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

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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 ¹⁴C-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 ¹⁴C-glucose into the microbial biomass. The result revealed that the extents of ¹⁴C-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 impose oxidative stress on soil microbial community.

Key words: N-PAHs, phenanthrene, ¹⁴C-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 (Fließbach 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 resired-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 (Fließbach 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 14C-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 14C-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 14C-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:
1/3 (100 g; n = 3) were amended with phenanthrene and four N-PAH standards (benzo[h]quinoline, 1,10-phenanthroline, 1,7-phenanthroline or 4,7-phenanthroline) dissolved in acetone to give concentration of 100 mg kg⁻¹. The amended soils were kept in the fume hood for 3 h to allow the carrier solvent volatilize, after which the soils were mixed with the remaining 1/3 (200 g). Blanks were prepared using un-amended soils. Soils amended with acetone only were also prepared to serve as a control. The amended soils were kept in amber glass jars and aged in the dark at 21 ± 1°C for 1, 30, 60 and 90 d. Soil moisture content was checked regularly and lost water was replenished with deionized water. After each ageing time (30 d interval), soils were analysed for microbial-substrate-mineralisation and biomass uptake. Extractability of phenanthrene and the N-PAH analogues from soil over time, and their percentage recoveries has been reported by Anyanwu and Semple (2015b, 2016a) (Table 1).

2.3 Mineralisation of 14C-glucose in soil.

The ability of indigenous soil microorganisms to mineralise 14C-glucose to 14CO₂ was assessed at 1, 30, 60 and 90 d contact time. Respirometric assays were carried out in modified 250 ml Schott bottles incorporating a Teflon-lined screw cap containing 1 M NaOH to trap any 14CO₂ (Reid et al., 2001). A slurry system with a solid: liquid ratio of 2:1 (20 g soil: 10 ml sterile water) was used to ensure complete 12/14C-glucose distribution. Standards were prepared in sterilized deionised water and delivered to give a 12C-glucose concentration of 3 mM glucose solution with an associated 14C-activity of 800 Bq per respirometer. Controls were also prepared. Respirometers were shaken at 100 rpm on an orbital shaker (Janke and Kunkel, IKA®-Labortechnik KS 510D), in the dark at 21±1°C. Sampling was carried out every 1, 2, 4, 6, 8, 12, 24 h and 2, 3, 4, 5 d with the vials containing trapped 14CO₂. Goldstar liquid scintillation cocktail was added to the vials. The vials were stored in the dark for 24 h before sample quantification was carried out by liquid scintillation counting (LSC) using standard calibration and quench correction techniques (Reid et al., 2001).

2.4 Uptake of 14C glucose into microbial biomass

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

a) Sample oxidation: The first sample was oven dried at 30°C and combusted in a sample oxidizer (Packard 307) to determine the level of 14C-activity remaining (i.e. residual 14C-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 CO₂ 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 K₂SO₄ (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 ¹⁴C-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 ¹⁴C-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 K₂SO₄, filtered (using Whatman No 1 filter papers) and analysed as per the un-fumigated extract.

2.5 Statistical analysis

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

\[ \text{¹⁴C-flush} = \text{¹⁴C-activity in fumigated soil} - \text{¹⁴C-activity in un-fumigated soil}. \]

\[ \text{¹⁴C-microbial biomass} = \frac{\text{¹⁴C-flush}}{k_{EC}}. \]

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 ¹⁴C-microbial biomass was re-calculated with the new coefficient. This process is based on the assumption that; the calculated ¹⁴C-labelled microbial-C is a representative of the total microbial biomass and that all the ¹⁴C-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).

\[ k_{EC} = \frac{(\text{¹⁴C-flush})}{(\text{¹⁴C-init} - \text{¹⁴C-respired} - \text{¹⁴C-activity in un-fumigated soil})}. \]

¹⁴C-flush and ¹⁴C-microbial biomass were later on expressed as percentages of the initial ¹⁴C-activity (¹⁴C_init).
3. Biophysical quotients (BQ) were calculated as:

\[ \text{BQ} = \frac{^{14}\text{CO}_2 \text{ respired}}{^{14}\text{C}-\text{microbial biomass (calculated from either fixed or variable } k_{\text{EC}})} \].

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 \(^{14}\text{C}-\text{glucose}\) was determined using analysis of variance (ANOVA). The statistical difference between the biomass calculated with fixed and variable \(k_{\text{EC}}\) 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 \(^{14}\text{C}-\text{glucose}\) to \(^{14}\text{CO}_2\) by soil microorganisms

The mineralisation of \(^{14}\text{C}-\text{glucose}\) 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 \(^{14}\text{C}-\text{substrate}\) (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 (% \(^{14}\text{CO}_2\) h\(^{-1}\)) recorded maximum values after 24 h following addition of \(^{14}\text{C}-\text{glucose}\) 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 \(^{14}\text{C}-\text{glucose}\) (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 \(^{14}\text{CO}_2\)-respired (%)) were determined (Table 2). The results revealed that the extents of mineralisation of \(^{14}\text{C}-\text{glucose}\) 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 \(^{14}\text{C}-\text{glucose}\) 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$_2$SO$_4$ at 30 d and 90 d.
3.3 Uptake of $^{14}$C-glucose into microbial biomass

The incorporation of the $^{14}$C-substrate into the microbial biomass in soils amended with 100 mg/kg single and binary mixtures of phenanthrene and the N-PAH analogues were calculated using fixed and variable $k_{EC}$, respectively (Table 2). Although not showing a consistent trend, the results showed that increase in fixed $k_{EC}$ values, lead to decrease in variable $k_{EC}$ values and vice versa in all the amendments, with the exception of B[h]Q amendment (Table 2). Statistical analysis of data showed statistically significant differences in the calculated data 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 consistent trend of higher values in the C-flush resulting in higher biomass values (fixed $k_{EC}$) and lower biomass values (variable $k_{EC}$) (Table 2).

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

4. Discussion

4.1 Mineralisation of $^{14}$C-glucose to $^{14}$CO$_2$ by soil microorganisms

The impact of single and binary mixtures of phenanthrene and its nitrogen-containing analogues on microbial utilisation of $^{14}$C-glucose in soil was studied over a 90-d incubation. Loss of phenanthrene, benzo[h]quinoline, 1,10-phenanthroline, 1,7-phenanthroline or 4,7-phenanthroline through volatilisation was considered minimal due to the sealed nature of the incubations (Hofman et al., 2008; Towell et al., 2011). From the results, mineralisation of $^{14}$C-glucose was greater in the N-PAHs amended soils than the control soil after 1 d; indicating that microorganisms utilized energy for cell maintenance rather than biosynthesis of new cells. This phenomenon
agrees with the observations of Bargett and Saggard (1994), Witter and Dahlin (1995), Chnader and Joergensen (2001), Boucard et al. (2008), and Bore et al. (2017). In support, Fließbach et al. (1994) reported that in heavily contaminated sites, soil respiration increased substantially compared to the corresponding low contaminated soils. Respiration has been linked as a process and microbial biomass as a pool to metabolic quotient for CO$_2$ ($q_{CO_2}$) by Anderson and Domsch (1986). Thus, it is widely accepted that a high $q_{CO_2}$ is a surprisingly common characteristic of soil microbial biomass in chronically contaminated soils (Fließbach et al., 1994). This has been suggested to be a useful indicator of oxidative stress in soils (Brookes, 1993; Mooshammer et al., 2017). In addition, Gunina et al. (2017) reported that an increased $q_{CO_2}$ indicates stress to the soil microbial community.

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

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

### 4.2 Uptake of $^{14}$C-glucose into soil microbial biomass

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

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

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

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

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

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

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Initial chemical conc (mg/kg)</th>
<th>Mean chemicals extracted (mg/kg)</th>
<th>Time (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Phen</td>
<td>100</td>
<td>78.00± 8.00</td>
<td>42.10±5.70</td>
</tr>
<tr>
<td>1,7-Phen</td>
<td>100</td>
<td>59.30± 9.00</td>
<td>54.40±12.50</td>
</tr>
<tr>
<td>B[h]Q</td>
<td>100</td>
<td>59.20± 9.00</td>
<td>89.40 ± 7.00</td>
</tr>
<tr>
<td>4,7-Phen</td>
<td>100</td>
<td>91.90± 4.20</td>
<td>41.70 ± 6.40</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Control</th>
<th>1,10-Phen</th>
<th>1,7-Phen</th>
<th>4,7-Phen</th>
<th>B[h]Q</th>
<th>Phen</th>
<th>1,10-Phen+Phen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (d)</td>
<td>Fastest rates (% h$^{-1}$)</td>
<td>Respired $^{14}$CO$_2$ (%)</td>
<td>C-flush$^b$</td>
<td>Biomass uptake$^b$ (fixed $k_{EC}$ = 0.35)</td>
<td>Biophysical quotient $^c$</td>
<td>Biomass uptake$^d$ (variable $k_{EC}$)</td>
<td>Biophysical quotient$^c$</td>
</tr>
<tr>
<td>1</td>
<td>0.46 ± 0.08</td>
<td>42.62 ± 2.20</td>
<td>2.59 ± 0.33</td>
<td>7.41 ± 0.94**</td>
<td>5.75 ± 2.32*</td>
<td>53.42 ± 6.81**</td>
<td>0.79 ± 0.32</td>
</tr>
<tr>
<td>1</td>
<td>0.43 ± 0.02</td>
<td>38.81 ± 2.84</td>
<td>1.49 ± 0.27</td>
<td>4.24 ± 0.77**</td>
<td>9.14 ± 0.00*</td>
<td>58.91 ± 10.78**</td>
<td>0.65 ± 0.26</td>
</tr>
<tr>
<td>1</td>
<td>0.43 ± 0.02</td>
<td>41.77 ± 1.99</td>
<td>2.27 ± 0.08</td>
<td>6.49 ± 0.22**</td>
<td>6.43 ± 0.01*</td>
<td>54.21 ± 1.87**</td>
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\[a \] C-flush = \[^{14}\text{C}\]-activity in fumigated soil - \[^{14}\text{C}\]-activity in un-fumigated soil  

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

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

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

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

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

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.