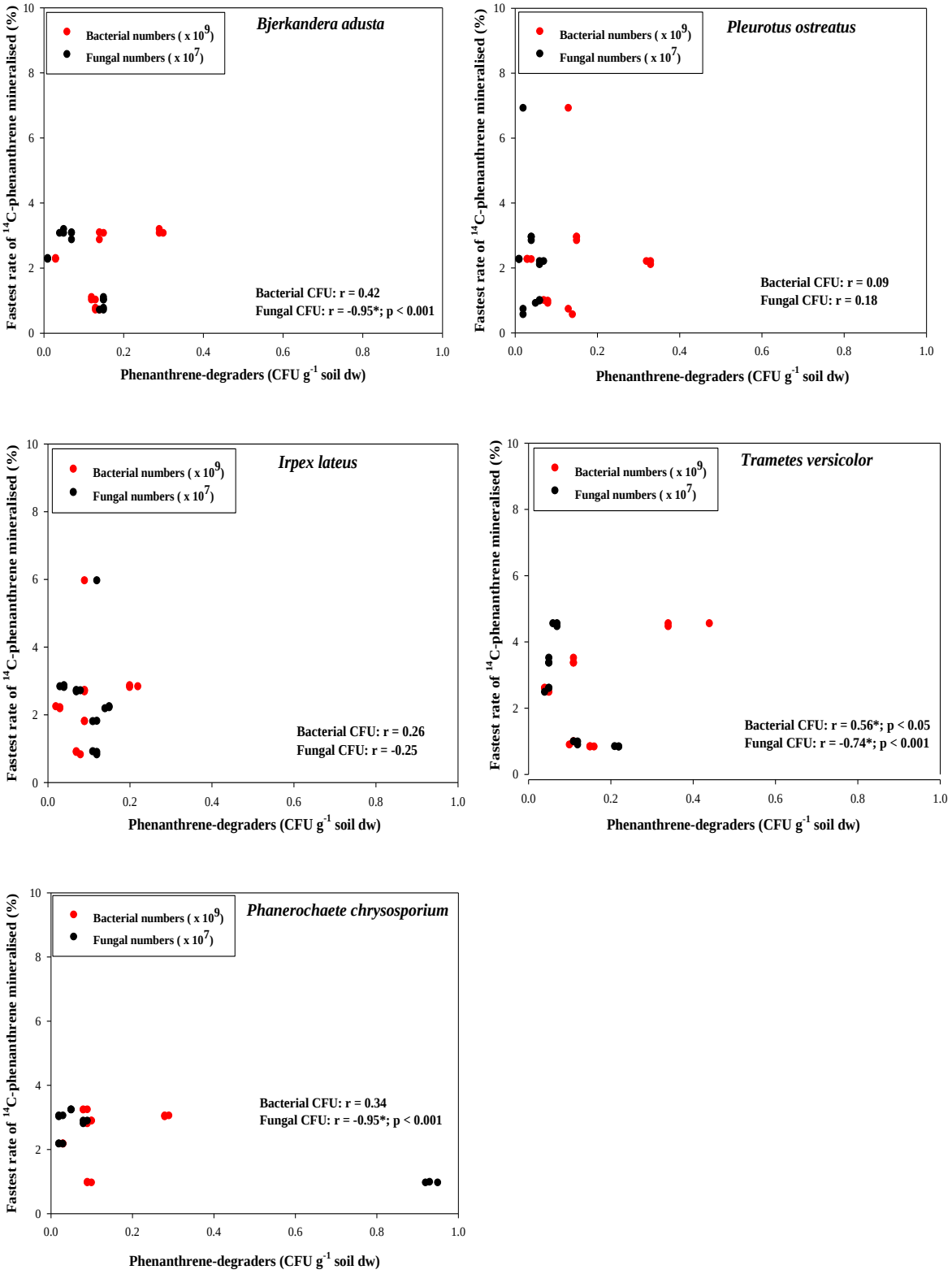

Figure S3. Relationships between the extents of ^{14}C-phenanthrene mineralised in amended soils with spawn spent brewery grains of *T. versicolor* (TV), *B. adusta* (BA), *P. chrysosporium* (PC), *P. ostreatus* (PO) and *I. lateus* (IR) and phenanthrene-degrading bacterial numbers.

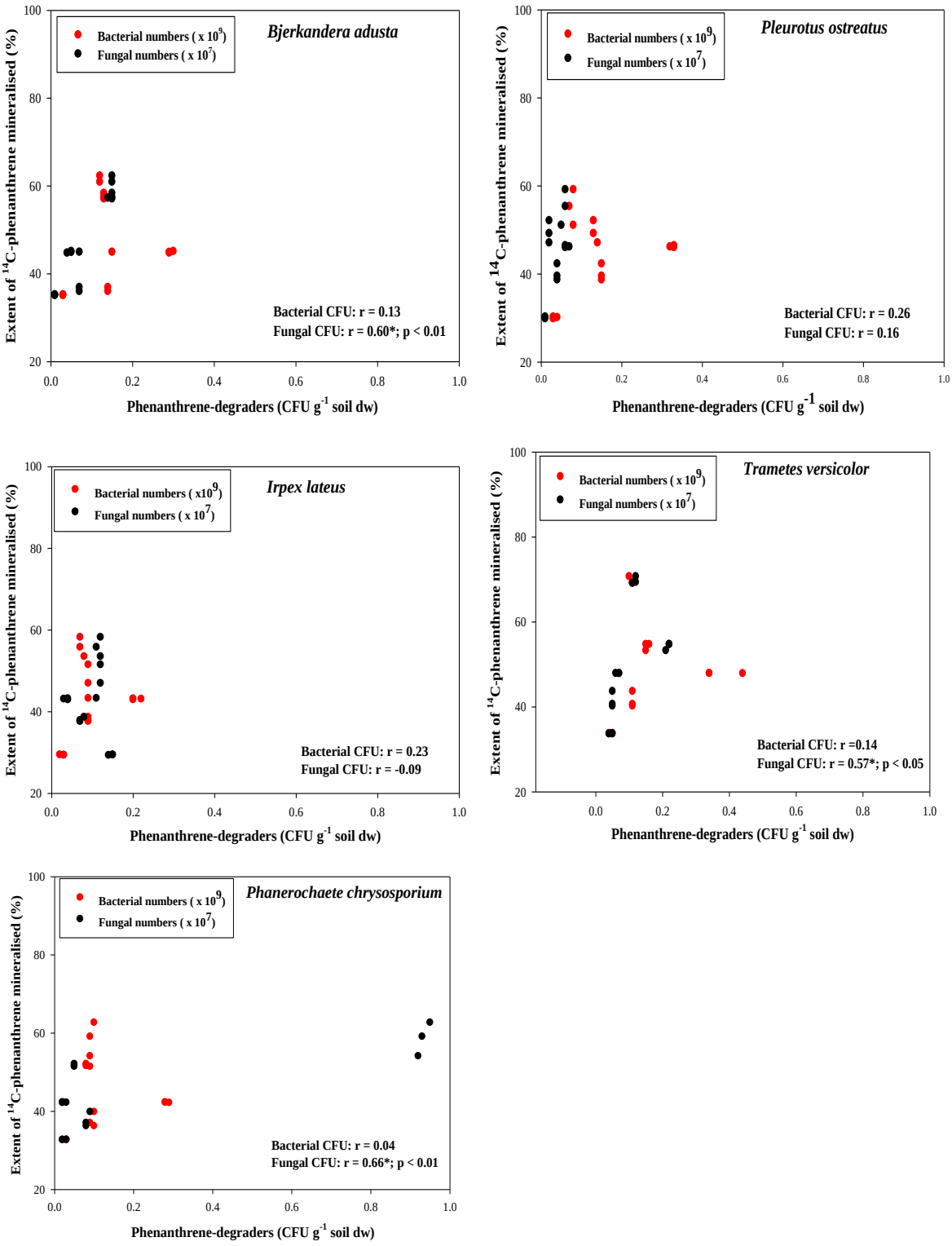

Figure S4. Relationships between the fastest rates of ^{14}C-phenanthrene mineralised in amended soils with spawn spent brewery grains of *T. versicolor* (TV), *B. adusta* (BA), *P. chrysosporium* (PC), *P. ostreatus* (PO) and *I. lateus* (IR) and phenanthrene-degrading fungal numbers.

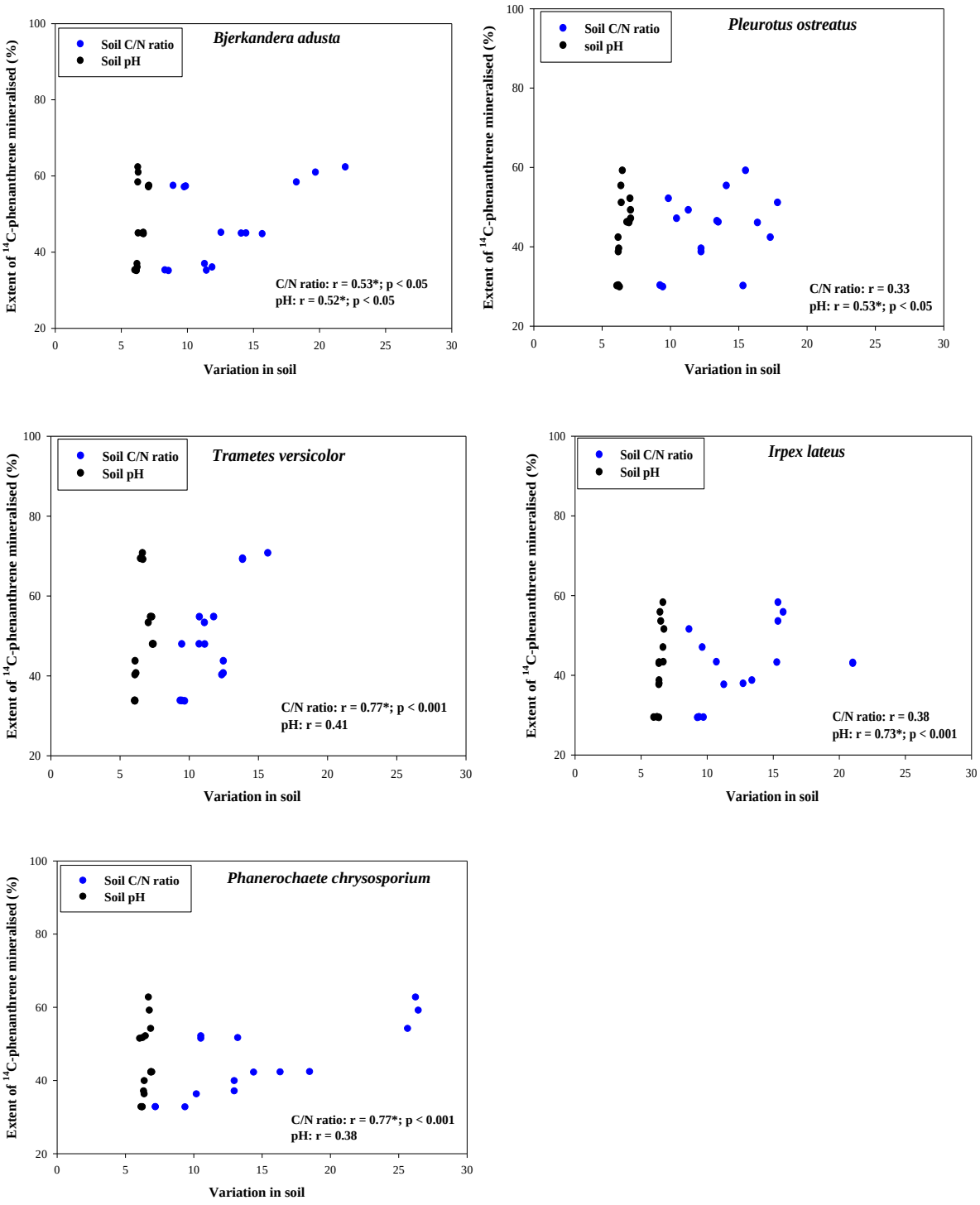

Figure S5. Relationships between the overall extents of ^{14}C-phenanthrene mineralised in amended soils with spawn spent brewery grains of *T. versicolor* (TV), *B. adusta* (BA), *P. chrysosporium* (PC), *P. ostreatus* (PO) and *I. lateus* (IR) and carbon-to-nitrogen ratios and pH levels.

Chapter 6

Impact of white-rot fungi immobilised on lignocellulosic waste on the biodegradation of ^{14}C-phenanthrene in contaminated soils.

Impact of white-rot fungi immobilised on lignocellulosic waste on the biodegradation of ^{14}C-phenanthrene in contaminated soils.

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6.1 Abstract

Biodegradation of polycyclic aromatic hydrocarbons (PAHs) after lignocellulose amendment depends on nutrient stimulation and the catabolic potential of soil microbes including their enzymes. In this study, we investigate the influence of five species of white-rot fungi (WRF) immobilised on spent brewery grains (SBG) with changes in the kinetics of ^{14}C-phenanthrene mineralisation (lag phase, fastest rate and overall extent), soil physiochemical (C/N ratio and pH) and biological (enzymatic and microbial) properties in contaminated soil over time (1, 25, 50, 75 & 100 d). Generally, immobilised WRF exhibited variations in all parameters tested in amended soils. Our results showed that all amended soils reduced the lag phases and increased the extents of biodegradation. We observed a greater reduction in the lag phase and higher rate of mineralisation in immobilised *Trametes versicolor*-amended soil compared to the other soil conditions. However, the soil amendment with WRFs (*Pleurotus ostreatus* and *Phanerochaete chrysosporium*) influenced biodegradation much more as compared to other fungal species. The C/N ratios were stimulated in most soil conditions; *P. chrysosporium* showed the highest increases in soil C/N ratio after amendment. The soil pH decreased over time in amended soils but remain unchanged with *T. versicolor* amendment. The highest extent of mineralisation was found at soil C/N ratio of 12.9 and pH 6.3. In addition, enzyme activities increased in amended soils and positively correlated with the extents of mineralisation in all amended soils.

Maximum ligninolytic enzyme activities were observed in *P. ostreatus*-amended soil. Microbial populations increased in all amended soils while PAH-degrading fungal numbers increased with increased soil-PAH contact time and strongly positively correlated with fastest rates of mineralisation. Our findings suggests that the five investigated WRFs are good lignocellulosic-immobilisers and could influence PAH degradation and soil properties (physicochemical and biological) in contaminated soil.

Key words: White-rot fungi, immobilisation, lignocellulose, enzymes, phenanthrene, soil

6.2 Introduction

The fate and degradation of polycyclic aromatic hydrocarbons (PAHs) in soil are influenced by the physicochemical properties of the molecules. As the ring number increases, PAHs become less mobile and biodegradable, thereby increasing their persistence in soil (Abdel-Shafy and Mansour, 2016; Ghosal *et al*., 2016). These include solid-liquid partition (Kd) and organic carbon-water partition coefficients (Koc), hydrophobibity, lipophilicity, and low aqueous solubility (Semple *et al*., 2003; 2007; Haritash and Kaushik, 2009; Couling *et al*., 2010; Yu *et al*., 2018). PAH molecules also display toxic, mutagenic and carcinogenic properties (Balmer *et al*., 2019), thus their presence poses a risk to human and environmental health (Lawal, 2017; Tongo *et al*., 2017; Sakshi *et al*., 2019).

Microbial degradation has been demonstrated as an important mechanism for the removal of PAHs and other organic contaminants in soil (Kim and Lee, 2009; Haritash and Kaushik, 2009; Ghosal *et al*., 2016). Bacterial and the fungal populations have been reported to play major roles in PAH degradation (Ghosal *et al*., 2016; Fernández-Luqueño *et al*., 2011). However, fungal degradation of PAHs is a more effective technique for soil biodegradation owing to their

vast hyphal network, extracellular enzymes, high surface area to volume ratio, easily adaptable to pH and temperature changes and presence of metal-binding proteins (Akhtar and Mannan, 2020). In addition, bacterial degradation of contaminants in soils often requires transport into cell membranes and this hydrophobic PAH transport process is rather slow (Schamfuß *et al*., 2013; Czaplicki *et al*., 2018). However, this can be overcome through the support of some hydrophilic filamentous fungi by active transportation of the contaminant through their hyphal network (fungal highways or pipelines) over a range of distance to bacteria cells (Kohlmeier *et al*. 2005; Schamfuß *et al*., 2013; Czaplicki *et al*., 2018). Fungal highway-like associations can also increase frequency of bacterial-PAH contact and facilitate the movement of indigenous bacteria, for example, the PAH-degrading bacteria into micro-zones of contaminated soil conditions for efficient PAH biodegradation (Wick *et al*., 2007; Furuno *et al*., 2012; Simon *et al*., 2015).

The basidiomycetes, especially the white rot fungi (WRF), which can be ligninolytic, have dual characteristics, being not only proficient degraders of lignocellulose biomass, but also active degraders of PAHs because both PAHs and lignin have aromatic structural components (Memić *et al*., 2020). Several species of WRF have been reported to efficiently degrade and mineralise PAHs especially those of the genera *Trametes*, *Bjerkandera*, *Phanerochaete*, *Irpex* and *Pleurotus* as well as the hyphomycetes *Penicillium* and *Aspergillus* (Quintella *et al*., 2019; Gao *et al*., 2010); however, depending on their ligninolytic enzyme complexes, the degradative function of each strain can potentially differ (Pozdnyakova, 2012; Cao *et al*., 2020). Lignin is degraded through ligninolytic mechanisms involving extracellular ligninolytic peroxidase enzymes (lignin peroxidase, manganese peroxidase, versatile peroxidase), and phenol oxidases (laccases), owing to their non-specific and oxidative properties, including the production of hydroxyl free radicals (Winquist *et al*., 2014; Akhtar and Mannan, 2020). As a result of this,

ligninolytic fungi have been shown to degrade low and high molecular weight PAHs, as well as other organopollutants including synthetic dyes, organochlorine pesticides, polychlorinated biphenyls (Young *et al*., 2015; Kadri *et al*., 2017; Al-Hawash *et al*., 2018; Pozdnyakova *et al*., 2018).

Lignocellulosic wastes can be applied to soil to deliver organic carbon, nitrogen and other nutrients to improve soil quality, support biological activity and stimulate microbial degradation of organic contaminants, such as PAHs (Lladó *et al*., 2015; Cao *et al*., 2016; Sigmund *et al*., 2018; Omoni *et al*., 2020b). Among the lignocellulosic waste materials, wheat straw, saw dust, sugarcane bagasse and corn cobs have been reported to stimulate both microbial and lignolytic activity (Dzionek *et al*., 2016). These waste residues can also enhance the survival of the fungal mycelia, optimizes enzyme secretion, protect both the fungi and enzyme from harsh environmental conditions and increase their viability and activity in soil (Dzionek *et al*., 2016). Previous studies however have focused more on WRF immobilised on lignocellulose residues for the degradation of chemical contaminants in wastewater treatment and agricultural pesticides (Gao *et al*., 2010; Dzionek *et al*., 2016). However, only a very few studies have investigated WRF immobilisation on lignocellulosic wastes (wheat straw, composted green wastes, saw dust and sugarcane bagasse) particularly for PAH degradation in soil (Dzul-Puc *et al*., 2005; Mohammadi and Nasernejad, 2009; Covino *et al*., 2010; Winquist *et al*., 2014). Further, to the authors' knowledge, there have been no published studies focussing on the the biodegradation of PAHs in soil by WRF immobilised on spent brewery grains (SBGs).

Therefore, this study investigated the impact of SBG-immobilised WRF on the biodegradation of ^{14}C- phenanthrene in five soils. In addition, the impact on the soil biological (enzyme and

microbial activities) and physicochemical properties (soil pH and C/N ratio) were investigated after 1, 25, 50, 75 and 100 d soil-PAH contact time.

6.3. Materials and method

6.3.1 Soil and lignocellulosic waste collection

A pristine soil (Dystric Cambisol, 5 –20 cm) from Myerscough Agricultural College, Preston, UK was used for this study. Soil was partially air-dried and then homogenised after sieving through a 2 mm mesh. Sieved soil was stored at 4 ^{o}C in the dark before use. The microbial and physicochemical properties have been determined previously (Couling *et al*., 2010a) and are presented in Table S1. Fresh SBGs were obtained from Lancaster Brewery, Lancaster, UK; the general properties are presented in Table S2 (Omoni *et al*., 2020b). The SBGs were stored at a temperature of 4 °C in a sterile sealed high-density polyethylene bag.

6.3.2 White rot fungi, culture conditions and preparation for immobilisation

All studied white rot fungi with most efficient capabilities for both ligninolysis and PAH mineralisation were supplied by the Czech Republic's Culture Collection of Basidiomycetes (CCBAS), Institute of Microbiology, Prague, Czech Republic. Strains included *Pleurotus ostreatus* (CCBAS 473), *Trametes versicolor* (CCBAS 614), *Phanerochaete chrysosporium* (CCBAS 570), *Irpex lateus* (CCBAS 196), and *Bjerkandera adusta* (CCBAS 232). The fungi were maintained on potato dextrose agar (PDA, 39 g l^{-1} pH 5.0) and incubated for 7 days (25 ± 1^{o}C). The cultures were subcultured routinely to maintain viability at every 15-20 days. Homogenised mycelial (Ultra-Turrax homogenizer at 10,000 rpm for 60s) were prepared from four mycelial plugs of 5 mm in diameter excised from actively growing edge of the fungus and

then transferred into 100 ml of potato dextrose broth (PDB, 24 g l^{-1} pH 5.0). The medium was devoid of nitrogen sources for efficient metabolism of the target contaminant. Mycelial suspensions were incubated at (25 ± 1^{o}C) under a rotary shaker at 150 rpm in the dark and pelleted mycelia were harvested by centrifuging at 3500 x *g*, for 10 mins at 4^{o}C (Thermo Scientific^{TM}, Sorvall TX-40R Cell Culture Centrifuge, UK). Supernatant was decarded and then pellets washed thrice with sterile distilled water after several resuspension of mycelial cells and centrifugation. The fungal biomass were measured (dry weights, dw) under the oven at 60^{o}C.

6.3.3 White-rot fungi immobilisation

The immobilisation of fungal cells on SBG was performed using a homogenised fungal mycelial suspension. The suspension was prepared by blending each of the fungal mycelium (0.01 g/g SBG dw) with sterile distilled water. Fresh brewery grains (250 g dw, autoclaved for 15 mins) were then mixed with mycelial suspensions (1:1, w/v) in a 500 ml Erlenmeyer flask and incubated for 24 hrs at 28 ^{o}C for sorption of fungal mycelia onto the SBGs. A moisture content of 60% was maintained in mycelial-SBG mixture before mixing into soil. The final moisture content was selected based on pre-study carried out to observe the marked cumulative CO_2 production, resulting from fungal growth (biomass), degradation and metabolism at various moisture contents (50, 60, 70, and 80%) of mycelial-SBG mixture over a 20 d incubation period (Figure S1). Fungal respiration was measured by CO_2 production using a Micro-Oxymax respirometer (automated Columbus Instrument), all of which was replicated in with triplicate incubations.

6.3.4 Soil spiking and immobilised spent brewery grains amendment

Sieved soil was rehydrated to 60% water holding capacity with deionized water and spiked with ^{12}C-phenanthrene (> 96%, HPLC-grade, Sigma-Aldrich, Germany) according to the method as previously described (Doick *et al*., 2003). Soil was amended with phenanthrene (100 mg/kg, dw) and homogenously mixed with 20% of an SBG-immobilised fungus (1:5 SBG:soil) for optimum PAH degradation, as reported previously (Omoni *et al*., 2020b). The SBG-immobilised fungus:soil mixtures, including the controls and blanks, were transferred to amber glass bottles and incubated in the dark (21 ± 1ºC) and aerated with humidified and nonsterile forced air at a flow rate of 0.35–0.40 ml min^{-1} g^{-1} soil. The soil conditions were incubated for 100 d and sampled at 1, 25, 50, 75 and 100 d. Non-destructive sampling was used in this experiment to maintain the same biological function and soil properties in all the soils at the various time points throughout the study. Respirometric soil-slurry assays were conducted to evaluate the influence of the SBG-immobilised fungi on the mineralisation of ^{12}C-/[9-^{14}C]-phenanthrene at each of the sample times (1, 25, 50, 75 and 100 days) in modified 250 ml Schott bottles (Teflon-lined screw cap) following the well-established method described by Semple *et al*. (2006). Briefly, at each contact time, 10 g ± 0.2 g (dw) of incubated soil was transferred into the respirometer, which contained 30 ml of deionized water (1:3 soil:liquid ratio) and added [9-^{14}C] phenanthrene standard (98.2 Bq g^{-1} soil) and incubated in the dark at 21 ± 1°C (n = 3). The respirometers (n = 3) contained $^{14}CO_2$ traps of 1 ml of 1 M NaOH solution and incubated by shaking at 100 rpm on a flat-bed orbital shaker in the dark at 21 ± 1°C for 18 days. But it should be noted that the respirometers were opened daily to replace the $^{14}CO_2$ traps, allowing an exchange of air into the respirometer.

The ^{14}C-phenanthrene activity on each $^{14}CO_2$ traps were quantified bihourly for 1 d and then daily by adding 5 ml of liquid scintillation counter (Goldstar-Meridian, UK) using the liquid scintillation analyzer (LSC, Canberra Packard Tri-Carb2250CA). Analytical blanks contained ^{12}C-phenanthrene (without amendment and ^{14}C-PAH), abiotic controls (autoclaved-sterilized soils) with ^{12}C-PAH but without ^{14}C-PAH and fungus) and controls with both ^{12}C and ^{14}C-phenanthrene (without organic amendments and fungus) were also prepared alongside the treatments. Here, the unamended control with ^{12}C and ^{14}C-phenanthrene was used as the control throughout the experiment.

6.3.5 Soil pH and C and N determinations

The changes in the soil pH for all soil conditions in soils were determined from 1:5 w/v (soil:water) slurry by a calibrated Jenway Model 3504 pH/conductivity meter. Carbon to nitrogen (C/N) ratios were analysed in soil samples by dry combustion method using an elemental analyser (Vario EL Cube, UK) according to the methodology described by Wilke, (2010).

6.3.6 Soil extraction and enzymes analyses

At each time point, 10 g (dw) of soil was homogenised in 25 ml potassium phosphate buffer (100 mM, pH 7.0), shaken on ice at 100 rpm for 60 mins and then the homogenate suspensions were centrifuged at 15,000 x g for 15 mins at 4^{o}C and repeated to completely remove cell debris (5000 x g, 15 mins, 4^{o}C), as previously described (Baldrian *et al*., 2000; Lang *et al*., 1998) with some minor modifications. Enzyme extract was then filtered with sterile hydrophobic

polytetrafluoroethylene (PTFE) syringe filter (0.45 µm pore size) and cleared filtrate assayed immediately for enzymes activity.

Ligninolytic enzyme activity (laccase, lignin peroxidase and manganese peroxidase) in soil was determined spectrophotometrically. Laccase (LAC) enzyme activity was assayed by ABTS (2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) oxidation, as described by Bourbonnais and Paice (1990- modified). Lignin peroxidase (LiP) activity was measured by veratryl alcohol oxidation method, as described by Tien and Kirk (1988). Manganese peroxidase (MnP) activity was determined by the oxidation of the phenol-red as substrate as described previously (Chan-Cupul, Heredia-Abarca, & Rodríguez-Vázquez, 2016). β-Glucosidase (βGA) activity was assayed using *p*–nitrophenyl–β–D-glucopyranoside (PNG) as substrate (Eivazi and Tabatabai, 1988), and phosphatase enzyme was measured using *p*–nitrophenyl phosphate (PNP) as substrate (Tabatabai and Bremner, 1969). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of the substrate per minute.

6.3.7 Quantification of microorganisms

Microbial numbers were measured in soils by dilution and spread plate method as described previously (Kästner *et al*., 1994; Okere *et al*., 2012; Omoni *et al*., 2020b) and presented as colony forming unit counts (CFUs g^{-1} soil dw). At each sample time point, soil (1.0 ± 0.01 g, dw) was collected and weighed and then used for the enumeration of both heterotrophic and PAH-degrading microbial numbers, respectively. The fungal numbers (heterotrophs and PAH-degraders) were quantified by the method described by Kästner *et al*. (1994) with some modifications. For PAH-degrading fungal CFUs, the minimal basal salt (MBS) medium was enriched with 50 mg l^{-1} ^{12}C-phenanthrene (carbon source) before inoculation. All inoculated

plates were incubated in triplicates and supplemented with antibacterial (penicillin-streptomycin-glutamate) and antifungal (fungizone) based on the target organism (Omoni *et al*., 2020a).

6.3.8 Statistical analyses

Data were statistically analysed using IBM SPSS version 25.0. Homogeneity of variance in the data were performed using Levene's and Shapiro-Wilk tests to check the normality of the data (Razali and Wah, 2011; Field, 2013). The data were transformed when necessary to ascertain ANOVA assumptions. Univariate analysis of variance (ANOVA) and the student's t-test was used to identify significant differences in the kinetics of ^{14}C-phenanthrene mineralisation (lag phases, fastest rates and extents), as well in the soil biological and physicochemical physical properties in immobilised WRFs-amended soils. Changes in these parameters over time were further analysed by Tukey's HSD post-hoc and Games-Howell tests. Pearson's correlations and linear regressions were applied to determine the relationships between the tested parameters in soil over time. Graphs were plotted with SigmaPlot 10.0 software.

6.4. Results

6.4.1 Impact of BSG-immobilised WRF on the mineralization of ^{14}C-phenanthrene in soil

The impact of SBG-immobilised WRF on the mineralisation of ^{14}C-phenanthrene was measured in soils at 1, 25, 50, 75 and 100 d of soil-PAH contact time (Table 1 and Figure 1). The lag phases were significantly reduced in most of the amended soils at all time points ($p < 0.05$), as compared to control soil. Following 1 d of soil-PAH contact time, the measured lag phases in all amendments were statistically similar ($p > 0.05$), except in soils amended with *B.*

adusta and *P. ostreatus,* which displayed significantly shorter lag phases ($p < 0.05$). Although the amended soils showed significantly extended lag phases of 2.93 d – 5.97 d ($p < 0.05$) after 1 day, compared to the other time points (25 d –100 d) where marked decreases were observed. Particularly, soil amended with *B. adusta* after 25 d showed statistically shorter lag phase compared to corresponding amendments and control. Similarly, SBG-immobilised *T. versicolor* reduced the length of the lag phases after 75 d and 100 d soil-PAH contact time. Furthermore, it was also observed that the soil amended with SBG-immobilised *T. versicolor* displayed both the longest and shortest lag phases, 5.97 ± 0.60 d and 0.19 ± 0.00 d after 1 d and 75 d, respectively. Specifically, the shortest lag phases for all SBG-immobilised fungal amendments were recorded at 75 d of soil incubation.

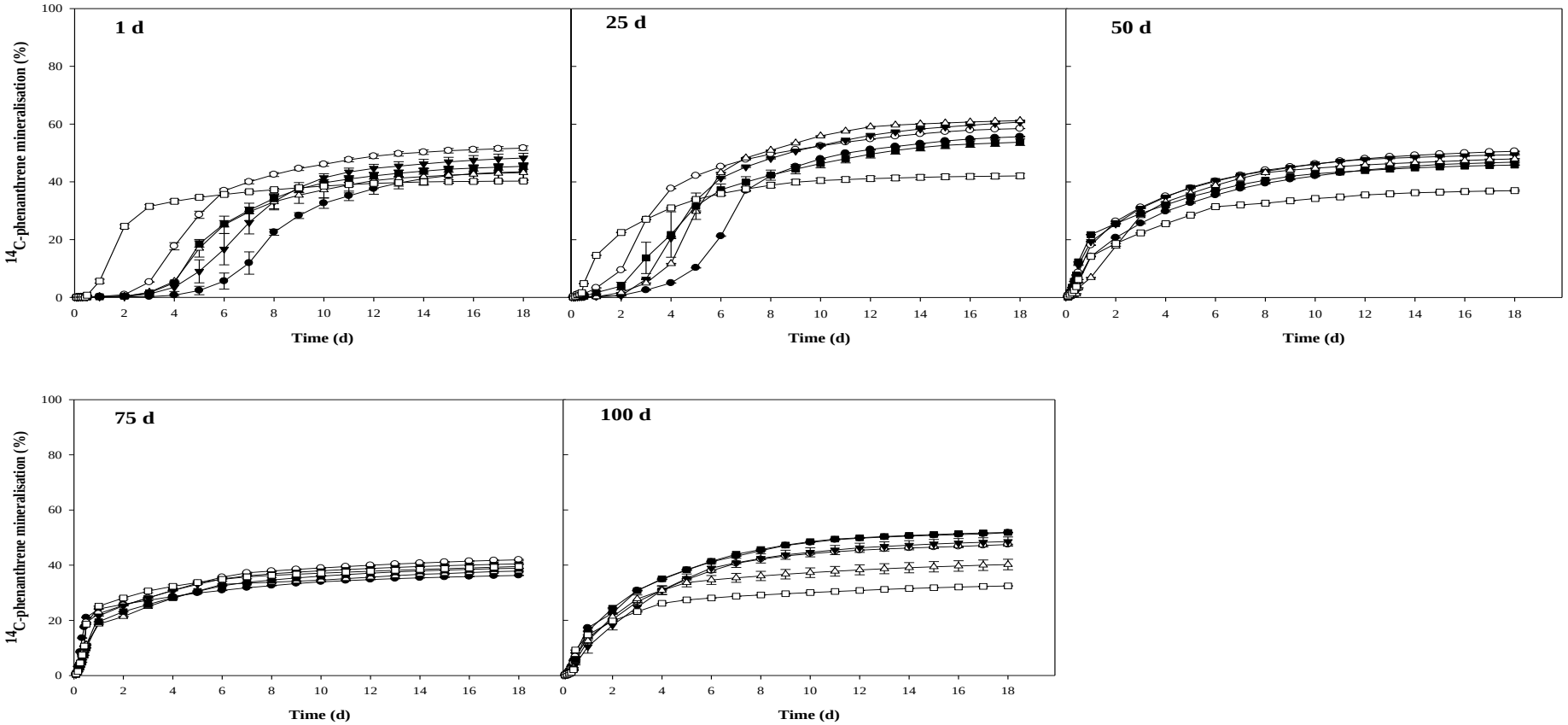

Figure 1. Catabolism of ^{14}C-phenanthrene in soils amended with immobilised fungi on spent brewery grains after 1, 25, 50, 75, and 100d soil-PAH contact time. Immobilised fungi: (●) *T. versicolor*, (○) *B. adusta*, (▼) *P. chrysosporium*, (△) *P. ostreatus*, (■) *I. lateus,* and control (unamended)(□). Values are mean ± SE (n = 3).

Table 1. Summary of the catabolic and microbial numbers (CFU/g) in soil amended with SBG-immobilised fungi after 18 days respirometric assay for different ageing periods after spiking with $^{12}/^{14}$C-phenanthrene. Values are mean ± standard error (n = 3).

Contact time (d)	Ligninolytic fungi	Lag phase (d)	Fastest rate (%$^{14}CO_2$ d^{-1})	Cumulative Extent (%)	Bacteria		Fungi	
					Total Heterotrophs	PAH-degraders	Total Heterotrophs	PAH-degraders
					CFU x 10^7 g^{-1} soil dw		CFU x 10^7 g^{-1} soil dw	
1	*T. versicolor*	5.97 ± 0.60d3	0.47 ± 0.14a3	43.52 ± 2.30ab3	1.52 ± 0.02d3	0.31 ± 0.00d3	52.8 ± 1.19e1	0.08 ± 0.00d5
	B. adusta	2.93 ± 0.02b4	0.52 ± 0.04a5	51.67 ± 0.77a2	1.67 ± 0.01c3	0.34 ± 0.00b2	97.9 ± 0.87a1	0.09 ± 0.00c5
	P. chrysosporium	4.53 ± 0.52cd3	0.44 ± 0.05a3	48.24 ± 1.54a2	1.48 ± 0.02d3	0.35 ± 0.01a3	85.5 ± 0.33c1	0.08 ± 0.00d5
	P. ostreatus	3.86 ± 0.04bc5	0.51 ± 0.10a4	43.20 ± 2.75ab2	1.86 ± 0.01a3	0.32 ± 0.00c3	76.6 ± 1.20d1	0.10 ± 0.00b4
	I. lateus	3.99 ± 0.01bc4	0.56 ± 0.04a4	45.37 ± 1.06ab2	1.75 ± 0.02b2	0.31 ± 0.00d2	97.2 ± 0.31b1	0.11 ± 0.00a4
	Control	6.12 ± 0.39a3	0.79 ± 0.01a5	40.27 ± 0.75b1,2	1.08 ± 0.00e2,3	0.01 ± 0.01e4	0.01 ± 0.00f3	0.05 ± 0.00e1
25	*T. versicolor*	4.64 ± 0.32d2	0.66 ± 0.00abc3	55.68 ± 0.17bc1	11.7 ± 0.18e1	0.34 ± 0.00a2	9.62 ± 0.15a2	0.33 ± 0.00a3
	B. adusta	1.27 ± 0.00a2	0.73 ± 0.01a4	58.48 ± 0.03ab1	28.9 ± 0.18a1	0.28 ± 0.00d3	7.70 ± 0.12b2	0.22 ± 0.00d4
	P. chrysosporium	2.77 ± 0.04c2	0.59 ± 0.09c3	60.70 ± 0.20a1	21.4 ± 0.29b1	0.30 ± 0.00c2	6.68 ± 0.14c2	0.18 ± 0.00e4
	P. ostreatus	3.00 ± 0.05c4	0.75 ± 0.00a3	61.24 ± 0.13a1	17.1 ± 0.17d2	0.34 ± 0.00a2	9.72 ± 0.10a2	0.24 ± 0.01c3
	I. lateus	1.83 ± 0.09ab4	0.62 ± 0.05bc4	53.76 ± 1.11c1	18.5 ± 0.25c1	0.32 ± 0.00b2	6.81 ± 0.12c2	0.26 ± 0.00b3
	Control	2.56 ± 0.34bc2	0.70 ± 0.01ab3	42.10 ± 0.61d1	1.27 ± 0.02f1	0.01 ± 0.00e2	0.03 ± 0.00d2	0.01 ± 0.00f3
50	*T. versicolor*	0.49 ± 0.03b1	1.32 ± 0.01c2	46.86 ± 0.27bc2,3	8.00 ± 0.12a2	1.47 ± 0.01a1	4.23 ± 0.09a3	0.53 ± 0.02b2
	B. adusta	0.41 ± 0.03ab2	1.78 ± 0.01b2	50.60 ± 0.11a2	4.30 ± 0.11b2	0.53 ± 0.03c1	2.61 ± 0.09c3	1.32 ± 0.02a1
	P. chrysosporium	0.38 ± 0.00a1	2.19 ± 0.05a2	49.40 ± 0.20ab2	2.18 ± 0.12c2	0.65 ± 0.02b1	1.98 ± 0.11d4	0.51 ± 0.01b2
	P. ostreatus	0.73 ± 0.06c3	1.02 ± 0.00d2	47.97 ± 0.20abc2	2.31 ± 0.09c3	0.52 ± 0.00c1	3.73 ± 0.07b3	0.40 ± 0.01c2
	I. lateus	0.31 ± 0.00a1	2.27 ± 0.02a1	45.86 ± 0.06c2	1.65 ± 0.09d2	0.41 ± 0.02d1	3.67 ± 0.15b3	0.37 ± 0.01c2
	Control	0.45 ± 0.04b2	1.22 ± 0.03c4	36.98 ± 2.89d2	0.04 ± 0.00e2	0.03 ± 0.00e1	0.02 ± 0.00e4	0.01 ± 0.00d5
75	*T. versicolor*	0.19 ± 0.00a1	2.64 ± 0.00c1	36.28 ± 0.09e4	0.94 ± 0.01a4	0.10 ± 0.00b4	4.56 ± 0.10a3	0.84 ± 0.01b1
	B. adusta	0.27 ± 0.06b1	4.38 ± 0.01a1	41.95 ± 0.18a4	0.27 ± 0.00c4	0.12 ± 0.00a4	1,29 ± 0.06cd3,4	0.96 ± 0.01a2
	P. chrysosporium	0.31 ± 0.03c1	1.37 ± 0.01c2	40.47 ± 0.11ab3	0.41 ± 0.01b4	0.08 ± 0.00d4	3.50 ± 0.07b3	0.85 ± 0.01b1
	P. ostreatus	0.26 ± 0.06b1	1.39 ± 0.07b1	37.87 ± 0.21d3	0.41 ± 0.01b4	0.08 ± 0.00d4	1.42 ± 0.03c4	0.56 ± 0.02c1
	I. lateus	0.34 ± 0.03d1,2	1.32 ± 0.00d3	38.87 ± 0.07cd3	0.25 ± 0.00c3	0.09 ± 0.00c3	1.02 ± 0.03de4	0.58 ± 0.02c1
	Control	0.26 ± 0.05b1	3.96 ± 0.02b1	39.53 ± 1.01bc1,2	0.02 ± 0.00d2,3	0.01 ± 0.00e3	0.03 ± 0.01e1	0.02 ± 0.00d2
100	*T. versicolor*	0.40 ± 0.01a1	1.39 ± 0.01c2	51.78 ± 0.06a1,2	0.53 ± 0.00a5	0.03 ± 0.00b5	0.32 ± 0.00a4	0.25 ± 0.00b4
	B. adusta	0.44 ± 0.04a2	1.17 ± 0.04c3	47.47 ± 0.13a3	0.49 ± 0.00b4	0.02 ± 0.00d5	0.24 ± 0.01b4	0.32 ± 0.00a3
	P. chrysosporium	0.62 ± 0.07b1	1.15 ± 0.13c2	48.52 ± 1.67a2	0.31 ± 0.01c4	0.03 ± 0.00a5	0.14 ± 0.00d5	0.26 ± 0.01b3
	P. ostreatus	0.49 ± 0.00a2	1.18 ± 0.04c1,2	40.22 ± 1.90b3	0.49 ± 0.00b4	0.02 ± 0.00e5	0.17 ± 0.00c4	0.24 ± 0.00c3
	I. lateus	0.48 ± 0.00a2	1.71 ± 0.02b2	51.83 ± 0.15a1	0.60 ± 0.01a3	0.03 ± 0.00c4	0.11 ± 0.00e5	0.15 ± 0.00d4
	Control	0.45 ± 0.03a2	3.58 ± 0.01a2	32.45 ± 0.01c3	0.01 ± 0.00d3	0.01 ± 0.00f4	0.01 ± 0.00f5	0.01 ± 0.00e4

*Mean values with the same lower-case letters within each aging time in amended soils are significantly different ($p < 0.05$) while different lower-case letters indicate significant differences ($p < 0.05$) within each aging time in amended soils (1d–100d).
*Mean values with the same numbers across the sampling points are significantly different ($p < 0.05$) while different numbers indicate significant differences ($p < 0.05$) across the sampling points in amended soils (1d–100d).

The fastest rates of mineralisation were also measured in soils amended with SBG-immobilised WRF at each time point (Table 1 and Figure 1). In general, the rates of mineralisation significantly increased ($p < 0.05$) as soil-PAH contact time increased in all of the amendment conditions but the increases were significantly lower ($p < 0.05$) than the control soil. Soil amended with SBG-immobilised *T. versicolor* recorded the highest rate of 4.38% d^{-1} closely followed by *B. adusta* (2.64% d^{-1}) at 75 d, while the lowest rate of 0.44% d^{-1} was observed in the SBG-immobilised *P. chrysosporium* amended soil at 1 d incubation time. Fastest rates of mineralisation were observed at 50 d(1.02 – 2.27 % d^{-1}) and 75 (1.32 – 4.38 % d^{-1}) with an average increase of 245 and 344% increase compared to 1-d soil incubation. In contrast, control soils showed significantly faster rates of mineralisation ($p < 0.05$) for most time points (1, 75 and 100 d), when compared to amended soils, except in soil amended with SBG-immobilised *B. adusta,* which displayed significantly faster rates at 75 d soil-PAH contact time ($p < 0.05$). After 50 d soil incubation, *I. lateus* and *P. chrysosporium* showed higher rates of mineralisation compared to unamended soil.

The effects of the different SBG-immobilised WRF on the overall extents of ^{14}C-phenanthrene mineralisation in soils were also measured over a 100 d incubation period (Table 1 and Figure 1). The greatest extents of ^{14}C-phenanthrene mineralisation were observed for all amended soils at 25 d (53.8–61.2%); immobilised *P. ostreatus* displayed the highest extent of mineralisation (61.2%). However, out of the five SBG-immobilised WRF *B. adusta* and *P. chrysosporium* exhibited significantly greater ($p < 0.05$) extents of mineralisation in most time points than all other amendments and control, although they were not significantly different ($p > 0.05$). Greater degrees of mineralisation were observed at almost all soil-PAH contact time points compared to the control soil ($p < 0.01$). For the SBG-immobilised WRF-amended soils, the extents of mineralisation increased from 1 d to 25 d contact time, then decreased through

50 d and 75 d soil incubation. However, greater extents of mineralisation ($p < 0.05$) were observed in SBG-immobilised WRF-amended soils after 100 d incubation, particularly in *T. versicolor* and *I. lateus* amended soils with significant increases by 30% and 25%, respectively, as compared to those observed at 75 d of soil-PAH contact time.

6.4.2 Influence of SBG-immobilised WRF on soil physicochemical properties

The effects of the SBG-immobilised WRF on the soil physicochemical properties (C:N ratio, and pH) were also measured (Figures 2 and 3). The soil C/N ratio was found to be 11.3 before the application of SBG-immobilised fungi and subsequently, the C/N significantly increased ($p < 0.05$) in most amendment conditions (especially in soil amended with immobilised *P. chrysosporium*) and at most time points (1 d and 25 d) compared to the unamended (control) soil (Figure 2). For example, C/N ratios were significantly higher in the soils containing SBG-immobilised *P. chrysosporium* followed by *B. adusta* after 1 d and 25 d soil-PAH contact time, respectively, when compared to the other amendment conditions and control soils. However, the soil C/N ratios were not significantly different among the amendment conditions and between the amendment conditions and control within and across most time points, except for the SBG-immobilised *P. chrysosporium* amended soil, which had a significantly higher C/N ratio in all of the soil-PAH contact time points ($p < 0.05$). Further, the SBG-immobilised WRFs showed significantly higher C/N ratios ($p < 0.05$) in most contact time in amended soils, especially *P. chrysosporium*-amended soils. Similarly, the highest C/N ratio (22.4) was found in soil amended with *P. chrysosporium* after 25 d aging. Significant decrease in the C/N ratios in all amended soils were noticeable from the other contact points onwards (50d–100 d) as was observed for the control soil.

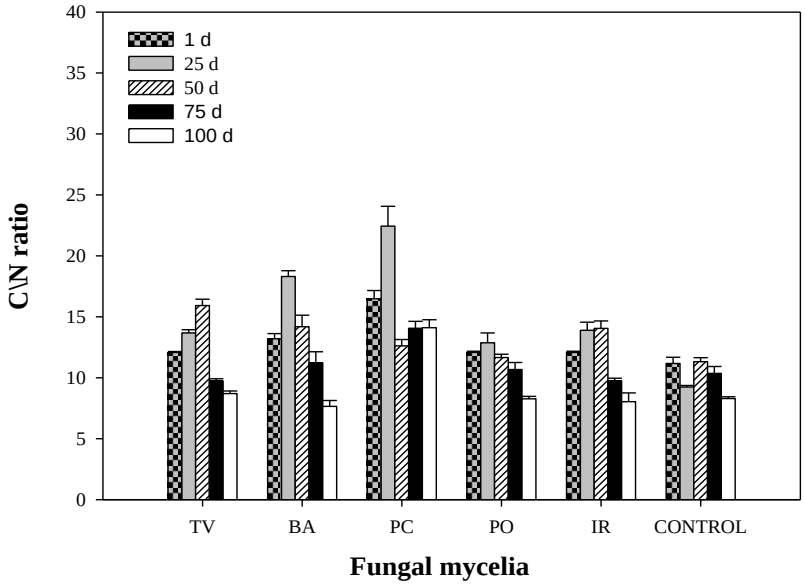

Figure 2. Changes in C/N ratio in amended soils with immobilised fungi on spent brewery grains after 1, 25, 50, 75, and 100d soil-PAH contact time. Values are mean ± SE (n = 3).

The SBG-immobilised WRF were found to influence the pH in the amended soils (Figure 3). It was observed that soil pH generally decreased in all of the amendment conditions with increased soil-PAH contact time, ranging from a maximum to a minimum pH value of 7.52 to 4.68. After 1 d incubation, the pH values for all soil conditions were significantly higher than the control ($p < 0.05$), but not after 75 d and 100 d, where the non-amended control showed a higher pH value than all the amended soils ($p < 0.05$). These two-time points (75 d and 100 d) showed acidic pH values of 4–5 in amended soils. *T. versicolor* amended soil exhibited a higher pH value at most time points in the experiment (50 d, 75 d and 100 d), followed by *P. chrysosporium* (50 d and 75 d). However, most time points did not show statistically different pH values ($p > 0.05$) in soils amended with WRFs. Furthermore, apart from *T. versicolor*-amended soil, all other soil conditions showed significant decrease ($p < 0.01$) in pH values over time ($100 < 75 < 50 < 25 < 1$). Moreover, the greatest ^{14}C-phenanthrene mineralisation was found at mean pH value of 6.3. It should be noted however that soil pH was strongly

positively correlated with the lag phases except for *T. versicolor* and negatively correlated with fastest rates of mineralisation in virtually all amended soils (Table S3-S7). The pH of soil amended with immobilised fungi (*B. adusta* and *P. ostreatus*) had significant positive correlations with overall extents of mineralisation (Table S4 and S6).

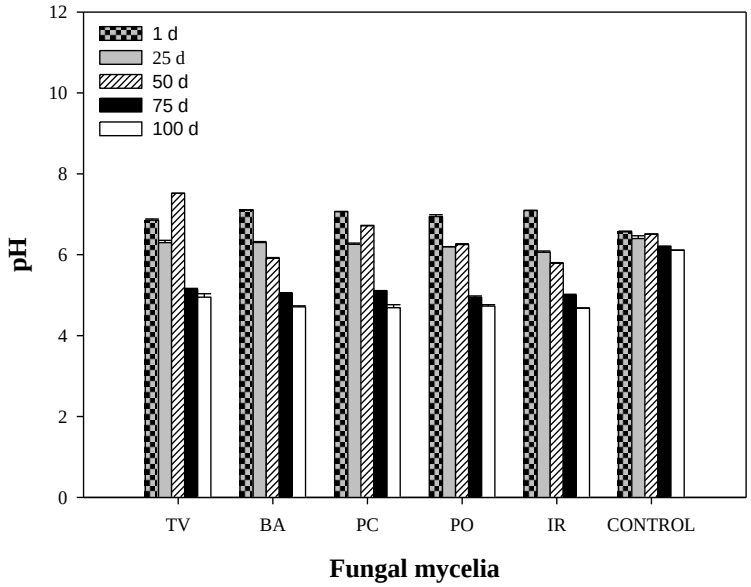

Figure 3. Changes in pH value in amended soils with immobilised fungi on spent brewery grains after 1, 25, 50, 75, and 100d soil-PAH contact time. Values are mean ± SE (n = 3).

6.4.3 Effects of SBG-immobilised WRFs on the enzyme activities in soil

The impact of SBG-immobilised WRF on ligninolytic enzyme activities was examined in phenanthrene-spiked soils over a 100 d study period (Figure 4(a-c). The ligninolytic systems (LAC, LiP and MnP) showed variations for all SBG-immobilised WRF-amended soils. For LAC enzyme, higher levels of activity were observed for all amended soils compared to the control, at most time points during the study (Figure 4a). The highest laccase activity was detected for *P. ostreatus* (2.52 U g^{-1}), closely followed by *T. versicolor* (1.61 U g^{-1}). After 1

day of soil incubation, LAC activity was significantly higher in *T. versicolor*-amended soil when compared to counterpart soils and control. Although, no enzyme activity was detected for treatments (*I. lateus* and *B. adusta*) while negligible enzyme activity was observed in *P. chrysosporium*-amended soil after 1 d soil aging. But not after 25 d and 50 d incubation, where enzyme activities were detected in both amended soils (*I. lateus* and *B. adusta*). Maximum LAC activity was detected after 25 d in all WRF-amended soils compared to the rest of the incubation period, however significant decreases were observed in WRF-amended soils from this point onward (50–100 d). No Lac activity was detected after 100 d soil incubation.

LiP activities of all SBG-immobilised WRF-amended soils were 5.76-fold higher after 25 d when compared to 1 d aging, especially in SBG-immobilised *P. ostreatus* amended soil with 14.3-fold higher than the other soil conditions. Higher LiP activities were measured in amended soils than the control ($p < 0.05$) for almost all time points (Figure 4b). *P. chrysosporium* (2.00 U g^{-1}) closely followed by *P. ostreatus* (1.10 U g^{-1}) soil conditions showed the highest LiP activities after 1 d incubation. Although LiP activities markedly varied in the different amendment conditions the enzyme levels obtained were much lower than those observed for LAC. It should be noted that only *B. adusta*-amended soil showed detection of LiP activities at all time points. Moreover, after 100 d, LiP activities further increased in all amended soils compared to 75 d soil-PAH contact time.

Compared to LAC and LiP enzymes measured, MnP activities were generally higher in SBG-immobilised WRF-amended soils; although, MnP activities showed similar patterns with those observed for LiP. The highest MnP activity of 3.84 U g^{-1} was observed in the presence of SBG-immobilised *P. ostreatus*. No MnP activity was measurable in the amended soils with *T. versicolor*, *P. chrysosporium* and *B. adusta* at the onset of incubation (1 d). However, MnPs

showed sharp increases from 1 d to 25 d incubation then decreased after 50 d –75 d followed by an increase at 100 d soil incubation. Further, MnP activity was detected in amended soils after 100 d incubation, while significantly higher MnP activities of more than 2.44-fold increase were detected in amended soils in comparison with 75 d soil-PAH contact time. Linear correlations and regressions showing the relationships of the ligninolytic enzyme activities in soils with the kinetics of mineralisation are presented (Table S3-S7, Figure S3-S7). No correlation was observed between the ligninolytic enzyme activities and the lag phases in amended soils, except for soil inoculated with *T. versicolor* for only laccase ($r = 0.54$, $p < 0.05$). Also, ligninolytic enzymes showed non-significant and weak negative correlations with fastest rates ($r = –0.16–0.58$) except *T. versicolor* and *I. lateus*-amended soils while the overall extents of ^{14}C-phenanthrene mineralisation was strongly positively correlated with ligninolytics ($r = 0.55–0.90$; $p < 0.05–0.001$).

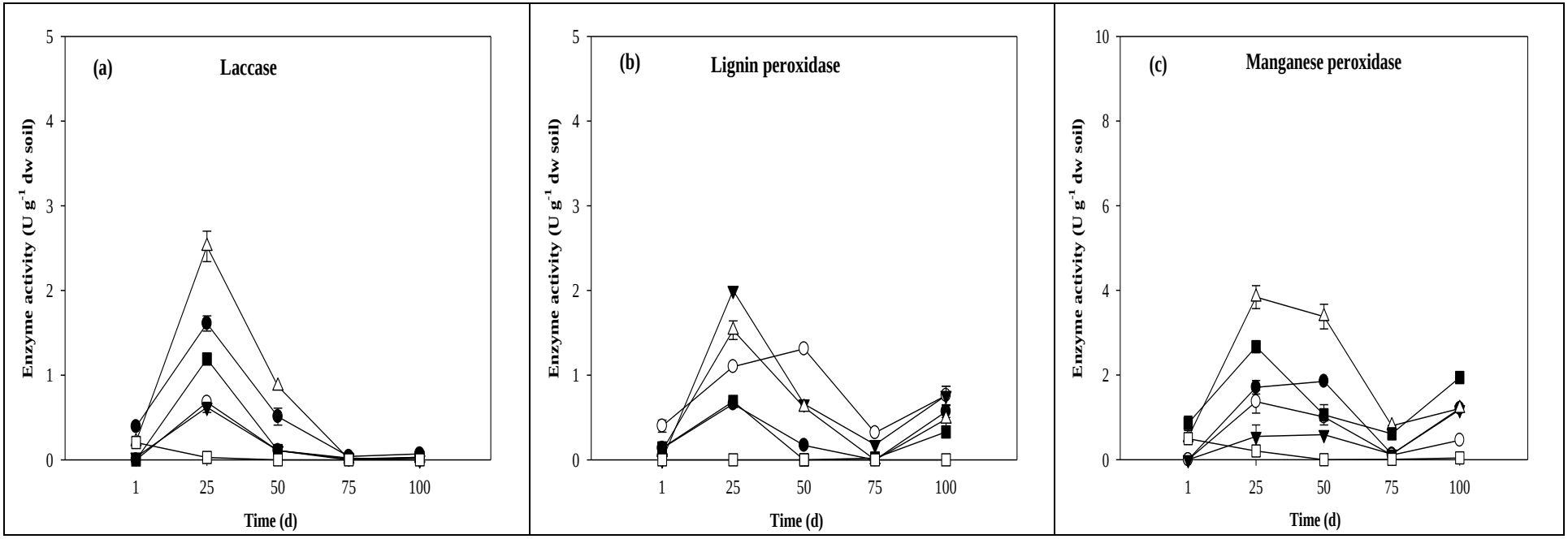

Figure 4. Immobilised fungi on spent brewery grains on the enzyme activities (a) Laccase; (b) Lignin peroxidase; (c) Manganese peroxidase in amended soils after 1, 25, 50, 75, and 100d soil-PAH contact time. Immobilised fungi: (●) *T. versicolor*, (○) *B. adusta*, (▼) *P. chrysosporium*, (△) *P. ostreatus*, (■) *I. lateus,* and control (unamended)(□). Values are mean ± SE (n = 3).

Nonligninolytic enzymes (βGA and ACP) levels were also measured over a 100 d period in amended soils and the results are presented in Figure S2 (a-b). The highest βGA activity were found in soils amended with *T. versicolor* (13.5 U g^{-1}). No significant differences were observed in βGA activity among the amendment conditions at most time points during the study (Figure S2a). For all amended soils, stimulated βGA activities were observed over time compared to control soil ($p < 0.001$). In contrast to the ligninolytic enzymes, increases in soil-PAH contact time did not affect the βGA activity which peaked at 50 d soil incubation. Compared to all the other enzymes assayed, the ACP activity was significantly higher ($p < 0.05$) in all amended soils and these enzyme levels were also significantly higher than the control ($p < 0.05$) (Figure S2b). Immobilised *B. adusta* and *P. chrysosporium* resulted in the highest ACP activity of 32.2 and 22.7 U g^{-1}, respectively. However, ACP activity was significantly reduced ($p < 0.05$) over time in amended soils. Furthermore, ACP activity in amended soils generally showed stronger correlations with the extent of mineralisation compared to βGA activity. Both enzymes displayed significantly positive correlations with the extents of mineralisation in almost all amended soils. Finally, ACP activity positively correlated with the lag phases ($r = 0.52–0.86$); *P. chrysosporium* amended soil showed the highest correlation coefficient ($r = 0.86$, $p < 0.001$) closely followed by *B. adusta* ($r = 0.76$, $p < 0.001$); but negatively correlated with fastest rates ($r = –0.52–0.67$) in all amended soils (Table S3-S7).

6.4.4 Quantification of culturable microbial numbers in soil

6.4.4.1 Bacterial populations

Bacterial numbers (CFU g^{-1} soil) were markedly influenced by the presence of SBG-immobilised WRF and soil-PAH contact time (Table 1). Furthermore, both heterotrophic and PAH-degrading bacterial numbers were consistently higher in all amended soils than the

control over time (1 d –100 d), where soils amended with *B. adusta* and *T. versicolor* presented the highest numbers of heterotrophs and PAH-degraders after 25 d and 50 d, respectively. Generally, the CFUs of total heterotrophs and PAH-degraders varied within and across BSG immobilised WRF-amended soils. At 25 d and 50 d soil incubations, the heterotrophic and PAH-degrading bacterial CFUs were significantly higher ($p < 0.05$) compared to other incubation periods, respectively. But not after 75 d –100 d where both heterotrophic and PAH-degrading bacterial numbers were significantly reduced ($p < 0.05$) in all amended soils. However, PAH-degrading bacterial numbers were significantly higher ($p < 0.05$) in all amendment conditions over time (Table 1). More so, PAH-degrading bacterial numbers were moderately positively correlated with the extents of ^{14}C-phenanthrene mineralised in *B. adusta* ($r = 0.45$, $p < 0.05$) and *P. ostreatus* ($r = 0.57$, $p < 0.05$) amended soils, while no correlations were found between PAH-degraders with lag phases and fastest rates in all WRF-amended soils, respectively (Table S3-S7). Additionally, no positive correlation between phenanthrene degrader CFUs and mineralisation kinetics was observed in *T. versicolor*-amended soil, despite the stimulated bacterial numbers (Table S3).

6.4.4.2 Fungal populations

The addition of SBG immobilised-WRF significantly stimulated the heterotrophic and PAH-degrading fungal numbers compared to the control soil throughout the investigation period (Table 1). *B. adusta* and *P. chrysosporium*-amended soils exhibited the highest fungal CFUs (heterotrophs and PAH-degraders) in soil after 24 hr incubation, respectively. The heterotrophic fungal numbers were significantly reduced after 25 d for the rest of the study period. In contrast to the heterotrophic bacteria CFUs, PAH-degrading fungal CFUs significantly increased in amended soils over time compared to 1 d soil-PAH contact time.

CFUs of heterotrophs and PAH degraders did not differ among the SBG-immobilised WRF amended soils at most of the time points. As observed in the bacterial numbers, *T. versicolor*-amended soil showed significantly higher heterotrophic fungal CFUs over time compared to the other amendment conditions. PAH-degrading fungal numbers had strong negative correlations with lag phases (r = –0.63–0.79), moderately correlated with the extents of ^{14}C-phenanthrene mineralisation (r = –0.30–0.66) in amended soils (Table S3-S7). However, we observed significant, positive correlations of PAH-degrading fungal numbers with fastest rates of mineralisation ($p < 0.01$); these linear relationships were stronger in soils amended with *T. versicolor* (r = 0.90, $p < 0.001$) closely followed by *P. ostreatus* (r = 0.82, $p < 0.001$).

6.5 Discussion

6.5.1 Mineralisation of ^{14}C-phenanthrene in amended soils using SBG-immobilised WRF

In this study, we found that SBG-immobilised WRFs resulted in reduced lag phases, decrease in fastest rates and increases in the extents of mineralisation of ^{14}C-phenanthrene in soils. However, the WRFs exhibited different biodegradation efficiencies in amended soils. The immobilisation was designed to improve PAH degradation in soil following available utilisable carbon and energy source (cellulose and hemicellulose) and fungal mycelial protection from PAH toxicity. Further, the fungal mycelia provided micro-habitats for the indigenous soil bacteria. The study shows that immobilised WRFs amendment showed significantly shorter lag phases in all amended soils over time, especially in soils amended with immobilised *T. versicolor* and *B. adusta*., indicating higher adaptation and catabolic action by these two fungal species and potentially enhancing the bioavailability of PAH compared to the other WRFs (Wang *et al*., 2012). These strains of WRFs are among the most efficient degraders of lignocellulose (Sánchez, 2009; Chen *et al*., 2010).

Furthermore, this present study showed that the fastest rates of ^{14}C-phenanthrene mineralisation in amended soils were significantly lower than those in the unamended soil. However, higher rates of mineralisation were seen after 50 d of soil incubation in amended soils with the exception of soil amended with *P*. ostreatus which show a reduced rates of mineralisation, suggesting higher SBG decomposition and release of more available nutrients for metabolic activities in soils. Oyelami *et al*. (2013) proposed that nutrient enrichment and microbial richness can enhance the rates of PAH biodegradation in soil. However, the reduction in the rate of mineralisation could be linked to microbial dynamics and changes in soil properties owing to more PAH partitioning with respect to increase in soil-PAH contact time (Northcott and Jones, 2001; Ogbonnaya and Semple, 2013). Our previous study demonstrated higher rates of PAH degradation by organic amendment in soils without fungal immobilisation (Omoni *et al*., 2020b). It must be noted however, that higher rates of mineralisation were found in soils amended with immobilised *T. versicolor* followed by *B. adusta*, indicating the potential of these fungal species in facilitating the rate of PAH uptake and degradation in soil. However, fungal hyphae can enhance biodegradation rate by increasing bioaccessibility of entrapped hydrophobic PAH in soil aggregates by hyphal penetration and reducing the distance for soil indigenous bacteria to the contaminant (Wick *et al*., 2007).

The extents of ^{14}C-phenanthrene mineralisation were hypothesised to depend on the ligninolytic potential of each fungus, ability of the mycelia to withstand harsh PAH condition and their positive synergistic fungal-bacterial interactions (Borràs *et al*., 2010; Wick *et al*., 2007). Phenanthrene have different metabolic pathways for its breakdown by WRFs (Acevedo *et al*., 2011). In this study, the addition of immobilised WRFs to PAH spiked soils increased the extents of ^{14}C-phenanthrene mineralisation in most contact points compared to our previous

study without fungal immobilisation (Omoni *et al*., 2020b). Here, it may be that the immobilised fungi aided higher transport of the phenanthrene to the mycelial network, and in turn, higher diffusion to bacterial and other cells in amended soils (Furuno *et al*., 2012). The extent of mineralisation was greatest for *P. ostreatus*-amended soil; it was also found that *B. adusta* and *P. chrysosporium* in soils consistently dispayed higher extents of mineralisation than their counterpart treatments in almost all contact points, suggesting that these two WRFs are better degraders. This might also be attributed to the WRFs facilitating accessible metabolisable carbon and energy sources to most soil bacteria leading to increase enzymes induction and cometabolism (Han *et al*., 2017; Harms *et al*., 2011). Furthermore, the differences between the treatment periods after each specific acclimation period may be likely attributed to the extent and ability of heavily forming strains (sporulation) in amended soils.

6.5.2 Effects of soil physicochemical properties on ^{14}C-phenanthrene mineralised using SBG-immobilised WRF

C/N ratio is a primary factor in determining the nutrient effect of PAH assimilation into microbial biomass during soil remediation (Leys *et al*., 2005). Lignocellulosic materials amended to soils would increased the C/N ratio (Hubbe *et al*., 2010) and C/N ratio around 9–10 promoted optimal PAH degradation (Riser-Roberts, 1998). In this study, soil C/N ratios increased in most of the amended soils; however, varied in amended soils over time, while soils amended with immobilised *P. ostreatus*, *B. adusta* and *P. chrysosporium* with C/N ratio of 12.9, 18.3 and 22.4, respectively, had the greatest extents of mineralisation after 25 d soil incubation. Furthermore, Teng *et al*. (2010) ascribed maximum PAH degradation in aged contaminated soils to a C/N ratio of 10:1 other than 25:1 or 40:1. This supports the results reported here where a C/N ratio of 12.9:1 resulted in higher extents of mineralisation than

higher C/N ratios (18.3 or 22.4). In addition, after 25 d incubation, the high C/N ratio found in amended soil with immobilised *P. chrysosporium* indicates more efficient delignification of the organic waste materials to release organic nutrients to the soil. However, we proposed here that the immobilised WRFs can facilitate PAH degradation with > 50% over a range of C/N ratio 13–22.

In contrast, the soil pH changes from neutral to acidic conditions after amendments; the highest rates and extents of mineralisation were noticeable at slightly acidic pH range (5.8–6.3) in amended soils, similar results within this pH range have been reported previously for enhanced PAHs biodegradation in soil (Bishnoi *et al*., 2008). This demonstrated that amendment with SBG-immobilised fungus may decreased the pH value in soil from slightly neutral condition, favouring the microbial catabolism of PAH. In addition, we also found that the soil pH decreased with increases in soil-PAH contact time, which may be due to increased production of more toxic metabolites from PAH mycotransformation (Ghosal *et al*., 2016) and/or production of tannic and humic acids during lignin biodegradation (Clemente, 2001) and likely greater CO_2 production dissolving in soil water to form weak organic acid such as in the closed system used in this study (Angert *et al*., 2015).

6.5.3 Influence of enzyme activities on the mineralisation of ^{14}C-phenanthrene in SBG-immobilised WRF-amended soil

In this study, ligninolytic enzymes (LAC, LiP and MnP) were all stimulated in amended soils. In particular, LiP enzyme was produced by the WRF evaluated (Rogalski *et al*., 1991; Nakamura *et al*., 1999; Rothschild *et al*., 2002; Asgher *et al*., 2011). However, MnP was the predominant ligninolytic enzyme in amended soils, which is in agreement with previously

reported results (Diez, 2010). Therefore, we proposed that the enzyme activities were likely influenced by the presence of SBG amendment. Depending upon the nutrients availability (high or low N), the WRFs may change their enzyme production to increase or decrease the enzyme pool in a system to maintain their supply of inorganic nitrogen, phosphorus and carbon (Olander and Vitousek, 2000). Ligninolytic enzymes also promoted the extents of PAH biodegradation in soils (Novotný *et al*., 2004; Kadri *et al*., 2017). In amended soils for example, the extents of ^{14}C-phenanthrene mineralisation decreased after 75 d soil incubation when compared to the other time points which were associated with the decline in ligninolytic enzymes activities. This was further evidence by the positive relationships found between the ligninolytic enzymes and the extents of $^{14}CO_2$ mineralised in soils. In particular, the high ligninolytic enzyme activities were observed in immobilised *P. ostreatus*-amended soil where the greatest extent of mineralisation was also found, indicating its high potential in ligninolytic enzymes production and PAH degradation in soil. Similar results are reported by others (Novotný *et al*., 1999; Pozdnyakova *et al*., 2010). Additionally, LAC activities correlated positively with the C/N ratios for all amended soils, indicating the role of C/N ratios in fungal laccase synthesis by WRF (Knežević *et al*., 2013).

6.5.4 Influence of microbial populations on the mineralisation of ^{14}C-phenanthrene in SBG-immobilised WRF-amended soil

The microbial numbers (heterotrophs and PAH-degraders) were significantly increased in all amended soils following the application of SBG-immobilised fungi. This may be due to increased microbial biomass carbon and nitrogen in soils (Awad *et al*., 2018). When soil is amended with organic substrates, respiration increases while microbial growth and activities are stimulated (Tejada *et al*., 2008; Omoni *et al*., 2020b). This high microbial numbers also

suggest the presence of biodegradable substrates in amended soils. Microbial numbers were generally high with immobilised *T. versicolor* compared to their counterpart strains in amended soils, however no positive relationship was found between the PAH-degrading bacterial numbers and the extents of mineralisation in soil but PAH-degrading fungal numbers showed a strong linear relationship. The diffusable toxic metabolites produced from PAH metabolism by WRF (Prenafeta-boldú *et al*., 2018) could have likely reduced the PAH-degrading bacterial numbers in soils. Moreover, PAH-degrading fungal numbers increased following increases in soil-PAH contact time in amended soils with the exception of 100 d soil incubation, which showed reduction in the fungal CFUs, suggesting a cooperative metabolic interaction with WRFs in soil and creating more available nutrient source for soil microbial activity.

6.6 Conclusions

Our study clearly indicate that all amended soils with SGB-immobilised WRFs enhanced the mineralisation of ^{14}C-phenanthrene in amended soils. The kinetics of mineralisation varied among all WRF-amended soils. Generally, soils amended with *T. versicolor* and *B. adusta* resulted in shorter lag phases and higher rates of mineralisation. The extents of mineralisation reduced over time in all amended soils, while the greatest extents of mineralisation were found in SBG-immobilised *P. ostreatus* and *P. chrysosporium*-amended soils. Soil amendment with immobilised WRF influenced the soil C/N ratios and pH level. SBG-immobilised *P. chrysosporium* increased the soil C/N ratio compared to the other soil treatments. Optimum mineralisation was found at soil C/N ratio of 12.9 and pH 6.3. Enzyme activities (ligninolytic and non-ligninolytic enzymes) increased in amended soils. Ligninolytics were positively correlated with the extents of $^{14}CO_2$ mineralised in all WRF-amended soils (Higher in *P. ostreatus*-amended soils). Microbial populations increased in all amended soils while PAH-

degrading fungal numbers increased with increased soil-PAH contact time. Therefore, the immobilisation of white-rot fungi on spent brewery grains further provided for a promising cheap and sustainable remedial strategy for contaminated soils.

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6.8 Supplementary Data

Table S1. Physicochemical properties of Myerscough soil used in the experiment (Data adapted from Couling *et al.*, 2010). Values are mean ± SEM (n=3)

Soil properties		Parameter Values
pH (in dH_2O)		6.50 ± 0.08
Moisture content (%)		21.07 ± 2.78
Microbial heterotrophic numbers (CFU g^{-1})		$2.17 \times 10^5 \pm 1.67 \times 10^4$
Elemental analysis	Total extractable carbon	1.80% ± 0.03
	Total extractable nitrogen	0.14% ± 0.01
	Total extractable organic carbon	1.60% ± 0.07
	Soil organic matter	2.70% ± 0.04
Soil particle size	Clay	19.5% ± 0.70
	Silt	20.0% ± 0.87
	Sand – Total	60.4% ± 1.20
	Coarse sand	0.12% ± 0.01
	Medium sand	6.90% ± 0.10
	Fine sand	53.3% ± 0.60
	Surface texture: clay loam	

Table S2. Physicochemical and microbial characteristics (mean ± SEM) of spent brewery grains (SBG) used in the experiment. Data adapted from Omoni *et al.,* 2010b.

Parameters measured	SBG
pH	5.00 ± 0.00
EC (mS)/25^{0}C	2.24 ± 0.00
Dry matter (%)	18.0 ± 0.58
Extractable N (NH_4-N) (mg/kg **dw**)	112.0 ± 15.0
Extractable N (NO_3-N) (mg/kg **dw**)	**bdl**
Extractable P (mg/kg **dw)**	762.7 ± 10.4
TOC (mg/kg)	382.0 ± 3.41
IC (mg/kg)	3.70 ± 0.12
TC (%)	47.4 ± 0.05
TN (%)	3.13 ± 0.02
TP (%)	0.41 ± 0.03
C:N	15.1 ± 0.12
THBC (CFU x 10^8 g^{-1} dw)	10.5 ± 1.33
TFC (CFU x 10^6 g^{-1} dw)	4.45 ± 0.07

*****bdl = below detection limit; dw = dry weight; EC= Electrical conductivity; TOC= Total organic carbon; IC= Iorganic carbon; TC= Total carbon; TN= total nitrogen; TP= Total phosphorus; THBC= Total heterotrophic bacterial count, TFC= Total fungal count

Table S3. Linear correlations between the biodegradation kinetics of ^{14}C-phenanthrene (lag, fastest rate and cumulative extent) and soil physical and biological properties (correlation coefficient, r). Data with statistical significance are indicated by $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) and represented in bold letters.

Trametes versicolor												
	Lag phase	Fastest rate	Cum. extent	LAC	LiP	MnP	βGA	ACP	C/N ratio	pH	PDBN	PDFN
Lag phase	1.00											
Fastest rate	0.78***	1.00										
Cum. extent	0.29	**0.59****	1.00									
LAC	**0.54***	**–0.58***	**0.64****	1.00								
LiP	0.15	**–0.48***	**0.82*****	**0.56***	1.00							
MnP	–0.22	–0.25	**0.72*****	**0.56***	**0.58***	1.00						
βGA	–0.36	0.40	–0.22	0.25	–0.33	0.37	1.00					
ACP	**0.49***	**–0.60****	**0.77*****	**0.92*****	**0.78*****	**0.55***	0.01	1.00				
C/N ratio	0.23	–0.39	0.20	**0.57***	–0.08	**0.53***	**0.57***	0.34	1.00			
pH	0.39	**–0.56***	0.10	0.37	–0.19	0.30	0.34	0.17	**0.90*****	1.00		
PDBN	–0.17	–0.14	0.06	0.16	–0.23	**0.56***	**0.60****	–0.03	**0.86*****	**0.83*****	1.00	
PDFN	**–0.68****	**0.90*****	**–0.54***	–0.24	**–0.46***	–0.03	**0.74*****	–0.36	–0.01	–0.25	0.14	1.00

*Cum. = cumulative; LAC = Laccase; LiP = Lignin peroxidase; MnP = Manganese peroxidase; βGA = β-glucosidase; ACP = C/N = Carbon-to-nitrogen; PDBN = Phenanthrene-degrading bacterial number; PDFN = Phenanthrene-degrading fungal number.

Table S4. Linear correlations between the biodegradation kinetics of ^{14}C-phenanthrene (lag, fastest rate and cumulative extent) and soil physical and biological properties (correlation coefficient, r). Data with statistical significance are indicated by $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) and represented in bold letters.

Bjerkandera adusta												
	Lag phase	Fastest rate	Cum. extent	LAC	LiP	MnP	βGA	ACP	C/N ratio	pH	PDBN	PDFN
Lag phase	1.00											
Fastest rate	**–0.61****	1.00										
Cum. extent	**0.46***	**–0.80*****	1.00									
LAC	0.04	–0.37	**0.80*****	1.00								
LiP	–0.16	–0.37	**0.55***	**0.65****	1.00							
MnP	–0.27	–0.32	**0.67****	**0.79*****	**0.66****	1.00						
βGA	–0.31	–0.30	**0.54***	**0.58***	**0.63****	**0.79*****	1.00					
ACP	**0.76*****	**–0.62****	0.28	–0.06	–0.12	–0.32	–0.40	1.00				
C/N ratio	0.30	–0.28	**0.76*****	**0.77*****	0.40	**0.63****	0.42	–0.08	1.00			
pH	**0.86*****	**–0.57***	**0.67****	0.29	0.05	0.10	0.04	0.40	**0.67****	1.00		
PDBN	0.27	–0.27	**0.45***	0.15	0.12	0.37	0.40	–0.28	**0.61****	**0.67****	1.00	
PDFN	**–0.68****	**0.64****	**–0.45***	–0.28	–0.05	0.13	0.24	**–0.89*****	–0.05	–0.35	0.39	1.00

*Cum. = cumulative; LAC = Laccase; LiP = Lignin peroxidase; MnP = Manganese peroxidase; βGA = β-glucosidase; ACP = C/N = Carbon-to-nitrogen; PDBN = Phenanthrene-degrading bacterial number; PDFN = Phenanthrene-degrading fungal number.

Table S5. Linear correlations between the biodegradation kinetics of ^{14}C-phenanthrene (lag, fastest rate and cumulative extent) and soil physical and biological properties (correlation coefficient, r). Data with statistical significance are indicated by $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) and represented in bold letters.

Phanerochaete chrysosporium												
	Lag phase	Fastest rate	Cum. extent	LAC	LiP	MnP	βGA	ACP	C/N ratio	pH	PDBN	PDFN
Lag phase	1.00											
Fastest rate	**–0.79*** **	1.00										
Cum. extent	0.40	–0.33	1.00									
LAC	0.28	–0.32	**0.84*** **	1.00								
LiP	0.02	–0.16	**0.86*** **	**0.88*** **	1.00							
MnP	**–0.45***	0.24	0.26	0.11	0.41	1.00						
βGA	0.01	0.26	**0.76*** **	**0.56****	**0.71****	0.37	1.00					
ACP	**0.86*** **	**–0.62****	**0.64****	**0.50****	0.29	–0.18	0.37	1.00				
C/N ratio	**0.53***	**–0.68****	**0.75*** **	**0.87*** **	**0.68****	–0.02	0.25	**0.63****	1.00			
pH	**0.63****	–0.12	0.36	0.27	–0.02	**–0.48***	0.37	**0.73*** **	0.25	1.00		
PDBN	0.14	0.41	0.29	0.19	0.06	–0.19	**0.62****	0.39	–0.06	**0.84*** **	1.00	
PDFN	**–0.75*** **	**0.64****	**–0.65****	–0.32	–0.31	–0.16	–0.36	**–0.85*** **	**–0.49***	–0.43	–0.13	1.00

*Cum. = cumulative; LAC = Laccase; LiP = Lignin peroxidase; MnP = Manganese peroxidase; βGA = β-glucosidase; ACP = C/N = Carbon-to-nitrogen; PDBN = Phenanthrene-degrading bacterial number; PDFN = Phenanthrene-degrading fungal number.

Table S6. Linear correlations between the biodegradation kinetics of ^{14}C-phenanthrene (lag, fastest rate and cumulative extent) and soil physical and biological properties (correlation coefficient, r). Data with statistical significance are indicated by $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) and represented in bold letters.

Pleurotus ostreatus												
	Lag phase	Fastest rate	Cum. extent	LAC	LiP	MnP	βGA	ACP	C/N ratio	pH	PDBN	PDFN
Lag phase	1.00											
Fastest rate	**–0.93*** **	1.00										
Cum. extent	**0.48***	–0.44	1.00									
LAC	0.42	–0.39	**0.95*** **	1.00								
LiP	0.29	–0.29	**0.92*** **	**0.93*** **	1.00							
MnP	0.05	–0.14	**0.82*** **	**0.83*** **	**0.85*** **	1.00						
βGA	–0.34	0.15	0.38	0.39	0.43	**0.72*** **	1.00					
ACP	**0.52***	**–0.67****	0.04	–0.09	0.02	–0.12	0.01	1.00				
C/N ratio	**0.63****	**–0.60*** **	**0.61****	**0.65****	0.37	0.43	0.09	–0.07	1.00			
pH	**0.83*** **	**–0.86*** **	**0.46***	0.36	0.18	0.23	0.03	**0.48***	**0.73*** **	1.00		
PDBN	0.40	**–0.55***	**0.57***	**0.51***	0.37	**0.64****	**0.57***	0.22	**0.70****	**0.80*** **	1.00	
PDFN	**–0.79*** **	**0.82*** **	–0.30	–0.19	–0.25	0.05	0.25	**–0.87*** **	–0.14	**–0.57***	–0.17	1.00

*Cum. = cumulative; LAC = Laccase; LiP = Lignin peroxidase; MnP = Manganese peroxidase; βGA = β-glucosidase; ACP = C/N = Carbon-to-nitrogen; PDBN = Phenanthrene-degrading bacterial number; PDFN = Phenanthrene-degrading fungal number.

Table S7. Linear correlations between the biodegradation kinetics of ^{14}C-phenanthrene (lag, fastest rate and cumulative extent) and soil physical and biological properties (correlation coefficient, r). Data with statistical significance are indicated by $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) and represented in bold letters.

Irpex lateus												
	Lag phase	Fastest rate	Cum. extent	LAC	LiP	MnP	βGA	ACP	C/N ratio	pH	PDBN	PDFN
Lag phase	1.00											
Fastest rate	**–0.79*** **	1.00										
Cum. extent	0.11	–0.15	1.00									
LAC	0.11	**–0.45***	**0.62****	1.00								
LiP	0.19	**–0.51***	**0.83*****	**0.87*****	1.00							
MnP	–0.02	–0.23	**0.90*****	**0.80*****	**0.93*****	1.00						
βGA	0.25	0.02	**0.50***	0.35	0.27	0.38	1.00					
ACP	**0.66****	**–0.52***	**0.71****	0.27	**0.59***	**0.54***	0.29	1.00				
C/N ratio	0.27	–0.12	0.13	**0.52***	0.16	0.17	**0.54***	0.02	1.00			
pH	**0.89*****	**–0.58***	0.04	0.19	0.06	–0.09	**0.45***	0.43	**0.63****	1.00		
PDBN	0.37	–0.08	0.11	0.36	0.02	0.04	**0.68****	0.06	**0.93*****	**0.75*****	1.00	
PDFN	**–0.63****	0.32	**–0.66****	–0.07	–0.40	–0.39	–0.39	**–0.94*****	0.01	**–0.44***	–0.12	1.00

*Cum. = cumulative; LAC = Laccase; LiP = Lignin peroxidase; MnP = Manganese peroxidase; βGA = β-glucosidase; ACP = C/N = Carbon-to-nitrogen; PDBN = Phenanthrene degrading bacterial number; PDFN = Phenanthrene-degrading fungal number

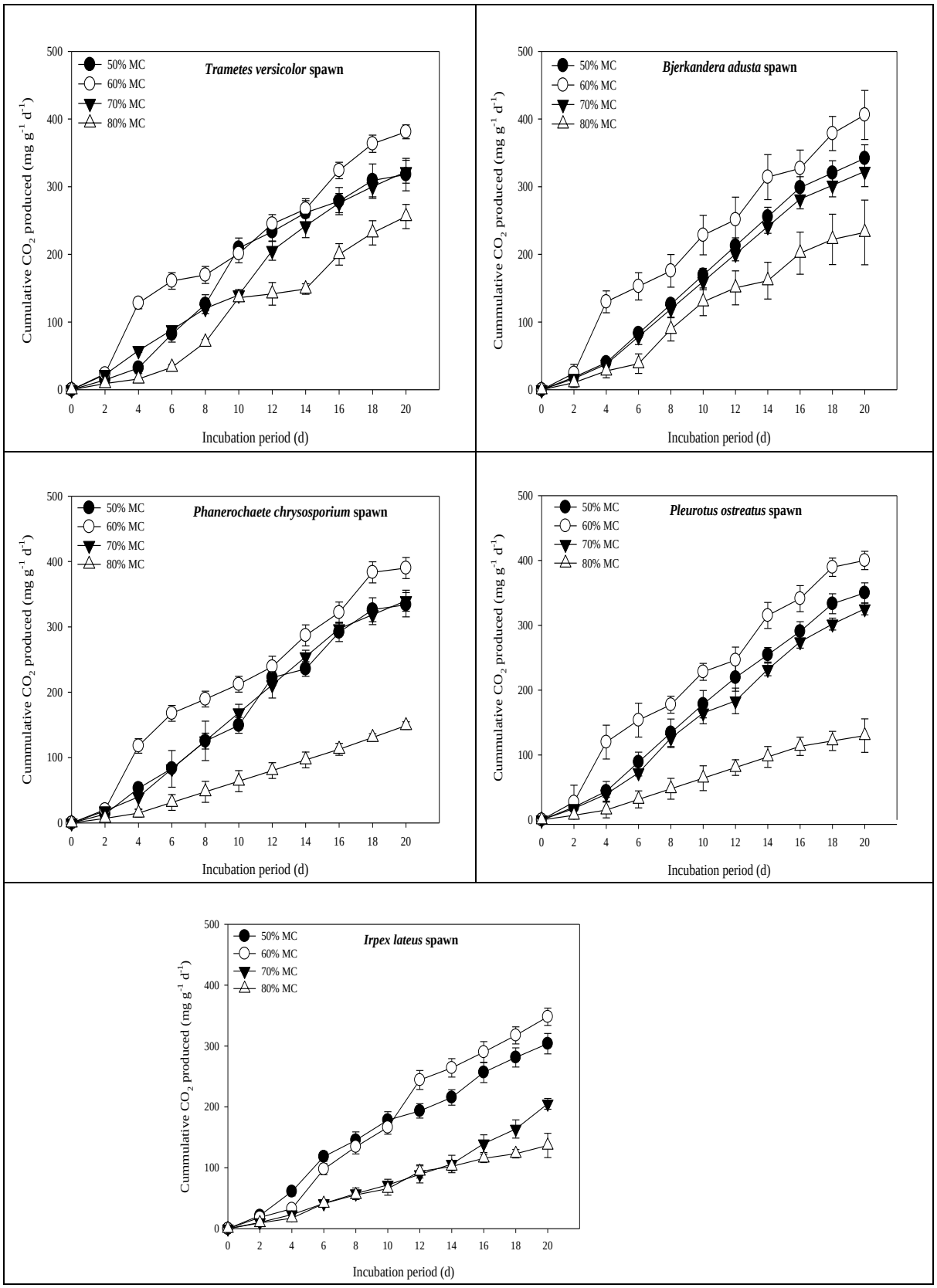

Figure S1. Cumulative CO_2 evolution during fungal spawn development (*Trametes versicolor*, *Bjerkandera adusta*, *Phanerochaete chrysopsorium*, *Pleurotus ostreatus* and *Irpex lateus*) after

amendment with spent brewery grains under different moisture contents (50, 60, 70 and 80%) over a 20 days incubation period.

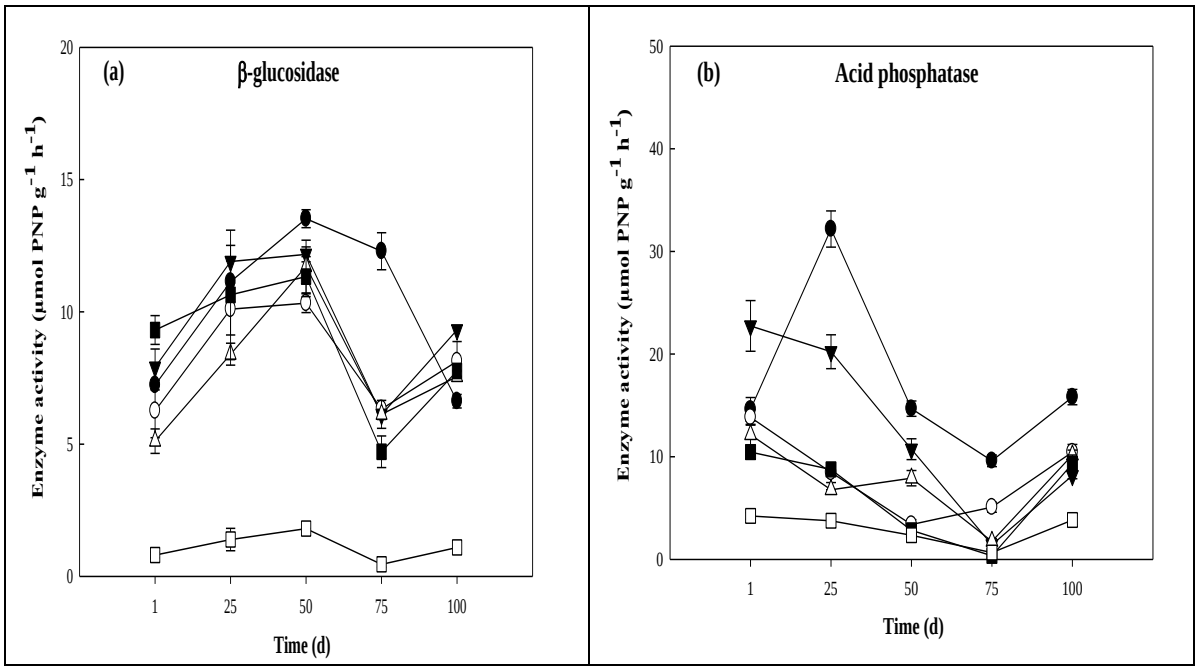

Figure S2 A. Level of β-glucosidase (µmol PNG g^{-1} h^{-1}) activity in amended soils with immobilised spent brewery grains. B. Level of phosphatase (µmol PNP g^{-1} h^{-1}) activity in amended soils with unspawned spent brewery grains after 1, 25, 50, 75, and 100d soil-phenanthrene contact time. Values are mean ± SE (n = 3).

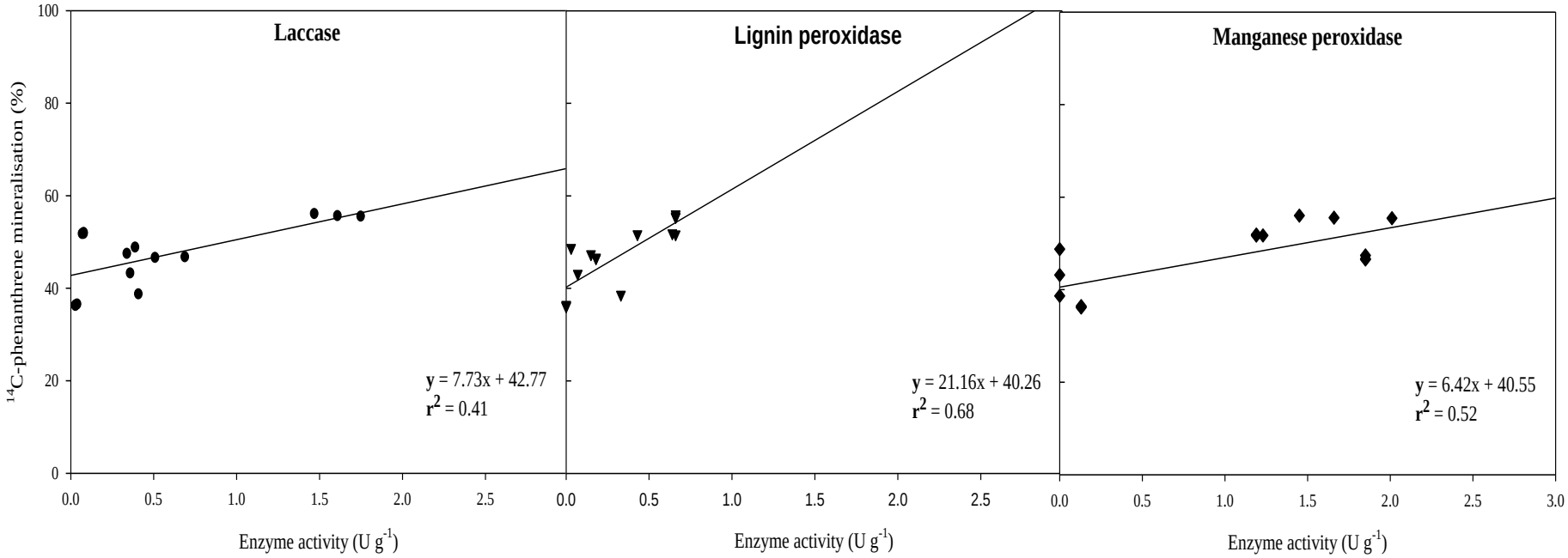

Figure S3. Linear regressions between the cumulative extents of ^{14}C-phenanthrene mineralised and the ligninolytic enzyme activities (laccase, lignin peroxidase and manganese peroxidase) in amended soils with white rot fungus *Trametes versicolor* immobilised on spent brewery grains over a 100 d incubation.

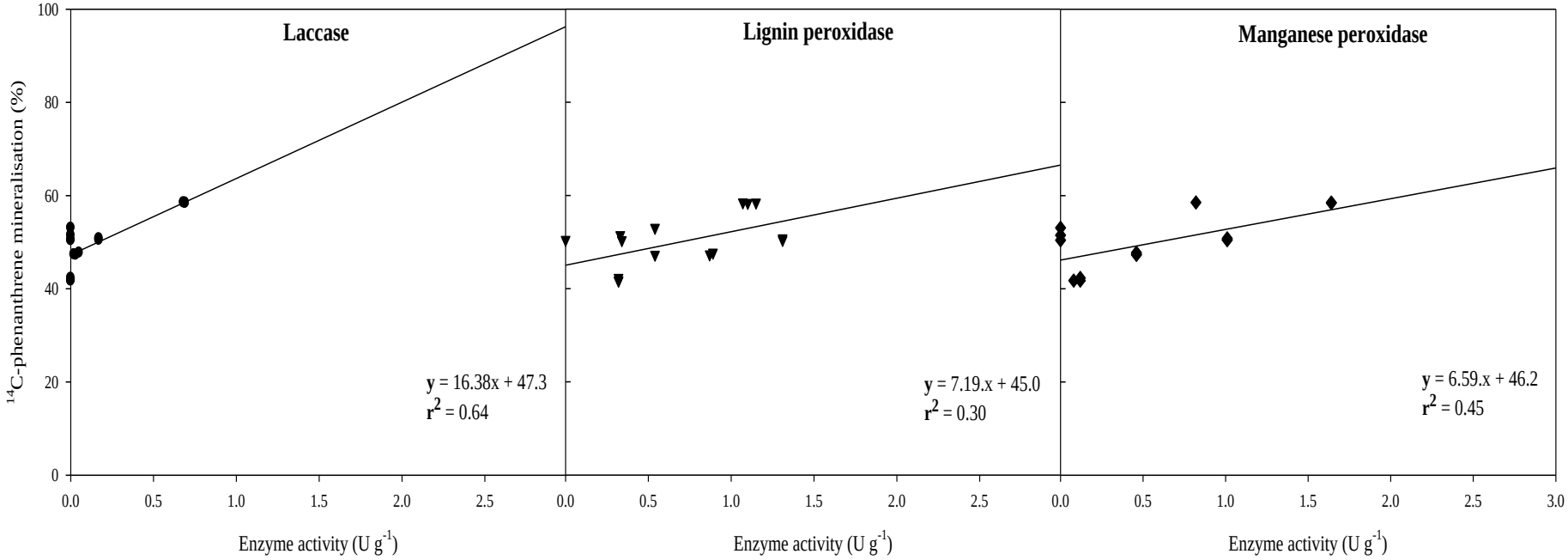

Figure S4. Linear regressions between the cumulative extents of ^{14}C-phenanthrene mineralised and the ligninolytic enzyme activities (laccase, lignin peroxidase and manganese peroxidase) in amended soils with white rot fungus *Bjerkandera adusta* immobilised on spent brewery grains over a 100 d incubation.

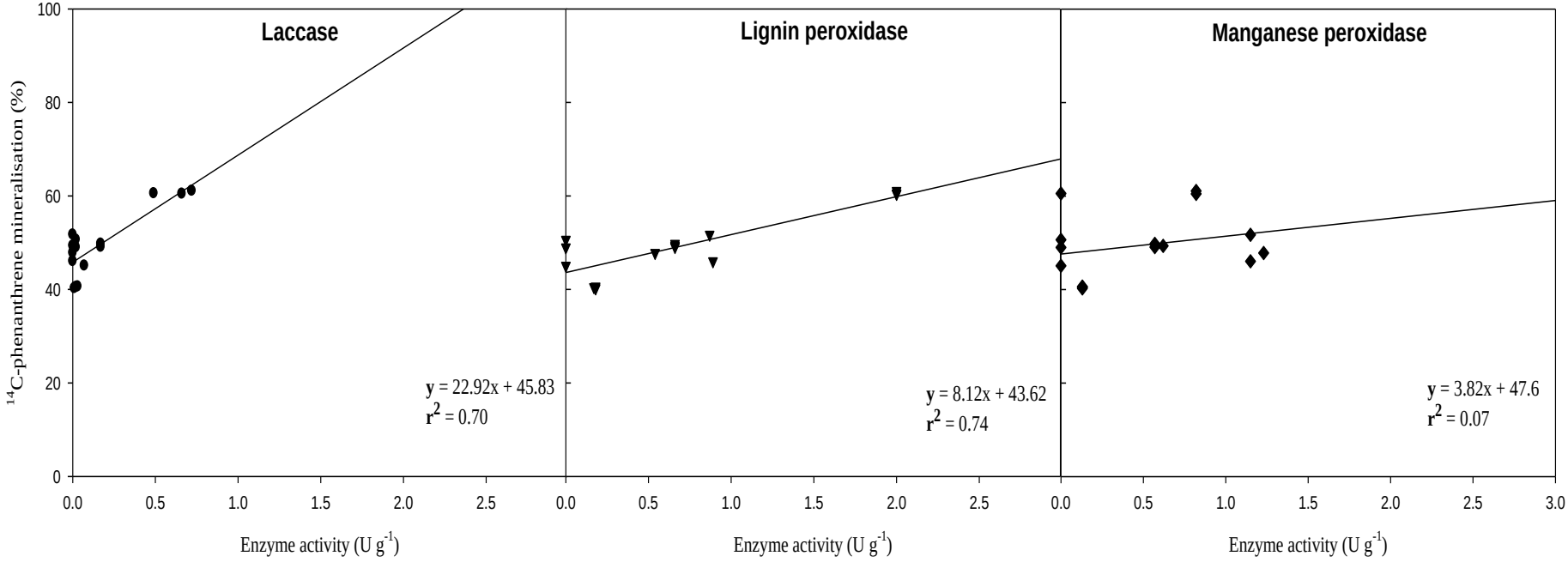

Figure S5. Linear regressions between the cumulative extents of ^{14}C-phenanthrene mineralised and the ligninolytic enzyme activities (laccase, lignin peroxidase and manganese peroxidase) in amended soils with white rot fungus *Phanerochaete chrysospsorium* immobilised on spent brewery grains over a 100 d incubation.

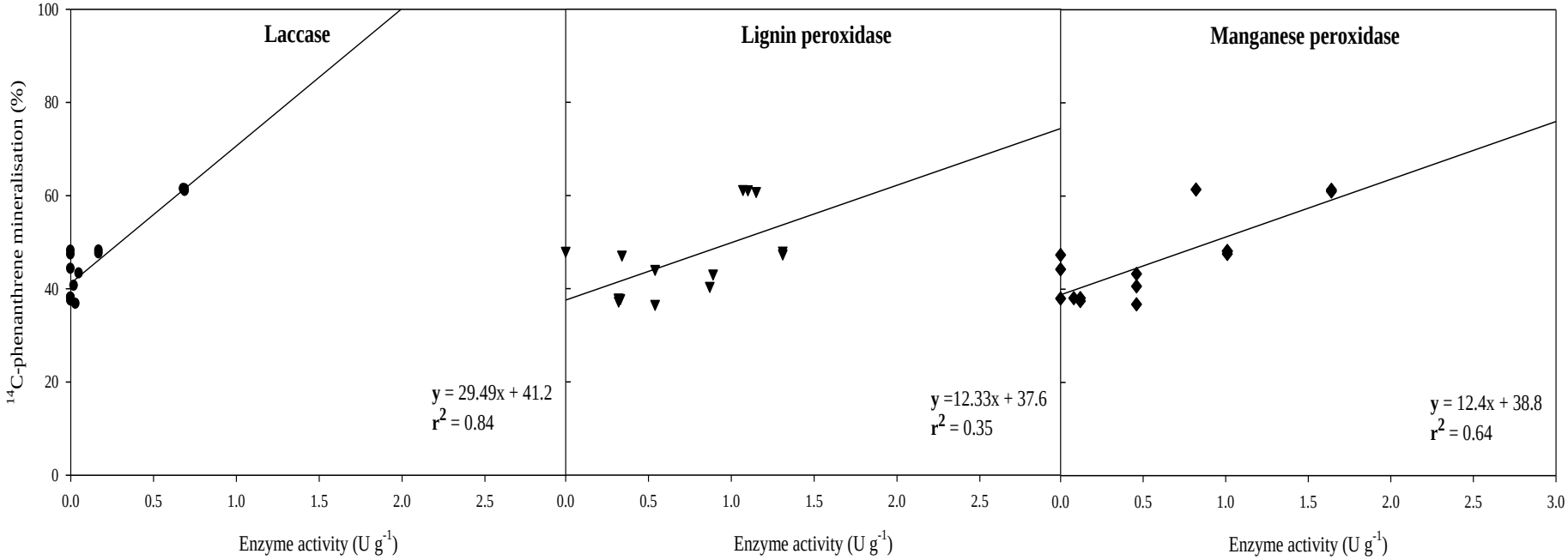

Figure S6. Linear regressions between the cumulative extents of ^{14}C-phenanthrene mineralised and the ligninolytic enzyme activities (laccase, lignin peroxidase and manganese peroxidase) in amended soils with white rot fungus *Pleurotus ostreatus* immobilised on spent brewery grains over a 100 d incubation.

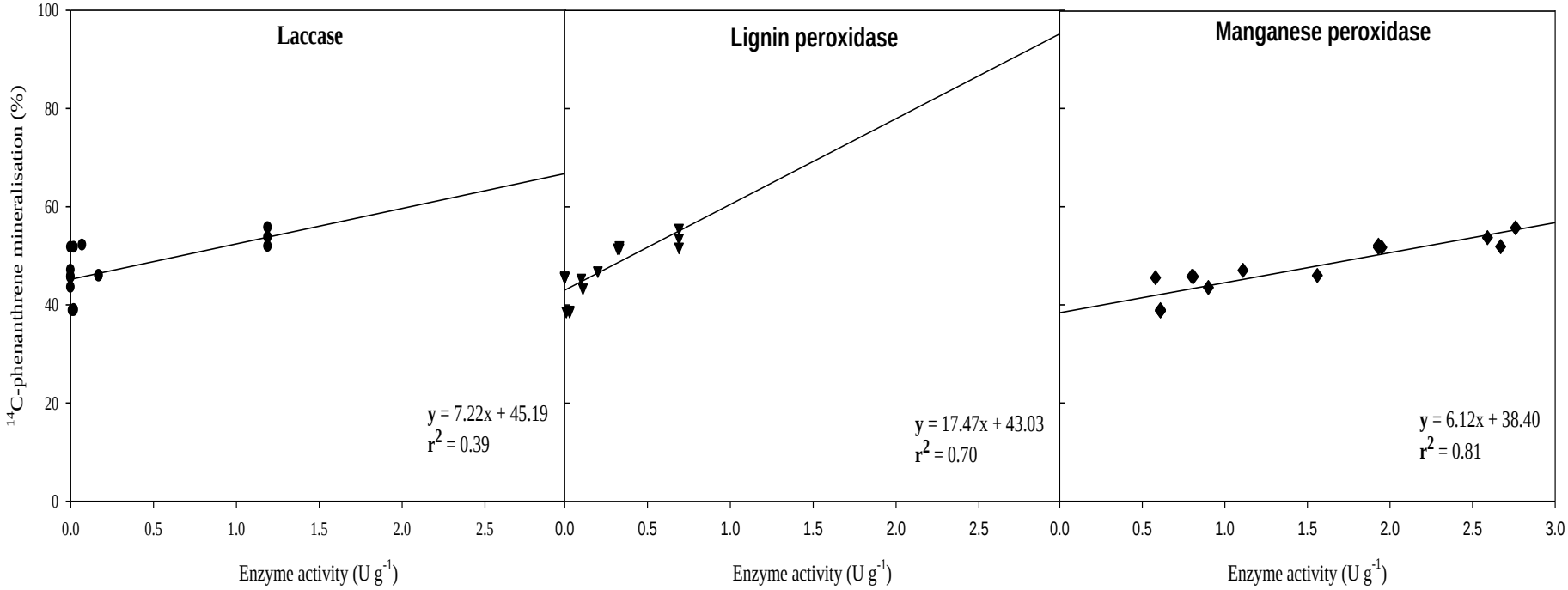

Figure S7. Linear regressions between the cumulative extents of ^{14}C-phenanthrene mineralised and the ligninolytic enzyme activities (laccase, lignin peroxidase and manganese peroxidase) in amended soils with white rot fungus *Irpex lateus* immobilised on spent brewery grains over a 100 d incubation.

Chapter 7

The impact of different nitrogen amendments on the biodegradation of ^{14}C-phenanthrene by endophytic fungal strains in liquid culture.

The impact of different nitrogen amendments on the biodegradation of ^{14}C-phenanthrene by endophytic fungal strains in liquid culture.

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7.1 Abstract

In this study, the biodegradation of phenanthrene was investigated in newly isolated endophytic fungal strains, *Fusarium* sp. (KTS01), *Trichoderma harzianum* (LAN03), *Fusarium oxysporum* (KTS02), *Fusarium oxysporum* (LAN04), and *Clonostachys rosea* (KTS05). This was performed under different carbon:nitrogen ratios (10:1, 20:1, and 30:1) using different nitrogen sources (urea and malt extract and ammonium nitrate) over a 30 d incubation period in both static and agitated liquid media. The kinetics of PAH biodegradaton mineralisation to CO_2 (lag phases, fastest rates, and overall extents) were measured for all of the fungal strains and nutrient conditions using ^{14}C-phenanthrene. All fungal strains were able to biodegrade ^{14}C-phenanthrene to $^{14}CO_2$ under the different nutrient amendments. However, ^{14}C-phenanthrene mineralisation varied for most of the fungal strains in static and agitated culture conditions. Greater extents of mineralisation were found in fungal cultures (strains KTS05 and KTS01) with C:N ratio of 10:1 in both static and agitated conditions, while the fungal strains (KTS05 and LAN03) showed the greatest phenanthrene mineralisation after N source amendments, particularly with malt extract. In addition, the phenanthrene mineralisation increased with higher C:N ratios for *Clonostachys rosea* (KTS05) only. Consequently, the results reported

here provide a promising potential for the endophytic fungal strains and the importance of nutrients amendments for the enhanced degradation of PAHs contaminated environments.

Keywords: Endophytic fungi, spent brewery grains**,** phenanthrene, nitrogen sources, C:N ratio

7.2 Introduction

The global increase in population growth, urbanization and industrialization have increasingly impacted the amounts of polycyclic aromatic hydrocarbons (PAHs) in the environment, resulting from increased levels of environmental pollution (Kim *et al*., 2013; Ramesh *et al*., 2012). Most PAHs have potentially adverse effects on human and environmental health because of their mutagenic, carcinogenic, and teratogenic properties associated with their exposure and biotransformation (Patel *et al*., 2020). PAHs are non-polar, organic compounds that are found in the environment either naturally or anthropogenically. Typically, an increase in molecular size of PAHs increases its hydrophobicity/lipophilicity, thus water solubility and biodegradability decrease, while octanol-water partition coefficient (K_{ow}), soil organic carbon normalised partition coefficient (K_{oc}) and soil/sediment-water distribution coefficient (K_d) values increase (Semple *et al*., 2003; Couling *et al*., 2010; Semple *et al*., 2013). Hence, PAHs become more resistant to microbial attack thus leading to their environmental persistence in soils, waters and sediments (Gupte *et al*., 2016).

Microbial degradation is one of the primary mechanisms for removing PAHs from contaminated soils, sediments, and waters (Ghosal *et al*., 2016). However, PAHs are known to be metabolised by microbial populations (bacteria and fungi) which play key roles in PAH

biodegradation (Peng *et al*., 2008; Brzeszcz and Kaszycki, 2018). Most biodegradation studies have focused on bacteria which often require contaminant transport into the cell for degradation and tend to have narrow degradative abilities (Cerniglia and Sutherland, 2010). However, filamentous fungi offer certain advantages over bacteria for biodegradation of organic contaminants since fungal mycelium can penetrate matrices, facilitate the transport of bacteria into micro-zones (fungal pipelines) and deploy extensive enzyme systems to biotransform PAHs co-metabolically (Furono *et al*., 2012; Anastasi *et al*., 2013; Simon *et al*., 2015). Additionally, most studies have also focused on PAH-degrading fungi, such as the basidiomycetes fungi that are indigenous to the soil environment, as they consist of versatile and metabolic capabilities for the removal of these ubiquitous contaminants (Arun *et al*., 2008). However, there are so far only a few studies that have examined certain groups of the basidiomycetes, such as the endophytic fungi - a highly diverse and specialised group of fungi - with the capacity to degrade organic compounds (Krishnamurthy and Naik, 2017). Soil may harbour a large number of uncultivated fungal populations and enzymes for the degradation of PAHs (Joutey *et al*., 2013). Previous studies have shown that endophytic fungi have lignin-degrading capability, thus contain two extracellular enzymatic systems, the hydrolytic enzymes, and the oxidative ligninolytic enzymes, which are involved in the biodegradation of PAHs (Noman *et al*., 2019).

Biodegradation of organic contaminants can be limited by certain environmental factors such as nutrients, temperatures, salinity, moisture, oxygen, and the pollutant concentration in soil, sediments, and water (Das *et al*., 2011). Biostimulation by nutrient supplementation is a widely used method for enhancing microbial degradation of organic compounds. It involves the modification of the environment by adding nutrients to stimulate indigenous microorganisms to enhance biodegradation processes (Yanto *et al*., 2017). Previous studies have shown

increases in PAH biodegradation following the addition of nutrients amendment into contaminated systems (Atagana *et al*., 2003; Bach *et al*., 2005; Sun *et al*., 2012). Further, certain organic nitrogen sources (including urea, malt extract, yeast extract, and peptone) and inorganic nitrogen sources (including ammonium nitrate, ammonium sulphate and ammonium chloride) have been reported to stimulate microbial growth and metabolism (Zhu *et al*., 2016; Nazifa *et al*., 2018; Vieira *et al*., 2018).

Similarly, fungal degradation of PAHs may also be limited by the lack of available nitrogen in the environment. The C:N ratio may be an important factor in controlling PAH biodegradation, but may vary among different microbial populations due to their nutritional requirements. Most degradation studies have reported a C:N ratio of 100:10:1 for optimal bacterial or fungal degradation of organic contaminants (Medaura *et al*., 2021). Although some studies have reported high, low, or imbalance C:N ratio as unfavourable for microbial growth and biodegradation (Zhou and Crawford, 1995; Atagana *et al*., 2005; Leys *et al*., 2005; García-Delgado *et al*., 2015). However, PAH mineralisation based on nutrient addition depends largely on the availability of nitrogen salts and amino acids in the culture medium (Leys *et al*., 2005). Many researchers have shown that appropriate C:N ratios may increase the rates and extents of PAH degradation in nutrient-deficient contaminated soils and sediments (Hupe *et al*., 1996; Atagana *et al*., 2005; Teng *et al*., 2010; Medaura *et al*., 2021). However, reports have been inconsistent because of the discrepancies in the C:N ratios; for example, C:N ratio of 10:1 (Teng *et al*., 2010), 25:1 (Atagana *et al*., 2005), and 12.5:1 (Hupe *et al*., 1996) are all reported to enhance biodegradation in soils.

Recently, interest in the catabolic potential of endophytic basidiomycetes has increased, especially in the genera of *Clonostachys*, *Fusarium*, *Trichoderma*, *Verticillium*, ***Xylaria,*** **and**

Phomopsis, which have shown high potential for hydrocarbon contaminants degradation, including the PAHs (Fu *et al*., 2018; Marín *et al*., 2018; Sim *et al*., 2019; Gupta *et al*., 2020). However, very few reports are available about the degradation capacity of endophytic basidiomycetes. Therefore, this present study investigated the PAH catabolic potential of endophytic fungal strains in liquid culture using ^{14}C-phenanthrene mineralisation as a measure of biodegradation. To address this aim, the objective was to assess the degradation ability of endophytic fungi following amendments with different C:N ratios and sources of nitrogen in liquid cultures. Further, the catabolic response of the endophytic isolates was assessed by measuring the ^{14}C-phenanthrene mineralisation kinetics (e.g. lag phase, maximum rate, and cumulative extent of $^{14}CO_2$) in liquid cultures.

7.3 Materials and Methods

7.3.1 Chemicals

Phenanthrene, sodium hydroxide, and N, N-dimethylformamide were purchased from Sigma Aldrich Co., Ltd, UK and [9-^{14}C] phenanthrene (radiochemical purity > 96%, specific activity = 55.7mCi mmol^{-1}) were obtained from American Radiolabeled chemical, USA. Goldstar liquid scintillation fluid was acquired from Meridian, UK. Malt extract broth (MEB), potato dextrose agar (PDA), and antibacterial agent (streptomycin-penicillin-glutamic) were purchased from Fisher Scientific, UK. Phenanthrene (^{12}C) was dissolved in acetone as a carrier solvent. Minimal basal salt solution (MBS) recipes (modified) contained the following (g/L): NaCl, $(NH_4)_2SO_4$, KNO_3, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, trace elements (LiCl ($LiBO_2$), $CaSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $Al_2(SO_4)_3 \cdot 16H_2O$ NiCl·$6H_2O$, $CoSO_4 \cdot 7H_2O$($CoNO_3$), KBr, KI, $MnCl_2 \cdot 4H_2O$, $SnCl_2 \cdot 2H_2O$, and $FeSO_4 \cdot 7H_2O$) (Vázquez-Cuevas *et al*., 2018).

7.3.2 Soil sample collection, strain isolation, and screening

Pristine soil (5–20cm depth) was collected from Myerscough Agricultural College (Preston, UK) (Figure S1). Microbial, physical and chemical properties of soil are presented in Table S1. Prior to rehydrating with deionized water back to its initial water holding capacity (60%), soils were homogenized and sieved through a 2 mm mesh. Soils were spiked with non-labeled phenanthrene (^{12}C) to obtain a final concentration of 100 mg/kg (dry weight) using acetone as carrier solvent following the methodology fully described by (Doick *et al*., 2003). This was done to feed and boost the fungal isolates with the potentials for phenanthrene growth and degradation. Served soils (10 ± 0.2 g, dw) of pristine soil sample was added to 100ml MBS solution (pH = 6.0) after sterilization (121^{o}C for 15 min) in 250-ml Erlenmeyer flasks supplemented with 10 µl ml^{-1} streptomycin-penicillin-glutamic to suppresses bacterial growth. This slurry was then incubated at 140 rev/min on a flat-bed orbital shaker at 21 ± 1°C in a controlled temperature environment for 10 days (logarithmic phase). Subsequently, 10% inoculum size was collected from the initial culture and transferred aseptically into fresh MBS solution for enrichment under the same culture conditions and phenanthrene concentration. The enrichment procedure was repeated twice in a fresh sterile medium for 4 weeks.

After enrichment, cultures were serially diluted and inocula size (100 ul) plated aseptically on MBS agar plates containing phenanthrene (5, 25, 50, and 100 mg l^{-1}) incubated every 2 weeks severally at 30^{o}C to selectively screen endophytic fungal isolates with the abilities to grow on the various concentration of phenanthrene without a source of nitrogen. Radial growth was observed daily over a period of up to 21 days for some selected colonies and their growth rates (diameter growth per day) were calculated (Table S2). Since most of the selected strains

showed good mycelial growth on agar plates (especially for 50 and 100 mg/L phenanthrene), they were further screened on their ability to effectively grow, colonise and penetrate lignocellulosic biomass (spent brewery grains) over a period of up to 14 days. Endophytic fungal strains that do not have the ability to delignify the spent brewery grains but apparently possessed the potential for phenanthrene degradation were not used in this experiment (data not shown). Only five fungal strains with both lignin-degrading and PAH-utilising abilities out of the twenty-three screened were selected and identified by their cultural characteristics, microscopic fungal structures, and taxonomic keys according to the morphologies of the cultured fungi and species' keys (Leslie and Summerell, 2006; Watanabe, 2010). Selected fungal endophytes were maintained on PDA agar prior to use. The general properties of spent brewery grains are presented in Table S3.

7.3.3 Molecular Identification of endophytic fungal strains

DNA was extracted from the five fungal mycelial strains by CTAB extraction method (Zhang *et al*., 2010). The extracted genomic DNA was amplified through polymerase chain reaction (PCR) using the PCR amplification DNA template. The primers DNA template pairs used were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR reaction was performed with 20 ng of genomic DNA in a 30µl reaction mixture using an *EF-Taq* DNA polymerase enzyme (SolGent, Korea). PCR amplification was set by activating the *EF-Taq* at 95^{o}C for 2 min, followed by 35 cycles of denaturation at 95^{o}C for 1 min, annealing at 55^{o}C for 1 min, polymerization at 72^{o}C for 1 min and the final extension with 1 cycle step at 72^{o}C for 10 min. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 cycle sequencing kit. Then the DNA containing the extension

products was added to Hi-Di formamide (Applied Biosystems, Foster City, CA). Thereafter, the mixture was incubated at 95^{o}C for 5 min, followed by 5 min on an ice bath, and then analyzed by ABI Prism 3730XL DNA analyzer sequencing machine (Applied Biosystems, Foster City, CA). Nucleotide blast was performed in the National Center for Biotechnology Information (NCBI) from its GenBank (https://www.ncbi.nlm.nih.gov/) to ascertain the degree of closeness to the sequence of other genes previously estimated. Sequence alignment was done by ClusterX (version 2.0). Evolutionary relationships of taxa and sequence analyses were constructed by MEGA software-version 6.0 (Tamura *et al*., 2011) using neighbour-joining method (Saitou and Nei, 1987) from 500 bootstraps (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site.

7.3.4 Strains preparation and treatment conditions for phenanthrene biodegradation

Strains of actively growing mycelial plugs (1cm x 1cm diameter) on PDA plates were excised and sub-cultured on MEB for enrichment. All fungal strains were incubated at 140 rpm at 21 ± 1°C for 7 days (without any carbon source). For phenanthrene mycodegradation, the mycelial pellets were aseptically filtered with sterile Whatman No. 1 and washed three times consecutively with sterile MBS solution, and used as fungal inocula for this study. The moisture content of fungal pellets was evaluated by oven-dried method with gentle heating at 40^{o}C for 24 hr. Static and agitated liquid degradation media for the fungal strains *Fusarium* sp. (KTS01), *Trichoderma harzianum* (LAN03), *Fusarium oxysporum* (KTS02), *Fusarium oxysporum* (LAN04) and *Clonostachys rosea* (KTS05) were prepared with three different C:N ratios (10:1, 20:1 & 30:1) and nitrogen sources: organic (urea (CH_4N_2O) and malt extract) and inorganic-ammonium nitrate (NH_4NO_3) in 250 ml modified Schott bottles (respirometer

bottles). The respirometers contain 30 ml of MBS solution supplemented with antibiotics (pH 6.0 ± 0.2) and ^{12}C-phenanthrene (100 mg/l) dissolved in N, N-dimethylformamide. The degradation media for the three C:N ratios (10, 20 and 30) were prepared and formulated with nitrogen contents of 0.251 g l^{-1} CH_4N_2O and 0.200 g l^{-1} of ammonium-nitrate and varying glucose concentrations of 3.30, 6.70 and 10.0 g l^{-1}, respectively. For the nitrogen sources, three other degradation culture media were supplied with 0.5 % urea, malt extract, and inorganic-ammonium nitrate, respectively. All culture media were amended with the same phenanthrene concentration (100 mg l^{-1}). All experimental media (adjusted to pH 6.0 ± 0.2) were homogenized and sterilized by autoclaving (121^{o}C for 10 min) prior to inoculation. However, urea was added aseptically as a sterile solution to media after sterilization.

7.3.5 Mineralisation of ^{14}C-phenanthrene in liquid cultures (static and agitated conditions)

To determine the mineralisation of ^{14}C-phenanthrene in fungal mycelia, ^{14}C-respirometry assays were monitored by incubation under stationary and agitated conditions at 21 ± 1°C in the dark after 0, 2, 5, 10, 15, 20, 25 and 30 days according to modified methods described previously (Samanta *et al*., 1999). Briefly, each respirometer (loosely capped) contained 30 ml of enriched MBS (pH 6.0) solution with a final contaminant concentration of 100 mg/l phenanthrene; and seeded with actively growing mycelial pellet to a final dry weight of 0.125 g per microcosm. Each respirometer was spiked with 667 Bq [9-^{14}C] phenanthrene dissolved in acetone as carrier solvent and incubated in the dark under stationary and agitated (140 rpm) conditions; $^{14}CO_2$ produced was trapped using 2 M NaOH in a 7 ml scintillation vial added to each respirometer. Schott bottles with ^{12}C and ^{14}C-phenanthrene with live mycelial pellets (without N amendments) and $HgCl_2$-killed mycelial cells containing ^{12}C and ^{14}C-phenanthrene

(without N amendments) were used as controls – biotic and abiotic, respectively. An analytical blank was prepared alongside with the controls which contain ^{12}C-phenanthrene (without mycelial pellets and ^{14}C-phenanthrene) only to measure the background activity. The killed mycelial cells were prepared by adding 0.7 g/l $HgCl_2$ powder to 5-day-old mycelial cells (Potin *et al*., 2004). At this period, the various treatments (both static and agitated) were incubated in triplicates along with their controls (after 0, 2, 5, 10, 15, 20, 25, and 30 days) at room temperature (21 ± 1°C). Thereafter, the $^{14}CO_2$ which was trapped in the 7 ml vial in each respirometer was assessed by a liquid scintillation analyzer (Canberra Packard Tricarb 2250CA, UK) after the addition of fresh liquid scintillation fluid (5 ml) to each trap and storage in the dark for 8hrs to prevent chemo-luminescence. The endpoints of this mineralisation assay were the lag phase (the time taken before ^{14}C-phenanthrene mineralisation reached 5%) prior to ^{14}C-phenanthrene mineralisation, fastest rate, and the cumulative extent of mineralisation of ^{14}C-phenanthrene after 30 d (Macleod and Semple, 2006; Omoni *et al*., 2020).

7.3.6 Data analysis

All the data analyses were analyzed using IBM SPSS (version 23.0), USA. Parametric student t-tests and One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were used to determine the effects of each isolated fungus on the lag phase, fastest rate, and overall extent of ^{14}C-mineralisation for both static and agitated conditions (95% confidence level). Data were graphically presented using SigmaPlot 10.0 software (Systat Software Inc., USA).

7.4 Results

7.4.1 Isolation and Identification of phenanthrene-degrading lignocellulosic fungi

Sequence analysis based on internal transcribed spacers 1 and 2 (ITS1 and ITS4) of the five fungal isolates was used in determining their relationships and relationship with their genus. Comparison of their sequences with closest relatives in the GenBank are presented in the phylogenetic tree (Figure 1). The results revealed that the identified fungal isolates have between 99.8 to 99.9% match with their close relatives retrieved from NCBI database (Table S4). Strain KTS01, LAN03, KTS02, LAN04 and KTS05 exhibited 99.9, 99.9, 99.8, 99.9 and 99.8% closest match and homologous similarities with *Fusarium* sp. R206 (accession No. JX559854.1), *Trichoderma harzianum* (accession No. KU896331.1), *Fusarium oxysporum* (accession No. HQ379659.1), *F. oxysporum* (accession No. AY928417.1) and *Clonostachys rosea* (accession No. MH855404.1), respectively (Figure 1 and Table S4). Other strains also clustered as a group around most of the new strains indicating an evolutionary relationship between them (Figure 1). The macroscopic and microscopic features of the identified strains are shown in Table S5. Most of the isolated strains belong to a large group of higher endophytic fungi, the Ascomycetes.

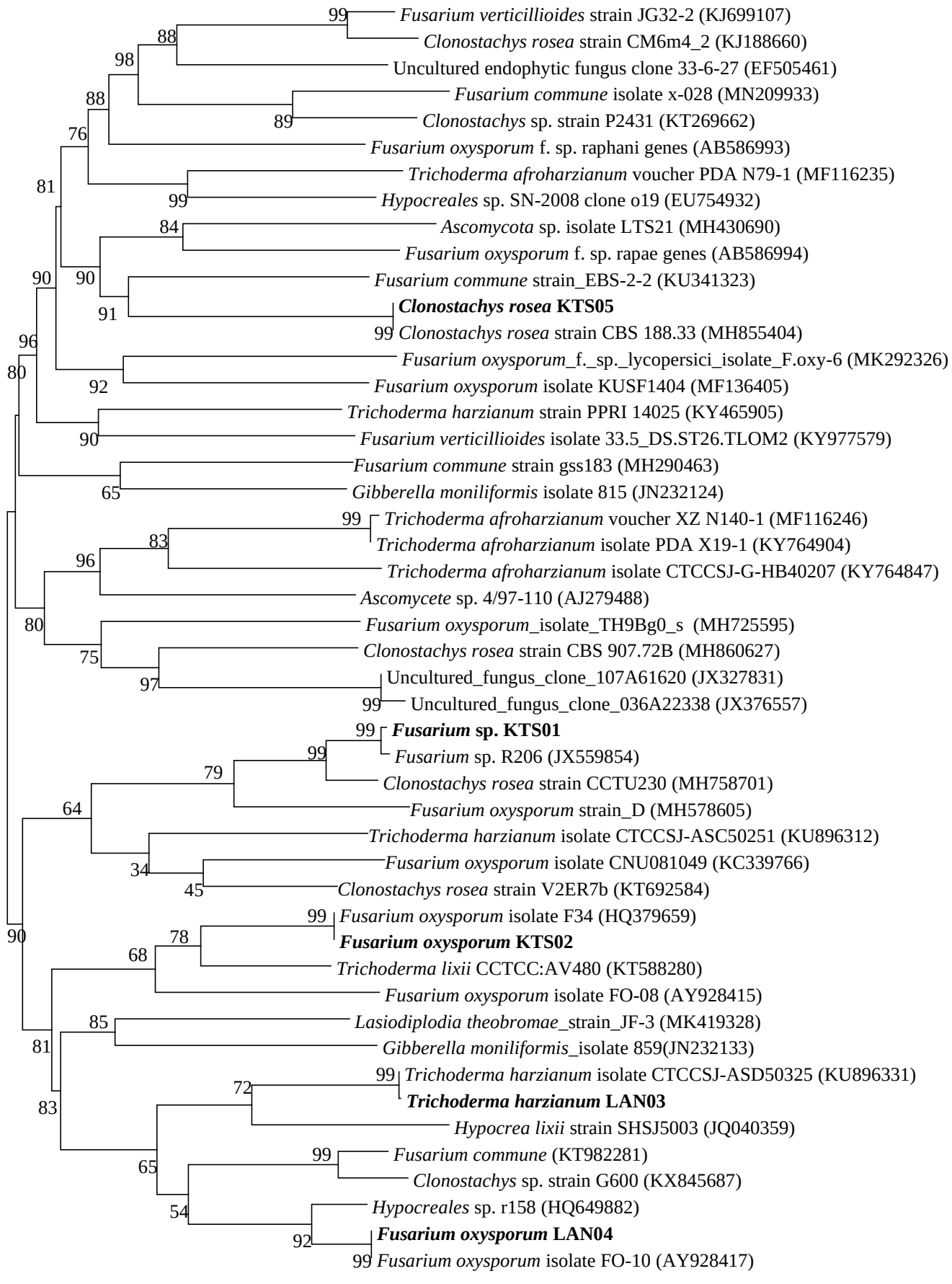

Fusarium verticillioides strain JG32-2 (KJ699107)
Clonostachys rosea strain CM6m4_2 (KJ188660)
Uncultured endophytic fungus clone 33-6-27 (EF505461)
Fusarium commune isolate x-028 (MN209933)
Clonostachys sp. strain P2431 (KT269662)
Fusarium oxysporum f. sp. raphani genes (AB586993)
Trichoderma afroharzianum voucher PDA N79-1 (MF116235)
Hypocreales sp. SN-2008 clone o19 (EU754932)
Ascomycota sp. isolate LTS21 (MH430690)
Fusarium oxysporum f. sp. rapae genes (AB586994)
Fusarium commune strain_EBS-2-2 (KU341323)
***Clonostachys rosea* KTS05**
Clonostachys rosea strain CBS 188.33 (MH855404)
*Fusarium oxysporum*_f._sp._lycopersici_isolate_F.oxy-6 (MK292326)
Fusarium oxysporum isolate KUSF1404 (MF136405)
Trichoderma harzianum strain PPRI 14025 (KY465905)
Fusarium verticillioides isolate 33.5_DS.ST26.TLOM2 (KY977579)
Fusarium commune strain gss183 (MH290463)
Gibberella moniliformis isolate 815 (JN232124)
Trichoderma afroharzianum voucher XZ N140-1 (MF116246)
Trichoderma afroharzianum isolate PDA X19-1 (KY764904)
Trichoderma afroharzianum isolate CTCCSJ-G-HB40207 (KY764847)
Ascomycete sp. 4/97-110 (AJ279488)
*Fusarium oxysporum*_isolate_TH9Bg0_s (MH725595)
Clonostachys rosea strain CBS 907.72B (MH860627)
Uncultured_fungus_clone_107A61620 (JX327831)
Uncultured_fungus_clone_036A22338 (JX376557)
***Fusarium* sp. KTS01**
Fusarium sp. R206 (JX559854)
Clonostachys rosea strain CCTU230 (MH758701)
Fusarium oxysporum strain_D (MH578605)
Trichoderma harzianum isolate CTCCSJ-ASC50251 (KU896312)
Fusarium oxysporum isolate CNU081049 (KC339766)
Clonostachys rosea strain V2ER7b (KT692584)
Fusarium oxysporum isolate F34 (HQ379659)
***Fusarium oxysporum* KTS02**
Trichoderma lixii CCTCC:AV480 (KT588280)
Fusarium oxysporum isolate FO-08 (AY928415)
*Lasiodiplodia theobromae*_strain_JF-3 (MK419328)
*Gibberella moniliformis*_isolate 859(JN232133)
Trichoderma harzianum isolate CTCCSJ-ASD50325 (KU896331)
***Trichoderma harzianum* LAN03**
Hypocrea lixii strain SHSJ5003 (JQ040359)
Fusarium commune (KT982281)
Clonostachys sp. strain G600 (KX845687)
Hypocreales sp. r158 (HQ649882)
***Fusarium oxysporum* LAN04**
Fusarium oxysporum isolate FO-10 (AY928417)
0.5

Figure 1. Phylogenetic tree of five isolated endophytic fungi compared to their closest relatives in the GenBank. The tree was constructed by neighbour-joining method from internal transcribed spacer sequence data (ITS 1 and ITS4) and 500 bootstrap replicates. Scale-bar and node labels represent 0.5 nucleotide substitution per nucleotide position and bootstrap support (%) respectively. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 48 nucleotide sequences (402 positions in the final dataset). The new endophytic fungal isolates in this study are highlighted in bold text.

7.4.2 Mineralisation of ^{14}C-phenanthrene in static and agitated liquid cultures under different CN ratios

The mineralisation of ^{14}C-phenanthrene was measured in static liquid cultures under various C:N ratios for the five endophytic fungal strains (KTS01, LAN03, KTS02, LAN04 and KTS05) after 0, 2, 5, 10, 15, 20, 25 and 30 days (Table 1 and Figure 2). The lag phases were significantly shortened ($p < 0.05$) for almost all C:N ratios (10:1, 20:1 and 30:1) investigated by the five fungal strains in both static and agitated cultures when compared to the controls (live and $HgCl_2$-killed mycelia cells). Overall, the shortest lag phases were observed in both agitated and static liquid cultures with a C:N of 10:1 compared to liquid cultures that have received C:N ratios of 20:1 and 30:1. With all liquid cultures (static and agitated) of the different fungal strains, the greatest reduction in the lag phases was noticeable in the culture of *Clonostachys* sp. KTS05 closely followed by *Fusarium* sp. KTS01 in static culture and *Fusarium* sp. KTS02 followed by *Clonostachys* sp in agitated culture throughout the experiment. It should be noted that the lag phases were exceeded in all C:N ratios examined for *Clonostachys* sp. KTS05 biodegradation. Increasing C:N ratio of the liquid cultures increases the lag phase to a certain extent (Figure 2). Generally, the C:N ratios in both static and agitated cultures significantly influenced ($p < 0.05$) the lag phase with all fungal strains for PAH degradation in the following order: 10:1 > 20:1 > 30:1. In addition, the lag phases increased for most fungal strains in both

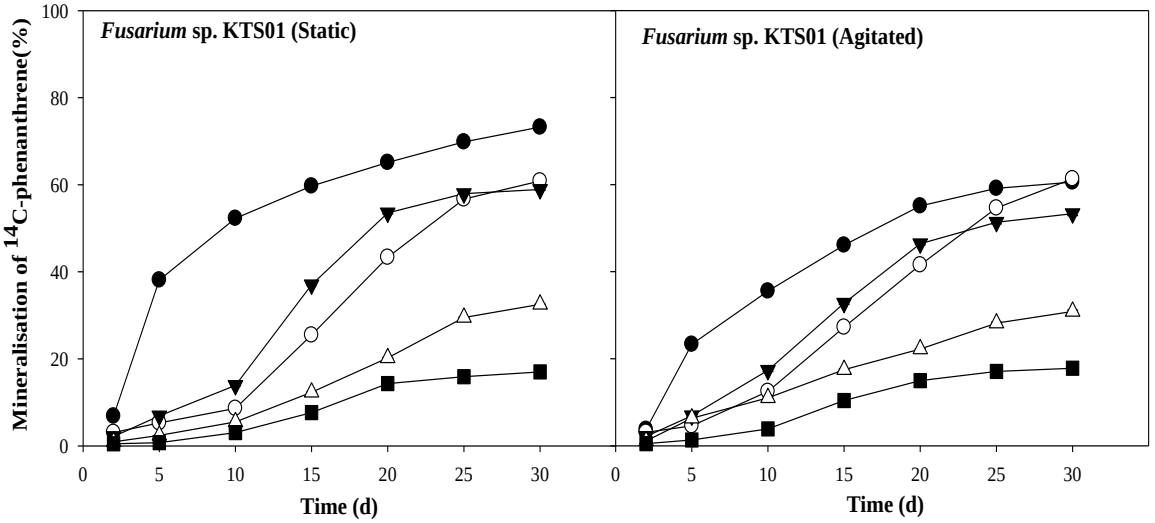
culture conditions (static and agitated) with C:N of 30:1 (Table 1). Moreover, the fungal strains varied in their preferences in adaptation to the different C:N ratios for PAH degradation in either static or agitated conditions. For example, there were observable significant differences ($p < 0.05$) between static and agitated cultures of the five fungal strains with C:N ratio of 10:1 except for *Clonostachys* sp. KTS05, which showed no statistical difference ($p > 0.05$) in the lag phases between the agitated and the static cultures. In addition, *Fusarium* sp. KTS01 and *Fusarium* sp. LAN04 showed significantly shorter lag phases in static than static cultures, while *T. harzianum* LAN03 and *Fusarium* sp. KTS02 showed significantly shorter lag phases in agitated compared to static cultures. Notably, *Fusarium* sp. KTS02 showed significantly reduced ($p < 0.05$) lag phases in agitated cultures in all C:N ratios studied when compared to static conditions. While liquid cultures with C:N 20:1 were found to have a significantly shorter ($p < 0.05$) lag phase in static than agitated liquid cultures in almost all of the pelleted fungal strains. Furthermore, some fungal strains displayed increased adaptation with the agitated than static conditions with C:N ratio of 30:1, and there were significantly reduced ($p < 0.05$) lag phases observed.

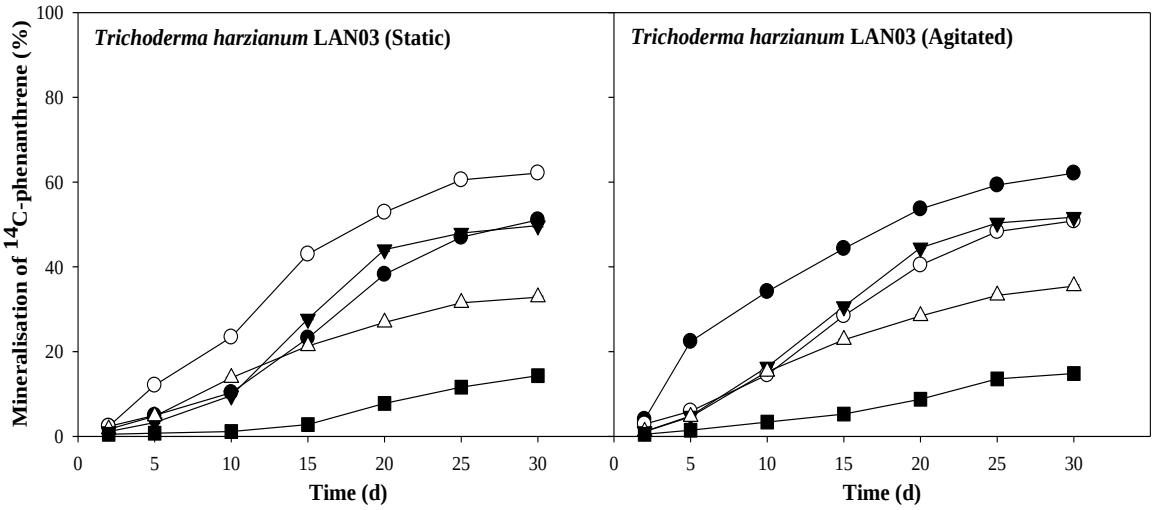
Enrichment of the liquid cultures with different C:N ratios (10:1, 20:1 and 30:1) resulted in faster rates of ^{14}C-phenanthrene mineralisation in both agitated and static conditions for all the five fungal strains investigated (Table 1 and Figure 2). In static cultures, the fastest rates were observed for *Fusarium* sp. KTS01 (10.41 ± 0.03 % $^{14}CO_2$ d^{-1}) followed by *Clonostachys* sp. KTS05 (6.53 ± 0.09 % $^{14}CO_2$ d^{-1}%) with C:N of 10:1 and 20:1, respectively; while *Fusarium* sp. KTS02, closely followed by *Fusarium* sp. KTS01 showed faster rates of mineralisation of 9.75 ± 0.07 and 6.52 ± 0.02 % $^{14}CO_2$ d^{-1} in the agitated cultures with C:N 10:1 (Table 1). In general, the rates of ^{14}C-phenanthrene mineralised were significantly higher than the control (nutrient-free, live and killed culture). The rates of mineralisation increased with the different

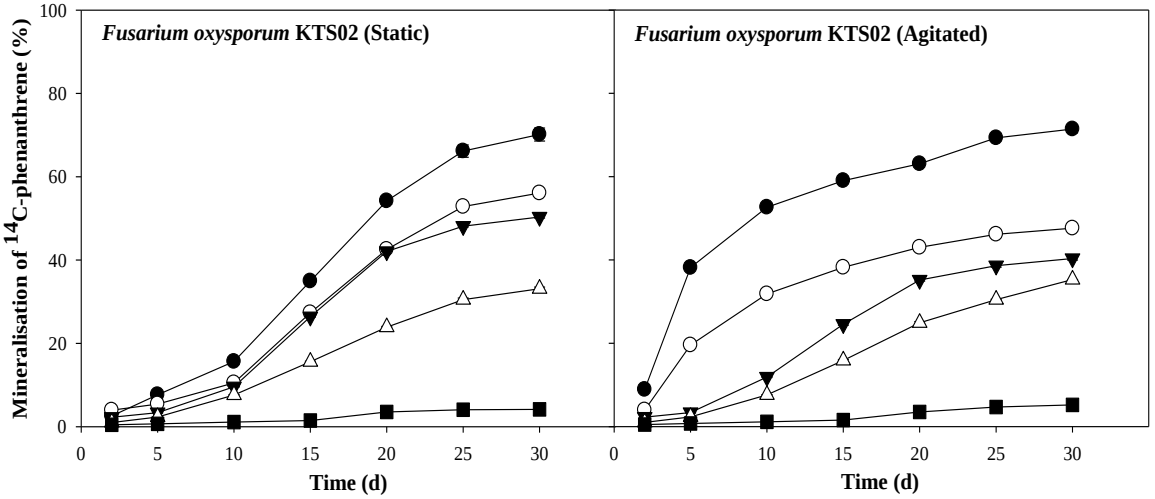
C:N ratios (10:1, 20:1 and 30:1) with all fungal strains in static conditions; however, no particular trend was observed among the fungal strains throughout the experiment (Figure 2). In comparison, agitated cultures of *Fusarium* sp. KTS02 followed *Clonostachys* sp. KTS05 exhibited also significantly faster rates of $^{14}CO_2$ mineralised ($p < 0.05$) in both cultures with C:N of 20 and 30 when compared to other counterpart strains, respectively. As observed with the lag phase for agitated cultures, when the C:N was increased (10:1 > 30:1), significantly reduced rates of mineralisation were observed after 30 days of incubation with the following order: C:N (10:1 < 20:1 < 30:1) (Table 1). Rates of mineralisation by the isolated fungal strains in this experiment were generally faster in static than agitated culture conditions.

To observe the potentials that the five isolated fungal strains with different C:N ratios (10:1, 20:1 and 30:1) can have on the cumulative extents of ^{14}C-phenanthrene mineralisation in both static and shaken conditions were also measured (Table 1 and Figure 2). The total extents of mineralisation by the fungal strains increased significantly ($p < 0.05$) which were highly dependent on the amount of C and N added to the degradation media (static and agitated) over time (Table 1). All isolated fungal strains exhibited high potentials using these metabolic substrates for the mineralisation of ^{14}C-phenanthrene in both static and agitated liquid culture conditions, being significantly higher ($p < 0.001$) than the fungal controls throughout the study. However, the observed extents of ^{14}C-phenanthrene mineralisation differed between the two culture conditions, greater extents of mineralisation were mainly observed for most fungal strains after 30 days of incubation in the static than the agitated condition. It should be noted that under static cultures, there was no particular trend in the extents of mineralisation as observed for all fungal strains with respect to the proportion of C:N ratios in the study (Table 1). However, *Fusarium* sp. KTS01 and *Clonostachys* sp. KTS05 mineralised up to 73.3 ± 0.58 % and 71.38 ± 0.67 % of ^{14}C-phenanthrene in static cultures amended with C:N 10:1 and 30:1,

respectively after 30 days. The greatest cumulative extent of mineralisation (73.25 ± 0.58 %) was observed for static culture of *Fusarium* sp. KTS01, however this changes with increasing C:N with the following trend (10:1 < 20:1 < 30:1), while there was an observable increase in the degradation performance (extent of mineralisation) with increasing C:N ratio in culture with *Clonostachys* sp. KTS05 (10:1 > 20:1 > 30:1). Comparatively, agitated culture seeded with mycelial pellets of *Fusarium* sp. KTS02 recorded the greatest extent of mineralisation (71.43 ± 0.95 %) for all C:N ratios and fungal strains explored; this was closely followed by *Clonostachys* sp. KTS05 (61.52 ± 0.95 %). Generally, the strain KTS05 influenced the extents of ^{14}C-phenanthrene mineralisation in agitated culture conditions (both in nutrient-amended and nutrient-free live mycelial cells) compared to the other fungal strains. Additionally, increases in the C:N ratios (10 > 30) of the culture systems led to an increase in the amount of $^{14}CO_2$ produced by *Clonostachys* sp. KTS05 in both static and agitated conditions compared to the other fungal strains throughout the study. Conversely, the extent of mineralisation was not sustained with increasing C:N ratios in culture seeded with the strain KTS02 which showed significant reductions by 33.3% and 43.5 % in C:N of 20 and 30, respectively when compared to C:N of 10:1. Furthermore, the fungal strains generally showed greater extents of mineralisation in the following order of C:N (10:1 > 20:1 > 30:1).


Fusarium sp. KTS01 (Static)
Fusarium sp. KTS01 (Agitated)
Mineralisation of ^{14}C-phenanthrene(%)
Time (d)


Trichoderma harzianum LAN03 (Static)
Trichoderma harzianum LAN03 (Agitated)
Mineralisation of ^{14}C-phenanthrene (%)
Time (d)


Fusarium oxysporum KTS02 (Static)
Fusarium oxysporum KTS02 (Agitated)
Mineralisation of ^{14}C-phenanthrene (%)
Time (d)

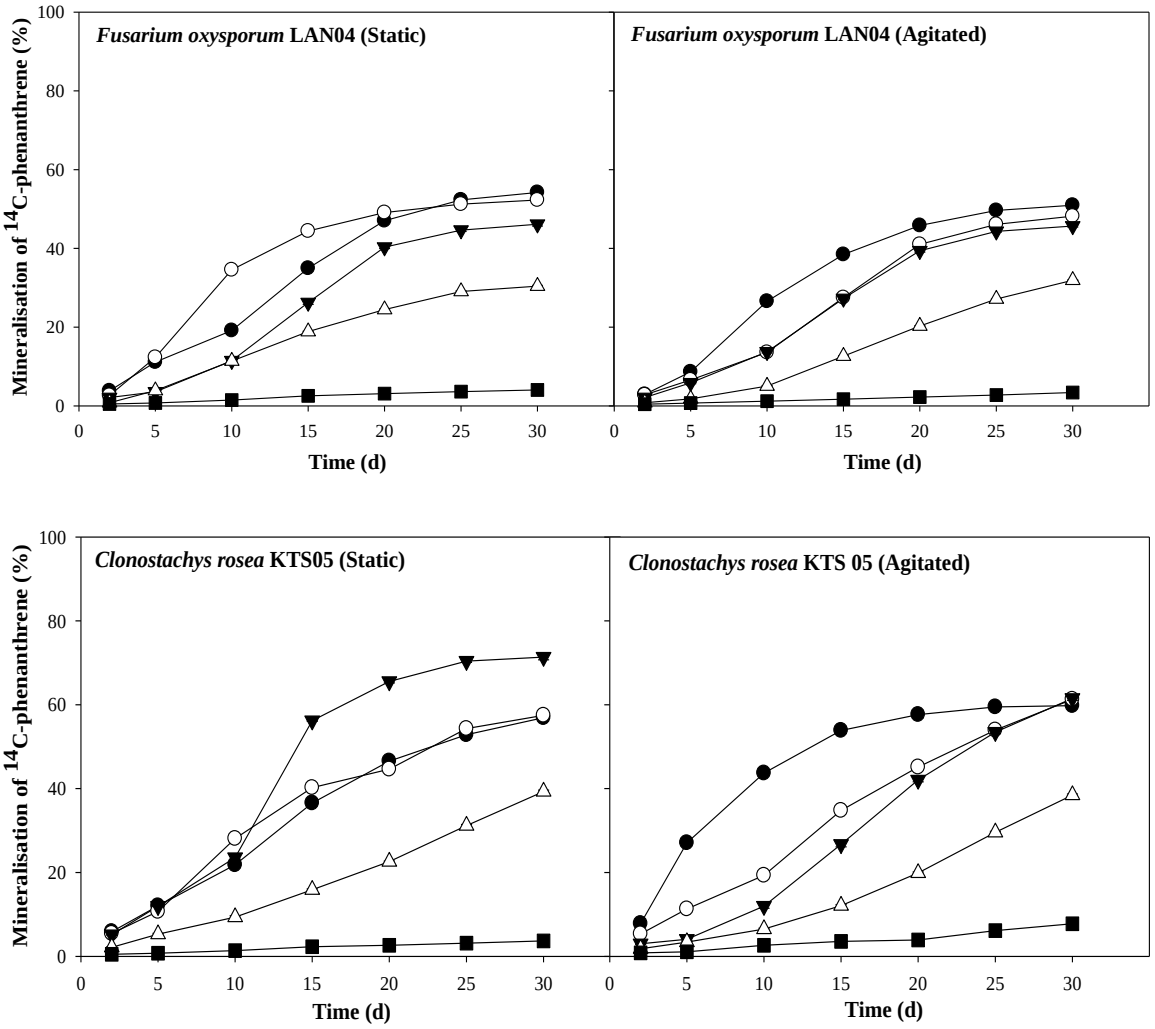


Figure 2. Development of ^{14}C-phenanthrene mineralisation by endophytic fungal strains after 30 days under different C/N ratios in static and agitated mycelia liquid cultures spiked with phenanthrene (50 mg/l): CN 10 (●), CN 20 (○), CN 30 (▼), Live fungal control (Δ), and $HgCl_2$-killed fungal control (■). Mineralisation showed triplicate samples (n = 3) of mean ± SE.

Table 1. Mineralisation kinetics of ^{14}C-phenanthrene in mycelia liquid cultures amended with different C:N ratio after 30 days respirometry assay (static and agitated conditions). Values are mean ± standard error (n = 3).

Endophytic fungal isolate	Isolate number	CN ratio	Lag phase (d)		Fastest rates (%$^{14}CO_2$ d^{-1})		Cumulative extent (%)	
			Static	Agitated	Static	Agitated	Static	Agitated
Fusarium sp.	KTS01	10:1	0.06 ± 0.00 aA1	0.09 ± 0.00 aB2	10.41 ± 0.03aA1	6.52 ± 0.02 aB2	73.25 ± 0.58 aA1,2	60.60 ± 0.11 aB2
		20:1	0.19 ± 0.01 aA1	0.24 ± 0.01 bB3	3.58 ± 0.04 c3,4	2.95 ± 0.02 cB3	60.87 ± 0.33 bB1	61.35 ± 0.07 abA1
		30:1	0.16 ± 0.01 aA1	0.16 ± 0.01 abA1	4.62 ± 0.02 bA2	3.09 ± 0.02 bB1	58.92 ± 0.19 bA2	53.32 ± 0.44 bB2
		Control A	0.49 ± 0.11 bB2	0.29 ± 0.06 bcA2	1.86 ± 0.02 dA1	1.74 ± 0.04 dB3	32.53 ± 0.04 cA2	30.87 ± 0.31 cB4
		Control B	0.52 ± 0.03 bB1	0.45 ± 0.01 dA1	1.33 ± 0.05 eA1	1.30 ± 0.03 eA1	0.21 ± 0.00 dB4	17.82 ± 0.40 dA1
Trichoderma harzianum	LAN03	10:1	0.21 ± 0.00 bB4	0.09 ± 0.00 aA2	3.01 ± 0.02 cB3	6.13 ± 0.05 aA3	51.08 ± 0.13 bB2,3	62.10 ± 0.06 aA2
		20:1	0.12 ± 0.00 aA2	0.17 ± 0.01 bB2	3.92 ± 0.02 aA2	2.79 ± 0.01 bB5	62.12 ± 0.07 aA1	50.85 ± 0.31 bB2
		30:1	0.27 ± 0.01 cB2	0.22 ± 0.01 bA2	3.62 ± 0.02 bA3	2.85 ± 0.02 bB2	49.73 ± 0.30 cB3,4	51.72 ± 0.13 bA2
		Control A	0.22 ± 0.01 bA1	0.44 ± 0.00 cB1	1.81 ± 0.02 dB1,2	2.13 ± 0.02 cA1	32.85 ± 0.25 dA2	35.47 ± 0.27 cB2
		Control B	0.72 ± 0.01 dB2	0.60 ± 0.02 dA2	1.00 ± 0.02 eA2	0.96 ± 0.02 dA2	14.34 ± 0.20 eA1	14.82 ± 0.37 dA2
Fusarium oxysporum	KTS02	10:1	0.13 ± 0.00 aB3	0.05 ± 0.00 aA1	3.87 ± 0.01 aB2	9.75 ± 0.07 aA1	70.14 ± 1.56 aA1	71.43 ± 0.95 aA1
		20:1	0.17 ± 0.00 aB3	0.09 ± 0.00 bA1	3.37 ± 0.02 bB4,5	5.20 ± 0.03 bA1	56.10 ± 0.17 bA2	47.62 ± 0.14 bB2
		30:1	0.26 ± 0.00 bB2	0.25 ± 0.00 cA2	3.37 ± 0.02 bA4	2.53 ± 0.02 cB4	50.33 ± 0.26 cA2,3	40.33 ± 0.35 cB3
		Control A	0.31 ± 0.00 cA1,2	0.31 ± 0.00 dA2	1.66 ± 0.02 cB2,3	1.81 ± 0.01 dA3	33.12 ± 0.41 dB2	35.28 ± 0.02 cA2
		Control B	1.84 ± 0.69 dA3	N/L	0.39 ± 0.02 dA3	0.39 ± 0.01 eA4	4.15 ± 0.10 eB2	5.22 ± 0.19 dA4
Fusarium oxysporum	LAN04	10:1	0.10 ± 0.00 Aa2	0.13 ± 0.00 aB3	3.16 ± 0.02 bB3	3.58 ± 0.02 aA4	54.21 ± 0.17 aA2,3	50.97 ± 0.33 aB3
		20:1	0.11 ± 0.00 aA2	0.16 ± 0.00 abB2	4.45 ± 0.03 aA1	2.79 ± 0.01 bB4	52.29 ± 0.14 bA3	48.20 ± 0.14 bB2
		30:1	0.25 ± 0.01 bB2	0.18 ± 0.01abA1	2.95 ± 0.02 cA5	2.71 ± 0.03 bB3	46.15 ± 0.46 cA3,4	45.67 ± 0.23 cB4
		Control A	0.24 ± 0.00 bA1	0.41 ± 0.00 cB1,2	1.52 ± 0.02 dA4	1.54 ± 0.01 cA4	30.41 ± 0.20 dB3	31.91 ± 0.09 dA3
		Control B	N/L	N/L	0.25 ± 0.03 eA4	0.22 ± 0.03 dB5	4.06 ± 0.16 eA2	3.40 ± 0.06 eB5
Clonostachys rosea	KTS05	10:1	0.07 ± 0.01 aA1	0.05 ± 0.00 aA1	3.14 ± 0.17 bB3	6.42 ± 0.01 aA2	56.91 ± 0.31 bB1,2	59.79 ± 0.60 aA2
		20:1	0.08 ± 0.00 bA2	0.08 ± 0.00 aA1	3.48 ± 0.02 bA3,4	3.10 ± 0.01 bB2	57.48 ± 0.59 bB2	61.29 ± 0.62 aA1
		30:1	0.20 ± 0.01 cA1	0.23 ± 0.01 bB2	6.53 ± 0.09 aA1	3.07 ± 0.03 bB1	71.38 ± 0.67 aA1	61.52 ± 0.46 aB1
		Control A	N/L	0.32 ± 0.01 cB1,2	1.72 ± 0.03 cB1,2	1.94 ± 0.02 cA2	39.31 ± 0.35 cA1	38.42 ± 0.20 bA1
		Control B		0.52 ± 0.03 dA2	0.25 ± 0.03 dB4	0.46 ± 0.02 dA3	3.68 ± 0.12 dB3	7.77 ± 0.07 cA13

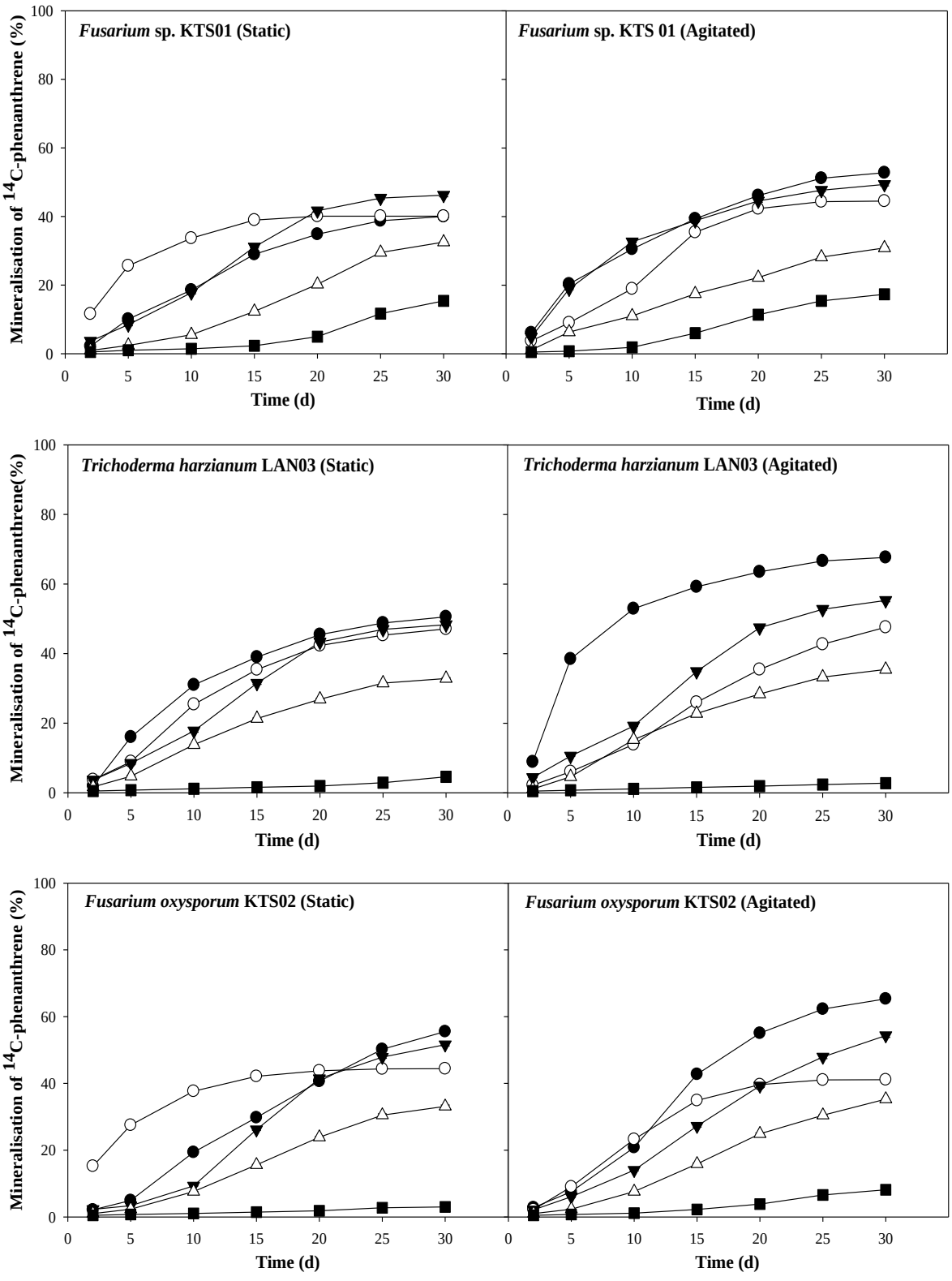
*Data presented for controls are the mean ± SEs of live fungal cells (control A) and $HgCl_2$-killed cells (control B) for the five isolated fungal strains.
*Values followed by different lowercase letters are significantly different ($p < 0.05$) within each fungal isolate with respect to the various CN ratio and controls, while different capital letters are significant different ($p < 0.05$) between culture conditions (static and agitated). Different numbers are significantly different ($p < 0.05$) across the five endophytic fungal isolates for each CN ratio.
*N/L means there was no lag phase that ism mineralisation did not reach or exceed 5% during the 14C-phenanthrene mineralisation in the liquid media.

7.4.3 ^{14}C-phenanthrene mineralisation in static and agitated liquid cultures under different N sources

The effects of fungal strains (KTS01, LAN03, KTS02, LAN04 and KTS05) on ^{14}C-phenanthrene mineralisation were also measured for 30 days under different N sources (malt extract, urea and ammonium nitrate) in both static and agitated culture conditions (Table 2 and Figure 3). All fungal strains in liquid cultures amended the various N sources showed significantly shorter ($p < 0.05$) lag phases than the controls under both static and agitated conditions throughout the experiment, respectively. However, the lag phases varied among the fungal strains under the different N amendments, with malt extract and NH_4NO_3 reducing the lag phases in most of the fungal agitated conditions but were not significantly different from each other. Furthermore, in static condition, most of the fungal strains displayed significantly shorter lag phases with urea after 30 d incubation, with observed lag phases before 5% mineralisations were reached. Generally, the shortest and longest lag phases were not associated with either a particular strain or an N source amendment throughout the study, with exception of the result observed for *T. harzianum* LAN03 (static, malt extract) and *Clonostachys* sp. KTS05 (agitated, urea) where significantly shorter lag phases ($p < 0.05$) were observed compared to other counterpart strains and controls (Table 2).

The fastest rates of ^{14}C-phenanthrene mineralisation were significantly influenced in static and agitated culture conditions by the addition of different N sources ($p < 0.001$) (Table 2 and Figure 3). For example, in both static and agitated conditions, there were significantly faster rates ($p < 0.001$) of mineralisation in all fungal cultures amended with the N sources than in the controls. Results indicate that static culture seeded with mycelial pellets of *Clonostachys* sp. KTS05 showed considerable increases in faster rates of ^{14}C-phenanthrene mineralisation compared with the other fungal strains throughout the study. Further, in static cultures, *Clonostachys* sp. KTS05 showed the fastest rate of $^{14}CO_2$ mineralised in urea (8.66 ± 0.06 % $^{14}CO_2$ d^{-1}) and NH_4NO_3 (4.71 ± 0.01 % $^{14}CO_2$ d^{-1}) after 30 d of incubation, while the lowest rate of mineralisation was consistently recorded for *Fusarium* sp. LAN04 in almost all N sources investigated. However, the fastest rates of $^{14}CO_2$ mineralised were generally higher in all fungal strains in liquid static cultures formulated with urea compared to the other N sources, apart from the fungus *T. harzianum* LAN03, which had a reduced fastest rate of mineralisation compared to the results observed with malt extract (Table 2). *Trichoderma* sp. strain LAN03 showed a significantly faster rate of mineralisation ($p < 0.05$) with malt extract as N source under static condition compared to the other fungal strains. In contrast, a significant increase ($p < 0.05$) in fastest rates of mineralisation were observed for all mycelial strains seeded in malt extract under agitated condition than those obtained by the other N sources with similar conditions (except *Clonostachys* sp. KTS05). Moreover, in comparison to agitated condition, all the fungal strains in static conditions significantly increased the rates of mineralisation in urea compared to other N sources. Conversely, the fungal strains with malt extract resulted in faster rates of mineralisation in agitated cultures than when incubated statically. Overall, the fastest (9.86 ± 0.02 % $^{14}CO_2$ d^{-1}, malt extract) and lowest (2.43 ± 0.02 % $^{14}CO_2$ d^{-1}, urea) rates of $^{14}CO_2$ mineralised were observed in agitated cultures of N sources seeded with strain LAN03 (*Trichoderma* sp.).

The amounts of ^{14}C-phenanthrene mineralised to $^{14}CO_2$ in static and agitated cultures were also influenced by all N amendments after 30 d incubation (Table 2 and Figure 3). As observed in the fastest rates of $^{14}CO_2$ mineralised, the cumulative extents of ^{14}C-phenanthrene mineralised by all fungal strains were significantly higher ($p < 0.001$) in both static and agitated culture conditions for all N amendments when compared to controls. After 30 d of incubation, in both static and agitated cultures, all fungal strains were found with significantly greater extents ($p < 0.05$) of mineralisation based on N sources (malt extract > NH_4NO_3 > urea), although the mineralisation curves for the fungal strains have not yet plateaued (Figure 3). The 0–30 d incubation period under agitated conditions showed that *Trichoderma* sp. LAN03 most influenced the overall extent of mineralisation. For example, *Trichoderma* sp. LAN03 showed consistently greater extents of mineralisation in culture with malt extract (67.68 ± 0.50 %), urea (47.60 ± 0.55 %) and NH_4NO_3 (55.31 ± 0.37 %) investigated. However, these observed extents of mineralisation were found to be statistically similar to those recorded for most fungal strains in agitated cultures over the 30 d incubation. In contrast, *Clonostachys* sp. KTS05 showed significantly higher extents of mineralisation ($p < 0.05$) in static cultures compared to the other fungal strains especially those amended with malt extract (60.48 ± 0.31 %) and NH_4NO_3 (59.22 ± 0.18 %). Notably, the extent of mineralisation was observed for *Clonostachys* sp. KTS05 closely followed *Fusarium* sp. KTS02 under static than agitated condition compared to their counterpart strains. The lowest extents of ^{14}C-phenanthrene mineralisation were consistently found in cultures seeded with *Fusarium* sp. LAN04 with all N amendments for static conditions.


Fusarium sp. KTS01 (Static)
Fusarium sp. KTS 01 (Agitated)
Trichoderma harzianum LAN03 (Static)
Trichoderma harzianum LAN03 (Agitated)
Fusarium oxysporum KTS02 (Static)
Fusarium oxysporum KTS02 (Agitated)
Mineralisation of ^{14}C-phenanthrene (%)
Mineralisation of ^{14}C-phenanthrene(%)
Time (d)
0
5
10
15
20
25
30
0
20
40
60
80
100

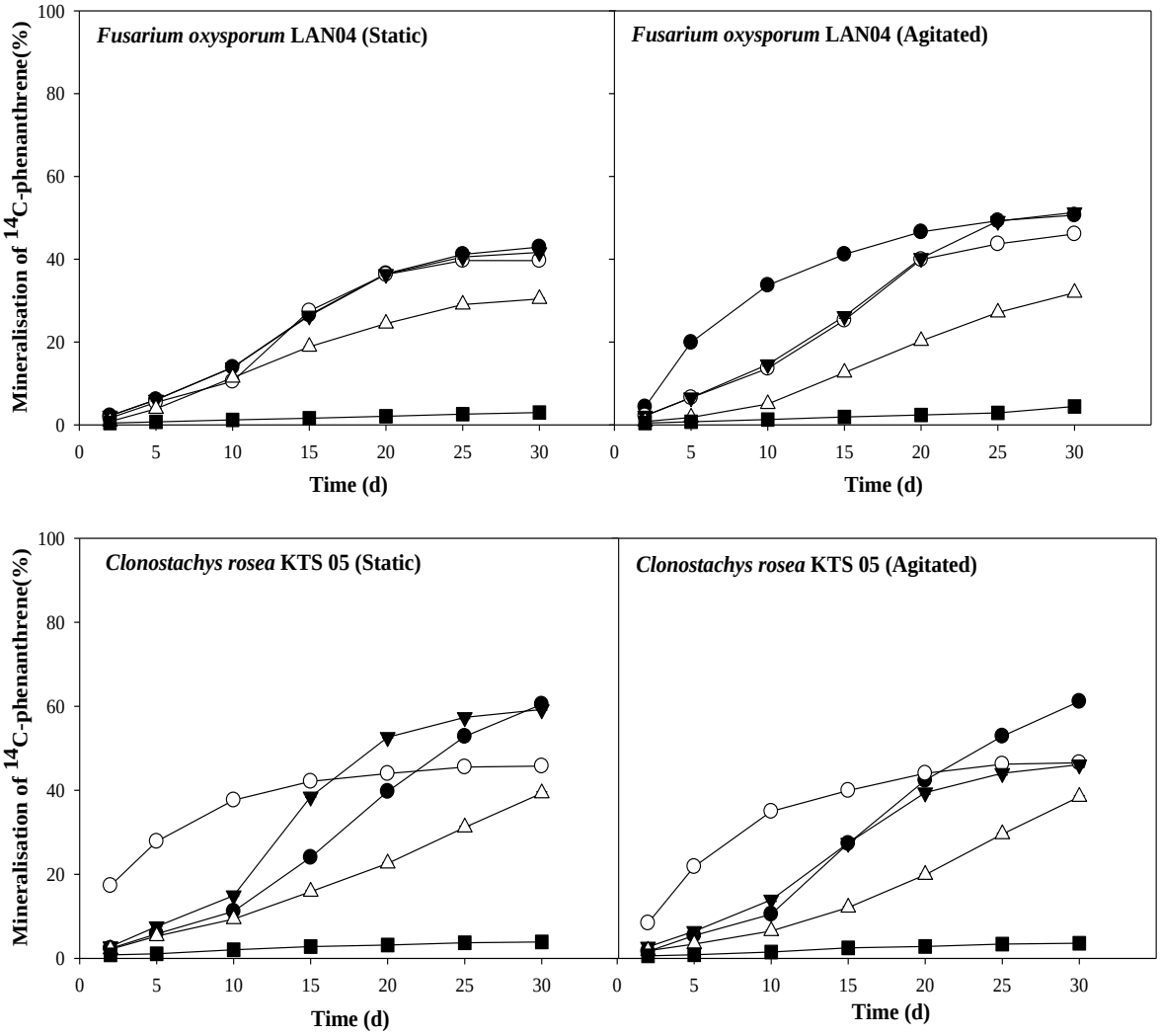


Figure 3. Development of ^{14}C-phenanthrene mineralisation by endophytic fungal strains after 30 days under different nitrogen sources in static and agitated mycelia liquid cultures spiked with phenanthrene (50 mg/l): Malt extract (●), Urea (○), Ammonium nitrate (▼), Live fungal control (Δ), and $HgCl_2$-killed fungal control (■). Mineralisation showed triplicate samples (n = 3) of mean ± SE.

Table 2. Mineralisation kinetics of ^{14}C-phenanthrene in mycelia liquid cultures amended with different nitrogen sources after 30 days respirometry assay (static and agitated conditions). Values are mean ± standard error (n = 3).

Endophytic fungal isolate	Isolate number	N source	Lag phase (d)		Fastest rates (%$^{14}CO_2$ d^{-1})		Cumulative extent (%)	
			Static	Agitated	Static	Agitated	Static	Agitated
Fusarium sp.	KTS01	Malt extract	0.13 ± 0.00 aB2	0.09 ± 0.00 aA1	2.62 ± 0.03 bB4	4.75 ± 0.04 aB3	40.01 ± 0.10 bB3,4	52.80 ± 0.09 aA1,2
		Urea	0.02a ± 0.00 A1	0.11 ± 0.00 aB2	5.79 ± 0.39 aA3	3.29 ± 0.02 bB2	40.09 ± 0.67 bB2,3	44.52 ± 0.17 cA1
		Ammon. nitrate	0.12 ± 0.02 aB1	0.09 ± 0.00 aA1	2.67 ± 0.02 bB3	4.69 ± 0.04 aA1	46.21 ± 0.47 aB3	49.37 ± 0.41 bA2
		Control A	0.49 ± 0.11 bB2	0.29 ± 0.06 bA2	1.86 ± 0.02 bA1	1.74 ± 0.04 cB3	32.53 ± 0.04 cA2	30.87 ± 0.31 dB4
		Control B	0.84 ± 0.01 cB1	0.59 ± 0.06 cA1	1.34 ± 0.03 cA1	1.13 ± 0.03 dB1	15.40 ± 0.26 dB1	17.33 ± 0.21 eA1
Trichoderma harzianum	LAN03	Malt extract	0.11 ± 0.00 aB1	0.09 ± 0.00 aA1	4.69 ± 0.04 aB1	9.86 ± 0.02 aA1	50.58 ± 0.34 aB2,3	67.68 ± 0.50 aA1
		Urea	0.10 ± 0.01 aB2	0.17 ± 0.01 bA3	3.30 ± 0.02 bA4	2.43 ± 0.02 cB4	47.08 ± 0.34 bA1	47.60 ± 0.55 cA1
		Ammon. nitrate	0.12 ± 0.01 aB1	0.09 ± 0.00 aA1	2.74 ± 0.02 cB3	3.13 ± 0.02 bA2	48.29 ± 0.26 bB3	55.31 ± 0.37 bA1
		Control A	0.22 ± 0.01 bA1	0.44 ± 0.00 cB1	1.81 ± 0.02 dB1,2	2.13 ± 0.02 dA1	32.85 ± 0.25 cB2	35.47 ± 0.27 dA2
		Control C	N/L	N/L	0.25 ± 0.03 eA3	0.25 ± 0.03 eA4	4.59 ± 0.08 dA2	2.79 ± 0.04 eB5
Fusarium oxysporum	KTS02	Malt extract	0.21 ± 0.00 bB4	0.17 ± 0.01 bA2	2.89 ± 0.02 cB3	4.39 ± 0.02 aA4	55.52 ± 0.17 aB1,2	65.32 ± 0.39 aA1
		Urea	0.03a ± 0.00 A1	0.13 ± 0.00 aB2	7.62 ± 0.06 aA2	2.84 ± 0.03 bB3	44.40 ± 0.38 cA1,2	41.12 ± 0.48 cB2
		Ammo. nitrate	0.27 ± 0.00 cB3	0.17 ± 0.01 cA2	3.37 ± 0.02 bA2	2.64 ± 0.02 cB4	51.61 ± 0.38 bB2	54.35 ± 0.56 bA1,2
		Control A	0.31 ± 0.00 dA1,2	0.31 ± 0.00 dA2	1.66 ± 0.02 dB2,3	1.81 ± 0.01 dA3	33.12 ± 0.41 dB3	35.28 ± 0.02 dA2
		Control D	N/L	N/L	0.26 ± 0.03 eB3	0.54 ± 0.02 eA2	3.02 ± 0.02 eB3	8.14 ± 0.28 eA2
Fusarium oxysporum	LAN04	Malt extract	0.17 ± 0.01 aA3	0.16 ± 0.01 aA2	2.53 ± 0.02 bB4	5.20 ± 0.04 aA2	42.95 ± 0.41 aB3	50.66 ± 0.56 aA2
		Urea	0.19 ± 0.00 aB3	0.16 ± 0.01 aA3	3.40 ± 0.02 aA4	2.93 ± 0.02 bB3	39.67 ± 0.31 bB3,4	46.11 ± 0.65 bA1
		Ammon. nitrate	0.17 ± 0.01 aA2	0.16 ± 0.01 aA2	2.50 ± 0.01 bA4	2.81 ± 0.03 bB3	41.64 ± 0.55 aB4	51.29 ± 0.30 aA1,2
		Control A	0.24 ± 0.00 bA1	0.41 ± 0.00 bB2	1.52 ± 0.02 cA4	1.54 ± 0.01 cA4	30.41 ± 0.20 cB3	31.91 ± 0.09 cA3
		Control B	N/L	N/L	0.22 ± 0.03 dB4	0.31 ± 0.01 dA3	2.99 ± 0.08 dB4	4.46 ± 0.11 dA3
Clonostachys rosea .	KTS05	Malt extract	0.18 ± 0.00 cA3	0.16 ± 0.01 bA2	3.14 ± 0.03 cB2	3.36 ± 0.02 bA5	60.48 ± 0.31 aA1	61.16 ± 0.20 aA1
		Urea	0.04a ± 0.00 A1	0.05 ± 0.01 aB1	8.66 ± 0.06 aA1	4.48 ± 0.03 aB1	45.77 ± 0.23 bA1,2	46.56 ± 0.38 bA1
		Ammon. nitrate	0.14 ± 0.00 bA1	0.16 ± 0.01 bB2	4.71 ± 0.01 bA1	2.71 ± 0.01 cB3	59.22 ± 0.18 aA1	46.11 ± 0.62 bB3
		Control A	0.20 ± 0.01 cA1	0.32 ± 0.01 cB1,2	1.72 ± 0.03 dB1,2	1.94 ± 0.02 dA2	39.31 ± 0.00 cA1	38.42 ± 0.20 cB1
		Control B	N/L	N/L	0.42 ± 0.02 eA2	0.30 ± 0.04 eB3	3.90 ± 0.30 dA3	3.59 ± 0.01 dA4

*Data presented for controls are the mean ± SEs of live fungal cells (control A) and $HgCl_2$-killed cells (control B) for the five isolated fungal strains.
*Values followed by different lowercase letters are significantly different ($p < 0.05$) within each fungal isolate with respect to the various CN ratio and controls, while different capital letters are significant different ($p < 0.05$) between culture conditions (static and agitated). Different numbers are significantly different ($p < 0.05$) across the five endophytic fungal isolates for each CN ratio.
* N/L means there was no lag phase that ism mineralisation did not reach or exceed 5% during the 14C-phenanthrene mineralisation in the liquid media.

7.5 Discussion

7.5.1 Effect of CN ratios on the mineralisation of ^{14}C-phenanthrene in agitated and static culture conditions

C:N ratios are vital for microbial growth, activity and metabolism where carbon is used as the energy source and nitrogen for the protein and nucleic acids synthesis (Mooshammer *et al*., 2014) as well as efficient biodegradation of organic compounds in soils and water (Lamar and White, 2001; Chen *et al*., 2008). The results in the present study revealed that all of the endophytic fungal strains were influenced by all C:N ratios amendments following decreases in the length of the lag phases, increases in the degradation rates and extents of ^{14}C-phenanthrene mineralisation in both static and agitated cultures over a 30 d incubation period. In general, the kinetics of mineralisation varied among the endophytic fungi in both agitated and static conditions.

The data showed that liquid cultures seeded with mycelial pellets of *Clonostachys* sp. KTS05 significantly reduced the lag phases with a C:N of 10:1 in static conditions compared to other fungal strains. In contrast, using the same C:N ratio (10:1), *Fusarium* sp. KTS02 showed a significantly shorter lag phase in agitated cultures. The reduced lag phases may have resulted from a rapid adaptation by the fungal strains in the presence of an appropriate C:N ratio,

suggesting that balance carbon and energy may be present for their cell growth (biomass) and metabolism (Mollea *et al*., 2005; Di Lonardo *et al*., 2020; Huang *et al*., 2020). Although optimal C:N ratio may likely vary for different culture conditions, an imbalance in the C:N ratio can reduce microbial function during PAH degradation (Nwinyi *et al*. 2016). Generally, the results showed significantly reduced lag phases by all fungal strains for the C:N amendments, however, this depends on the incubation conditions for some fungal strains. For example, the some of the fungal strains displayed shorter lag phases at C:N ratio of 20:1 with static conditions while a C:N ratio of 30:1 for agitated cultures, suggesting that nutrient optimisation for a fungus, including growth and activities may be related to the culture condition during biodegradation studies.

The fastest rates of ^{14}C-phenanthrene mineralisation showed similar trends as observed for lag phases with increasing C:N ratios in agitated conditions. However, in static conditions, the rates of mineralisation tend to be faster in higher C:N ratios (20 and 30) than in a lower C:N ratio (10). This result suggested that high C:N ratios in static cultures increased the rates of phenanthrene mineralisation. Although solubilisation and lowered surface tension by agitation can facilitate the interphase mass transfer of PAH into aqueous phases during PAH degradation (Ding *et al*., 2008; Kadri *et al*., 2017). The results suggest that at a high C:N ratio, phenanthrene was more highly metabolised by the fungal strains in static than in agitated conditions thus the faster rates of mineralisation, which were particularly evident in the culture of *Fusarium* sp. KTS01. However, the rates of mineralisation were high in almost all fungal strains in agitated cultures with C:N ratio of 10:1 compared to the other C:N ratio amendments, indicating that incubation condition to a larger extent, depending on the fungal strains, would facilitate their growth and metabolism.

Both static and agitated cultures increased significantly the extents of ^{14}C-phenanthrene mineralisation by the fungal strains, and this was also observed across all C:N ratios in liquid cultures. However, the static condition promoted greater extents of mineralisation than the agitated condition for most fungal strains. The formation of mycelial mats and likely increase contact between mycelial cells and phenanthrene in static cultures was suggested to have facilitated the extents of mineralisation. More so, in static cultures, ligninolytic enzymes by the endophytic fungi are more likely to be synthesised due to an increase in the contact area between mycelial cells without shear stress (Ding *et al*., 2008; Acevedo *et al*., 2011). We also found that the extents of mineralisation in most fungal strains were significantly higher in static than in agitated conditions under C:N ratio of 10:1 compared to the other C:N ratios in most time points, indicating an optimal C:N ratio for PAH biodegradation either in water or sediment matrices (Chen *et al*., 2008). Further, *Fusarium* spp and *Clonostachys* sp displayed higher extents of mineralisation than their counterpart strains in both static and agitated conditions. Some past research has demonstrated efficient degradation of organic contaminants, including the PAHs by *Fusarium* spp (Chulalaksananukul *et al*., 2006; Jacques *et al*., 2008; Pozdnyakova *et al*., 2019). However, several species of *Fusarium* such as *F*. *oxysporum* are known soil-borne plant pathogens which can cause devastating vascular wilt in many plant species (Lopez Berges *et al*., 2012; Srinivas *et al*., 2019). *Clonostachys* sp. KTS05 displayed greater extents of mineralisation in both static and agitated cultures with higher C:N ratios (especially 30:1), indicating the catabolic potential of this strain in high C:N ratio systems. Notably, this is the first investigative work reporting the potential of *Clonostachys* sp. in PAH degradation.

7.5.2 Effect of N-source amendment on the mineralisation of ^{14}C-phenanthrene in agitated and static culture conditions

Amending the mycelial cultures with various organic and inorganic nitrogen amendments resulted in significantly reduced lag phases in both static and agitated conditions. These data were expected since rapid microbial growth can occur by available sources of nitrogen especially for efficient degradation of organic contaminant (Crisafi *et al*., 2016). However, the lag phases in the different amended cultures with N source varied with the fungal strains. For example, most of the fungal strains in agitated conditions showed greater reductions in the lag phases under nitrogen sources (malt extract and NH_4NO_3) than in urea, whereas the length of the lag phases was reduced before mineralisation began after urea addition in static cultures. In static and agitated cultures, *T. harzianum* LAN03 and *Clonostachys* sp. KTS05 resulted in reduced lag phases compared to the other strains when the cultures were amended with malt extract and urea, respectively, indicating stimulatory effects of these nutrients and adaptation of the endophytic fungal strains.

The data showed that all N sources markedly influenced the fastest rates of ^{14}C-phenanthrene mineralisation in the liquid microcosms with fungal mycelia (both static and agitated conditions). Addition of an N source to fungal cultures enhanced the rate of hydrocarbon degradation as reported by previous researchers (Röling *et al*., 2002; Hamzah *et al*., 2012; Hamzah *et al*., 2018). The fastest rates of mineralisation varied between the fungal strains in terms of incubation conditions in liquid cultures with the N sources. In static cultures amended with urea, the fungal strains (in particular, strain KTS05) generally displayed higher rates of mineralisation than the other N source; whereas the agitated cultures with malt extract resulted in faster rates of $^{14}CO_2$ mineralised by most of the fungal strains, especially with *T. harzianum*

LAN03. This may be attributed to differences in nutrition (growth and metabolism) by fungal species for PAH degradation (Joutey *et al*., 2013). In particular, both fungal strains (KTS05 and LAN03) showed their best forms of nitrogen for increased rates of phenanthrene degradation under different incubation conditions.

Clonostachys sp. KTS05 and *T. harzianum* LAN03 were the most effective degraders in the liquid cultures amended with N sources for phenanthrene degradation. In most cases, the results showed differences in the fungal strains in the extents of mineralisation by the different N amendments. This may be attributed to differences in their mycelial network, enzyme secretion, growth rates with the target contaminant, and hence the different behaviour observed (Mollea *et al*., 2005; Kadri *et al*., 2017). Compared to their counterpart strains, strains KTS05 and LAN03 exhibited greater extents of mineralisation in static and agitated cultures, respectively. These observations, however, are consistent with previous studies on the removal of organic contaminants in liquid microcosms (Mollea *et al*., 2005; Chang *et al*., 2020). In addition, malt extract cultures amended with all mycelial strains; however, had significant influence on the extent of mineralisation in both static and agitated cultures. Generally, in terms of N source, the agitated cultures displayed greater extent of mineralisation compared to the static cultures for most fungal strains. This clearly indicates that agitated cultures with N amendment (especially malt extract) would promote higher phenanthrene degradation by most endophytic fungal strains (Hadibarata *et al*., 2009; Vitali *et al*., 2006). Agitated conditions could have led to more oxygen availability, PAH solubility and mass transfer into the aqueous phase for fungal uptake (Johnsen *et al*., 2005).

7.6 Conclusions

Overall, the kinetics of ^{14}C-phenanthrene mineralisation (lag phases, fastest rates and extents) were influenced after nutrients amendments in all fungal liquid cultures (both static and agitated conditions), implying that nutrients addition could promote the stimulatory effects on PAH biodegradation. The results of the study showed however, that the kinetics of ^{14}C-phenanthrene mineralisation varies for the fungal strains under the different nutrient forms amended to liquid cultures as well as the culture conditions. This evidence supports the view that most fungal catabolism is related to their culture conditions during microcosm degradation studies. Nutrient amendment with C:N ratio of 10 showed higher phenanthrene mineralisation while the fungal cultures (strains KTS05 and KTS01) showed greater influence on phenanthrene mineralisation in both static and agitated conditions. Specifically, fungal strains (KTS05 and LAN03) greatly influenced phenanthrene mineralisation after N source amendments, particularly in malt extract. Based on these findings the endophytic fungal strains cannot only be used for pre-treatment studies involving organic materials but also as potential PAH-degraders when supplied with nutrients in bioremediation studies. This study further demonstrates the importance of nutrients amendments for enhanced PAH degradation in liquid cultures and thus appropriate carbon/nitrogen ratios and nitrogen sources are important for fungal growth, uptake and metabolism of PAHs.

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7.8 Supplementary Data

Table S1. Physicochemical properties of Myerscough soil used in the experiment (Data adapted from Couling *et al.*, 2010). Values are mean ± SEM (n = 3).

Soil properties		Parameter Values
pH (in dH_2O)		6.50 ± 0.08
Moisture content (%)		21.07 ± 2.78
Microbial heterotrophic numbers (CFU g^{-1})		$2.17 \times 10^5 \pm 1.67 \times 10^4$
Elemental analysis	Total extractable carbon	1.80% ± 0.03
	Total extractable nitrogen	0.14% ± 0.01
	Total extractable organic carbon	1.60% ± 0.07
	Soil organic matter	2.70% ± 0.04
Soil particle size	Clay	19.5% ± 0.70
	Silt	20.0% ± 0.87
	Sand – Total	60.4% ± 1.20
	Coarse sand	0.12% ± 0.01
	Medium sand	6.90% ± 0.10
	Fine sand	53.3% ± 0.60
	Surface texture: clay loam	

Table S2. Radial mycelial growth measurements of selected isolated endophytic fungi in various phenanthrene concentrations (mean ± SE; n = 5). Data are the first 6 days of fungal growth on agar plates.

Fungal Isolates	Radial growth rates (mm/day)					Mean growth rate (mm/day)
	5 mg/L	10 mg/L	25 mg/L	50 mg/L	100 mg/L	
KTS01	1.45 ± 0.12	1.44 ± 0.09	1.26 ± 0.02	1.07 ± 0.02	0.92 ± 0.03	1.23 ± 0.06
LAN03	1.32 ± 0.07	1.25 ± 0.03	1.18 ± 0.00	0.95 ± 0.05	0.83 ± 0.13	1.11 ± 0.06
KTS02	1.11 ± 0.05	1.17 ± 0.01	1.07 ± 0.09	0.89 ± 0.07	0.55 ± 0.04	0.96 ± 0.05
LAN04	1.23 ± 0.11	1.23 ± 0.01	1.13 ± 0.10	0.77 ± 0.01	0.64 ± 0.11	1.00 ± 0.07
KTS05	1.51 ± 0.03	1.38 ± 0.10	1.32 ± 0.02	1.20 ± 0.08	1.07 ± 0.03	1.30 ± 0.05
Mean	1.32 ± 0.08	1.29 ± 0.05	1.19 ± 0.05	0.98 ± 0.06	0.80 ± 0.07	1.12 ± 0.06

*Values followed by different lowercase letters are significantly different at $p < 0.05$ (Tukey's HSD Test).

Table S3. Physicochemical and microbial characteristics (mean ± SEM) of spent brewery grains (SBG) used in the experiment (Data adapted from Omoni *et al.*, 2020).

Parameters measured	SBG
pH	5.00 ± 0.00
EC (mS)/25^{0}C	2.24 ± 0.00
Dry matter (%)	18.0 ± 0.58
Extractable N (NH_4-N) (mg/kg dw)	112.0 ± 15.0
Extractable N (NO_3-N) (mg/kg dw)	bdl
Extractable P (mg/kg dw)	762.7 ± 10.4
TOC (mg/kg)	382.0 ± 3.41
IC (mg/kg)	3.70 ± 0.12
TC (%)	47.4 ± 0.05
TN (%)	3.13 ± 0.02
TP (%)	0.41 ± 0.03
C:N	15.1 ± 0.12
THBC (CFU x 10^8 g^{-1} dw)	10.5 ± 1.33
TFC (CFU x 10^6 g^{-1} dw)	4.45 ± 0.07

*****bdl = below detection limit; dw = dry weight; EC= Electrical conductivity; TOC= Total organic carbon; IC= Iorganic carbon; TC= Total carbon; TN= total nitrogen; TP= Total phosphorus; THBC= Total heterotrophic bacterial count, TFC= Total fungal count

Table S4. Results from a NCBI BLAST on nucleotide sequences (ITS 1 and IT4) of the isolated phenanthrene-degrading ligninolytic fungi with GenBank database

Strain No.	Length (bp)	Genbank (NCBI)		ITS sequence similarity (%)	Possible Identity
		Closest match	Accession number		
KTS01	1156	*Fusarium* sp. R206	JX559854.1	99.9	*Fusarium* sp.
LAN03	592	*Trichoderma harzianum*	KU896331.1	99.9	*Trichoderma harzianum*
KTS02	541	*Fusarium oxysporum*	HQ379659.1	99.8	*Fusarium oxysporum*
LAN04	527	*Fusarium oxysporum*	AY928417.1	99.9	*Fusarium oxysporum*
KTS05	540	*Clonostachys rosea*	MH855404.1	99.8	*Clonostachys rosea*

Table S5. Cultural and microscopic characteristics of the different PAH- lignocellulosic fungi isolated from pristine soil

S/No	Identified mycelial code	NCBI identification with 98-100% homologous identity	Genbank Accession number	Macroscopic features	Microscopic features with lactophenol cotton blue stain
1	KTS01	*Fusarium* sp.	MH550475.1	White fluffy mycelium colonies with pink-coloured mycelium (aerial). Pellets appeared are also purple coloured	Few microconidia with thin wall (reniform-oval with two cells), no macroconidia. Septate hypha with short conidiophores that are monophialides which extend singly from the conidiophores. Terminal chlamydospores are seen.
2	LAN03	*Trichorderma harzianum*	KU896312.1	Grey-brown colonies (cottony) Yellow showing shades of light green and white mycelia	Long, many slender hyphae that are non-septate. Conidia are thin and oval and are hydra-shaped. No chlamydospores observed.
3	KTS02	*Fusarium* oxysporum	AY928417.1	White, cotton-like (fluffy) colonies	Non-pigmented (hyaline) hyphae that are septate. Short condiophores with intercalary chlamydospores. Monophialides are seen.
4	LAN04	*Fusarium* oxysporum	MF136405.1	White, cottony colonies, appeared in purple mycelial pellets	Very few microconidia with a thin wall (reniform-oval with two cells), no macroconidia. Septate hypha with short conidiophores that are monophialides which extend singly from the conidiophores. Terminal chlamydospores are seen.
5	KTS05	*Clonostachys rosea*	KT269662.1	Yellow-white colonies appear white cottony (thick) at the surface with wrinkled centre.	Coniodiophores and conidia are borne on sticky ball at the tip of phialides, having verticillium-like condiophores

Note: *Fusarium* spp appeared purple or pink on potato dextrose agar (PDA) but these colours were not very visible on malt extract agar (MEA) but most of the pelleted forms appear pink or purple on potato dextrose broth (PDB).

Chapter 8

General Discussion, Conclusions and Future work

8. Discussion, Conclusions and future work

8.1 Discussion

Soils contaminated with HOCs, such as the PAHs, have been widely studied due to their persistence in the environment and the potential risks these organic contaminants pose to human and environmental health (Idowu *et al.*, 2019; Sakshi *et al.*, 2019). Further, soils can act as sinks for these organic contaminants in the environment through sorption to soil mineral and organic fractions (Lee *et al.*, 2014). Therefore, this influences their mobility, bioavailability, and biodegradation and, as a result, may influence the persistence of these contaminants in soil (Riding *et al.*, 2013). The presence of PAHs and other contaminants can affect microbial growth, proliferation, enzymatic function, nitrification, as well as microbial populations and genetic diversity in soil (Mohd Kamil and Talib, 2016; Ghosal *et al.*, 2016).

While it is acknowledged that biodegradation is an important loss process contributing to the removal of organic contaminants from soil, it may be affected by low nutrient and organic carbon concentrations in contaminated soil (Zhang *et al.*, 2012); further, the catabolism of organic chemicals by microorganisms depends largely on nutrient bioavailability (Azubuike *et al.*, 2016). It is recognised that nutrient bioavailability is by far the most important factor for microbial degradation of PAHs in soils. The presence of readily available nutrients and organic matter, electron donors (energy sources), and suitable environmental factors (e.g., oxygen, pH, nutrient concentrations), as well as the presence of the right microorganisms or competent microorganisms are amongst the major factors that can impact on the biodegradation of PAHs and can also determine the overall success of a remediation process (Kim *et al.* 2014; Latinwo and Agarry, 2015).

In the first part of this thesis, the influence of increasing waste-to-soil ratios (1:10, 1:5, 1:2, 1:1 & 2:1) of two organic waste materials, spent brewery grains (SBG) and spent mushroom compost (SMC) were investigated to ascertain the optimal mix ratio for ^{14}C-phenanthrene biodegradation in amended soil spiked with ^{12}C-phenanthrene (100 mg/kg) (Chapter 3). The two organic materials (SBG and SMC) showed higher mineralisation in amended soils. The addition of organic materials in soils can influence the physicochemical and biological properties of soil (Chojnacka *et al.*, 2020) and consequently, promotes the indigenous microbial population with capacity to degrade organic contaminants from the soil (García-Delgado *et al.*, 2015; Kästner and Miltner, 2016). In addition, soils amended with smaller amounts (1:10 & 1:5) of both SBG and SMC showed significantly reduced lag phases, faster rates and greater levels of phenanthrene mineralisation in amended soils compared to the larger dose rates (1:1, 1:2 & 2:1). The results confirm the stimulatory effect of the organic waste materials on the biodegradation of phenanthrene with lower amendment ratios (Abioye *et al.*, 2012; Adam *et al.*, 2015; Sigmund *et al.*, 2018). For example, Abioye *et al.* (2012) reported that the addition of smaller amounts of organic amendments to soil (1:10) largely increased degradation rates, despite the sequestration of the chemicals in the soil.

Data showed that there was no measurable lag phase in soils amended with 2:1 SBG:soil ratio and significantly lower rates and extents were observed in amended soils throughout the study. Therefore, microcosms with the higher amendment (2:1) could have limited oxygen transport for microbial activity, hence reduced mineralization, due to the nature of water-saturated bulky material formed after amendment. In addition, this could also be linked to high sorptive capacity of the high organic matter present in the higher dose of the organic materials for phenanthrene (Rhodes *et al.*, 2008; Okere *et al.*, 2017) and consequently, this reduces the

bioavailability of the target contaminant for microbial degradation (Namkoong *et al.*, 2002; Semboung Lang *et al.*, 2016).

The assessment of the microbial activity in amended soils showed that the microbial numbers increased after application of the organic materials to PAH contaminated soil compared with unamended controls (Sigmund *et al.*, 2018). In addition, bacterial CFUs (heterotrophic and phenanthrene-degraders) were higher in amended soils (1:10 and 1:5); however, phenanthrene-degrading bacterial numbers did not statistically correlate with fastest rate of ^{14}C-phenanthrene mineralized but show significantly weak but negative correlations with the total extents of ^{14}C-phenanthrene mineralization. This low response of phenanthrene-degraders after the addition of organic materials to contaminated soil could have affected PAH catabolism (Carmichael and Pfaender, 1997). However, higher dose application (1:2 and 1:1) in both SBG and SMC-amended soils showed higher fungal numbers (heterotrophs and phenanthrene-degraders) over time but these results did not correlate with either the fastest rates or extents of mineralisation in this study.

Based on these findings, a second study of ^{14}C-phenanthrene mineralisation was carried in the presence of pyrolyzed organic materials (biochar). The aim of this study was also to investigate the impact of increasing amounts (0, 0.01, 0.1, 0.2, 0.5 and 1.0%) of two types of biochar (so-called enhanced and non-enhanced) in the biodegradation of ^{14}C-phenanthrene from soil (Chapter 4). This was done to show that the presence of pyrolyzed organic materials (enhanced and nonenhanced) will promote higher phenanthrene degradation. Enhanced (or cultured) biochar were seeded with inoculants which are designed to potentially stimulate microbial activity and promote biological function in soil. This cultured biochar was enriched with some microbial inoculants: the arbuscular mycorrhizal fungi *such as Glomus* spp (> 450

propagules/g), *Ascophyllum nodosum* and *Trichoderma* spp (>1x10^9 CFU/g) and wormcasts. Here, the overall findings in this study show that EbioC amendments stimulated the mineralisation of ^{14}C-phenanthrene in soils to greater levels than the NEbioC amendments. The higher mineralisation of ^{14}C-phenanthrene after amendment with immobilised biochar (EBioC) can be attributed to more bioaccessible fractions of the sorbed-PAH substrate on the immobilised biochar surface and may have resulted from an increase in soil metabolic activities (Xiong *et al.*, 2017; Zhang *et al.*, 2018). Xiong *et al.* (2017) stated that the mass transfer of PAHs to degrading cells in biochar-amended soil could be facilitated by dissolved and solid sorbing matrices for PAH degradation. Furthermore, biochar application and soil-contaminant contact time influenced the lag phase, rates and extents of ^{14}C-phenanthrene mineralised compared to control soils, which were particularly evident in the lower amendments (0.1%, 0.2%, 0.01%) in both types of biochar. For example, increasing the length of soil-phenanthrene interactions also increased ^{14}C-phenanthrene mineralisation in soil amended with smaller amounts, especially EBioC. Therefore, the immobilised inoculum on the EbioC may have provided additional support for the indigenous microbial population than NEbioC which led to shorter lag phases, increases in fastest rates and extents of ^{14}C-phenanthrene mineralisation in amended soil in this study. EbioC was also suggested to accelerate nutrient uptake and release, improved oxygen and water holding capacity and support metabolic activities and processes in soil rhizosphere (Drobek *et al.*, 2019).

As mentioned before in our previous study with SBG and SMC, the addition of larger amounts (0.5% and 1%) of both biochar types (EBioC and NEbioC) increased the lag phases, reduced the rates and extents of ^{14}C-phenanthrene in amended soils. The reduced mineralisation in the larger amounts (1.0% > 0.5%) of both biochar types supports the sorptive effects of biochars, which can reduce mass transfer and bioaccessibility of PAHs to soil microorganisms (Rhodes

et al., 2012; Anyika *et al.*, 2015; Ogbonnaya *et al.*, 2016). It was suggested that the larger amounts of biochar increased the number of microporous sites for contaminant sorption in biochar-amended soils. Although the biochar amendments (EBioC and NEbioC) increased the total numbers of heterotrophs (bacteria and fungi) but caused a decrease in phenanthrene degraders, suggesting reduced bioaccessibility/desorbable (biodegradable) fractions of the PAH in soil as a carbon source for the PAH degraders. However, in this present study, significant negative correlations were observed between phenanthrene degraders and mineralisation (rates and extents) for most biochar-amended soil. This may be as a result of either the microbes are not synthesizing the required enzymes for the target contaminant (however, this was not measured) or they were partially or fortuitously metabolizing the phenanthrene in the presence of non-target carbon substrate (in this case, the biochar) (Namkoong *et al.*, 2002; Das *et al.*, 2011). On the contrary, it was observed that the extent of mineralisation in EBioC (0.2%) showed significant positive correlations with both bacterial and fungal degrading CFUs, while positive correlation was also observed with fungal degraders only in NEbioC-amended soil with 0.01%. Therefore, the results suggests that the presence of PAH degrading populations may not be too adequate to indicate the degradation of a contaminant in soil but their high CFUs could contribute to the extent of PAH degradation and provide also useful information on the degradability of the contaminant by indigenous microorganisms in soils.

To date, few studies have previously reported the impact of soil amendments with fungal pre-treated organic materials and in particular the lignocellulosic ones, on the biodegradation of HOCs in soil (Arum Sari *et al.*, 2014; Andriani and Tachibana, 2016). In this study, lignocellulosic waste material (SBG) was first pre-treated with five white-rot fungi (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Irpex lateus*, *Pleurotus ostreatus*, and

Bjerkandera adusta) to enhance the carbon, energy and nutrients prior to soil amendment (Chapter 5). This was performed to further increase the bioactivities and biodegradation of phenanthrene in soil. It was carried out based on the results obtained from the previous studies with organic amendment only without white-rot fungi treatment (Chapter 3 and 4). The results showed that pre-treated SBG influenced the mineralisation of ^{14}C-phenanthrene, stimulate the enzyme activities and microbial population in all amended soils. However, this depends on the fungal strain used for the pre-treatment of SBG before soil amendment. Data also showed that fungal pre-treated SBG-amended soils generally caused reductions in lag phases as well as higher rates and extents of ^{14}C-phenanthrene mineralisation in the following trend *T. versicolor* > *B. adusta* > *P. chrysosporium* = *P. ostreatus* > *I. lateus*. The trend suggests a higher metabolism of SBG as carbon and energy source for PAH mineralisation, higher enzymes secretion and capacity to degrade PAHs (Andriani and Tachibana, 2016), faster adaptation to phenanthrene and possible synergistic interactions with soil indigenous microorganisms by each fungus (Kästner and Miltner, 2016; Han *et al.*, 2017; Omoni *et al.*, 2020a).

In addition, higher extents of mineralisation were generally found between slightly acidic (6.3) and neutral pH (7.2) in amended soils. However, the soil pH reduced with increases with contact time (75–100 d). The extents of ^{14}C-phenanthrene mineralisation were positively correlated ($p < 0.05$) with soil pH in most fungal pre-treated SBG-amended soils. Results showed that higher C/N ratios were observed for *P. chrysosporium* followed by *P. ostreatus* throughout the study period. For the enzyme activities assayed, both ligninolytic (LAC, LiP and MnP) and non-ligninolytic (βGA and ACP) enzymes were stimulated in all amended soils. SBG pre-treated *T. versicolor* followed by *B. adusta* and *P. chrysosporium* showed higher levels of both soil enzyme activities (ligninolytic and non-ligninolytic). The higher mineralisation of ^{14}C-phenenthrene by both fungi (*B. adusta* and *T. versicolor*) can be attributed

to the high secretion of ligninolytic enzymes in amended soils, especially the MnPs (Lladó *et al.*, 2013; Andriani and Tachibana, 2016). However, there were slight variations in the amount of ligninolytic enzymes secreted by the fungal strains. Results also showed that higher MnP activity was detected for all amendment conditions compared to other ligninolytic enzymes in amended soils at almost all time points (Novotný *et al.*, 2004; Pozdnyakova, 2012). Overall, the ligninolytic enzymes generally decreased, while non-ligninolytic enzymes increased as the extent of mineralisation diminished in all amended soils over time. The study also confirms the hypothesis that all fungal strains used in the pre-treatment of SBG would have a positive influence on the microbial activities (bacteria and fungi) in amended soils which were showed by the positive correlations observed in most amended soils between the phenanthrene-degraders (especially fungi) and mineralisation (rates and extents). The C/N ratios in amended soils (*B. adusta*, *P. chrysosporium* and *T. versicolor*) showed significant correlations with phenanthrene-degrading fungal numbers.

Due to PAH toxicity to microorganisms in contaminated soils, lignocellulosic waste materials can further be immobilised with microbes for enhanced PAH biodegradation in soil (Arum Sari *et al.*, 2014; Kheirkhah *et al.*, 2020; Kumar and Chandra, 2020). However, the white-rot fungi (WRF) have dual characteristics as proficient degraders of lignocellulose biomass and active degraders of PAHs (Memić *et al.*, 2020). Immobilisation of white-rot fungi on lignocellulosic materials can enhance the survival of the fungal mycelia, optimizes enzyme secretion, protect both the fungi and enzyme from harsh environmental conditions and increase their viability and activity in soil (Andriani and Tachibana, 2016; Dzionek *et al.*, 2016). In this study, SBG was used as a support material for immobilisation of five white-rot basidiomycetes mentioned earlier were assessed for phenanthrene degradation in soil (Chapter 6). Results showed that all amended soils with immobilised fungi reduced the lag phases and increased the extents of

biodegradation. In particular, soils amended with immobilised *T. versicolor* and *B. adusta* resulted in shorter lag phases and higher rates of mineralisation compared to the other soil conditions. However, the presence of immobilised WRFs (*Pleurotus ostreatus* and *Phanerochaete chrysosporium*) showed higher biodegradation as compared to other fungal species. The study suggests that these two immobilised fungi aided higher transport of the phenanthrene to the mycelial network, and in turn, higher diffusion to bacterial and other cells in amended soils (Furuno *et al.*, 2012). The fungus *P. chrysosporium* was the most stimulator of soil C/N ratio in amended soil.

The result showed that the soil pH decreased over time in amended soils. The highest rates and extents of mineralisation were noticeable at slightly acidic pH range (5.8–6.3) in amended soils, which agrees to those reported previously for enhanced PAHs biodegradation in soil (Bishnoi *et al.*, 2008). The changes in the soil pH in the present study suggests the production of toxic metabolites, tannic and humic acids as well as organic acid during mycodegradation of PAH (Ghosal *et al.*, 2016). Enzyme activities increased in amended soils and positively correlated with the extents of mineralisation in all amended soils. Soil amended with *P. ostreatus*-amended soil had the maximum ligninolytic enzyme activities. This result was supported by similar studies during PAH biodegradation (Novotný *et al.*, 1999; Pozdnyakova *et al.*, 2010). It was shown that the addition of SGB-immobilised fungi to the contaminated soil increased the microbial populations (bacteria and fungi) in all amended soils, although the values varied among the soil conditions. PAH-degrading fungal numbers increased with increased soil-PAH contact time. It was observed that microbial numbers were generally high with immobilised *T. versicolor* compared to their counterpart strains in amended soils.

One important factor that may promote phenanthrene degradation in soils amended with organic waste materials will involve seeding the waste materials with indigenous microorganisms that possess dual characteristics such as (i) proficient lignocellulose degraders and (ii) phenanthrene degraders. In this study the catabolic evolution (^{14}C-phenanthrene to $^{14}CO_2$) of five selected and screened indigenous endophytic fungal strains: *Fusarium* sp. (KTS01), *Trichoderma harzianum* (LAN03), *Fusarium oxysporum* (KTS02), *Fusarium oxysporum* (LAN04), and *Clonostachys rosea* (KTS05) under different nitrogen (N) amendments were investigated under different carbon/nitrogen ratios (C:N ratios of 10, 20, and 30 to 1) and nitrogen sources (urea and malt extract and ammonium nitrate) in both static and agitated culture conditions (Chapter 7).

The results revealed that the identified fungal isolates have between 99.8 to 99.9% match with their close relatives retrieved from NCBI database and are members of endophytic ascomycetes. Interestingly, these fungal strains have been reported to show both hydrocarbonoclastic and ligninolytic activity (Mollea *et al.*, 2005; Ding *et al.*, 2008; Acevedo *et al.*, 2011; Noman *et al.*, 2019). Generally, results showed that there were variations in the amounts of ^{14}C-phenanthene mineralisation for most of the fungal strains under all N amendments as well in both static and agitated culture conditions throughout this study. Although static condition promoted greater extents of mineralisation than the agitated condition for most fungal strains which suggests the formation of mycelial mats and likely increase contact between mycelial cells and phenanthrene without shear stress in static cultures and as a result, ligninolytic enzymes may be synthesised (Ding *et al.*, 2008; Acevedo *et al.*, 2011). Also, there were faster rates of mineralisation in all static fungal cultures amended with C/N than agitated culture conditions. In contrast, for N source amendment static and agitated cultures for most fungal strains resulted in faster rates of mineralisation especially for urea and

malt extract, respectively. In this study, agitated cultures amended with N source displayed greater extents of mineralisation compared to the static cultures for most fungal strains. This may likely suggest more oxygen availability, PAH solubility and mass transfer into the aqueous phase for fungal uptake (Johnsen *et al.*, 2005).

The results also showed that incubation condition to a larger extent, depending on the fungal strains, would facilitate their growth and metabolism. In both static and agitated liquid cultures, *Clonostachys rosea* KTS05 and *Fusarium* sp. KTS01 showed significantly shorter lag phases, higher faster rates and greater extents of mineralisation after amendment with C/N ratio of 10:1 compared to the other fungal strains and C/N ratios except for *Fusarium* sp. KTS02 where significantly faster rate than *Fusarium* sp. KTS01 was observed in the agitated culture with the same C/N ratio (10:1). The reduced lag phases may have resulted from a rapid adaptation by the fungal strains in the presence of an appropriate C:N ratio, suggesting that balance carbon and energy may be present for their cell growth (biomass) and metabolism (Di Lonardo et al. 2020; Huang et al. 2020). Interestingly, *Fusarium* spp has been reported to efficiently degrade organic contaminants, including the PAHs (Chulalaksananukul et al. 2006; Pozdnyakova et al. 2019), while this study is the first investigative work reporting the potential of *Clonostachys* sp. in PAH degradation.

For N source amendments, fungal cultures of *Clonostachys rosea* KTS05 and *Trichoderma harzianum* LAN03 showed greater phenanthrene mineralisation in static and agitated conditions, respectively, particularly with malt extract. These observations are consistent with previous studies on the removal of organic contaminants in liquid microcosms by these two fungal strains (Mollea et al. 2005; Chang et al. 2020). Malt extract as N source amendment in both static and agitated cultures is proposed to promote higher phenanthrene mycodegradation

(Hadibarata et al. 2009; Vitali et al. 2006). The differences in biodegradation kinetics observed for all fungal strains may be attributed to differences in their mycelial network, enzyme secretion, growth rates with the target contaminant, and hence the different behaviour observed (Mollea *et al.*, 2005; Kadri *et al.*, 2017). The results suggest the importance of appropriate and specific nutrients amendments for enhanced fungal growth, uptake and metabolism of PAHs.

8.2 Conclusions

The application of organic amendments into the soil represents a viable, sustainable and low-cost approach for (i) promoting the microbial activities in soil contaminated with organic contaminants, and (ii) the improvement of the biodegradation efficiency by indigenous microbes in nutrient-poor contaminated soil. The major findings of this study were that the addition of organic residues - spent brewery grains and spent mushroom compost at 10 % and 20 % and biochars (enhanced and nonenhanced) at 0.01 %, 0.1 and 0.2 % application rates can effectively promote the biodegradation kinetics of ^{14}C-phenanthrene mineralisation and microbial numbers (heterotrophs and PAH-degraders) in soils studied over time. Another finding from this study is that large application rates of organic waste materials can potentially lower PAH mobility, bioavailability and consequently, lead to reductions in the degradation of PAHs in soil. Considering these results, it is possible to enhance the biodegradation of organic contaminants in soil either through the action of immobilising selected fungi on the support material (spent brewery grains) or pre-treating same organic material with the selected fungi prior to soil amendments. Higher extents of mineralisation, microbial numbers and some lignin-modifying and accessory (non-ligninolytic) enzyme activities were increased in amended soils. However, the rates of mineralisation decreased with increasing soil-

phenanthrene interaction over time in most of the amended soils. This study showed that *T. versicolor* and *B. adusta* are more efficient degraders of the PAH in both soils' microcosms (immobilisation and pre-treatment). Also, the finding also revealed that the presence of supplemented nitrogen source can also stimulate higher mycodegradation of PAHs. Therefore, this thesis presents the impacts of organic amendments and some key mechanisms to improve and promote the microbial degradation of PAHs in contaminated soils.

8.3 Recommendations for future research

The addition of fungi immobilised and fungal-pre-treated on spent brewery grains showed significant changes in soil pH after amendment and these values significantly reduced with increases in soil-phenanthrene contact time. The reasons for the reduction and possible metabolites produced which could have resulted in changes in soil pH should be examined before and after amendments to ascertain the metabolites causing a rise in pH level, either it is eliminated or perhaps the addition of a buffer solution to resist any change in soil pH for optimal PAH degradation (Zhang *et al.*, 2011; Neina, 2019).

Although soil amendment with organic materials, in particular, spent brewery grains showed high potentials for the degradation of PAHs and a viable source of nutrient for increasing the biological activities in soil. However, the components of spent brewery grains that are responsible for these positive effects are unknown. In a study carried out by Sun *et al.* (2014) investigated the sorption behaviour of rice straw and its main constituents (lignin, cellulose, and hemicellulose) and in the presence of other co-existing organic pollutants, phenanthrene, benzo[a]pyrene, phenol, and pentachlorophenol. They found that pyrene showed the greatest sorption on lignin due to its greater aromaticity and smaller polarity, and the sorption

coefficient was almost two orders of magnitude greater than those on cellulose and hemicellulose. Similarly, in the presence of phenanthrene, the inhibition was greater on cellulose and hemicellulose than on lignin. This result was attributed to sufficient sorption sites on lignin which made it less important for competitive sorption to occur. In addition, the existence of phenol promoted the sorption of pyrene on rice straw and lignin but inhibited the sorption on cellulose and hemicellulose. In a similar study by Wang *et al.* (2007), the affinity of pyrene, phenanthrene and naphthalene towards lignin was much higher than the chitins and celluloses owing to lack of aromatic carbon compounds in chitins and celluloses. Hence, they conclude that bioavailability of the PAHs in the soil was affected by the presence of lignin. The investigation of each purified constituents and in combination of spent brewery grains and other organic materials in PAH biodegradation in soil may provide more insight for the result obtained in this thesis.

Immobilisation of lignocellulosic and PAH-degrading enzymes might be more useful in the degradation of PAHs in soil (Mester and Tien, 2000; Cajthaml, 2008; Pozdynakova, 2012). However, this technique may be difficult when apply practically in a complex and heavy hydrocarbon contaminated matrix like those encountered during field bioremediation. Therefore, more research is needed to ascertain if these materials would promote higher PAH biodegradation in real life scenarios. In addition, the research was performed under semi-controlled conditions; however, to further evaluate the potential of these organic wastes (spent brewery grains) and the fungal treatment techniques, studies need to be carried out either in the field or in windrows of hydrocarbon contaminated soils to further ascertain the potentials of soil organic amendments.

Microbial numbers obtained from culture-dependent method can only quantify the microbial activities within an environmental sample but lacked the capacity to detect the dominant species in a microbial community. Thus, further studies should involve a culture-independent method such as metagenomics- the *Illumina-based 16S rRNA gene sequencing* which lead to a better understanding of the changes in microbial community and the active microorganisms potentially responsible in PAH degradation after organic amendments in soil. For example, high-throughput sequencing can give us information about the species richness and their distribution and provides also useful information on the functional genes of the new microbial communities (Leff *et al.*, 2015). This method is an unbiased culture-and PCR-independent method for microbial community structure.

Next generation sequencing (NGS) technologies can provide information about the role of key enzymes/functional genes involved in the degradation of the organic contaminant and other environmental samples (Bharagava *et al*., 2019). In addition, culture-based studies are poorly suited for revealing synergistic interactions (bacteria-fungi etc) in the contaminated soils such as several fungal acting as pipelines or highways for bacteria involved for the contaminant degradation (Schamfuß *et al*., 2013). This technique (NGS) can reveal the degradative genes and key actors involved as well new microbial communities useful in PAH biodegradation in soil. Information about the highly prevalent PAH-degrading microbes or genes derived from the metagenomic communities in contaminated soils can help to improve bioremediation. Therefore, the understanding of the biodegradation process will improve our knowledge on how to promote and efficiently monitored a target(s) hydrophobic organic contaminant in soil.

However, some researchers have reported a gap between these two methods (culture-dependent and culture-independent) but no study has fully investigated the biases associated with these

two methods of culturing microorganisms in a bioremediation study (Stefani *et al.*, 2015). In this study, it was observed that specific application rates of spent mushroom compost and biochar increased the mineralisation of PAH (Omoni *et al.*, 2020b). Therefore, the application of mixtures of spent brewery grains with spent mushroom compost and biochar, respectively might result in a synergistic effect on the extent of degradation of PAHs in soil. The same approach can also be applied using fungal mycelia immobilised on these substrates and fungal-pre-treated substrates. Further studies should examine the application of these two organic materials and their mixtures for better PAH biodegradation in soil.

Soil-phenanthrene contact time reduced the mineralisation of PAHs and biological activities in soil; thus, greater PAHs degradation and increased soil biological activities can be achieved with surfactant amendment to PAHs contaminated soils in order to increase the bioavailability of PAHs by reducing interfacial tension and improving its water solubility as well amend microbes in the soil (Bezza, 2016; Patel *et al.*, 2020; Guo and Wen, 2021). The enzymes tend to decrease activity following increases in soil-PAH interaction in this thesis which indicates that either the fungal secretion of enzymes was affected by decreased bioavailability, thus the use of surfactant may increase the secretion and stimulation of enzymatic action (Guo and Wen, 2021).

Selected white-rot fungi with high degradative capacity for both PAHs and lignocellulose should be investigated in the remediation of hydrocarbons contaminated soils following amendments. More so, other lignin-degrading accessory enzymes such as aryl alcohol oxidase, glyoxal oxidase cellobiose dehydrogenase, superoxide dismutase, quinone reductase that cannot degrade lignin on their own but are important to complete the process of lignin and PAH

degradation should be examined after lignocellulosic amendment in soil to observe their potential roles during the degradation process (Janusz *et al.*, 2017).

Soil pH (acidity or alkalinity) can be neutralised or adjusted by the addition of lime especially in large scale or windrows remediation (Bamforth and Singleton, 2005; Tomei and Daugulis, 2013) or the addition of buffer solution should be considered and investigated for enhanced bioremediation of fresh and aged contaminated soils after organic wastes amendment.

To further ascertain the potentials of the organic materials in promoting PAH biodegradation, studies should investigate and compare amendments with standard N and P additions and these organic wastes in contaminated soils. Also, C:N:P ratios suitable for biodegradation of organic contaminants in soil should be examined from a mixture of these organic materials (Huang *et al*., 2020; Medaura *et al*., 2021). And these strategies should also be investigated on mixed and aged PAH contaminated soils to observe their roles in bioremediation (Winquist *et al.*, 2014; García-Delgado *et al.*, 2015; Andriani and Tachibana, 2016; Lukic´ *et al*., 2016).

To scaled up the treatment methods, the addition of organic and/or inorganic co-contaminants to displaced PAHs from limited absorption sites should be added (White *et al.*, 1999; Stroud *et al.*, 2009; Yu *et al.*, 2018). This will further increase the bioaccessibility and mobility of the contaminant to the microbial uptake and metabolism.

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Appendix

Impact of digestate and its fractions on mineralization of ^{14}C-Phenanthrene in aged soil.

Impact of Digestate and its fractions on mineralization of ^{14}C-Phenanthrene in Aged Soil

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1. Abstract

The impact of whole digestate (WD) and its fractions (solid [SD] and liquid [LD]) on ^{14}C-phenanthrene mineralization in soil over 90 d contact time was investigated. The ^{14}C-phenanthrene spiked soil was aged for 1, 30, 60 and 90 d. Analysis of water-soluble nitrogen, phosphorus, total (organic and inorganic) carbon, and quantitative bacterial count were conducted at each time point to assess their impact on mineralization of ^{14}C-phenanthrene in soils. Indigenous catabolic activity (total extents, maximum rates and lag phases) of ^{14}C-phenanthrene mineralization were measured using respirometric soil slurry assay. The soil amended with WD outperformed the SD and LD fractions as well as showed a shorter lag phase, higher rate and extent of mineralization throughout the study. The digestates improved ($P<0.05$) the microbial population and nutritive content of the soil. However, findings showed that spiking soil with phenanthrene generally reduced the growth of microbial populations from 1 to 90 d and gave a lower nutritive content in comparison with the non-spiked soil. Also, soil fertility and bacteria count were major factors driving ^{14}C-phenanthrene mineralization. Particularly, the non-phenanthrene degraders positively influenced the cumulative mineralization of ^{14}C-phenanthrene after 60 d incubation. Therefore, the digestates (residue from anaerobic digestion) especially WD, which enhanced ^{14}C-phenanthrene mineralization of the soil without minimal basal salts medium nor additional degraders should be further exploited for sustainable bioremediation of PAHs contaminated soil.

Keywords: soil fertility; phenanthrene; mineralization; degraders; heterotrophs; digestate

2. Introduction

Environmental pollution is a global concern particularly due to increasing persistent organic pollutants (POPs) in soil. One of such group of contaminants is polycyclic aromatic hydrocarbons (PAHs), found in alarming concentrations and consequently potentially harmful to both the population and ecosystem (Steffen et al. 2007; Ibeto et al. 2019). Depending on the number and arrangement of their fused rings, PAHs are known to have carcinogenic and mutagenic effects, and are potent immunosuppressants (Rajendran et al. 2013). PAHs deposition into the environment are usually from natural (petrogenic sources through thermal geologic production) and anthropogenic (e.g. incomplete combustion of organic matter and fossil fuels) sources (Tang et al. 2005; Oyelami et al. 2015). In the soil, PAHs become persistent and recalcitrant to microbial degradation. This could be attributed to their low aqueous solubility, polarity, lipophilicity adsorption to soil micropores and matrices (Macleod and Semple 2000; Northcott and Jones 2000; Wu et al. 2013).

Microbial degradation of PAHs through mineralization is a well-known approach to remediating soils polluted with PAHs (Peng et al. 2008; Ghosal et al. 2016). This is because of the degrading effect of some microbial enzymes on PAHs (Rhodes et al. 2010; Obuekwe and Semple 2013; Ogbonnaya et al. 2016; Umeh et al. 2018). However, soil nutrients are essential for microbial activities and degradation of PAHs (Chiu et al. 2009). These nutrients are abundant in biodegradable wastes sourced from farms and food industries. A more readily available form of the nutrients is abundant in digestate, compost, sewage sludge and farmyard manure (Chiu et al. 2009; Agamuthu et al. 2013). Several studies have reported the potential of nutrients from biodegradable waste for the mineralization of PAHs in soil (Christensen et al. 2004; Zhang et al. 2012; Chen et al. 2015; Kästner and Miltner 2016). Digestate, a residue

from anaerobic digestion has gained more attention as nutrient-rich (nitrogen and mineral elements) organic fertilizer in nutrient-poor agricultural soils (Nkoa 2014; Möller 2015; Fagbohungbe et al. 2019). In agricultural soils, depending on the nutrient requirement and planting season, the digestate can be applied in different forms: whole, solid and liquid fractions (Ibeto et al. 2020; Johansen et al. 2013; Liedl et al. 2006; Tiwary et al. 2006). The application of digestate to enhance soil remediation is a favourable development as it is expected to further broaden its current use. In developing countries, these cheap, value-added, nutrient-rich and abundant sources of microbial nutrients indiscriminately end up in the receiving environment. However, the application of digestate could potentially influence biological and microbial proliferation in soil matrices, especially in the oil polluted areas, where there is staggering soil contamination. Digestate is a potential microbial stimulator and could facilitate the rates and extents in which PAHs can be metabolized in poor-nutrient contaminated soils (Leahy and Colwell 1990). Presently, there is no published literature on either the use of digestate or its fractions on the mineralization of PAHs in soil. Therefore, this study aimed to investigate (i) changes in soil properties in digestate amended phenanthrene spiked and non-spiked soil (ii) the impact of digestate forms: WD, SD, and LD on ^{14}C-phenanthrene mineralization in soil and (iii) the catabolic activities through microbial numbers (heterotrophic and phenanthrene-degrading bacteria) in both amended and unamended, ^{12}C-phenanthrene spiked and non-spiked soils.

3. Materials and methods

3.1 Materials

Non-labeled phenanthrene (^{12}C) and sodium hydroxide were obtained from Sigma Aldrich Co., Ltd, UK. Radio-labeled phenanthrene 9-^{14}C (radiochemical purity > 96%, specific activity =

55.7mCi $mmol^{-1}$) was obtained from American Radio labeled chemical, USA. General-purpose microbiological agar (agar-agar), plate count agar (PCA), Ringer's solution tablet (general purpose grade), minimal basal salt solution (MBS) recipes, Amphotericin-B and sodium hydroxide were purchased from Fisher Scientific, UK, and Goldstar liquid scintillation fluid was acquired from Meridian, UK. Anaerobic digestate: WD and SD were collected from Cockerham Green Energy Ltd, Lancaster, UK. LD was obtained from mechanically separating WD using a centrifuge at 3600 rpm for 10 mins.

3.2 Digestate and Soil Analysis

Digestate and soil properties: pH, electrical conductivity, moisture content, loss on ignition, water-soluble N- nitrate and ammonium (NO_3^-, NH_4^+), phosphate (PO_4^{3-}), total organic carbon (TOC), total carbon (TC) and inorganic carbon (IC) were determined using standard methods. pH and EC were analysed using a 1:2.5 and 1:5 soil/digestate: Milli Q water proportion (dry weight:volume), respectively. Samples were shaken for 30 mins at 100 rpm and then centrifuged at 3600 rpm for 10 mins prior to analysis. Moisture content was analysed by oven drying at 105 ˚C till constant weight while the organic matter content indicated by loss on ignition (LOI) of each soil was measured after combustion at 550 ˚C in a furnace for 24 h (APHA 1998).

The samples were extracted using a 1:4 soil/digestate: Milli Q water (weight: volume) then shaken for 1 hr at 120 rpm using a horizontal shaker, centrifuged for 10 mins at 3600 rpm, filtered through a 0.45 µm membrane filter, before NO_3^-, NH_4^+, and PO_4^{3-} analysis (Alef and Nannipieri 1995; Forster 1995). The water-soluble N and P of soil extracts were then

determined using autoanalyzer model 3HR (AAR 3HR) (Haney et al. 2008), while the total organic and inorganic carbon were measured using Shimadzu TOC-L CPH (Siudek et al. 2015).

3.3. Soil preparation

A pristine uncontaminated soil classified as clay-loamy (Towell et al. 2011a) was obtained from Myerscough Agricultural College (Preston, UK) for this study. The soil was collected from a depth of approximately 5–30 cm, air-dried and sieved through a 2 mm mesh to remove large organic fragments/stones and stored at 4 ^{o}C until required. During the experimental setup, the soil was prepared into three portions as follows: a) spiked with 100 mg kg^{-1} non-labeled (^{12}C) phenanthrene, b) spiked with 100 mg kg^{-1} non-labeled (^{12}C) phenanthrene and 93.3 Bq g^{-1} radio-labeled (^{14}C) phenanthrene and c) Non-spiked soil (Vázquez-Cuevas et al. 2018). To achieve the required concentration of 100 mg kg^{-1} phenanthrene in the soil, one-quarter of soil (700 g) was spiked with ^{12}C phenanthrene (dissolved in acetone:soil ratio of 1:20. Acetone serves as a carrier) and then homogenized with other parts and allowed to volatilize for 2–3h in a fume hood. Thereafter, the soils (both ^{12}C-phenanthrene-spiked and non-spiked) were amended with 0.1% digestate (WD, SD, and LD) to-soil concentration while the unamended soil spiked with ^{12}C-phenanthrene served as the control. The application rate was based on the optimal mix ratio from a pre-study carried out on different digestate amendment-to-soil concentrations (0.01, 0.1, 1 and 10%) using the WD and how each amended soil influenced the cumulative extent of ^{14}C-phenanthrene mineralization after 14 d soil-PAH contact time (Supplementary material; Figure S1, Tables S1, and S2). The amended, unamended, spiked and non-spiked soils were then kept in paraffin sealed 500 ml amber glass jars and incubated in the dark at 21 ± 1°C for 1, 30, 60 and 90 d time points or contact times.

3.4 ^{14}C-phenanthrene mineralization in soils

To assess the digestate-assisted microbial degradation, mineralization of ^{14}C-phenanthrene were assessed after each incubation time point (1, 30, 60 and 90 d) in the amended soil (Reid et al. 2001; Doick and Semple 2003). The soils were spiked with ^{14}C-phenanthrene Standard (93.3 Bq g^{-1}dry wt $soil^{-1}$) and incubated in a rotary shaker at 100 rpm and 21 ± 1°C for 14 d. Respirometric soil-slurry assays (in triplicates) were carried out in 250 mL Schott bottles, incorporated with a suspended 7 ml glass scintillation vial containing 1 ml 1 M NaOH solution that served as a $^{14}CO_2$ trap. Each respirometer contained 10 ± 0.2 g soil and 30 mL of deionized water to achieve a liquid:solid ratio of 3:1, which is recommended for improved reproducibility and greater overall extents of mineralization (Doick and Semple, 2003). The $^{14}CO_2$ mineralized in the trap was assessed daily by addition of Goldstar liquid scintillation cocktail followed by counting using a Packard Canberra Tri-Carb 2250CA liquid scintillation counter with standard calibration and quench correction techniques. The biodegradation parameters assessed in this study were (i) lag phase, defined as the time taken to reach 5% mineralization considering the upper and lower contact times and values obtained from the scintillation counter. (ii) the fastest rate is the maximum rate of $\%^{14}CO_2$ evolution d^{-1} determined from the increase in mineralization between each sampling point and (iii) the cumulative extent of mineralisation is expressed as a percentage of the initial ^{14}C-phenanthrene, that has been mineralised to $^{14}CO_2$ during each sampling time. It is the cumulative mineralization for the 14 days incubation (respirometry assay). Control soil (pristine soil with $^{12}C/^{14}$C-phenanthrene but without amendment) and an analytical blank (pristine soil without ^{14}C-phenanthrene and amendment) were also set up during the 14 d experiment.

3.5 Quantitative bacterial enumeration

Bacterial numbers (heterotrophic and phenanthrene-degrading bacteria) in each soil sample were determined by spread plate techniques (Oyelami et al. 2013). Soil (1.0 ± 0.1 g dry wt) before respirometry was extracted with Ringer's solution in 1:10 mixture (soil: ringer solution). Thereafter, the mixture was serially diluted before uniformly spreading 0.1 ml on plate count agar (heterotrophs) supplemented with amphotericin-B (5µl ml^{-1}) and MBS agar plates (phenanthrene-degraders) supplemented with both amphotericin-B (5µl ml^{-1}) and ^{12}C-phenanthrene amendment (0.05 mg ml^{-1}). MBS solution Agar plates were incubated at 29 ±1°C, and CFUs were counted after 48 h and 5 d for heterotrophic and phenanthrene-degrading bacteria respectively, for the different soil-PAH contact times (1, 30, 60 and 90 d).

3.6 Statistical analysis

Using SPSS (version 22), data was statistically analyzed by analysis of variance (ANOVA) and Tukey's Post Hoc test where equal variance was assumed while Welch ANOVA and Games Howell's Post Hoc test were used where equal variance was not assumed. Post-hoc test ($P<0.05$) was used to determine any significant differences in means of amended soils (WD, SD and LD) for the lag phases, fastest rates, extents of mineralization, and bacteria (total heterotrophs and phenanthrene degraders) count at each time point (1, 30, 60 and 90 d). Also, the influence of digestate-amendment on soil properties compared with control (without amendment) was also analyzed. Spearman's correlation was used to ascertain the relationship between bacterial numbers, soil properties and ^{14}C-mineralization. Data was presented as mean ± standard error and the graphs were plotted using SigmaPlot 10.0 version.

4. Results

4.1 Physicochemical properties of the amended PAH spiked and non-spiked soils

Table 1 shows differences between the properties of the digestates. Fibre digestate had the highest organic matter content with the lowest pH while whole digestate had the highest pH. The soil had very low organic matter (5.05%) which indicates the need for the amendments.

Table 1: Physicochemical properties of the soil and digestates

Material	pH	Electrical Conductivity (ms/cm)	Moisture Content (%)	Organic matter (%)
WD	8.36±0.01	10.44±0.18	89.43±0.34	72.86±0.22
SD	7.96±0.03	2.82±0.02	73.81±0.54	86.21±0.60
LD	8.29±0.02	10.95±0.05	94.23±0.15	65.19±1.13
Control Soil	6.29±0.02	0.05±0.00	17.16±0.15	5.05±0.10

The amount of water-soluble N (NH_4^+-N and NO_3^--N), P (PO_4^{3-}-P) and carbon (TC, IC, TOC) in phenanthrene-spiked and non-spiked digestate amended soils after 1, 30, 60 and 90 d soil contact times are shown in Table 2. The non-amended soil (5.61 ± 0.10 mg/l) and WD (5.21 ± 0.32 mg/l) for phenanthrene spiked and non-spiked soils respectively had the highest NH_4^+ concentrations after 30 d incubation. Also, the other treatments (SD and LD) had a higher amount of NH_4^+ after 30d compared to the other time points. The NO_3^--N content increased in all the phenanthrene-spiked soils ($p < 0.05$) with increase in contact time, except at 30 d where NO_3^--N was only detected in soil amended with WD. In contrast, a noticeably higher NO_3^--N content in all non-phenanthrene digestate amended soils was observed after 1 d soil incubation

and the highest amount was 19.55 ± 1.58 mg/l for LD amended soil. Both phenanthrene and non-phenanthrene spiked digestate amended soils showed no noticeable effect on PO_4^{3-}-P level throughout the studied period. However, after 1 and 30 d of soil-phenanthrene contact time, the PO_4^{3-}-P level was significantly higher in non-phenanthrene spiked than in spiked soils (except for WD after 30 d). For TOC, the highest concentration was observed for SD in both spiked (470.79 ± 7.89 mg/l) and non-spiked (530.60 ± 30.33 mg/l) soils after 30 d of soil interaction with digestate. However, as the soil-phenanthrene contact increased, the TOC significantly decreased ($p < 0.05$) in WD and SD amended phenanthrene spiked and non-spiked soils.

Table 2. Physicochemical properties of PAH spiked and non-spiked soils with different digestate forms (1 – 90 d incubation)

		Phenanthrene Spiked Soil (mg/kg soil dw)				**Non-Spiked Soil (mg/kg soil dw)**			
Soil contact time (days)	Amendment	NH^{+}_4	NO_3^-	PO_4^{3-}	Total Organic carbon	NH^{+}_4	NO_3^-	PO_4^{3-}	Total Organic carbon
1	Whole	2.34 ± 0.18	2.66 ± 0.26	2.30 ± 0.12	133.64 ± 1.43	2.86 ± 0.19	16.41 ± 2.07	2.92 ± 0.10	85.35 ± 7.51
	Solid	4.15 ± 0.46	2.47 ± 0.09	2.36 ± 0.02	120.86 ± 8.46	2.62 ± 0.05	14.41 ± 1.10	3.17 ± 0.05	89.09 ± 10.91
	Liquid	2.94 ± 0.20	3.56 ± 0.36	2.52 ± 0.16	97.31 ± 2.65	3.21 ± 0.16	19.55 ± 1.58	3.24 ± 0.07	83.74 ± 11.24
	Control	2.49 ± 0.06	0.08 ± 0.02	2.79 ± 0.10	82.23 ± 1.30	3.71 ± 0.23	9.07 ± 1.23	2.82 ± 0.12	80.95 ± 0.88
30	Whole	4.37 ± 0.29	0.05± 0.01	2.28 ± 0.07	440.39 ± 27.32	5.21 ± 0.32	6.80 ± 0.51	2.15 ± 0.02	507.5 ± 15.82
	Solid	4.68 ± 0.12	ND*	2.18 ± 0.12	470.79 ± 7.89	4.25 ± 0.94	9.78 ± 0.52	2.37 ± 0.16	530.60 ± 30.33
	Liquid	4.73 ± 0.10	ND*	2.08 ± 0.02	116.91 ± 16.60	5.11 ± 0.38	2.94 ± 0.51	2.40 ± 0.13	507.89 ± 33.06
	Control	5.61 ± 1.83	ND*	1.99 ± 0.06	75.37 ± 0.64	2.02 ± 0.02	6.09 ± 0.39	2.17 ± 0.07	92.87 ± 6.85
60	Whole	2.40 ± 0.44	4.15 ± 0.28	2.88 ± 0.02	155.09 ±20.75	2.66 ± 0.11	8.37 ± 0.47	2.61 ± 0.13	224.53 ± 20.41
	Solid	2.55 ± 0.16	2.72 ± 0.19	2.92 ± 0.08	131.08 ± 13.28	4.78 ± 0.68	6.79 ± 0.31	2.41 ± 0.13	173.10 ± 8.78
	Liquid	2.06 ± 0.21	4.25 ± 0.27	2.99 ± 0.07	177.06 ± 10.44	2.32 ± 0.06	8.43 ± 0.28	2.18 ± 0.08	171.65 ± 19.27
	Control	2.00 ± 0.15	1.45 ± 0.08	2.85 ± 0.02	169.28 ± 13.68	2.61 ± 0.50	5.58 ± 0.31	1.98 ± 0.04	160.79 ± 3.52
90	Whole	0.28 ± 0.02	5.82 ± 0.14	2.39 ± 0.11	193.91 ± 4.95	1.91 ± 0.14	6.31 ± 0.27	2.36 ± 0.08	159.87 ± 1.23
	Solid	0.22 ± 0.02	4.02 ± 0.14	2.56 ± 0.09	172.86 ± 0.85	0.27 ± 0.04	6.23 ± 0.44	2.50 ± 0.01	175.39 ± 14.01
	Liquid	0.08 ± 0.04	5.77 ± 0.54	2.48 ± 0.08	139.14 ± 3.68	0.23 ± 0.04	6.34 ± 0.36	3.00 ± 0.08	161.11 ± 4.36
	Control	0.92 ± 0.30	2.77 ± 0.12	2.46 ± 0.02	137.65 ± 5.61	0.19 ± 0.02	6.21 ± 0.24	2.89 ± 0.04	148.96 ± 3.36

ND* = non-detected; Values are mean ± standard error (n = 3)

4.2 Mineralization of ^{14}C-Phenanthrene in Soil

The catabolic response to WD, SD, and LD on ^{14}C-phenanthrene mineralization was investigated for 14 d after 1, 30, 60, 90 d soil-phenanthrene contact times (Figure 1 and Table 3). The results showed that the digestate amended soils generally influenced the lag phase, mineralization rate and the total extent of ^{14}C-mineralization. There was a shorter lag phase, higher rate and longer extent of ^{14}C-phenanthrene mineralization after 60 d contact time. Following 1 d time point, lag phases were significantly shorter with increasing contact time for all fractions of digestate-amended soils than control soil except after the 60d. The length of the lag phase varied depending on the amended digestate fraction. The data showed that WD had a shorter lag phase compared to the other fractions (WD < LD < SD) in the PAH-amended soils ($p < 0.05$). The shortest lag phase was observed at 60 d for SD (0.38 ± 0.00) and longest (3.58 ± 0.01) for control after 1 d incubation. More so, control soils generally had a longer lag phase compared to soil amendments except for 90 d time point.

The results of maximum rates of ^{14}C-phenanthrene mineralization in digestate amended-phenanthrene spiked soil showed that SD had the fastest rate of ^{14}C-phenanthrene mineralization (1.09 ± 0.02) compared to those of WD and LD. The amendments had significant effect ($p < 0.05$) on maximum rates of ^{14}C-phenanthrene mineralization during soil ageing. Infact, significantly higher rates of mineralization were observed for all treatments after 60 d of soil incubation.

Table 3. Lag phases, maximum rates and cumulative extents of ^{14}C-phenanthrene mineralization during the 90 d aging period

Phenanthrene Spiked Soil				
Soil contact time (days)	Amendment	Lag phase (before Mineralization $^{14}CO_2$ ≥5%) d	Fastest rate (% $^{14}CO_2$ d^{-1})	Cumulative Extent (%)
1	Whole	2.33 ± 0.00	13.91 ± 0.10	29.93 ± 0.12
	Solid	3.38 ± 0.00	8.79 ± 0.02	23.39 ± 0.21
	Liquid	2.67 ± 0.01	7.51 ± 0.08	25.91 ± 0.15
	Control	3.58 ± 0.01	5.82 ± 0.02	14.18 ± 0.05
30	Whole	0.68 ± 0.01	12.59 ± 0.48	26.36 ± 0.34
	Solid	0.86 ± 0.01	9.41 ± 0.18	23.74 ± 0.84
	Liquid	0.74 ± 0.02	10.13 ± 0.09	25.23 ± 0.41
	Control	2.91 ± 0.07	4.40 ± 0.59	9.34 ± 0.04
60	Whole	0.40 ± 0.00	22.81 ± 0.07	31.26 ± 0.21
	Solid	0.38 ± 0.00	26.08 ± 0.48	28.58 ± 0.61
	Liquid	0.40 ± 0.00	22.03 ± 0.90	29.94 ± 0.11
	Control	1.32 ± 0.03	7.89 ± 0.99	16.80 ± 0.07
90	Whole	0.85 ± 0.01	8.75 ± 0.18	23.86 ± 0.33
	Solid	0.82 ± 0.01	9.37 ± 0.39	22.71 ± 0.33
	Liquid	0.76 ± 0.08	9.94 ± 0.22	21.25 ± 0.51
	Control	0.58 ± 0.02	17.80 ± 0.83	20.47 ± 0.06

Values are mean ± standard error (n = 3)

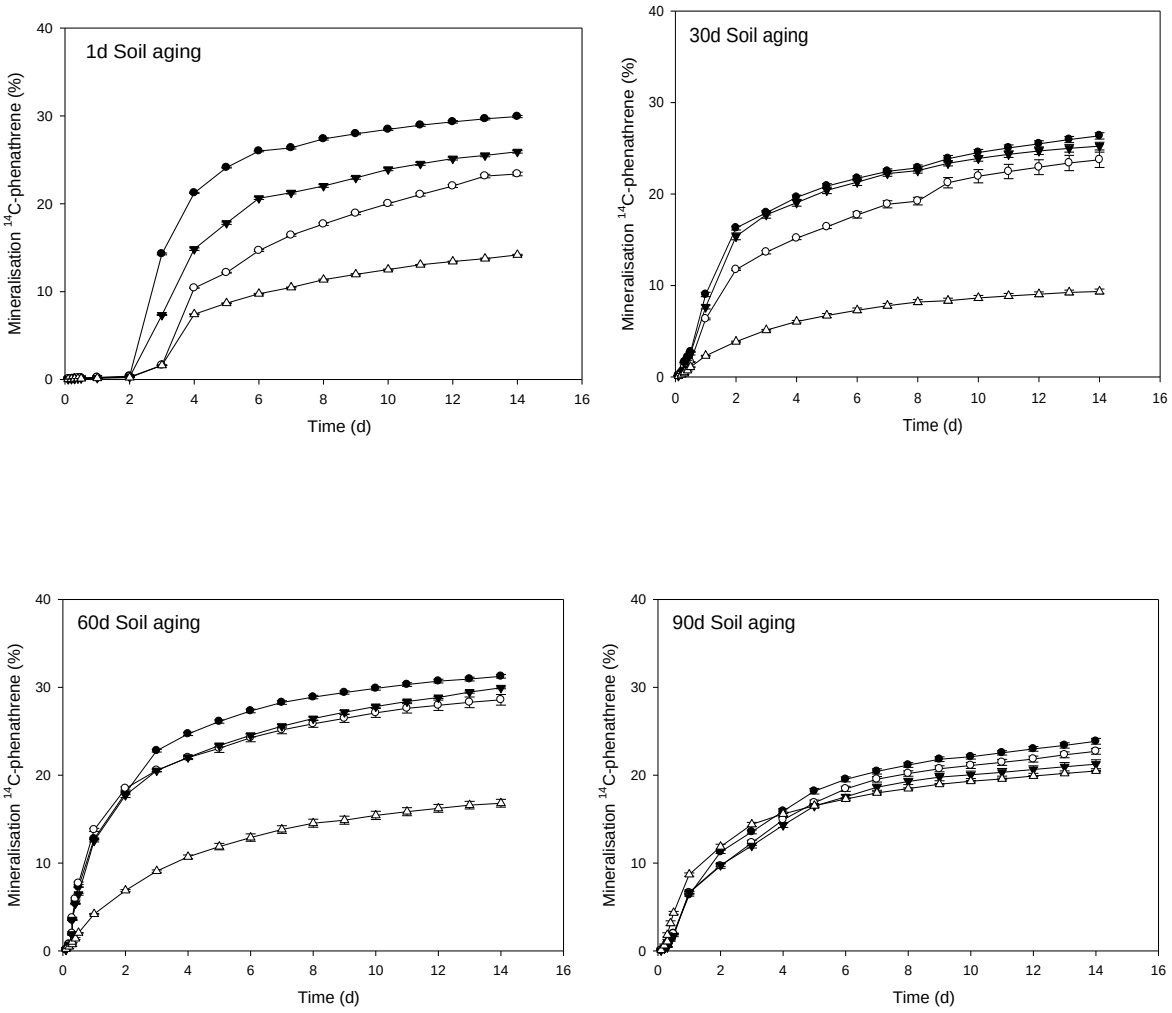

Figure 1. Evolution of $^{14}CO_2$ from the catabolism of ^{14}C-phenanthrene in 100mg/kg phenanthrene spiked soil amended with WD, SD and LD. [WD (●); SD (○); LD (▼); control (Δ)]. Error bars represent standard error of mean (SEM) of triplicate samples (n = 3).

Results showed that the amount of ^{14}C-phenanthrene mineralized in WD amended soils were consistently higher than other treatments (SD and LD) throughout the study period. Extent of ^{14}C-phenanthrene mineralized in digestate-amended soils ranged from 21.25 to 31.26% and this was significantly higher ($p < 0.05$) than the unamended soil. The effect of the digestate fractions in PAH-spiked soils with increasing contact time showed there was no consistent trend in the cumulative extent of mineralization (1 d to 90 d).

Correlation studies showed some relationships between mineralization and soil properties. There was a strong positive correlation between NH_4^+-N nitrification and maximum rate of mineralization after 1 d ($r = 0.974$, $p = 0.000$) contact time. NO_3^--N availability in soil showed a strong significant positive correlation with cumulative extent of mineralization after 60 d ($r = 0.884$, $p = 0.001$). More so, TOC correlated positively with cumulative extent after 90 d ($r = 0.720$, $p = 0.008$) and phosphate correlated with maximum rate after 30d ($r=0.709$, $p=0.01$) incubation periods.

4.3 Enumeration of soil bacteria

Total heterotrophs and phenanthrene degraders were studied in both spiked and non-spiked digestate amended soils. Results showed significantly higher CFUs for heterotrophs at all time points for both spiked and non-spiked amended soils when compared to the soil without any amendment and phenanthrene (control). Based on the treatment of soil with phenanthrene, the heterotrophic and phenanthrene-degrading bacterial numbers were higher in spiked soil than in non-spiked soil including control soil (Table 4).

Table 4. Colony forming units (CFUs) of total heterotrophs and phenanthrene degraders in amended PAH spiked and non-spiked soils during the 90 d aging period

		Phenanthrene Spiked Soil (CFU x 10^7 g^{-1} soil dw)		**Non-Spiked Soil (CFU x 10^6 g^{-1} soil dw)**	
Soil contact time (days)	Amendment	Heterotrophs	PHE-degraders	Heterotrophs	PHE-degraders
1	Whole	39.0 ± 1.8	1.77 ± 0.56	187.9 ± 4.5	1.39± 0.07
	Solid	27.4 ± 1.8	1.07 ± 0.53	63.6 ± 4.5	1.48 ± 0.09
	Liquid	24.0 ± 2.5	1.19 ± 0.69	137.2 ± 4.6	1.97 ± 0.05
	Control	21.7 ± 0.7	0.93 ± 0.21	60.8 ± 1.1	4.37 ± 0.02
30	Whole	33.9 ± 0.7	0.23 ± 0.01	46.1 ± 3.3	1.93 ± 0.22
	Solid	15.4 ± 0.7	0.17 ± 0.01	72.9 ± 4.0	1.59 ± 0.09
	Liquid	13.8 ± 0.9	0.20 ± 0.01	91.3 ± 2.2	2.11 ± 0.04
	Control	4.5 ± 0.2	0.13 ± 0.01	28.7 ± 1.8	0.69 ± 0.01
60	Whole	49.8 ± 0.0	0.32 ± 0.03	22.5 ± 0.2	2.84± 0.08
	Solid	40.2 ± 3.2	0.30 ± 0.02	8.9 ± 0.3	0.82 ± 0.03
	Liquid	41.0 ± 0.8	0.31 ± 0.02	11.3 ± 0.1	0.95 ± 0.02
	Control	17.2 ± 3.2	0.25 ± 0.01	53.1 ± 0.2	0.63± 0.01
90	Whole	26.9 ± 0.2	0.35 ± 0.01	111.0± 1.5	2.40 ± 0.11
	Solid	27.0 ± 0.7	0.58 ± 0.01	72.4 ± 3.5	4.03 ± 0.03
	Liquid	40.3 ± 1.0	0.59 ± 0.03	100.2 ± 2.1	2.61 ± 0.10
	Control	16.7 ± 0.7	0.50 ± 0.05	68.8± 1.2	2.27 ± 0.05

Values are mean ± standard error (n = 3)

Soils amended with digestate increased the phenanthrene-degraders in both phenanthrene spiked and non-spiked soils with WD and LD recording higher phenanthrene-degrading CFUs at most time points during the study period. The data also showed that digestate amended soils with phenanthrene exhibited higher CFU of phenanthrene-degrading bacterial numbers at all time points compared to amended soil without phenanthrene. However, this bacterial numbers decrease with time in spiked soil as compared to 1 d contact time, while results from digestate amended soils without phenanthrene subsequently showed an increase in the CFUs of

phenanthrene-degraders. For amended conditions, WD (spiked soil) recorded the highest number of degraders (1.77 x 10^7 ± 0.56 CFU g-1 soil) at 1d time point, while the lowest phenanthrene degrading numbers (0.08 x 10^7 ± 0.03) was observed for SD after 60 d time point. Both soils (spiked and non-spiked) displayed an increase CFUs after 90 d as compared to 30 and 60 d incubation. The results of the Spearman correlation showed a negative correlation (r= -0.935, p=0.000, r= -0.935, p=0.000 and r= -0.715, p=0.009) between the phenanthrene degraders and lag phase for time points 1, 30 and 60 d respectively. As the lag phase shortened, phenanthrene degrading number increased in amended soils. Also, CFUs of phenanthrene degraders and heterotrophs correlated positively with cumulative extent and maximum rate after 1 d and 30 d, while only heterotrophs had a strong positive correlation with the cumulative extent at time point 3 which had the optimum mineralization for all amended soils.

5. Discussion

5.1 Influence of incubation time on soil properties in phenanthrene spiked and non spiked soils

This study was designed to compare the impact of whole, liquid and fibre digestate on phenanthrene mineralization in soil by measuring the lag phases, rates and extents of mineralization, heterotrophs, phenanthrene degraders and the soil physicochemical properties. The results revealed that all digestate forms in soil varied with soil properties. The digestate (WD and LD) amended phenanthrene spiked soils resulted in higher NO_3^--N over time. However, the non-phenanthrene spiked soil amended with LD showed highest NO_3^--N which was significantly higher ($p < 0.05$) than that of the spiked soil. Also, nitrate levels in non-spiked soils were higher at all time points than spiked soils. The conversion of NH_4^+-N to NO_3^--N by

nitrification depends on the bacterial strain, soil type and soil conditions (pH, temperature, moisture content and oxygen concentration (Ghaly and Ramakrishnan 2013). Therefore, it could be concluded that the addition of phenanthrene to soil partially inhibited microbial growth which may have played an important role delaying the nitrification process after 30 d in the spiked soil.

Microbial degradation of organic contaminants depends on the bioavailability of accessible carbon, nitrogen, and phosphorus (Leys et al. 2015). Comparing the phenanthrene spiked and nonspiked soil, digestate amendment was found to have a higher effect on phosphate level after 1 d and 30 d incubation in non-phenanthrene spiked soil, although it was observed that increasing contact time (after 60 d) resulted to a higher level of phosphate in amended spiked soils (especially in WD and SD). TOC was higher ($P<0.05$) in spiked than non-spiked for the 1^{st} and 90^{th} (apart from LD) days. It plays an important role in the partition and retention of PAHs in soil, especially at high concentration in soil (Okere, et al. 2017; Nam et al. 2009).

5.2 ^{14}C-phenanthrene biodegradation in digestate amended soils

The influence of digestate and its fractions (WD, SD and LD) on the lag phase, mineralization rates and overall cumulative extent of mineralization in phenanthrene spiked soil were studied over time (1, 30, 60 and 90 d). Generally, the digestate amendments in soil shortened the lag phases, increased the fastest rate and extents of ^{14}C-phenanthrene mineralization with increase in soil contact time specifically from 1 d to 60 d in comparison with the control (unamended soil). Studies have also shown that nutrients amendments in PAH contaminated soil have the potential to stimulate biodegradation through the supplier of nutrient and microbial inoculates (Namkoong et al. 2002; Chiu et al. 2009; García-Delgado et al. 2015). Hence, the addition of

digestate in the soil could enhance the degree of biodegradation of organic contaminants. Therefore biodegradation potential of PAH in soil may be influenced by the amount of nutrient and organic carbon in contaminated soil (Zhang et al. 2012).

The lag phase is an indication of the microflora adaptation or acclimatization to the presence of ^{14}C -phenanthrene, resulting in increased mineralization (Couling et al. 2010; Rhodes et al. 2010). Addition of digestate to soil had an effect on the adaptation of the soil microbial community as revealed from the data obtained in this study. Soil amendment with digestate and its fractions after 1 d soil-phenanthrene contact time, influenced the lag phase. Furthermore, WD treated soil had significantly shorter lag phase than the other fractions (SD and LD), however, the shortest lag phase was observed for SD after 60 d incubation. The lag phase is the time for microbial adjustment to a new soil environment and therefore a reduction in lag phase consequently revealed microbial adaption (Macleod and Semple 2002). As the length of the lag phase reduced over time, there was an increase in the number of phenanthrene-degraders which is further evidence from the strong negative correlation observed between lag phase and phenanthrene-degraders in amended soil. Other studies have reported similar results over time (Umeh et al. 2018; Oyelami et al. 2013; Oyelami et al. 2015).

Micronutrients from organic amendments could enrich and facilitate mineralization of ^{14}C-phenanthrene in soil. For instance, Horel and Schiewer (2009) reported that the addition of N and P releasing fertilizers increased respiration by 76% and 119% respectively for over 4-month period in contaminated Alaskan soil. In this study, nutrient treatment in the form of N and P from the digestates had a stimulatory effect on phenanthrene mineralization compared to the control soil except for 90 d.

WD showed a significantly faster rate of mineralization of ^{14}C-phenanthrene with stronger catabolic potential compared to the other amendments after 1 and 30 d of soil incubation. This can be attributed to the higher number of phenanthrene degraders and available nitrogen. From the data obtained, the number of PAH degrading bacteria for WD increased after 1 d incubation with a corresponding lower lag phase. In general, digestate amended soils for most soil-phenanthrene contact time did not influence the rate of mineralization. This may be attributed to the phenanthrene fraction that was rapidly desorbable in the aqueous phase in the soil for microbial attack and sequestration (Ogbonnaya et al. 2014). It can also be attributed to the differences in the digestates' nutrient content, microbial preferred nutrient forms and uptake in soil matrices. PAHs tend to be sequestered over time in soils with high TOC, thereby reducing their bioaccessibility/bioavailability to microbial degradation (Okere et al. 2017). Puglisi et al. (2007) and Lukic´ et al. (2016) reported a decrease in microbial degradation due to increased soil sorption of a contaminant in organic waste amended soil. Also, PAHs biodegradation can be limited by essential nutrients depletion, especially in soils with high organic carbon contents (Towell et al. 2011b). However, the TOC of WD did not increase the lag phase. The potential to accelerate biodegradation through a general stimulation of the microbial biomass can be achieved with a high TOC content (Kogel-Knabner 2002). This suggests that the numbers of phenanthrene degraders were probably enhanced by the presence of organic carbon which subsequently led to a higher level of ^{14}C-phenanthrene mineralization/catabolic activity. Some studies have suggested that the organic nutrient addition to soil promotes diverse soil microbial communities, improve soil nutrition and health (Harrison and Bardgett 2010; Omoni et al. 2015; Martínez-García et al. 2018).

The addition of digestate to spiked soil had an effect on the cumulative extent of mineralization; however, this was inconsistent over time especially in SD and LD amended soils. Optimal

mineralization was observed in soil amended with WD (31.26%) following 60 d soil incubation, with the lowest lag phase, fastest rate and cumulative extent for all treatments ($p < 0.05$).

Total heterotrophs and phenanthrene degraders were studied in the spiked and non-spiked digestate amended soils. In the spiked soil, the total heterotrophs were higher than the phenanthrene degraders at all incubation periods; whilst phenanthrene-degraders were higher in nonspiked than spiked soil. This may be due to the toxic nature of the contaminant on the indigenous microbial populations present in the soil. Both spiked and non-spiked soils with WD amendment had the highest phenanthrene degraders in this study. Microbial adaptation to the contaminant is very important in any degradation process (Couling et al. 2010; Rhodes et al. 2010). Correlation studies showed a positive relationship between the rate and extent of ^{14}C-phenanthrene mineralization and the bacterial number (total heterotrophic and phenanthrene-degrading bacteria) after 1 and 30 d only. This reveals the potential of the microbes and their likely catabolic enzymes for phenanthrene degradation (Das et al. 2011). Also, heterotrophs influence the extent of $^{14}CO_2$ mineralized as found from the strong positive correlation after 60 d incubation (which showed the optimal degradation).

Bacterial numbers obtained showed that the phenanthrene degraders and total heterotrophs were affected by phenanthrene addition since the soil microbial numbers increased in non-spiked soils compared to spiked soils. This could be attributed to the differences in availability of nitrogen between both soils, which can affect microbial communities and has been reported in other studies (Harrison and Bardgett 2010; Nakhro and Dkhar 2010). The definitive trend was not apparent between ^{14}C-hydrocarbon mineralization parameters (lag phases, rates, and extents of mineralization) in the control soils and soil nutrient content (available N and P).

Moreover, in some instances, there was no significant effect on the overall extents of ^{14}C-hydrocarbon mineralization from addition of nutrients to the soil by the digestate. Several studies have reported similar observations in amended soil (Chaineau et al. 2005; Chaillan et al. 2006; Ramírez et al. 2008). This has been related to availability of nutrients, soil heterogeneity, toxicity of nutrient intermediaries and presence of nitrogen-fixing bacteria (Seklemova et al. 2001; Sarkar et al. 2005). Nevertheless, the extent and rate of degradation may also depend on the structure and concentrations of contaminant, microbial community and amendment type (Oyelami et al. 2013).

6. Conclusion

This study is the first report on the impact of digestate and its fractions on soil fertility, total heterotrophs and phenanthrene degraders, for phenanthrene mineralization. Digestate amendments improved microbial populations and the soil fertility which positively influenced rate and extent of ^{14}C-phenanthrene mineralization. However, spiking with phenanthrene reduced the microbial growth in the soil. Mineralization was optimum at 60d soil-phenanthrene contact time with a corresponding increase in available nitrate and phosphate levels. The extent of mineralization decreased with increasing contact time, but this varied for the digestate and its fractions. WD amended soil performed better than SD and LD throughout the incubation period. Furthermore, the bacterial number was a major influence driving the extents of ^{14}C-phenanthrene mineralization, although phenanthrene degraders were not directly related to mineralization. Heterotrophic bacterial numbers which positively correlated with cumulative extent of mineralization, were the determinants of PAH mineralization after 60 d incubation. The addition of minimal basal salts to digestate amended soil to further enhance the growth of

indigenous phenanthrene degraders should be studied for the remediation of PAHs in contaminated soil.

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8. Supplementary Data

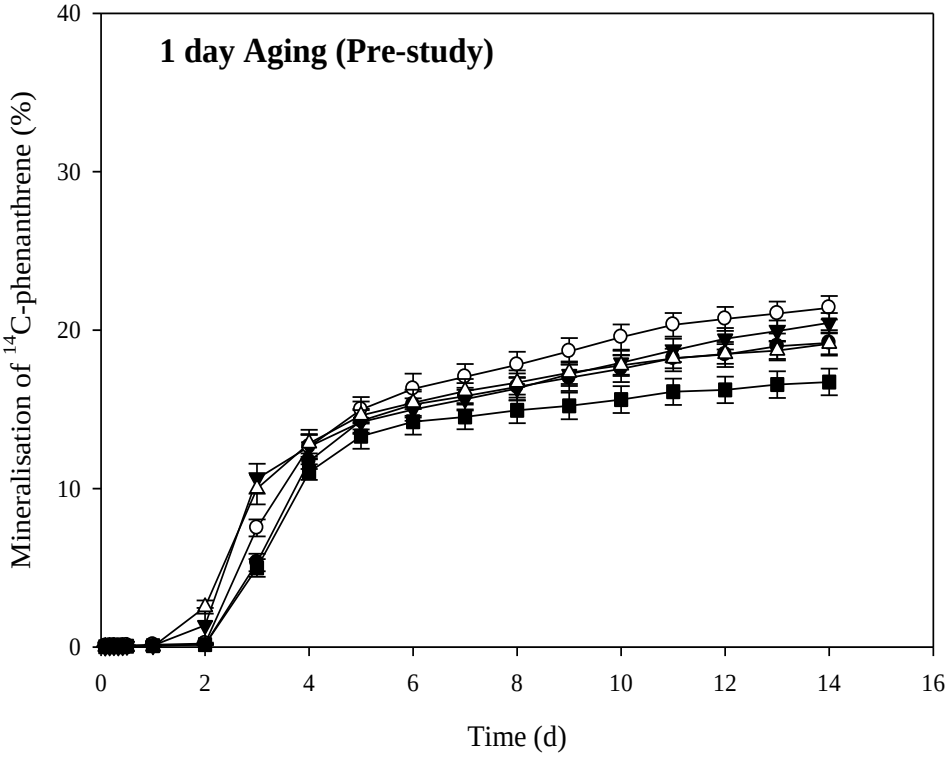

Figure S1: Evolution of $^{14}CO_2$ from the catabolism of ^{14}C-phenanthrene in soil amended with whole digestate. The 1-90 incubation graphs show: 0.01% (●), 0.1% (○), 1% (▼)10% (Δ) and control (■)

Table S1: Catabolism of ^{14}C-phenanthrene mineralized in soil amended with varying concentrations of whole digestate

Soil-PAH aging (d)	Amended concentrations (%)	Lag phase ($^{14}CO_2$ ≥5%) (d)	Fastest rate rate (% $^{14}CO_2$ d^{-1})	Cumulative Extent (%)
1	0	3.02 ± 0.10	6.11 ± 0.28	16.73 ± 0.71
	0.01	3.03 ± 0.10	6.46 ± 0.27	19.19 ± 0.80
	0.1	2.63 ± 0.07	7.30 ± 0.54	21.41 ± 0.74
	1	2.38 ± 0.11	9.25 ± 0.69	20.46 ± 0.62
	10	2.33 ± 0.07	7.47 ± 0.79	19.14 ± 0.66

Values are mean ± standard error (n = 3)

Table S2. Inorganic and Total carbon contents of the amended soils

	TC	TIC	TC	TIC	TC	TIC	TC	TIC
	1st Time point		2nd Time point		3rd Time point		4th Time point	
WD	101.55±8.77	16.19±1.27	546.03±16.93	38.48±1.16	230.76±19.79	6.23±0.61	161.31±1.54	1.47±0.31
FD	102.39±12.78	13.31±1.86	570.38±30.06	39.86±0.30	178.74±10.35	5.62±2.45	177.00±13.85	1.664±0.20
LD	96.76913.05	13.03±1.80	543.96±38.81	36.01±5.75	174.59±19.46	2.91±0.26	162.64±4.32	1.50±0.05
PWD	141.31±1.42	7.726±5.44	509.20±214.65	68.74±22.88	157.67±75.66	2.56±2.27	195.75±70.86	1.79±0.61
PFD	127.20±9.37	6.33±5.13	539.65±200.76	68.84±23.8	133.47±69.27	2.38±2.08	174.59±66.06	1.69±0.59
PLD	101.63±3.77	4.32±1.12	135.26±20.93	18.40±4.37	179.04±10.32	2.01±0.45	140.55±3.66	1.39±0.15
P Control	91.19±0.31	8.96±0.97	85.18±0.01	9.81±0.66	182.20±2.321	2.3±0.15	143.12±2.85	1.29±0.14
P Blank	85.85±3.6	6.28±0.40	160.67±5.77	19.05±3.11	171.89±13.72	2.60±0.20	138.98±5.57	1.296±0.10
No P Blank	86.88±0.09	5.93±0.95	103.24±7.25	10.37±0.40	163.30±3.48	2.48±0.05	150.12±3.31	1.18±0.10

Values are mean ± standard error (n = 3)

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