SOIL BIOLOGY AND BIOCHEMISTRY

Impact of single and binary mixtures of phenanthrene and N-PAHs on microbial utilisation of ^{14}C-glucose in soil

Ihuoma N. Anyanwu[a, b] and Kirk T. Semple[a,*]

[a] Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, United Kingdom.

[b] Department of Biological Sciences, Federal University Ndufu-Alike Ikwo, P.M.B 1010, Abakaliki, Ebonyi State, Nigeria.

Corresponding author: k.semple@lancs.ac.uk

Abstract

Microbes are susceptible to contaminant effects, and high concentration of chemicals in soil can impact on microbial growth, density, viability and development. The impact of single and binary mixtures of phenanthrene and its nitrogen-containing polycyclic aromatic hydrocarbon analogues (N-PAHs) on microbial metabolism of ^{14}C-glucose in soil was measured over a 90 d soil-contact time. Impacts were assessed by measuring the rates and mean overall extents of mineralisation (%), as well as the incorporation of ^{14}C-glucose into the microbial biomass. The result revealed that the extents of ^{14}C-glucose mineralisation were consistently greater in N-PAH amended soils than the control and phenanthrene soils with increased incubations. This indicates a trend of increasing diversion of C from biosynthesis to maintenance requirement by soil microorganisms. Furthermore, biomass uptake in the amended soils showed reduced substrate utilization (fixed-k_{EC}), suggesting that N-PAHs decreased the amount of substrate-C that was incorporated into the microbial biomass. This however, signifies that N-PAHs imposes oxidative stress on soil microbial community.

Key words*:* N-PAHs, phenanthrene, ^{14}C-biomass uptake, mineralisation, k_{EC} coefficient.

1. Introduction

The importance of microbial activity in the cycling of organic matter and regulating active nutrient pools suggests that the effects of stress on microbial community will fundamentally impact on crops, natural vegetation and ecosystem productivity (Killham, 1985; Anyanwu and Semple, 2016a; Siles and Margesin, 2017). Soil microorganisms are very sensitive to environmental stress or change, and this often results in the diversion of carbon from biosynthesis to maintenance of cells (Bargett and Saggar, 1994; Anyanwu and Semple, 2016a). Thus, soil microbial biomass measurements are important in ascertaining the extent of chemical stress and/or disturbance on soil ecosystem and the time dependence of microbial recovery. Most studies have used respiration rate (Fournier et al., 1992; Nakamoto and Wakahara, 2004; Anyanwu and Semple, 2016a; Sun et al., 2017; Xu et al., 2017) and changes in biomass (Anyanwu and Semple, 2016a; Mehnaz et al., 2017; Siles and Margesin, 2017). Using a ^{14}C-substrate, the influence of synthetic and organophosphate sheep dip formulations (Boucard et al., 2008), pesticides (Fournier et al., 1992), heavy metals (Bargett and Saggar, 1994; Bogomolov et al., 1996), sewage sludge (Flieβbach et al., 1994; Witter and Dahlin, 1995) and the ratio of ^{14}C-biomass-incorporated with ^{14}C-respired (Sparling and West, 1989; Sparling et al, 1990; Gunina et al., 2017), have been determined on soil microbial activity. The approach of using ^{14}C-glucose as a substrate to determine the ratio of respired-C, to biomass-incorporated C, has shown that microorganisms in contaminated soils are less efficient in the utilization of substrates for biomass synthesis and spend more energy in the maintenance requirements (Bargett and Saggar, 1994; Witter and Dahlin, 1995; Anyanwu and Semple, 2016a; Gunina et al., 2017). Thus, leading to a decrease in the ratio, increases in stress, faster respiration, reduced efficiency of fresh substrate incorporation into new soil microbial biomass and increased microbial turnover in contaminated soils (Fliebβach et al., 1994; Bargett and Saggar, 1994; Witter and Dahlin, 1995; Boucard et al., 2008; Gunina et al., 2017; Bore et al., 2017). These studies have revealed that the growth, activity and physiological conditions of soil microbial community may be altered and/or destroyed by the presence of contaminants.

Persistent contaminants are of particular concern due to their toxicity and widespread pollution that has occurred during production, spills, combustion and disposition (Beelen and Doelman, 1997; Anyanwu and Semple, 2015a); examples include metals, pesticides and polycyclic aromatic hydrocarbons (PAHs). However, for sustainable environmental policies and regulations, risk assessment of other persistent contaminants such as, the nitrogen-containing polycyclic aromatic hydrocarbons (N-PAHs) in the environment is of great importance. N-PAHs are chemicals present in most contaminated sites worldwide and represent two-thirds of known organic xenobiotic

chemically synthesized (Rajasekhar et al., 2000; Anyanwu and Semple, 2015a). For example, they are used as industrial solvents, dyes, explosives, pharmaceuticals and pesticides (Kaiser et al., 1996). The US Environmental Protection Agency (USEPA) and International Agency for Research on Cancer (IARC) classified N-PAHs as probable human carcinogens (IARC, 2012). Furthermore, many of these N-PAHs are antimicrobial (Vance et al., 1986; Ferraz et al., 2017); therefore, their accumulation is a major threat to microbes because they have the potency of inducing oxidative stress to soil microorganisms and other biotas.

Despite the widespread uses of N-PAHs, and previous N-PAHs studies in literature (Anyanwu and Semple, 2015a; 2015b; 2016a; Anyanwu et al., 2017), there has not been information of their impacts on microbial utilization of ^{14}C-glucose and/or synthesis of cell biomass in soil. Functionally, microbes can act as relevant indicators of environmental pollution; as a result, there is great need to assess the impact of N-PAHs on soil microbial metabolism and biosynthesis of cell biomass. In this study therefore, the impact of single and binary mixtures of phenanthrene and its nitrogen-containing analogues on microbial utilization of ^{14}C-glucose was investigated over a 90 d incubation period in soil using respirometric assays.

2. Materials and Methods

2.1 Chemicals

Phenanthrene (Phen), 1,10-phenanthroline (1,10-Phen), 1,7-phenanthroline (1,7-Phen), 4,7-phenanthroline (4,7-Phen) and benzo[h]quinoline (B[h]Q) and radiolabelled ^{14}C-glucose were obtained from Sigma-Aldrich, UK. Goldstar liquid scintillation cocktails were supplied by Meridian Biotechnologies Ltd, UK.

2.2 Soil preparation

A pristine agricultural soil from Myerscough, UK, collected from the top layer of field under pasture, from a depth of approximately 5-20 cm was prepared for the study (n = 3). The soil texture was sandy-loam (19.5% clay, 60.4% sand, 20.0% silt), with organic matter content of 2.7%; total nitrogen of 0.14%; total organic carbon of 1.6% and pH 6.5. The soil was thoroughly homogenized, air dried at room temperature and sieved with 2 mm mesh size. The soil was rehydrated with deionised water back to 45% water holding capacity (WHC) and amended with phenanthrene and the N-PAH analogues as described in Doick et al. (2003). Soil samples were placed in bowls:

87 $^{1}/_{3}$ (100 g; n = 3) were amended with phenanthrene and four N-PAH standards (benzo[h]quinoline, 1,10-
88 phenanthroline, 1,7-phenanthroline or 4,7-phenanthroline) dissolved in acetone to give concentration of 100 mg
89 kg^{-1}. The amended soils were kept in the fume hood for 3 h to allow the carrier solvent volatilize, after which the
90 soils were mixed with the remaining $^{2}/_{3}$ (200 g). Blanks were prepared using un-amended soils. Soils amended
91 with acetone only were also prepared to serve as a control. The amended soils were kept in amber glass jars and
92 aged in the dark at 21 ± 1°C for 1, 30, 60 and 90 d. Soil moisture content was checked regularly and lost water
93 was replenished with deionized water. After each ageing time (30 d interval), soils were analysed for microbial-
94 substrate-mineralisation and biomass uptake. Extractability of phenanthrene and the N-PAH analogues from soil
95 over time, and their percentage recoveries has been reported by Anyanwu and Semple (2015b, 2016a) (Table 1).
96

97 *2.3 Mineralisation o f ^{14}C-glucose in soil.*

98 The ability of indigenous soil microorganisms to mineralise ^{14}C-glucose to $^{14}CO_2$ was assessed at 1, 30, 60 and 90
99 d contact time. Respirometric assays were carried out in modified 250 ml Schott bottles incorporating a Teflon-
100 lined screw cap containing 1 M NaOH to trap any $^{14}CO_2$ (Reid et al., 2001). A slurry system with a solid: liquid
101 ratio of 2:1 (20 g soil: 10 ml sterile water) was used to ensure complete $^{12/14}$C-glucose distribution. Standards were
102 prepared in sterilized deionised water and delivered to give a ^{12}C-glucose concentration of 3 mM glucose solution
103 with an associated ^{14}C-activity of 800 Bq per respirometer. Controls were also prepared. Respirometers were
104 shaken at 100 rpm on an orbital shaker (Janke and Kunkel, IKA®-Labortechnik KS 510D), in the dark at 21±1°C.
105 Sampling was carried out every 1, 2, 4, 6, 8, 12, 24 h and 2, 3, 4, 5 d with the vials containing trapped $^{14}CO_2$.
106 Goldstar liquid scintillation cocktail was added to the vials. The vials were stored in the dark for 24 h before
107 sample quantification was carried out by liquid scintillation counting (LSC) using standard calibration and quench
108 correction techniques (Reid et al., 2001).
109

110 *2.4 Uptake of ^{14}C glucose into microbial biomass*

111 After each 5 d incubation, soil samples from respirometers were divided into three portions and analysed as
112 follows:

113 a) *Sample oxidation:* The first sample was oven dried at 30°C and combusted in a sample oxidizer (Packard
114 307) to determine the level of ^{14}C-activity remaining (i.e. residual ^{14}C-activity in soil). Soil (1 g), plus

200 µl of combustAid was combusted for 3 min. Carbon-sorb-E (10 ml) and Permaflour-E (10 ml) was used as CO_2 trap and scintillation fluid, respectively. Sample quantification was carried out using LSC.

b) *Un-fumigated extraction:* The second sample (~4 g) was immediately extracted with 0.5 M K_2SO_4 (50 ml, pH 7) by shaking on an orbital shaker at 100 rpm for 30 min. The soil solutions were filtered using Whatman No 1 filter papers and an aliquot of 5 ml supernatant was added to 15 ml scintillation cocktail. The quantification of ^{14}C-activity was carried out using the LSC.

c) *Fumigated extraction:* The third sample (~4 g) was placed in a desiccator and fumigated with ethanol-free chloroform for 24 h to measure the ^{14}C-activity within microbial biomass. After fumigation, the samples were vented to remove chloroform residuals in the soil. After venting, samples were extracted with 0.5 M K_2SO_4, filtered (using Whatman No 1 filter papers) and analysed as per the un-fumigated extract.

2.5 Statistical analysis

The proportion of ^{14}C-glucose incorporated into the microbial biomass was calculated as in Sparling et al. (1990) and Boucard et al. (2008).

^{14}C-flush = ^{14}C-activity in fumigated soil – ^{14}C-activity in un-fumigated soil.

^{14}C-microbial biomass = ^{14}C-flush ÷ $k_{EC.}$

1. A fixed k_{EC} coefficient (0.35) was used to convert C-flush into microbial biomass Sparling et al., 1990; Boucard et al., 2008).
2. Variable k_{EC} coefficients were also calculated from each amendment, at all the ageing times, and the ^{14}C-microbial biomass was re-calculated with the new coefficient. This process is based on the assumption that; the calculated ^{14}C-labelled microbial-C is a representative of the total microbial biomass and that all the ^{14}C-activity not taken into account by mineralisation and un-fumigated soil extraction has been incorporated into the microbial biomass with negligible amount of extracellular metabolite (Sparling et al., 1990; Boucard et al., 2008).

   k_{EC} = (^{14}C-flush) ÷ ($^{14}C_{init.}$ – ^{14}C-respired – ^{14}C-activity in un-fumigated soil).

   ^{14}C-flush and ^{14}C-microbial biomass were later on expressed as percentages of the initial ^{14}C-activity ($^{14}C_{init.}$).

3. Biophysical quotients (BQ) were calculated as:

BQ = $^{14}CO_2$ respired ÷ ^{14}C-microbial biomass (calculated from either fixed or variable k_{EC}).

Following blank corrections, data was statistically analysed using SigmaStat 3.5. Statistical significant differences between the impacts of phenanthrene, N-PAHs, and soil contact time on soil microbial activity following addition of ^{14}C-glucose was determined using analysis of variance (ANOVA). The statistical difference between the biomass calculated with fixed and variable k_{EC} was also determined. Results are statistically significant when $p<0.05$. Data was presented as mean ± SE and graphs were plotted using Sigma-Plot 10.0 version.

3. Results

3.1 Mineralisation of ^{14}C-glucose to $^{14}CO_2$ by soil microorganisms

The mineralisation of ^{14}C-glucose in the presence of 100 mg kg^{-1} phenanthrene and its N-PAH analogues was measured (Fig. 1 and 2). Upon the addition of glucose, there was a considerable increase in % mineralisation in the presence of the amended chemicals. However, the mineralisation of the ^{14}C-substrate (glucose) in the presence of benzo[h]quinoline (B[h]Q) soil was reduced at 1 d compared to the control soils (Fig. 1 and 2).

The fastest rates of mineralisation were determined (Table 2), and the fastest rates (% $^{14}CO_2$ h^{-1}) recorded maximum values after 24 h following addition of ^{14}C-glucose in all the amendments at all of the time points with the exception of 4,7-Phen, B[h]Q and Phen, which recorded their fastest rates 48 h after addition of ^{14}C-glucose (30 d) (Table 2). Furthermore, 1,10-Phen (single amendment) and 1,10-Phen + Phen (binary mixtures) recorded maximum fastest rates at 6 h (90 d). From the data, the fastest rates followed a trend of decreased values with increases in the soil-contact time. However, 1,7-Phen and B[h]Q amendments showed a dramatic rise of 50% and 70%, respectively, after 90 d (Table 2).

The extents of mineralisation (total $^{14}CO_2$-respired (%)) were determined (Table 2). The results revealed that the extents of mineralisation of ^{14}C-glucose appeared to be consistently greater in amended soils than the control soils with increase in ageing time; with the exception of 1,10-Phen, 4,7-Phen and Phen amended soils (90 d). The overall extent of mineralisation followed a trend of increased ^{14}C-glucose mineralisation at 1 d in all the amendments. Among the N-PAHs, however, B[h]Q soils recorded increased mineralisation with increase in soil-contact time, but, this declined a little at 90 d (Table 2).

While the extents of mineralisation in the single amendments displayed decreased and increased values, a consistent decrease in ^{14}C-glucose mineralisation was observed in the binary mixtures over time (Table 2). Analysis of data among the treatment groups showed no statistically significant differences between the mean values at 1 d ($p>0.05$); however, statistically significant differences was observed after 30 d ($p<0.05$) (Table 2). Furthermore, statistical analysis of data showed statistically significance differences between phenanthrene and N-PAH amended soils over time ($p<0.05$). In addition, incubation times were observed to affect the % mineralisation of ^{14}C-glucose in all the amendments at all the time points ($p<0.001$).

3.2 Impact of phenanthrene and N-PAHs on the k_{EC} coefficients

The impact of 100 mg/kg phenanthrene and its nitrogen-containing analogues on the k_{EC} coefficients was calculated (Table 2). It was noted that the fumigation-extraction released 0.3–15% of the incorporated microbial-C giving the calculated k_{EC} ranges of 0.003–0.149. However, variation among the chemical amendments was observed in the calculated k_{EC} values obtained after the fumigation-extraction (Table 2). Furthermore, the calculated k_{EC} values (0.003–0.149) were lower than the fixed k_{EC} value (0.35) in all the amendments (Table 2). Although the data showed a disparaging statistical difference, all the amendments showed a similar trend of low k_{EC} values at 1 d and 60 d and high k_{EC} values at 30 d and 90 d (with the exception of Phen and 1,7-Phen + Phen chemicals). Also, the presence of 1,10-Phen and 1,7-Phen in soil recorded lower k_{EC} coefficients (30 d and 60 d) compared to the control soil values (Table 2).

Soils amended with binary mixtures of phenanthrene and N-PAHs recorded low, but varying k_{EC} values compared to the control soils at all the time points. For example, while control fixed k_{EC} values ranged from 3.06 ± 0.87 – 19.92 ± 3.65, values of 0.66 ± 0.13 – 15.08 ± 3.47 and 1.12 ± 0.11 – 9.93 ± 1.22 (fixed k_{EC}) were recorded in the single amendments and binary mixtures, respectively. Also, while control values for variable k_{EC} = 46.67 ± 8.55 – 63.45 ± 18.1, values range of 34.03 ± 3.11 – 62.45 ± 11.22 and 41.45 ± 1.93 – 59.37 ± 9.45 (variable k_{EC}) were measured (single amendments and binary mixtures, respectively) (Table 2). In addition, the calculated k_{EC} values of 0.003 ± 0.00 – 0.140 ± 0.03 (single amendments), and 0.007 ± 0.00 – 0.013 ± 0.00 (binary mixtures) was obtained, while, control values = 0.016 ± 0.00 – 0.149 ± 0.02 (Table 2). Furthermore, incubation time was noted to have statistically significant effect on the k_{EC} values in binary mixtures ($p<0.001$). Although not consistent, there was a trend of increases in the extraction efficiency of K_2SO_4 at 30 d and 90 d.

198

199 *3.3 Uptake of ^{14}C-glucose into microbial biomass*

200 The incorporation of the ^{14}C-susbstrate into the microbial biomass in soils amended with 100 mg/kg single and
201 binary mixtures of phenanthrene and the N-PAH analogues were calculated using fixed and variable k_{ECS},
202 respectively (Table 2). Although not showing a consistent trend, the results showed that increase in fixed k_{EC}
203 values, lead to decrease in variable k_{EC} values and vice versa in all the amendments, with the exception of B[h]Q
204 amendment (Table 2). Statistical analysis of data showed statistically significant differences in the calculated data
205 for both the fixed and variable k_{EC} values obtained after 1 d – 90 d soil-contact time ($p<0.001$). Also, there was a
206 consistent trend of higher values in the C-flush resulting in higher biomass values (fixed k_{EC}) and lower biomass
207 values (variable k_{EC}) (Table 2).

208 The BQs (using biomass values calculated with fixed and variable k_{ECs}) was determined, and the results differed
209 significantly in all the amendments at all the time points (Table 2). For example, BQs calculated with the fixed
210 k_{EC} varied widely compared to that of the variable k_{EC}. Furthermore, the amended soils recorded significant BQ
211 values at 1 d compared with the control soils ($p<0.05$); and Phen amendment recorded the highest BQ value of
212 54.76% (Table 2). A trend of high BQ values at 1 d and 60 d (fixed k_{EC}), and low values at 30 d and 90 d (variable
213 k_{EC}) was observed in all of the amendments (Table 2). Among the chemical amendments, B[h]Q showed a
214 consistent increase in BQ value with increased ageing, recording values >1 ($p<0.05$). Although showing high
215 variability, the calculated BQs recorded high values with increased ageing in the binary mixtures.

216

217 **4. Discussion**

218 *4.1 Mineralisation of ^{14}C-glucose to $^{14}CO_2$ by soil microorganisms*

219 The impact of single and binary mixtures of phenanthrene and its nitrogen-containing analogues on microbial
220 utilisation of ^{14}C-glucose in soil was studied over a 90-d incubation. Loss of phenanthrene, benzo[h]quinoline,
221 1,10-phenanthroline, 1,7-phenanthroline or 4,7-phenanthroline through volatilisation was considered minimal due
222 to the sealed nature of the incubations (Hofman et al., 2008; Towell et al., 2011). From the results, mineralisation
223 of ^{14}C-glucose was greater in the N-PAHs amended soils than the control soil after 1 d; indicating that
224 microorganisms utilized energy for cell maintenance rather than biosynthesis of new cells. This phenomenon

225 agrees with the observations of Bargett and Saggar (1994), Witter and Dahlin (1995), Chnader and Joergensen
226 (2001), Boucard et al. (2008), and Bore et al. (2017). In support, Flieβbach et al. (1994) reported that in heavily
227 contaminated sites, soil respiration increased substantially compared to the corresponding low contaminated soils.
228 Respiration has been linked as a process and microbial biomass as a pool to metabolic quotient for CO_2 (qCO_2)
229 by Anderson and Domsch (1986). Thus, it is widely accepted that a high qCO_2 is a surprisingly common
230 characteristic of soil microbial biomass in chronically contaminated soils (Flieβbach et al., 1994). This has been
231 suggested to be a useful indicator of oxidative stress in soils (Brookes, 1993; Mooshammer et al., 2017). In
232 addition, Gunina et al. (2017) reported that an increased qCO_2 indicates stress to the soil microbial community.

233 In this study, N-PAHs (B[h]Q amendment) recorded low mineralisation at 1 d; this is an evidence of reduced
234 microbial substrate utilization efficiency under chemical stress. Hattori (1992) and Molaei et al. (2017)
235 documented that initial microbial respiratory responses are the most sensitive in quantifying the impact of
236 contaminants following their introduction into soil. However, the consistent increase in B[h]Q mineralisation after
237 30 d could be attributed to oxidative stress and/or chemical bioavailability, due to its lower K_{ow} (Anyanwu and
238 Semple, 2015b, 2016a); since the total concentration did not exceed that of other amended soils.

239 A decline in mineralisation (%) was observed over time. The notable decline may be as a result of chemical
240 sequestration (into soil organic matter) there by rendering the contaminants less available to microorganisms
241 (Semple et al., 2007), microbial degradation (Anyanwu and Semple, 2015b; 2016a) and/or adaptation to toxicity
242 (Granato et al., 2017; Anyanwu and Semple, 2017b). Organic contaminants are known to be retained within the
243 soil through chemical or physical sequestration processes, such as binding, sorption to clay and/or soil organic
244 matter as well as occlusion within the 3-dimensional structure of the soil (Semple et al., 2007). Furthermore,
245 factors which include, soil organic matter content and physico-chemical properties of the chemical (aqueous
246 solubility, polarity, hydrophobicity, molecular structure, K_{ow}, and lipophilicity) are known to control the fate and
247 behaviour of organic contaminants (N-PAHs) in soil (Anyanwu and Semple, 2015b; 2017b; Zhu et al., 2017;
248 Doley et al., 2017).

249

250 *4.2 Uptake of ^{14}C-glucose into soil microbial biomass*

251 The study revealed that microbial uptake in the chemically amended soils did, however, show reduced substrate
252 utilization. Thus, the amount of glucose incorporated was lower in amended soils. Studies have shown that

253 microorganisms subjected to stress exhibit a higher ratio of respired-C to biomass-incorporated-C; indicating a
254 reduced microbial substrate utilization efficiency under chemical stress and a change in community structure
255 following substrate addition (Bargett and Saggar, 1994; Witter and Dahlin, 1995; Frostegård et al., 1996; Knight
256 et al., 1997; Boucard et al., 2008; Gunina et al., 2017). Killham (1985) recorded that increasing stress often causes
257 a reduction in soil respiration, soil dehydrogenase activity and an increase in the ratio of respired-C to biomass
258 incorporated-C. Furthermore, the decreased biomass uptake observed with N-PAHs (B[h]Q) over time may be
259 attributed to microbial toxicity and/or oxidative stress as shown by the consistent increase in BQ to >1. (It should
260 be noted that BQ values >1 signifies oxidative stress to microbial community). McGrath et al. (1995) observed
261 that long-term exposure results in decreased soil microbial biomass. In this study, it may be because
262 microorganisms differ in their sensitivity to chemicals and prolonged N-PAHs exposure may have increased the
263 mortality of cells due to disturbance in the normal functioning, and/or gradually changed the community sizes due
264 to alterations in viability or competence (Van Beelen and Doelman, 1997; Giller et al., 1998; Anyanwu and
265 Semple 2016a; Molaei et al., 2017; Siles and Margesin, 2017).

266 Biomass uptake varied significantly over time; and the variations among chemicals were observed to be
267 consistent. This confirms the findings of Chander and Brookes (1991); Bardgett and Sagger (1994); Boucard et
268 al. (2008). Despite the variations, however, it could be concluded that soil microorganisms subjected to long term
269 N-PAH exposure, may not be able to maintain the same overall biomass as in un-contaminated soil.

270

271 *4.3 Impact of phenanthrene and its nitrogen-containing analogues on the k_{EC} coefficient*

272 The K_{EC} coefficient is related to the extractability from the soil of the microbial-C after it has been released from
273 dead fumigated cells. The k_{EC} coefficient, which is used to convert the C-flush of oxidizable organic-C to
274 microbial-C, allows for the incomplete release and extraction of microbial-C, and was obtained by calibrating
275 against alternative methods to estimate the microbial-C (Sparling et al., 1990).

276 Variation in k_{EC} coefficients (fixed and variable) was observed. The observed variations in k_{EC} coefficients after
277 fumigation are consistent with the findings in water content (Sparling and West, 1989; Ross, 1990 b) and sheep
278 dip formulation (Boucard et al., 2008). The cause is not known, however, difference in chemical amendments
279 may be attributable. This portrays the impact of contaminants on soil microbial uptake and further showed that
280 the fixed k_{EC} coefficient (0.35), fails to consider the impact of contaminated sites on soil microorganism; thus

overestimating (and/or underestimating) the biomass uptakes in contaminated soils (if the biomass uptakes calculated with the variable k_{EC} values are considered to be more accurate). In addition, the calculated k_{EC} coefficient showed that PAH and N-PAH contaminants can greatly affect the amount of substrate-C extracted by 0.5 M K_2SO_4 after fumigation.

In this present study, it could be that: (1) N-PAHs may have impacted the k_{EC} coefficients by influencing the factors that modify the toxicity of contaminants in soil; such as, physico-chemical properties and/or the physiological state of the microbes (Boucard et al., 2008; Siles and Margesin, 2017); or (2) The impact of N-PAHs may have resulted in a possible reduction in the efficiency of chloroform disintegration of the microbial cell membrane (lysis) or interference with the K_2SO_4 extraction (Sparling et al., 1990; Joergensen et al., 1995; Badalocco et al., 1997; Boucard et al., 2008). However, N-PAHs bioavailability and/or differences in microbial community structure between soils that vary in their sensitivity to chemical toxicity (Butler et al., 2011), could be an important factor in explaining the variability in k_{EC} coefficients.

5. Conclusions

In this current study, the presence of N-PAHs resulted in alterations to soil microbial activity and functions. It could be that the increased energy requirement for repair and maintenance probably was the main reason for the increased respiration, but synergistic process cannot be neglected. However, the study was unable to ascertain if the biomass uptakes in the chemically amended soils were characterized by either a low substrate utilization efficiency or death rate; if stress increased the burden of the microbial community. Nevertheless, B[h]Q, may have persistent deleterious impacts on soil microorganisms. From an ecotoxicity perspective, future investigations should consider the impact of these contaminants on changes in the soil microbial community structure. Further studies could also investigate the development of bacterial and fungal degrading populations within the microbial community which may be able to exploit the C and N for their metabolic needs.

Acknowledgment

This work was funded by Petroleum Technology Development Fund (PTDF), Nigeria.

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444 **Table 1.** Extractability (%) of phenanthrene and its N-PAHs from soil over time

Chemical	Initial chemical conc (mg/kg)	Mean chemicals extracted (mg/kg)			
		Time (d)			
		0	30	60	90
Phen	100	78.00± 8.00	42.10±5.70	11.30 ± 3.10	3.10 ± 0.30
1,7-Phen	100	59.30± 9.00	54.40±12.50	49.60 ± 4.00	38.00 ±8.00
B[h]Q	100	59.20± 9.00	89.40 ± 7.00	64.10 ± 6.00	58.70 ± 4.20
4,7-Phen	100	91.90 ± 4.20	41.70 ± 6.40	29.30 ± 4.00	29.60 ± 3.90

445 Source: Anyanwu and Semple (2015b).

Table 2. Distribution of ^{14}C-glucose in soils amended with 100 mg/kg single and binary mixtures of phenanthrene and its N-PAH analogues after 5 d

Chemical	Time (d)	Fastest rates (% h^{-1})	Respired $^{14}CO_2$ (%)	C–flush[a]	Biomass uptake[b] (*fixed* k_{EC} = 0.35**)**	Biophysical quotient [c]	Biomass uptake[d] (*variable* k_{EC})	Biophysical quotient[e]	Calculated k_{EC}[f]
Control	1	0.46 ± 0.08	42.62 ± 2.20	2.59 ± 0.33	7.41 ± 0.94**	5.75 ± 2.32*	53.42 ± 6.81**	0.79 ± 0.32	0.048 ± 0.00
	30	0.25 ± 0.01	35.07 ± 0.45*	3.63 ± 0.57	10.36 ± 1.63**	3.38 ± 0.27	54.02 ± 8.52**	0.64 ± 0.05	0.067 ± 0.01
	60	0.18 ± 0.03	31.07 ± 2.00*	1.07 ± 0.31	3.06 ± 0.87**	10.15 ± 2.28	63.45 ± 18.1**	0.48 ± 0.11	0.016 ± 0.00
	90	0.18 ± 0.00	28.42 ± 1.26*	6.97 ± 1.28	19.92 ± 3.65**	1.42 ± 0.34	46.67 ± 8.55**	0.60 ± 0.14	0.149 ± 0.02
1,10-Phen	1	0.43 ± 0.02	38.81 ± 2.84	1.49 ± 0.27	4.24 ± 0.77**	9.14 ± 0.00*	58.91 ± 10.78**	0.65 ± 0.26	0.025 ± 0.00
	30	0.29 ± 0.02	36.98 ± 0.81*	2.96 ± 0.02	8.46 ± 0.06**	4.72 ± 0.03	51.90 ± 0.39**	0.71± 2.02	0.057 ± 0.00
	60	0.23 ± 0.03	31.83 ± 1.55*	0.77 ± 0.36	2.21 ± 1.04**	14.42 ± 0.00	61.00 ± 23.82**	0.52 ± 0.05	0.012 ± 0.00
	90	0.15 ± 0.03	23.13 ± 1.70*	2.35 ± 0.04	6.72 ± 0.10**	3.44 ± 0.08	56.01 ± 0.85**	0.41 ± 1.98	0.04 2 ± 0.00
1,7-Phen	1	0.43 ± 0.02	41.77 ± 1.99	2.27 ± 0.08	6.49 ± 0.22**	6.43 ± 0.01*	54.21± 1.87**	0.76 ± 1.06	0.041 ± 0.00
	30	0.30± 0.03	36.90 ± 1.27*	2.82 ± 0.32	8.05 ± 0.90**	4.58 ± 0.00	51.35 ± 5.79**	0.71 ± 0.21	0.054 ± 0.00
	60	0.21 ± 0.02	36.83 ± 2.06*	1.67 ± 0.74	4.77 ± 2.11**	7.72 ± 0.00	53.72 ± 23.83**	0.68 ± 0.08	0.031 ± 0.01
	90	0.50 ± 0.07	34.74 ± 2.43*	5.28 ± 1.22	15.08 ± 3.47**	2.30 ± 0.00	37.49 ± 8.63**	0.92 ± 0.28	0.140 ± 0.03
4,7-Phen	1	0.26 ± 0.03	33.51 ± 2.93	0.31 ± 0.06	0.90 ± 0.16**	37.24 ± 0.03*	62.45 ± 11.22**	0.53 ± 0.26	0.005 ± 0.00
	30	0.22 ± 0.03	35.31 ± 1.78*	3.03 ± 0.44	8.65 ± 1.25**	4.08 ± 0.00	56.11 ± 8.16**	0.62 ± 0.21	0.053 ± 0.01
	60	0.20 ± 0.00	33.57 ± 0.27*	1.27 ± 0.16	3.64 ± 0.44**	9.22 ± 0.00	57.71 ± 7.10**	0.58 ± 0.03	0.022 ± 0.00
	90	0.17 ± 0.04	27.31 ± 2.35*	2.21 ± 0.62	6.31 ± 1.77**	4.32 ± 0.00	39.41 ± 11.08**	0.69 ± 0.21	0.056 ± 0.01
B[h]Q	1	0.37 ± 0.05	39.61 ± 1.83	0.53 ± 0.09	1.52 ± 0.25**	26.01 ± 0.01*	56.72 ± 9.43**	0.69 ± 0.19	0.009 ± 0.00
	30	0.28 ± 0.03	42.52 ± 2.73*	3.06 ± 0.61	8.75 ± 1.72**	4.85 ± 0.00	46.12 ± 9.11**	0.92 ± 0.29	0.066 ± 0.01
	60	0.34 ± 0.05	44.62 ± 2.30*	1.13 ± 0.47	3.22 ± 1.33**	13.86 ± 0.00	41.51 ± 17.26**	1.07 ± 0.13	0.027 ± 0.01
	90	0.70 ± 0.05	37.01 ± 2.25*	2.50 ± 0.23	7.16 ± 0.65**	5.17 ± 0.01	34.03 ± 3.11**	1.08 ± 0.72	0.073 ± 0.00
Phen	1	0.35 ± 0.02	36.42 ±1.21	0.23 ± 0.00	0.66 ± 0.13**	54.76 ± 0.17*	60.99 ± 1.27**	0.59 ± 0.95	0.003 ± 0.00
	30	0.27 ± 0.01	37.17 ± 0.92*	3.32 ± 0.18	9.48 ± 0.51**	3.91 ± 0.00	42.74 ± 2.31**	0.86 ± 0.39	0.077 ± 0.00
	60	0.23 ± 0.01	32.01 ± 2.47*	1.39 ± 1.03	3.96 ± 2.94**	8.07 ± 0.00	58.41 ± 43.33**	0.54 ± 0.05	0.023 ± 0.01
	90	0.19 ± 0.01	26.78 ± 0.21*	2.19 ± 0.35	6.27 ± 1.00**	4.27 ± 0.00	51.48 ± 8.24**	0.52 ± 0.02	0.042 ± 0.00
1,10-Phen+Phen	1	0.41 ± 0.07	41.82 ± 3.49	0.64 ± 0.08	1.82 ± 0.21**	22.02 ± 0.03*	50.59 ± 6.09**	0.82 ± 0.57	0.012 ± 0.00
	30	0.26 ± 0.04	36.20 ± 2.80*	1.53 ± 0.56	4.39 ± 1.58**	8.25 ± 0.00	55.02 ± 19.92**	0.65 ± 0.14	0.027 ± 0.01

	60	0.25 ± 0.02	36.33 ± 1.44*	1.13 ± 1.16	3.73 ± 3.31**	9.74 ± 0.00	53.70 ± 47.77**	0.67 ± 0.03	0.024 ± 0.02
	90	0.15 ± 0.03	23.87 ± 0.87*	2.47 ± 0.42	7.05 ± 1.20**	3.38 ± 0.00	50.07 ± 8.55**	0.47 ± 0.10	0.049 ± 0.00
1,7-Phen+Phen	1	0.44 ± 0.10	42.72 ± 2.11	0.77 ± 0.11	2.19 ± 0.31**	19.51 ± 0.01*	55.99 ± 8.12**	0.79 ± 0.25	0.013 ± 0.00
	30	0.26 ± 0.04	38.02 ± 1.10*	2.56 ± 0.18	7.32 ± 0.51**	5.19 ± 0.00	52.74 ± 3.70**	0.72 ± 0.29	0.048 ± 0.00
	60	0.25 ± 0.03	36.41 ± 2.38*	1.44 ± 0.61	4.10 ± 1.74**	8.87 ± 0.00	53.44 ± 22.77**	0.68 ± 0.10	0.026 ± 0.01
	90	0.40 ± 0.04	31.99 ± 2.22*	2.50 ± 0.12	7.16 ± 0.33**	4.47 ± 0.03	41.45 ± 1.93**	0.77 ± 1.14	0.060 ± 0.00
4,7-Phen+Phen	1	0.53 ± 0.02	44.20 ± 0.72	0.52 ± 0.08	1.47 ± 0.23**	29.98 ± 0.00*	59.37 ± 9.45**	0.74 ± 0.07	0.008 ± 0.00
	30	0.27 ± 0.01	39.88 ± 1.00*	3.30 ± 0.28	9.44 ± 0.79**	4.22 ± 0.00	53.39 ± 4.49**	0.74 ± 0.22	0.061 ± 0.00
	60	0.21 ± 0.03	35.89 ± 1.93*	1.89 ± 1.19	5.41 ± 3.39**	6.63 ± 0.00	57.95 ± 36.37**	0.61 ± 0.05	0.032 ± 0.02
	90	0.28 ± 0.05	31.21 ± 1.62*	3.02 ± 0.61	8.63 ± 1.73**	3.61 ± 0.00	52.73 ± 10.57**	0.59 ± 0.15	0.057 ± 0.01
BhQ+Phen	1	0.43 ± 0.04	43.89 ± 1.91	0.39 ± 0.04	1.12 ± 0.11**	39.21 ± 0.03*	54.64 ± 5.66**	0.80 ± 0.33	0.007 ± 0.00
	30	0.31 ± 0.04	42.91 ± 1.73*	2.38 ± 0.22	6.79 ± 0.61**	6.31 ± 0.00	49.32 ± 4.48**	0.86 ± 0.38	0.048 ± 0.00
	60	0.37 ± 0.03	43.00 ± 2.00*	2.42 ± 0.14	6.92 ± 0.39**	6.21 ± 0.01	50.66 ± 2.92**	0.84 ± 0.68	0.047 ± 0.00
	90	0.34 ± 0.05	32.63 ± 2.34*	3.48 ± 0.43	9.93 ± 1.22**	3.28 ± 0.00	44.93 ± 5.55**	0.72 ± 0.24	0.077 ± 0.00

[a] C-flush = ^{14}C-activity in fumigated soil–^{14}C- activity in un-fumigated soil

[b] ^{14}C-microbial biomass = ^{14}C-flush/fixed k_{EC} 0.35

[c] BQ = ^{14}C respired/^{14}C in biomass (using fixed k_{EC} 0.35)

[d] ^{14}C-microbial biomass = ^{14}C-flush/variable k_{EC}

[e] BQ = ^{14}C respired/^{14}C in biomass (using variable k_{EC})

[f] k_{EC} = (^{14}C-flush)/(initial ^{14}C-activity added–^{14}C respired–^{14}Cactivity in un-fumigated soil)

Conc = 100 mg/kg

n = 3

* = $p<0.05$

** = $p<0.001$

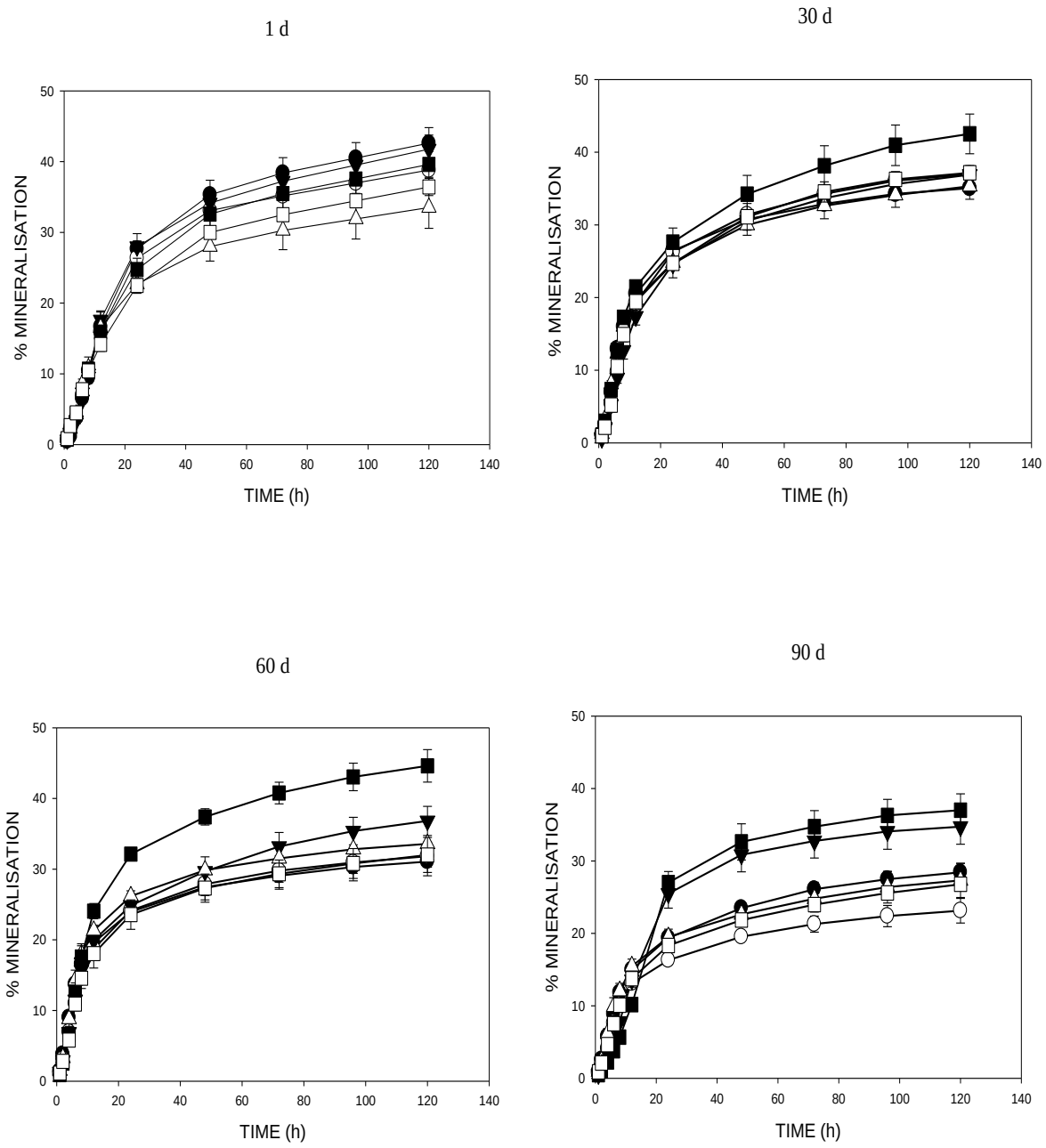

Fig. 1. Microbial mineralisation of ^{14}C-glucose in soils amended with phenanthrene and its N-PAH analogues (single amendments). The 1–90 d incubation graphs shows: control (●), 1,10-Phen (○), 1,7-Phen (▼), 4,7-Phen (Δ), B[h]Q (■) and Phen (□). Conc = 100 mg/kg.

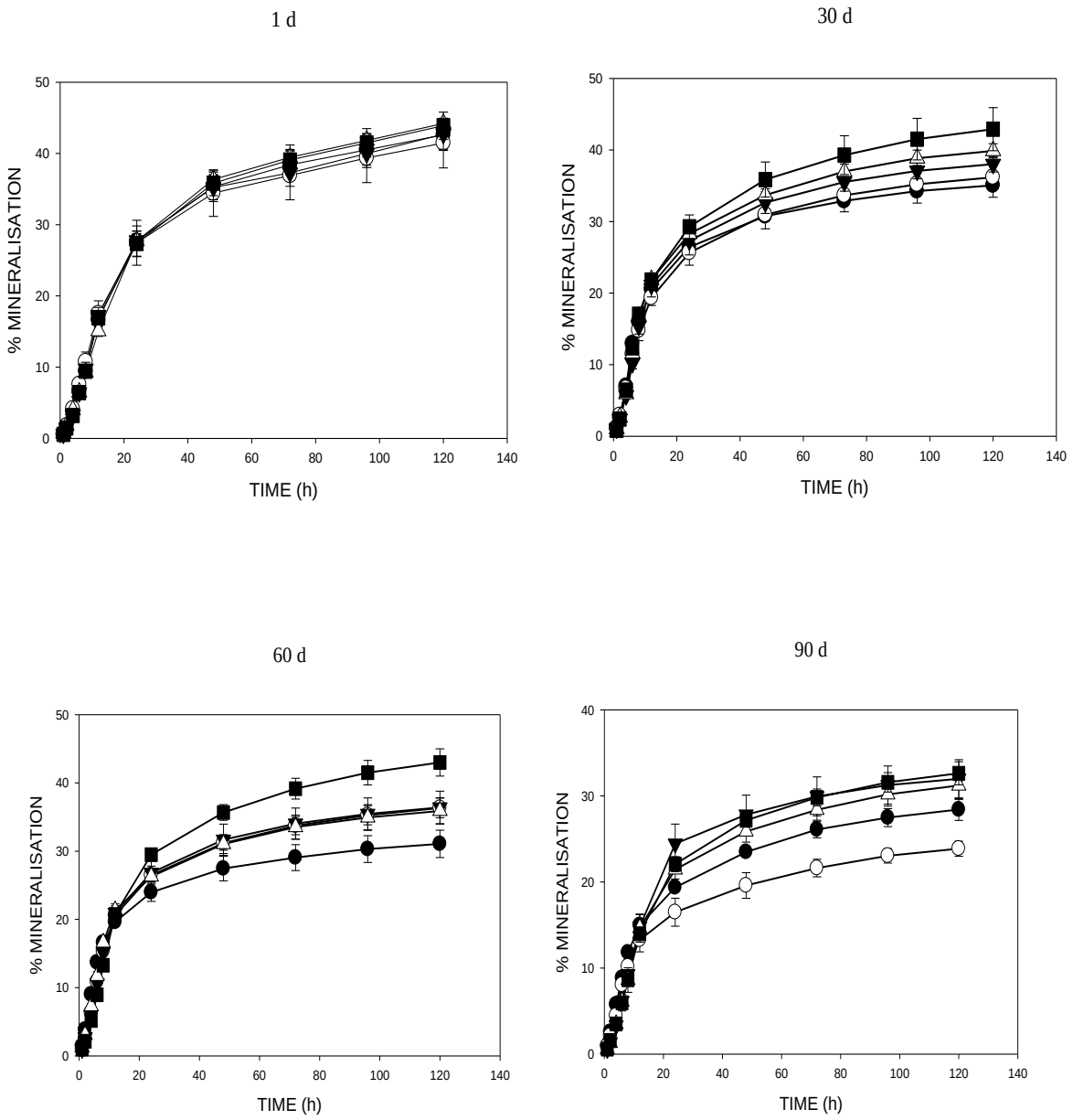

Fig. 2. Microbial mineralisation of ^{14}C-glucose in soils amended with phenanthrene and its N-PAH analogues (binary mixtures). The 1–90 d incubation graphs shows: control (●), 1,10-Phen + Phen (○), 1,7-Phen + Phen (▼), 4,7-Phen + Phen (Δ) and B[h]Q + Phen (■). Conc = 100 mg/kg.