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

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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

1. 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

51 manure (Chiu et al. 2009; Agamuthu et al. 2013). Several studies have reported the potential
52 of nutrients from biodegradable waste for the mineralization of PAHs in soil (Christensen et
53 al. 2004; Zhang et al. 2012; Chen et al. 2015; Kästner and Miltner 2016). Digestate, a residue
54 from anaerobic digestion has gained more attention as nutrient-rich (nitrogen and mineral
55 elements) organic fertilizer in nutrient-poor agricultural soils (Nkoa 2014; Möller 2015;
56 Fagbohungbe et al. 2019). In agricultural soils, depending on the nutrient requirement and
57 planting season, the digestate can be applied in different forms: whole, solid and liquid
58 fractions (Ibeto et al. 2020; Johansen et al. 2013; Liedl et al. 2006; Tiwary et al. 2006). The
59 application of digestate to enhance soil remediation is a favourable development as it is
60 expected to further broaden its current use. In developing countries, these cheap, value-added,
61 nutrient-rich and abundant sources of microbial nutrients indiscriminately end up in the
62 receiving environment. However, the application of digestate could potentially influence
63 biological and microbial proliferation in soil matrices, especially in the oil polluted areas,
64 where there is staggering soil contamination. Digestate is a potential microbial stimulator and
65 could facilitate the rates and extents in which PAHs can be metabolized in poor-nutrient
66 contaminated soils (Leahy and Colwell 1990). Presently, there is no published literature on
67 either the use of digestate or its fractions on the mineralization of PAHs in soil. Therefore,
68 this study aimed to investigate (i) changes in soil properties in digestate amended
69 phenanthrene spiked and non-spiked soil (ii) the impact of digestate forms: WD, SD, and LD
70 on ^{14}C-phenanthrene mineralization in soil and (iii) the catabolic activities through microbial
71 numbers (heterotrophic and phenanthrene-degrading bacteria) in both amended and
72 unamended, ^{12}C-phenanthrene spiked and non-spiked soils.
73

74 ## 2. Materials and methods

75 *2.1 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.

2.2 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).

2.3. 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 ºC 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.

126

127 *2.4 ^{14}C-phenanthrene mineralization in soils*

128 To assess the digestate-assisted microbial degradation, mineralization of ^{14}C-phenanthrene
129 were assessed after each incubation time point (1, 30, 60 and 90 d) in the amended soil (Reid
130 et al. 2001; Doick and Semple 2003). The soils were spiked with ^{14}C-phenanthrene Standard
131 (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.
132 Respirometric soil-slurry assays (in triplicates) were carried out in 250 mL Schott bottles,
133 incorporated with a suspended 7 ml glass scintillation vial containing 1 ml 1 M NaOH
134 solution that served as a $^{14}CO_2$ trap. Each respirometer contained 10 ± 0.2 g soil and 30 mL of
135 deionized water to achieve a liquid:solid ratio of 3:1, which is recommended for improved
136 reproducibility and greater overall extents of mineralization (Doick and Semple, 2003). The
137 $^{14}CO_2$ mineralized in the trap was assessed daily by addition of Goldstar liquid scintillation
138 cocktail followed by counting using a Packard Canberra Tri-Carb 2250CA liquid scintillation
139 counter with standard calibration and quench correction techniques. The biodegradation
140 parameters assessed in this study were (i) lag phase, defined as the time taken to reach 5%
141 mineralization considering the upper and lower contact times and values obtained from the
142 scintillation counter. (ii) the fastest rate is the maximum rate of $\%^{14}CO_2$ evolution d^{-1}
143 determined from the increase in mineralization between each sampling point and (iii) the
144 cumulative extent of mineralisation is expressed as a percentage of the initial ^{14}C-
145 phenanthrene, that has been mineralised to $^{14}CO_2$ during each sampling time. It is the
146 cumulative mineralization for the 14 days incubation (respirometry assay). Control soil
147 (pristine soil with ^{12}C/^{14}C-phenanthrene but without amendment) and an analytical blank
148 (pristine soil without ^{14}C-phenanthrene and amendment) were also set up during the 14 d
149 experiment.

150 *2.5 Quantitative bacterial enumeration*

151 Bacterial numbers (heterotrophic and phenanthrene-degrading bacteria) in each soil sample
152 were determined by spread plate techniques (Oyelami et al. 2013). Soil (1.0 ± 0.1 g dry wt)
153 before respirometry was extracted with Ringer's solution in 1:10 mixture (soil: ringer
154 solution). Thereafter, the mixture was serially diluted before uniformly spreading 0.1 ml on
155 plate count agar (heterotrophs) supplemented with amphotericin-B (5µl ml^{-1}) and MBS agar
156 plates (phenanthrene-degraders) supplemented with both amphotericin-B (5µl ml^{-1}) and ^{12}C-
157 phenanthrene amendment (0.05 mg ml^{-1}). MBS solution Agar plates were incubated at 29
158 ±1°C, and CFUs were counted after 48 h and 5 d for heterotrophic and phenanthrene-
159 degrading bacteria respectively, for the different soil-PAH contact times (1, 30, 60 and 90 d).
160
161 *2.6 Statistical analysis*
162 Using SPSS (version 22), data was statistically analyzed by analysis of variance (ANOVA)
163 and Tukey's Post Hoc test where equal variance was assumed while Welch ANOVA and
164 Games Howell's Post Hoc test were used where equal variance was not assumed. Post-hoc
165 test ($P<0.05$) was used to determine any significant differences in means of amended soils
166 (WD, SD and LD) for the lag phases, fastest rates, extents of mineralization, and bacteria
167 (total heterotrophs and phenanthrene degraders) count at each time point (1, 30, 60 and 90 d).
168 Also, the influence of digestate-amendment on soil properties compared with control (without
169 amendment) was also analyzed. Spearman's correlation was used to ascertain the relationship
170 between bacterial numbers, soil properties and ^{14}C-mineralization. Data was presented as
171 mean ± standard error and the graphs were plotted using SigmaPlot 10.0 version.
172
173 **3. Results**
174 *3.1 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

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

Table 1: Physicochemical properties of the soil and digestates

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

191 incubation and the highest amount was 19.55 ± 1.58 mg/l for LD amended soil. Both
192 phenanthrene and non-phenanthrene spiked digestate amended soils showed no noticeable
193 effect on PO_4^{3-}-P level throughout the studied period. However, after 1 and 30 d of soil-
194 phenanthrene contact time, the PO_4^{3-}-P level was significantly higher in non-phenanthrene
195 spiked than in spiked soils (except for WD after 30 d). For TOC, the highest concentration
196 was observed for SD in both spiked (470.79 ± 7.89 mg/l) and non-spiked (530.60 ± 30.33
197 mg/l) soils after 30 d of soil interaction with digestate. However, as the soil-phenanthrene
198 contact increased, the TOC significantly decreased ($p < 0.05$) in WD and SD amended
199 phenanthrene spiked and non-spiked soils.
200

201

202 **Table 2**

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)

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

3.2 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

Phenanthrene Spiked Soil				
Soil contact time (days)	Amendment	Lag phase (before Mineralization $^{14}CO_2 \geq 5\%$) 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

228

229 Values are mean ± standard error (n = 3)

230

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

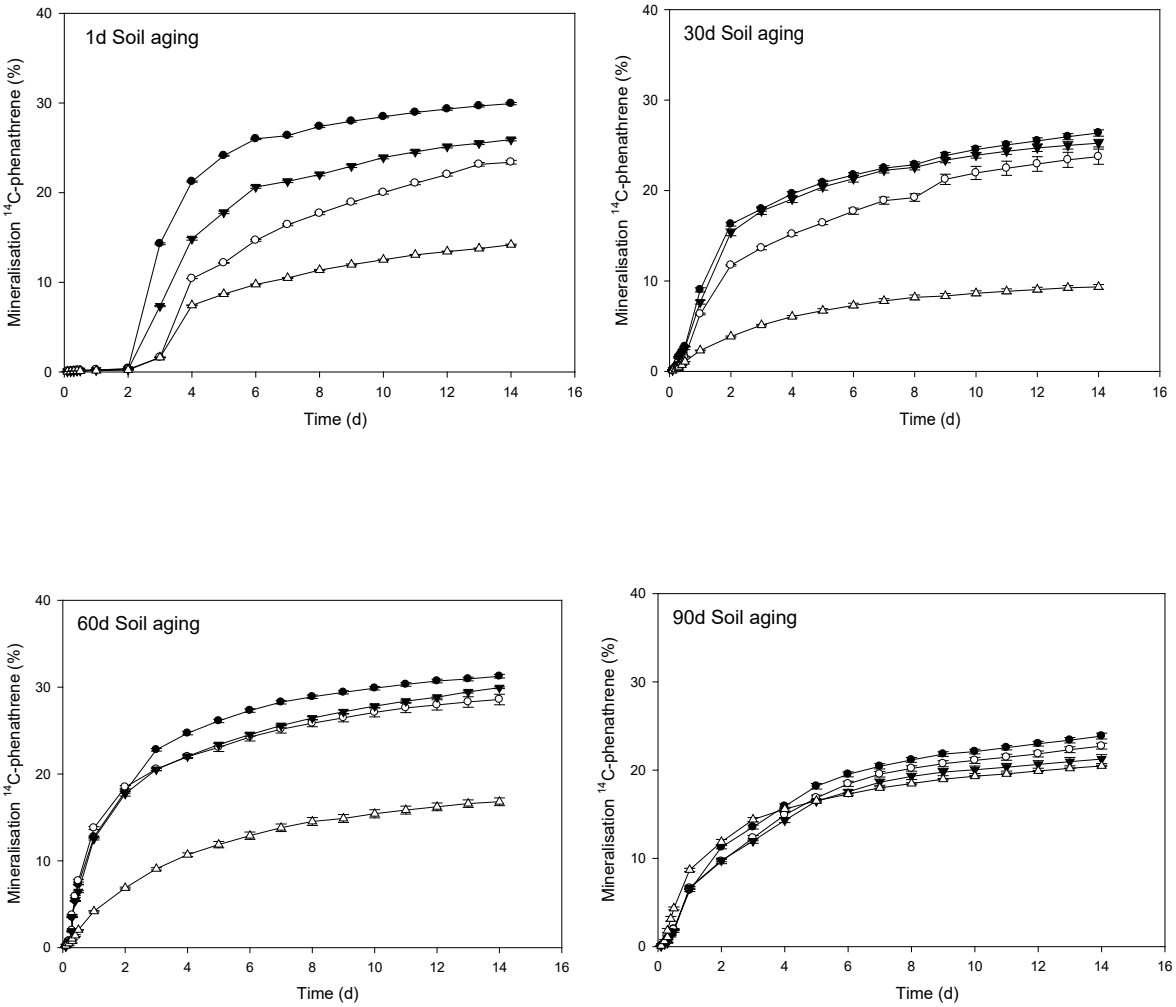

Figure 1. Evolution of $^{14}CO_2$ from the catabolism of ^{14}C-phenanthrene in 100mg/kg phenanthrene spiked soil amended with WD, SD and LD. [WD (●); SD (○); LD (▼); control (Δ)]. Error bars represent standard error of mean (SEM) of triplicate samples (n = 3).

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.

3.3Enumeration 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

		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)

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

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 ($1.77 \times 10^7 \pm 0.56$ CFU g-1 soil) at 1d time point, while the lowest phenanthrene degrading numbers ($0.08 \times 10^7 \pm 0.03$) 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.

4.0 Discussion

4.1 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 1st and 90th (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).

4.2 ^{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

320 in soil contact time specifically from 1 d to 60 d in comparison with the control (unamended
321 soil). Studies have also shown that nutrients amendments in PAH contaminated soil have the
322 potential to stimulate biodegradation through the supplier of nutrient and microbial inoculates
323 (Namkoong et al. 2002; Chiu et al. 2009; García-Delgado et al. 2015). Hence, the addition of
324 digestate in the soil could enhance the degree of biodegradation of organic contaminants.
325 Therefore biodegradation potential of PAH in soil may be influenced by the amount of
326 nutrient and organic carbon in contaminated soil (Zhang et al. 2012).
327 The lag phase is an indication of the microflora adaptation or acclimatization to the presence
328 of ^{14}C -phenanthrene, resulting in increased mineralization (Couling et al. 2010; Rhodes et al.
329 2010). Addition of digestate to soil had an effect on the adaptation of the soil microbial
330 community as revealed from the data obtained in this study. Soil amendment with digestate
331 and its fractions after 1 d soil-phenanthrene contact time, influenced the lag phase.
332 Furthermore, WD treated soil had significantly shorter lag phase than the other fractions (SD
333 and LD), however, the shortest lag phase was observed for SD after 60 d incubation. The lag
334 phase is the time for microbial adjustment to a new soil environment and therefore a reduction
335 in lag phase consequently revealed microbial adaption (Macleod and Semple 2002). As the
336 length of the lag phase reduced over time, there was an increase in the number of
337 phenanthrene-degraders which is further evidence from the strong negative correlation
338 observed between lag phase and phenanthrene-degraders in amended soil. Other studies have
339 reported similar results over time (Umeh et al. 2018; Oyelami et al. 2013; Oyelami et al.
340 2015).
341 Micronutrients from organic amendments could enrich and facilitate mineralization of ^{14}C-
342 phenanthrene in soil. For instance, Horel and Schiewer (2009) reported that the addition of N
343 and P releasing fertilizers increased respiration by 76% and 119% respectively for over 4-
344 month period in contaminated Alaskan soil. In this study, nutrient treatment in the form of N

345 and P from the digestates had a stimulatory effect on phenanthrene mineralization compared
346 to the control soil except for 90 d.
347 WD showed a significantly faster rate of mineralization of ^{14}C-phenanthrene with stronger
348 catabolic potential compared to the other amendments after 1 and 30 d of soil incubation. This
349 can be attributed to the higher number of phenanthrene degraders and available nitrogen.
350 From the data obtained, the number of PAH degrading bacteria for WD increased after 1 d
351 incubation with a corresponding lower lag phase. In general, digestate amended soils for most
352 soil-phenanthrene contact time did not influence the rate of mineralization. This may be
353 attributed to the phenanthrene fraction that was rapidly desorbable in the aqueous phase in the
354 soil for microbial attack and sequestration (Ogbonnaya et al. 2014). It can also be attributed to
355 the differences in the digestates' nutrient content, microbial preferred nutrient forms and
356 uptake in soil matrices. PAHs tend to be sequestered over time in soils with high TOC,
357 thereby reducing their bioaccessibility/bioavailability to microbial degradation (Okere et al.
358 2017). Puglisi et al. (2007) and Lukic´ et al. (2016) reported a decrease in microbial
359 degradation due to increased soil sorption of a contaminant in organic waste amended soil.
360 Also, PAHs biodegradation can be limited by essential nutrients depletion, especially in soils
361 with high organic carbon contents (Towell et al. 2011b). However, the TOC of WD did not
362 increase the lag phase. The potential to accelerate biodegradation through a general
363 stimulation of the microbial biomass can be achieved with a high TOC content (Kogel-
364 Knabner 2002). This suggests that the numbers of phenanthrene degraders were probably
365 enhanced by the presence of organic carbon which subsequently led to a higher level of ^{14}C-
366 phenanthrene mineralization/catabolic activity. Some studies have suggested that the organic
367 nutrient addition to soil promotes diverse soil microbial communities, improve soil nutrition
368 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

394 (available N and P). Moreover, in some instances, there was no significant effect on the
395 overall extents of ^{14}C-hydrocarbon mineralization from addition of nutrients to the soil by the
396 digestate. Several studies have reported similar observations in amended soil (Chaineau et al.
397 2005; Chaillan et al. 2006; Ramírez et al. 2008). This has been related to availability of
398 nutrients, soil heterogeneity, toxicity of nutrient intermediaries and presence of nitrogen-
399 fixing bacteria (Seklemova et al. 2001; Sarkar et al. 2005). Nevertheless, the extent and rate of
400 degradation may also depend on the structure and concentrations of contaminant, microbial
401 community and amendment type (Oyelami et al. 2013).
402
403 **5. Conclusion**
404 This study is the first report on the impact of digestate and its fractions on soil fertility, total
405 heterotrophs and phenanthrene degraders, for phenanthrene mineralization. Digestate
406 amendments improved microbial populations and the soil fertility which positively influenced
407 rate and extent of ^{14}C-phenanthrene mineralization. However, spiking with phenanthrene
408 reduced the microbial growth in the soil. Mineralization was optimum at 60d soil-
409 phenanthrene contact time with a corresponding increase in available nitrate and phosphate
410 levels. The extent of mineralization decreased with increasing contact time, but this varied for
411 the digestate and its fractions. WD amended soil performed better than SD and LD throughout
412 the incubation period. Furthermore, the bacterial number was a major influence driving the
413 extents of ^{14}C-phenanthrene mineralization, although phenanthrene degraders were not
414 directly related to mineralization. Heterotrophic bacterial numbers which positively correlated
415 with cumulative extent of mineralization, were the determinants of PAH mineralization after
416 60 d incubation. The addition of minimal basal salts to digestate amended soil to further
417 enhance the growth of indigenous phenanthrene degraders should be studied for the
418 remediation of PAHs in contaminated soil.

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