International Journal of Environmental Bioremediation & Biodegradation, 2015, Vol. 3, No. 3, 66-78
Available online at http://pubs.sciepub.com/ijebb/3/3/1

DOI:10.12691/ijebb-3-3-1

The Effect of Flavonoids on the Microbial Mineralisation of Polycyclic Aromatic Hydrocarbons in Soil

Aniefiok E. Ite[1,2,3,*], Kirk T. Semple[1]

[1]Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, United Kingdom
[2]Research and Development, AkwaIbom State University, P.M.B. 1167, Uyo, AkwaIbom State, Nigeria
[3]Faculty of Natural and Applied Sciences, AkwaIbom State University, P.M.B. 1167, Uyo, AkwaIbom State, Nigeria
*Corresponding author: aniefiokite@yahoo.co.uk

Abstract The effect of flavonoids (flavone, morin hydrate and 3-hydroxyflavone)on the microbial mineralisation of polycyclic aromatic hydrocarbons (PAHs) in soil slurry by the indigenous microbial communities has been investigated. The rates and extents of ^{14}C–PAHs (^{14}C–naphthalene, ^{14}C–phenanthrene and ^{14}C–pyrene)mineralisation in artificially spiked soils were monitored in the absence and presence of flavonoids applied at three different concentrations (50, 100 and 200 µg kg^{-1}) either as single compounds or as a mixture of flavonoids (flavone, morin hydrate and 3-hydroxyflavone at a 1:1:1 ratio). Respirometric and microbial assays were monitored in fresh (0 d) and pre–incubated (28 d) artificially spiked soils following amendment with flavonoids. The highest extents of^{14}C–naphthalene, ^{14}C–phenanthrene and ^{14}C–pyrenemineralisation (P< 0.001) were obtained in fresh artificially spiked soils amended with 100 µg kg^{-1}3-hydroxyflavonecompared to those obtained in unamended soils. However, amendment of fresh artificially spiked soils with higher concentrations of morin hydrate (≥ 100 µg kg^{-1}) inhibited mineralisation of^{14}C–pyrene by indigenous microbial communities.Apart from naphthalene and pyrene spiked soils amended with morin hydrate, pre–incubated artificially spiked soils amended with 100 µg kg^{-1} flavonoids had the highest extents of microbial mineralisation of PAHs compared to those obtained in unamended soils. The results obtained showed enhanced microbial mineralisation of ^{14}C–PAHs in fresh artificially spiked soils amended with 50 – 100 µg kg^{-1} flavonoidsand in addition, low molecular weight PAHs (containing < 4 aromatic rings) exhibited higher extents of microbial mineralisation compared to high molecular weight PAH.Depending on its (bio) available concentration and solubility in soil, flavonoids can either stimulate or inhibit mineralisation of PAHs by indigenous soil microbial communities. This study suggests that phytochemical–microbe interactions are essential drivers of ecosystem functions relevant for the biodegradation of petroleum–derived or organic contaminants in soils.

Keywords: *microbial mineralisation, PAHs, flavone, morin hydrate, 3-hydroxyflavone, soil*

Cite This Article: Aniefiok E. Ite, and Kirk T. Semple, "The Effect of Flavonoids on the Microbial Mineralisation of Polycyclic Aromatic Hydrocarbons in Soil." *International Journal of Environmental Bioremediation & Biodegradation*, vol. 3, no. 3 (2015): 66-78. doi: 10.12691/ijebb-3-3-1.

1. Introduction

Petroleum hydrocarbon contamination is a widespread and well recognised environmental problem in most oil–producing countries, causing acute and diffuse contamination of soil and sediments, surface and groundwater, marine environment and terrestrial ecosystems [1,2]. Microbial transformation plays a major role in degradation of most petroleum–derived contaminants, such as benzene, toluene, ethylbenzene and xylenes (BTEX), aliphatic and polycyclic aromatic hydrocarbons [2]. The main pathway for polycyclic aromatic hydrocarbons (PAHs) removal from environmental compartments is considered to be microbial transformation and growth–linked mineralisation by indigenous microbial communities [2,3,4]. Over the last few decades, biological and/or microbial degradation of PAHs in soils and sediments has been studied extensively [5-13]. It is widely known that PAHs can be resistant to biodegradation and may persist in the natural environment because of their hydrophobic nature, resulting in low water solubility and a tendency to be adsorbed to the soil and sediment matrices [14,15,16]. Biodegradation of organic contaminants in soil can be limited by factors such as adsorption, mass transfer and bioavailability, while combined plant–microbial systems can lead to more efficient growth–linked mineralisation in the root zone or rhizosphere [17]. Several researcheshave demonstrated that interactionsbetween plants and microorganisms are essential for thebiologicaland/or microbial transformation of organic contaminants in soil [17-22].

Plants employ several mechanisms to remediate soils and/or ecosystem contaminated with petroleum–derived or organic contaminants. Phytoremediation studies have demonstrated enhanced biodegradation of organic contaminants in the rhizosphere of certain plants [23,24,25]. For example, Yoshitomi and Shann [24] attributed enhanced polycyclic aromatic hydrocarbons (PAHs) degradation in the rhizosphere to the stimulation effect of plant root exudates. Some studies have shown the positive effect of root exudates, including phenolic

compounds, terpenes and flavonoids, on microbial activity in soil and on biodegradation of polychlorinated biphenyls(PCBs) [26,27,28]. It is widely known that roots secrete an enormous number of chemical compounds (phytochemicals) into the root zone orrhizosphere where the secreted chemicals mediate multi-partite interactions and alter their immediate environments around plant roots [29,30]. Plant secreted chemical compounds, including salicylic acid, monoterpenes and flavonoids, may facilitate transformation of organic contaminants with similar structures [18]; induce genes encoding enzymes involved in the degradation process, increase contaminant bioavailability, and/or increase in microbial activity and selective enrichment in the numbers of microbial communities that are capable of degrading specific contaminants[17,19,20]. In a study using a 'rhizosphere metabolomic' approach, Narasimhan *et al.* [31] identified a large majority of aromatic acids or phenylpropanoids, including flavonoids, in plant exudates. Flavonoids, which are common to vascular plants other than legumes [32], are known components of plant root exudates [33], plant tissue and plant based products.

Flavonoids, being *O*-containing heterocyclic compounds, belong toa diverse group of polyphenolic secondary metabolic compounds which has a general C_6–C_3–C_6 carbon framework (Figure 1). Flavonoids are produced by the phenylpropanoid biosynthetic pathway in plants and accumulate inthe vacuoles for release in exudates [32]. Based on the biosynthetic pathways, flavonoidscan be divided into about30 classes depending on the structure of the propane fragment (–C_3–) [34]. From further elucidation of the degree of oxidation of the C_3 unit, flavonoids could also be classified into six major subclasses: chalcones, flavones, flavonols, flavandiols, anthocyanins and proanthocyanidins, and a seventh subclass called aurones. In few studies [35,36], it has been demonstrated that some flavonoids can promote movement of microbial communities towards plant and stimulate microbial activities in rhizosphere. In a review, Cesco*et al.* *[33]* summarized the types and concentrations of flavonoids in root exudates and most of these were from plants grown in solution. Flavonoid concentrations vary widely and are dependent on plant growth conditions, sampling techniques, nutrient supply and plant species [33]. However, the fate of flavonoids in the rhizosphere depends on the physical–chemical properties of the soil and the presence of active microbial communities. Depending on their modifications, flavonoids persistence in the rhizosphere soils varies and can be < 72 h, depending on the chemical structure of the flavonoid [37]. Indigenous soil microbial communities undoubtedly influence the quality and quantity of flavonoids, both through modification of root exudation patterns and microbial catabolism of exudates [38,39]. Therefore, plant–secreted flavonoids may influence the population structure of rhizosphere associated microbial communities, stimulate the overall population size and activity of indigenous soil microbial communities that have the capability to degrade specific organic contaminants.

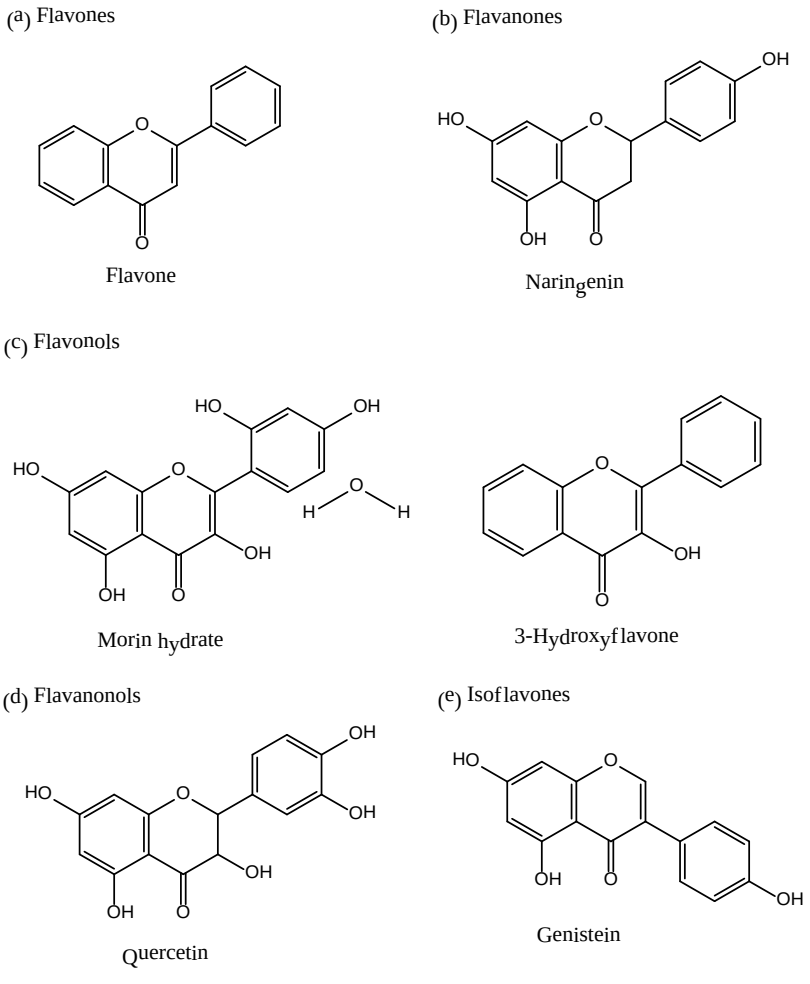

Figure 1. Chemical structures of some of the major groups of flavonoids

The hypotheses for this study were: (i) the presence of flavonoids above a threshold concentration in contaminated soil may stimulate microbial activity and subsequent prolific microbial population growth, and (ii) the addition of appropriate concentration of flavonoids or a mixture of flavonoidsmay increase bioavailability of organic contaminant, intrinsic microbial catabolic activity and enhance microbial mineralisationof PAHs in soil by active indigenous microbial communities. To address these hypotheses, the following aims were considered: (i) assessment of the microbial mineralisation of ^{14}C–PAHs(^{14}C–naphthalene, ^{14}C–phenanthrene and ^{14}C–pyrene) in soils, (ii) monitoring of microbial mineralisation of PAHs in artificially spiked soils in the absence and presence of flavonoids at three different concentrations (50, 100 and 200 µg kg^{-1}) either as single compounds or as a mixture of flavonoids(flavone, morin hydrate and 3-hydroxyflavone at a 1:1:1 ratio) and (iii) monitoring of PAHs (naphthalene, phenanthrene and pyrene) mineralisation by indigenous microbial communities and enumeration of microbial cell numbersin fresh (0 d) and pre–incubated (28 d) artificially spiked soils following flavonoids amendment.

2. Materials and Methods

2.1. Materials

Naphthalene (>96%); [UL–^{14}C] naphthalene (specific activity = 2–10 mCi mmol^{-1}, radiochemical purity > 95%), phenanthrene (>96%); [9–^{14}C] phenanthrene (specific activity = 50 mCi mmol^{-1}, radiochemical purity 99.6%), and pyrene (>98%); [4,5,9,10-^{14}C]pyrene (specific activity = 7.5 mCi mmol^{-1}, radiochemical purity 98.6%)were obtained from American Radiolabeled Chemicals (ARC) UK. Sigma–Aldrich UK suppliedflavone (≥98%), morin hydrate (>95%) and 3-hydroxyflavone (≥98%).Fisher Scientific UK supplied the nutrient agar, sodium hydroxide (NaOH) used for the CO_2 traps and the mineral basal salts (MBS) solution reagents. Ringer's solution pellets and plate count agar (PCA) powder were obtained from Oxoid Ltd, UK. Fisher Scientific UK supplied toluene and acetone used forvarious experimental procedures. Schott Duran® bottles (250 ml) with Teflon™ lined screw caps were supplied by Schott UK and the metal fittings used to make the respirometers were supplied by RS Components Ltd, UK. The 7 ml glass scintillation vials and Goldstar liquid scintillation cocktail were supplied by Meridian UK.

2.2. Soil Sampling and Analysis

A pristine agricultural soil (DystricCambisol) was collected from the A horizon (5 – 20 cm depth) from Myerscough Agricultural College, Lancashire, UK. Prior to spiking, the soil was air–dried for 24 h and subsequently homogenised by passing through a 2 mm mesh sieveto remove stones and residual plant materials [21]. The field moisture content was determined in triplicate by oven drying at 105 °C for 24 h [40]. Soil texture was determined using sedimentation, 40 g air dried soil and thesoil pH was determined using a calibrated pH meter (Model 657R-00), ratio 10 g soil:25 ml dH_2O. The total extractable organic carbon content was determined using loss on ignition method (450°C for 24 h) and a Carlo Erba CHNS–OEA 1108 CN–Elemental analyzer was used to determine the total carbon and nitrogen contents. In addition, the phosphate content was determined by acid digestion with HNO_3 and a phosphate reducing agent (neutralized with NaOH) was used to develop the characteristic blue colour for spectrometric determination at 882 nm (Cecil CE 1011 UV Spectrometer). All measurements were triplicate determinations.

2.3. Soil Spiking with Target Polycyclic Aromatic Hydrocarbons (PAHs)

Prior to spiking, soilsamples were rehydrated with deionised water to 70% soil water–holding capacity. Samples of the soil (240 g wet weight) were then spiked with ^{12}C–labelled naphthalene, phenanthrene and pyrene using acetone as the carrier solvent to give a final ^{12}C–hydrocarbon concentration of 20 mg kg^{-1} (dry weight). Each soils piked mixture was then blended following the method developed by Doick *et al.* *[41]*. In addition, controlsconsisting of rehydrated soil (120 g wet weight) only were produced as analytical blanks. The artificially spiked soils and controls were stored in amber glass jars (in triplicates) with loosely fitted Teflon–lined™ screw caps to allow ambient oxygen exchange. The samples of artificially spiked soil and controls were incubated in darkness at 21.5 ± 0.5°C and 45% relative humidity for 0 to 28 d. The pre–incubated artificially spiked soils were sampled at two time intervals (0and 28 d) for both respirometric assays and microbial analysis.

2.4. Mineralisation of ^{14}C–PAHsin Soil

The extents of mineralisation of ^{14}C–PAHs (naphthalene, phenanthrene and pyrene) in the artificially spiked soils were measured (in triplicate) through the evolution of $^{14}CO_2$ producedusing respirometric assays [42]. The mineralisation assay was performed in respirometers which consist of modified 250 ml Schott Duran® bottles containing 10 g ± 0.1 g soil (wet weight) and 30 ml autoclaved minimal basal salt solution [41]. Each of the respirometers was spiked with naphthalene, phenanthrene and pyrene standards prepared in toluene to deliver ^{12}C–PAHs (naphthalene, phenanthrene and pyrene) concentration of 10 mg kg^{-1} soil dry weight with an associated ^{14}C–activity of 83 Bq g^{-1} soil dry weight. A 7 ml scintillation vial containing 1 ml NaOH (1 M) solution was suspended from the lid of each respirometer to trap $^{14}CO_2$ that evolved as a result of microbial mineralisation of^{14}C–PAHs. In addition, unamended respirometers were prepared as outlined above, with 10± 0.1 g naphthalene, phenanthrene and pyrene spiked soils (wet weight) and 30 ml of autoclaved MBS solution.

To investigate techniques to optimise the microbial mineralisation of target organic contaminants, respirometers were also prepared as above with the following treatments: (i) fresh artificially spiked soils (0 d) amended with 50, 100 or 200 µg kg^{-1}flavonoids (flavone, morin hydrate and 3-hydroxyflavone) and a mixture of flavonoids (flavone, morin hydrate and 3-hydroxyflavone at a 1:1:1 ratio) using toluene as the carrier solvent, (ii) pre-incubated artificially spiked soils(28 d) amended with 50, 100 or 200 µg kg^{-1}flavonoids (flavone, morin hydrate and 3-hydroxyflavone) and a mixture of flavonoids prior to mineralisation assays, and (iii) artificially spiked soils with no flavonoids

amendment were used as controls to assess any increase in rates and extents of microbial mineralisation of the target organic contaminants.

Respirometers containing only 10 ± 0.1 g rehydrated soil (wet weight) and 30 ml of autoclaved MBS solution were also prepared as analytical blanks. During the 14 d mineralisation assay sampling period, respirometers were placed securely on an orbital shaker (SANYO Gallenkamp) at 21°C and shaken at 100 rpm to agitate and ensure adequate mixing of the soil slurry. The $^{14}CO_2$ traps were replaced every 24 h. Scintillation fluid (5 ml) was added to each spent $^{14}CO_2$ trap and stored in darkness overnight to reduce the effects of chemilumiescence [21]. The trapped ^{14}C-activity was counted using a Packard Canberra Tri-Carb 2300TR liquid scintillation counter and quantified using standard counting protocols with an automatic quench correction [43].The lag phases, rates and extents of ^{14}C–naphthalene, ^{14}C–phenanthrene and ^{14}C–pyrene mineralisation in the soil slurries were calculated based on the percentage of trapped $^{14}CO_2$ over the total pool of ^{14}C–labelled carbon.

2.5. Enumeration of Total Heterotrophic and PAH–degrading Bacteria

The number of total heterotrophic bacteria (THB) and indigenous naphthalene, phenanthrene and pyrene–degrading bacteria were evaluated following standard aseptic plate count techniques [44]. In brief, 1 ± 0.1 g soil was extracted with 10 ml quarter–strength sterile Ringer's solution following proper mixing and 0.1 ml extracts were then serially diluted with Ringer's solution. Serial dilutions of bacterial suspension (0.01 ml) were inoculated onto plate count agar for THB and agar–agar plates amended with 0.1% naphthalene, phenanthrene and pyrene as the sole carbon source for naphthalene, phenanthrene and pyrene–degrading bacteria. The inoculated plates were incubated at 25 ± 0.5 °C and the cell number of THB was counted after 48 h and > 7 d for naphthalene, phenanthrene and pyrene–degrading bacteria. The microbial cell number was expressed as colony–forming units per gram soil dry weight (CFU g^{-1}).

2.6. Statistical Analysis of Data

Statistical analysis of the results after blank correctionwas performed at various time intervals and statistically verified using *t*–tests after normality and equal variance tests (Tukey test, $P \leq 0.05$) using statistical software –SigmaStat®, Version 3.5 (Systat Software Inc., USA). PAHsmicrobial mineralisation profiles in artificially spiked soils in the absence and presence of flavonoidsare presented using graphing software package – SigmaPlot®, Version 12.5 (Systat Software Inc., USA).

Table 1. Physicochemical and microbial characteristics of Myerscough soil. Values are the mean (*n = 3*) ± standard errors of the mean (SEM)

Parameter		Value
pH in (dH_2O)		6.50 ± 0.08
Moisture content (%)		34.87 ± 0.89
Maximum water holding capacity (%)		38.03 ± 0.02
Elemental analysis		
	Total extractable organic carbon (%)	1.65 ± 0.01
	Total extractable carbon (%)	1.70 ± 0.09
	Total extractable nitrogren (%)	0.14 ± 0.01
	Soil organic matter (%)	2.71 ± 0.04
	Phosphorus (µg g^{-1})	997.00 ± 0.01
	C:N ratios	11.8:1
Particle analysis		
	Clay (%)	19.50 ± 0.70
	Silt (%)	20.00 ± 0.90
	Sand - Total (%)	60.40 ± 1.40
	Coarse sand	0.12 ± 0.01
	Medium sand	6.90 ± 0.10
	Fine sand	53.30 ± 0.60
Microbial analysis		
	Heterotrophs (CFU g^{-1})	$5.28\times10^4\pm 0.00\times10^0$
	Pyrene degraders (CFU g^{-1})	$3.24\times10^4\pm 3.33\times10^4$
	Phenanthrene degraders (CFU g^{-1})	$4.04\times10^4\pm 3.33\times10^4$
	Naphthalene degraders (CFU g^{-1})	$5.14\times10^4\pm 3.33\times10^4$

3. Results

3.1. Mineralisation of ^{14}C–PAHs in Soil

The physicochemical and microbiological properties of the soil are presented in Table 1. The ability of the indigenous soil microbial communities to mineralise ^{14}C–naphthalene, ^{14}C–phenanthrene and ^{14}C–pyrene were measured in fresh artificially spiked soils (Figure 2 – Figure 4 [A, B & C] and Table 2) and pre–incubated artificially spiked soils (Figure 2 – Figure 4 [D, E & F] and Table 3) in the absence and presence of flavonoids.The lag phases (time taken to achieve 5% mineralisation) in

fresh artificially spiked soils significantly ($P < 0.001$) varied between each of the target organic contaminants (51.93 ± 1.27 to 64.44 ± 1.93 h in systems with naphthalene; 112.17 ± 0.92 to 167.28 ± 0.25 h in systems with phenanthrene and 140.43 ± 3.49 to 201.57 ± 3.16 h in systems with pyrene; Table 2). In systems containing pyrene, amendment with 100 µg kg^{-1} morin hydrate resulted in statistically ($P < 0.05$) longer lag phases compared to the unamended systems (control) and fresh artificially spiked soils amended with 50 µg kg^{-1} morin hydrate. Apart from artificially spiked soils amended with 200 µg kg^{-1} 3–hydroxyflavone, flavonoids amendment did not affect lag phases ($P > 0.05$) in systems containing naphthalene. However, shorter lag phases were often measured in fresh artificially spiked soilsamended with 3–hydroxyflavone and the mixture of flavonoids (flavone, morin hydrate and 3-hydroxyflavone at a 1:1:1 ratio). After 28 dpre–incubation, there were significant ($P < 0.001$) reductions in lag phases in artificially spiked soils containing naphthalene (4.55 ± 0.02 to 7.13 ± 0.01 h) and phenanthrene (4.48 ± 0.02 to 6.55 ± 0.12 h) compared to systems containingpyrene (13.29 ± 0.83 to 28.34 ± 3.16 h)as presented in Table 3. The pre–incubated artificially spiked soils amended with 50, 100 and 200 µg kg^{-1} flavonoidsexhibited similar ($P > 0.05$)lag phases in systems containing naphthalene and phenanthrene. Although there were no statistical reductions of lag phases in soils in the absence and presence of flavonoids amendment ($P > 0.05$), longer lag phases were measured in fresh artificially spiked soils amended with morin hydrate. After 28 d pre–exposure, lag phases were significantly ($P < 0.001$) shorter in pre–incubated artificially spiked soils (Table 3) compared to those measured in fresh artificially spiked soils (Table 2)

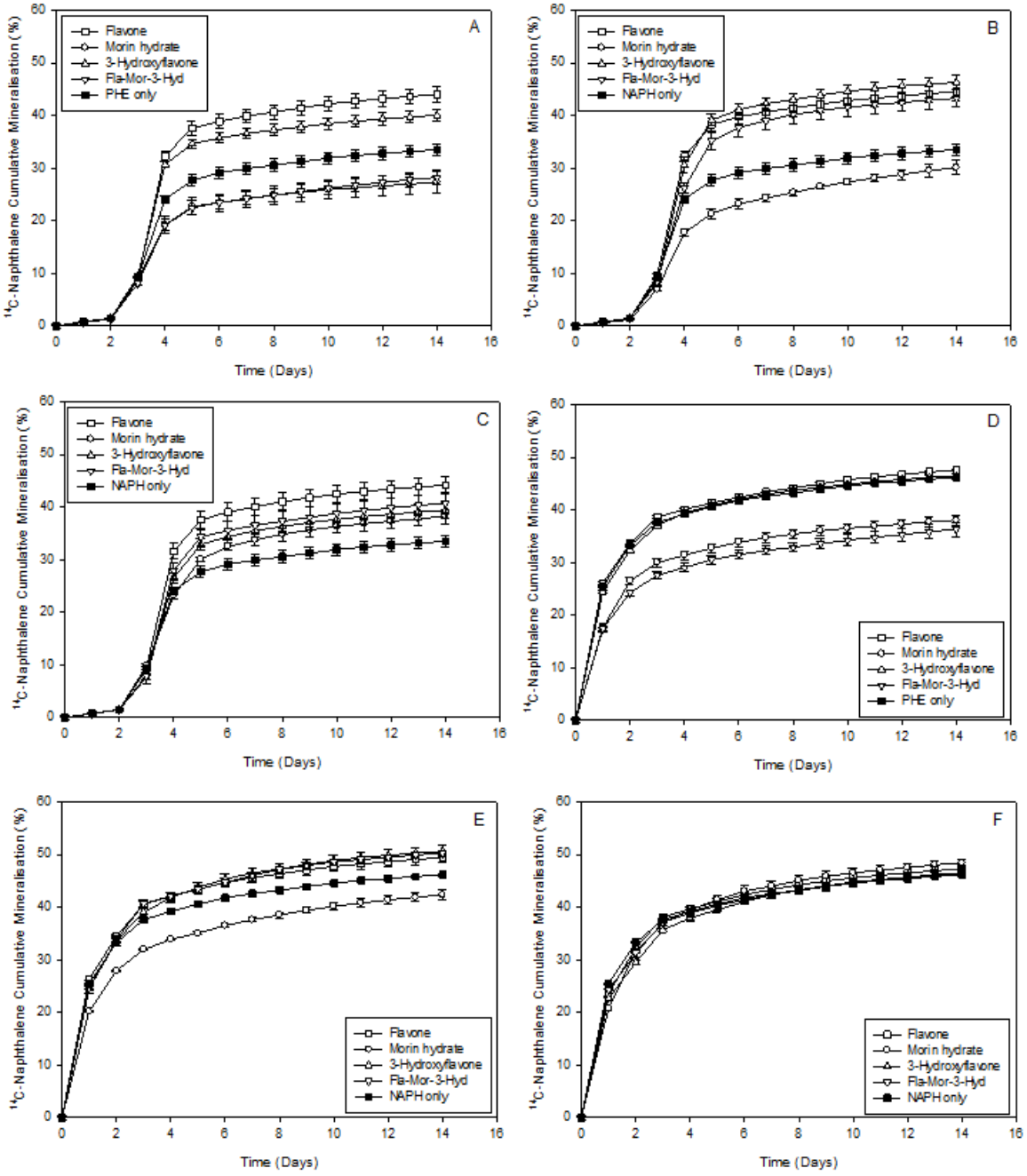

Figure 2. Development of microbial mineralisation of ^{14}C–naphthalene in fresh artificially spiked soils amended with flavonoids at concentrations of (A) 50 µg kg^{-1}, (B) 100 µg kg^{-1} and (C) 200 µg kg^{-1} and pre–incubated artificially spiked soils amended with flavonoids at concentrations of (D) 50 µg kg^{-1}, (E) 100 µg kg^{-1} and (F) 200 µg kg^{-1}. Error bars are the standard error of mean (SEM) and data are presentated as means ($n = 3$)

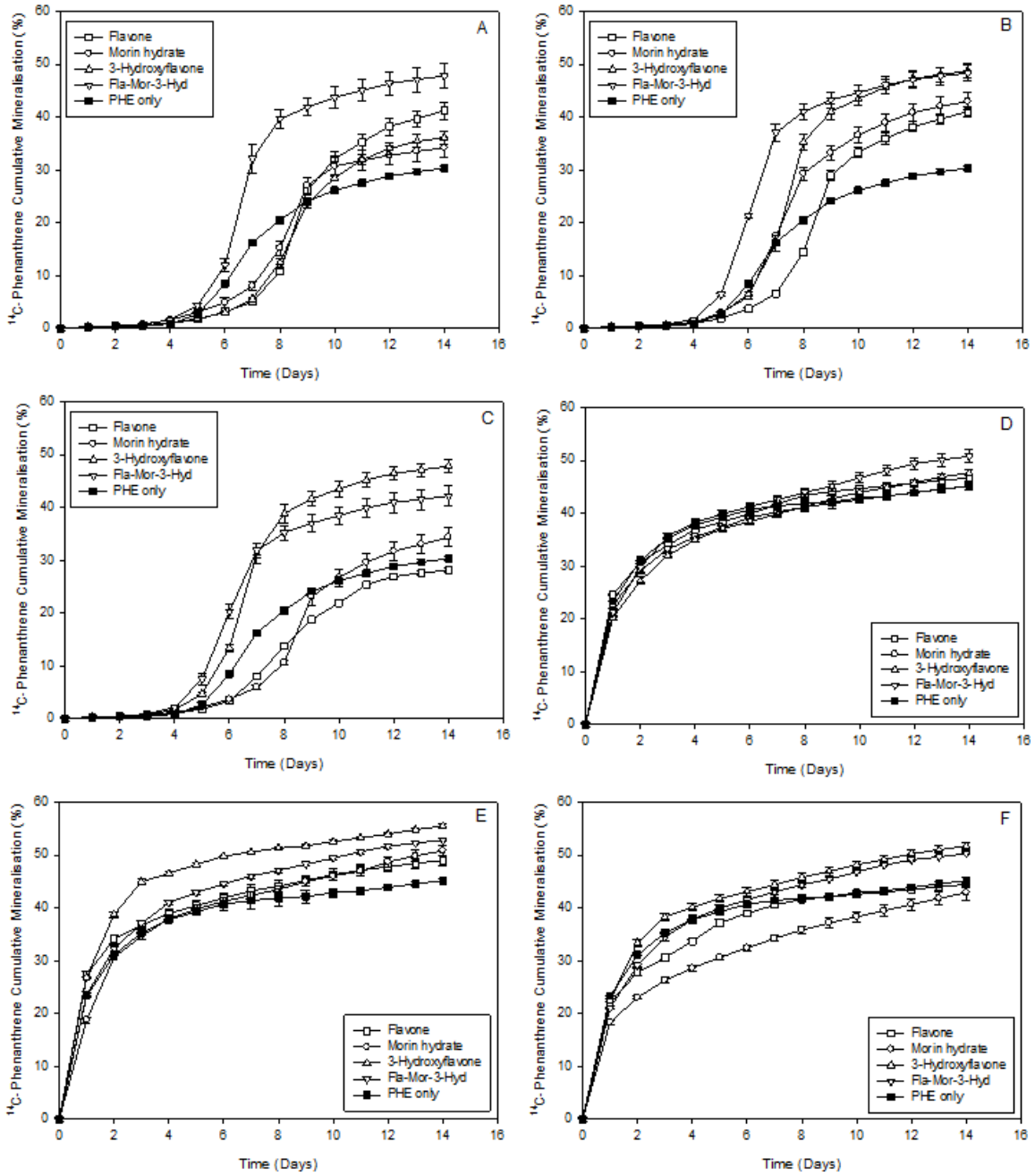

Figure 3. Development of microbial mineralisation of ^{14}C–phenanthrene in fresh artificially spiked soils amended with flavonoids at concentrations of (A) 50 µg kg $^{-1}$, (B) 100 µg kg $^{-1}$ and (C) 200 µg kg $^{-1}$ and pre–incubated artificially spiked soils amended with flavonoids at concentrations of (D) 50 µg kg $^{-1}$, (E) 100 µg kg $^{-1}$ and (F) 200 µg kg $^{-1}$. Error bars are the standard error of mean (SEM) and data are presentated as means (n = 3)

The rates of ^{14}C–PAHs mineralisation in fresh artificially spiked soils in the absence and presence of flavonoids amendment varied from 0.44 $^{14}CO_2$–0.97 $^{14}CO_2$% h^{-1} in systems with naphthalene; 0.24 $^{14}CO_2$–0.84 $^{14}CO_2$ % h^{-1} in systems with phenanthrene; and 0.19 $^{14}CO_2$– 0.19 $^{14}CO_2$ % h^{-1} in systems with pyrene (Table 2). Apart from^{14}C–phenanthrene, the rates of ^{14}C–naphthalene and ^{14}C–pyrene mineralisation were not significantly ($P > 0.05$) enhanced in soils amended with morin hydrate compared to controls. The fastest rates of ^{14}C–PAHs mineralisation were measured in fresh artificially spiked soils amended with 50 – 100 µg kg^{-1}flavone, 3–hydroxyflavone and the mixture of flavonoids (flavone, morin hydrate and 3-hydroxyflavone at a 1:1:1 ratio) as presented in Table 2. However, fresh artificially spiked soils amended with 50 – 200µg kg^{-1}morin hydrate generally exhibited the lowest rates of ^{14}C–PAHs mineralisation ($P < 0.05$) compared to other flavonoids amendment and unamended conditions. After 28 dpre–exposure, therates of mineralisation in pre–incubated artificially spiked soilsin the absence and presence of flavonoids amendment varied from 0.73 $^{14}CO_2$– 1.10 $^{14}CO_2$ % h^{-1} in systems with naphthalene; 0.76 $^{14}CO_2$–1.14 $^{14}CO_2$ % h^{-1} in systems with phenanthrene; and 0.18 $^{14}CO_2$– 0.38 $^{14}CO_2$ % h^{-1} in systems with pyrene (Table 3). The maximum rates of ^{14}C–PAHs mineralisation in pre–incubated artificially spiked soils (Table 3) were significantly ($P< 0.001$) higherthan those measured in fresh artificially spiked soils (Table 2). With exception of morin hydrate, flavonoids amendment revealed subtle differences in the rates of PAHs mineralisation between various treatments (Table 3). The rates of ^{14}C–naphthalene and ^{14}C–phenanthrene mineralisation in soils were significantly ($P < 0.001$) higher compared to pre–incubated artificially spiked soilscontaining ^{14}C–pyrene.

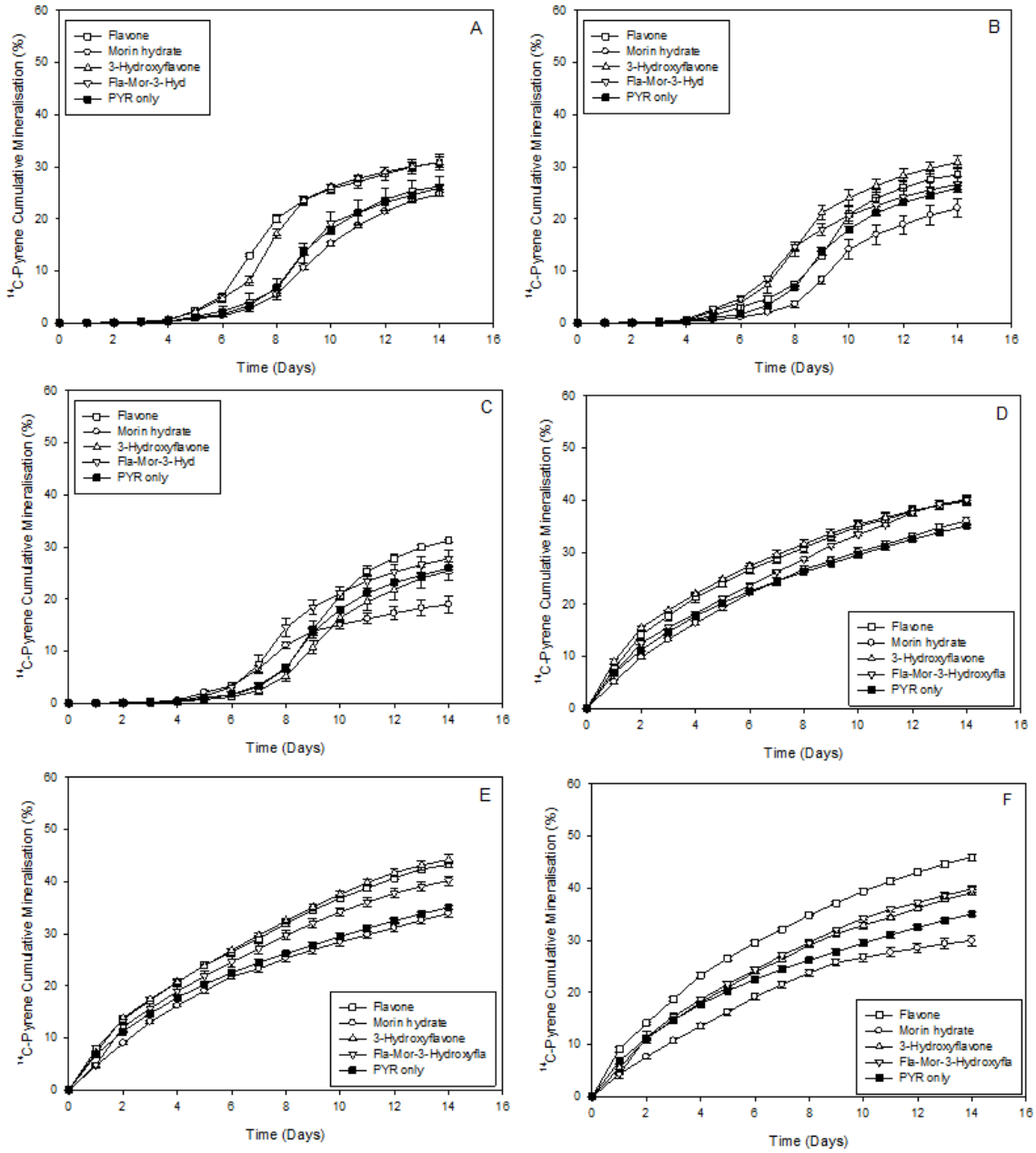

Figure 4. Development of microbial mineralisation of ^{14}C– pyrene in fresh artificially spiked soils amended with flavonoids at concentrations of (A) 50 µg kg $^{-1}$, (B) 100 µg kg $^{-1}$ and (C) 200 µg kg $^{-1}$ and pre–incubated artificially spiked soils amended with flavonoids at concentrations of (D) 50 µg kg $^{-1}$, (E) 100 µg kg $^{-1}$ and (F) 200 µg kg $^{-1}$. Error bars are the standard error of mean (SEM) and data are presentated as means (n = 3)

The extents of ^{14}C–PAHs mineralisation in fresh artificially spiked soilsin the absence and presence of flavonoids amendmentranged from 27.30 ± 1.98 to 46.30 ± 1.27 % in systems with naphthalene; 28.10 ± 0.33 to 48.73 ± 1.35 % in systems with phenanthrene; and 19.01 ± 1.64 to 30.88 ± 1.26 % in systems with pyrene (Figure 2 – Figure 4 [A, B and C] and Table 2). Fresh artificially spiked soils amended with 100 µg kg^{-1}3–hydroxyflavonehad the highest extents of ^{14}C–naphthalene (46.30 ± 1.27), ^{14}C–phenanthrene (48.73 ± 1.35) and ^{14}C–pyrene (30.88 ± 1.26) mineralisation compared to umamended soils (controls).Apart from artificially spiked soilsamended with morin hydrate, there were no significant (P> 0.05) differences in the extents of ^{14}C–naphthalene and^{14}C–phenanthrene mineralisation in fresh artificially spiked soils amended with flavonoids (Table 2). However, the extents of^{14}C–pyrene mineralisationin fresh artificially spiked soilswere significantly (P< 0.05) inhibited following amendment with > 100 µg kg^{-1}morin hydrate. After 28 dpre–exposure, extents of ^{14}C–PAH smineralisation in pre-incubated artificially spiked soils in the absence and presence of flavonoids ranged from 36.30 ± 1.37 to 50.59 ± 1.07 % in systems with naphthalene; 42.86 ± 0.79 % to 55.48 ± 0.32 in systems with phenanthrene; and 29.96 ± 1.64 to 44.30 ± 0.88 % in systems with pyrene (Figure 2 – Figure 4 [D, E and F]; Table 3). There were significant (P< 0.001) increases in the extents of^{14}C–naphthalene (4.55 ± 0.02 % to 7.13 ± 0.01 %) and ^{14}C–phenanthrene (4.48 ± 0.02 % to 6.55 ± 0.12 %) mineralisation compared to extent of ^{14}C–pyrene (13.29 ± 0.83 % to 28.34 ± 3.16 %) mineralisation in pre-incubated artificially spiked soils in the absence and presence of flavonoids (Table 3). Apart from the pre-incubated artificially spiked soils containing ^{14}C–phenanthrene amended with 100 µg kg^{-1} morin hydrate, there was no statistical significant (P > 0.05) enhancement

in the extents of ^{14}C–naphthalene and ^{14}C–pyrenemineralisation following morin hydrate amendments. Although soil–contaminant pre–exposure significantly ($P< 0.05$) increased the extents of ^{14}C–PAH mineralisation in soils, there were no significant ($P > 0.05$) differences in the extents of ^{14}C–naphthalene, ^{14}C–phenanthrene and ^{14}C–pyrene mineralisation in artificially spiked soils amended with flavonoids. The highest extents of ^{14}C–PAHS mineralisation were measured in pre–incubated artificially spiked soils amended with 50 – 100 µg kg^{-1} flavone, 3–hydroxyflavone and the mixture of flavonoids (flavone, morin hydrate and 3-hydroxyflavone at a 1:1:1 ratio). Overall, the extents of mineralisation increased in the order of naphthalene ≥ phenanthrene > pyrene and pre-incubated artificially spiked soils exhibited higher extents of ^{14}C–PAH mineralisationcompared to freshartificially spiked soils.

Table 2. Microbial Mineralisation of ^{14}C–PAHs (^{14}C–naphthalene, ^{14}C–phenanthrene and ^{14}C–pyrene) in fresh artificially spiked soils(0 d) amended with flavonoids at concentrations of 50 µg kg $^{-1}$, 100 µg kg $^{-1}$and 200 µg kg $^{-1}$. Values are the mean (n=3) ± standard error of the mean (SEM)

	Treatment conditions	Lag Phase (h)			
		Unamended	50 µg kg^{-1}	100 µg kg^{-1}	200 µg kg^{-1}
Naphthalene	Unamended soil	58.87 ± 0.09			
	Flavone		59.35 ± 0.02	58.15 ± 0.60	58.99 ± 0.98
	Morin hydrate		61.35 ± 0.91	64.44 ± 1.93	58.52 ± 0.17
	3-Hydroxyflavone		58.71 ± 0.10	59.47 ± 0.72	51.93 ± 1.27
	1:1:1 mixture of the three flavonoids		61.85 ± 0.79	59.50 ± 0.40	60.67 ± 2.30
Phenanthrene	Unamended soil	131.06 ± 2.38			
	Flavone		167.28 ± 0.25	153.48 ± 0.42	152.81 ± 0.06
	Morin hydrate		134.47 ± 0.66	133.53 ± 0.46	162.63 ± 2.11
	3-Hydroxyflavone		163.90 ± 3.59	135.44 ± 4.09	120.82 ± 0.98
	1:1:1 mixture of the three flavonoids		122.94 ± 0.27	112.49 ± 1.00	112.17 ± 0.92
Pyrene	Unamended soil	181.61 ± 2.89			
	Flavone		141.44 ± 2.21	164.86 ± 7.73	182.87 ± 5.95
	Morin hydrate		185.83 ± 1.86	201.57 ± 3.16	155.35 ± 3.96
	3-Hydroxyflavone		140.43 ± 3.49	151.65 ± 4.95	185.98 ± 8.61
	1:1:1 mixture of the three flavonoids		166.32 ± 4.10	144.97 ± 3.79	153.16 ± 6.77

	Treatment conditions	Maximum Rate (% h^{-1})			
		Unamended	50 µg kg^{-1}	100 µg kg^{-1}	200 µg kg^{-1}
Naphthalene	Unamended soil	58.87 ± 0.09			
	Flavone		59.35 ± 0.02	58.15 ± 0.60	58.99 ± 0.98
	Morin hydrate		61.35 ± 0.91	64.44 ± 1.93	58.52 ± 0.17
	3-Hydroxyflavone		58.71 ± 0.10	59.47 ± 0.72	51.93 ± 1.27
	1:1:1 mixture of the three flavonoids		61.85 ± 0.79	59.50 ± 0.40	60.67 ± 2.30
Phenanthrene	Unamended soil	131.06 ± 2.38			
	Flavone		167.28 ± 0.25	153.48 ± 0.42	152.81 ± 0.06
	Morin hydrate		134.47 ± 0.66	133.53 ± 0.46	162.63 ± 2.11
	3-Hydroxyflavone		163.90 ± 3.59	135.44 ± 4.09	120.82 ± 0.98
	1:1:1 mixture of the three flavonoids		122.94 ± 0.27	112.49 ± 1.00	112.17 ± 0.92
Pyrene	Unamended soil	181.61 ± 2.89			
	Flavone		141.44 ± 2.21	164.86 ± 7.73	182.87 ± 5.95
	Morin hydrate		185.83 ± 1.86	201.57 ± 3.16	155.35 ± 3.96
	3-Hydroxyflavone		140.43 ± 3.49	151.65 ± 4.95	185.98 ± 8.61
	1:1:1 mixture of the three flavonoids		166.32 ± 4.10	144.97 ± 3.79	153.16 ± 6.77

	Treatment conditions	Cumulative Extents (%)			
		Unamended	50 µg kg^{-1}	100 µg kg^{-1}	200 µg kg^{-1}
Naphthalene	Unamended soil	33.47 ± 1.17			
	Flavone		43.98 ± 1.41	44.47 ± 1.57	44.15 ± 1.64
	Morin hydrate		27.30 ± 1.98	30.07 ± 1.39	38.17 ± 1.31
	3-Hydroxyflavone		40.03 ± 1.15	46.30 ± 1.27	39.36 ± 1.49
	1:1:1 mixture of the three flavonoids		28.24 ± 1.37	43.24 ± 1.64	40.65 ± 1.16
Phenanthrene	Unamended soil	30.32 ± 0.50			
	Flavone		41.24 ± 1.63	41.05 ± 0.98	28.10 ± 0.38
	Morin hydrate		34.21 ± 1.94	42.97 ± 1.70	34.32 ± 1.74
	3-Hydroxyflavone		36.08 ± 1.18	48.73 ± 1.35	47.79 ± 1.18
	1:1:1 mixture of the three flavonoids		47.75 ± 1.34	48.28 ± 1.56	42.17 ± 1.93
		Cumulative Extents (%)			
Pyrene	Unamended soil	25.99 ± 0.43			
	Flavone		30.94 ± 1.41	28.53 ± 1.50	31.25 ± 0.21
	Morin hydrate		24.76 ± 0.44	22.07 ± 1.76	19.01 ± 1.64
	3-Hydroxyflavone		30.86 ± 1.04	30.88 ± 1.26	25.43 ± 1.87
	1:1:1 mixture of the three flavonoids		26.31 ± 1.89	26.69 ± 1.60	27.82 ± 1.60

Table 3. Microbial Mineralisation of ^{14}C–PAHs (^{14}C–naphthalene, ^{14}C–phenanthrene and ^{14}C–pyrene) in pre–incubated artificially spiked soils (28 d) amended with flavonoids at concentrations of 50 µg kg $^{-1}$, 100 µg kg $^{-1}$ and 200 µg kg $^{-1}$. Values are the mean (*n*=3) ± standard error of the mean (SEM)

	Treatment conditions	Lag Phase (h)			
		Unamended	50 µg kg^{-1}	100 µg kg^{-1}	200 µg kg^{-1}
Naphthalene	Unamended soil	4.72 ± 0.00			
	Flavone		4.55 ± 0.02	4.57 ± 0.04	5.14 ± 0.18
	Morin hydrate		7.13 ± 0.01	5.97 ± 0.01	5.92 ± 0.02
	3-Hydroxyflavone		4.91 ± 0.10	5.06 ± 0.21	5.29 ± 0.06
	1:1:1 mixture of the three flavonoids		6.90 ± 0.15	4.87 ± 0.08	4.92 ± 0.07
Phenanthrene	Unamended soil	5.21 ± 0.13			
	Flavone		4.89 ± 0.06	4.38 ± 0.18	5.58 ± 0.20
	Morin hydrate		5.13 ± 0.00	6.26 ± 0.19	6.55 ± 0.12
	3-Hydroxyflavone		5.94 ± 0.08	4.48 ± 0.02	5.39 ± 0.04
	1:1:1 mixture of the three flavonoids		5.76 ± 0.16	5.19 ± 0.11	5.73 ± 0.01
Pyrene	Unamended soil	17.06 ± 1.65			
	Flavone		15.54 ± 0.03	14.04 ± 0.01	13.55 ± 0.96
	Morin hydrate		24.64 ± 1.89	27.31 ± 0.08	28.34 ± 0.51
	3-Hydroxyflavone		13.29 ± 0.83	16.88 ± 0.22	20.97 ± 0.13
	1:1:1 mixture of the three flavonoids		17.73 ± 0.13	25.85 ± 1.06	23.84 ± 3.48

	Treatment conditions	Maximum Rate (% h^{-1})			
		Unamended	50 µg kg^{-1}	100 µg kg^{-1}	200 µg kg^{-1}
Naphthalene	Unamended soil	1.06 ± 0.00			
	Flavone		1.09 ± 0.01	1.10 ± 0.01	0.99 ± 0.03
	Morin hydrate		0.73 ± 0.03	0.84 ± 0.00	0.86 ± 0.02
	3-Hydroxyflavone		1.02 ± 0.02	1.02 ± 0.04	0.94 ± 0.01
	1:1:1 mixture of the three flavonoids		0.73 ± 0.01	1.03 ± 0.03	1.02 ± 0.01
Phenanthrene	Unamended soil	0.97 ± 0.02			
	Flavone		1.02 ± 0.01	1.14 ± 0.03	0.92 ± 0.03
	Morin hydrate		0.94 ± 0.03	0.78 ± 0.02	0.76 ± 0.01
	3-Hydroxyflavone		0.84 ± 0.01	1.12 ± 0.00	0.94 ± 0.01
	1:1:1 mixture of the three flavonoids		0.89 ± 0.02	0.98 ± 0.02	0.88 ± 0.00
Pyrene	Unamended soil	0.29 ± 0.02			
	Flavone		0.32 ± 0.00	0.33 ± 0.03	0.38 ± 0.02
	Morin hydrate		0.22 ± 0.02	0.20 ± 0.01	0.18 ± 0.00
	3-Hydroxyflavone		0.38 ± 0.02	0.30 ± 0.00	0.24 ± 0.01
	1:1:1 mixture of the three flavonoids		0.29 ± 0.00	0.30 ± 0.01	0.30 ± 0.01

	Treatment conditions	Cumulative Extents (%)			
		Unamended	50 µg kg^{-1}	100 µg kg^{-1}	200 µg kg^{-1}
Naphthalene	Unamended soil	46.14 ± 0.28			
	Flavone		47.55 ± 0.21	49.37 ± 1.00	47.26 ± 0.78
	Morin hydrate		38.05 ± 0.96	42.28 ± 0.91	48.41 ± 0.76
	3-Hydroxyflavone		46.46 ± 0.46	50.59 ± 1.07	46.43 ± 0.74
	1:1:1 mixture of the three flavonoids		36.30 ± 1.37	50.24 ± 0.45	46.51 ± 0.78
Phenanthrene	Unamended soil	45.13 ± 0.87			
	Flavone		46.66 ± 0.64	48.95 ± 1.01	44.43 ± 0.54
	Morin hydrate		45.09 ± 0.66	50.80 ± 0.96	42.86 ± 0.79
	3-Hydroxyflavone		47.56 ± 0.55	55.48 ± 0.32	51.66 ± 0.62
	1:1:1 mixture of the three flavonoids		50.75 ± 1.25	52.82 ± 0.32	50.29 ± 0.16
Pyrene	Unamended soil	35.10 ± 0.02			
	Flavone		40.13 ± 0.84	43.28 ± 0.39	46.02 ± 0.65
	Morin hydrate		36.04 ± 0.70	33.94 ± 0.68	29.96 ± 0.99
	3-Hydroxyflavone		39.84 ± 0.87	44.30 ± 0.88	39.19 ± 0.45
	1:1:1 mixture of the three flavonoids		40.18 ± 0.09	40.18 ± 0.90	39.88 ± 0.70

Table 4. Microbial cell numbers of total heterotrophic and PAHs degrading microbial communities after mineralisation assays in fresh (0 d) artificially spiked and pre–incubated (28 d) artificially spiked soils amended with flavonoids at concentrations of 50 µg kg $^{-1}$, 100 µg kg $^{-1}$ and 200 µg kg $^{-1}$. Values are the mean (*n=3*) ± standard error of the mean (SEM)

(A) Fresh artificially spiked soils (0 d)		Microbial cell numbers (CFU g^{-1})				
		Degraders (Unamended soils)	Heterotroph (Unamended soil)	Degraders (50 µg kg^{-1})	Degraders (100 µg kg^{-1})	Degraders (200 µg kg^{-1})
Naphthalene	Unamended soil	$7.33\times10^{6} \pm 5.77\times10^{2}$				
	Flavone		$8.99\times10^{6} \pm 3.33\times10^{4}$	$1.16\times10^{8} \pm 0.00\times10^{0}$	$1.31\times10^{8} \pm 0.00\times10^{0}$	$1.12\times10^{8} \pm 5.77\times10^{4}$
	Morin hydrate		$8.99\times10^{5} \pm 3.33\times10^{2}$	$8.49\times10^{7} \pm 3.33\times10^{5}$	$8.96\times10^{7} \pm 5.77\times10^{4}$	$7.89\times10^{7} \pm 0.00\times10^{0}$
	3-Hydroxyflavone		$1.49\times10^{7} \pm 3.33\times10^{4}$	$9.09\times10^{8} \pm 0.00\times10^{0}$	$9.46\times10^{8} \pm 0.00\times10^{0}$	$8.09\times10^{8} \pm 5.77\times10^{4}$
	1:1:1 mixture of the three flavonoids		$6.86\times10^{7} \pm 0.00\times10^{0}$	$7.86\times10^{8} \pm 8.82\times10^{5}$	$8.99\times10^{7} \pm 3.33\times10^{4}$	$6.26\times10^{8} \pm 0.00\times10^{0}$
Phenanthrene	Unamended soil	$6.56\times10^{6} \pm 0.00\times10^{0}$				
	Flavone		$8.99\times10^{6} \pm 3.33\times10^{4}$	$1.06\times10^{8} \pm 0.00\times10^{0}$	$1.21\times10^{8} \pm 0.00\times10^{0}$	$8.96\times10^{7} \pm 1.53E7$
	Morin hydrate		$8.99\times10^{5} \pm 3.33\times10^{2}$	$8.69\times10^{7} \pm 3.33\times10^{4}$	$8.70\times10^{7} \pm 5.77\times10^{5}$	$7.98\times10^{7} \pm 0.00\times10^{0}$
	3-Hydroxyflavone		$7.49\times10^{7} \pm 3.33\times10^{2}$	$6.06\times10^{8} \pm 0.00\times10^{0}$	$7.06\times10^{8} \pm 0.00\times10^{0}$	$5.69\times10^{8} \pm 0.00\times10^{0}$
	1:1:1 mixture of the three flavonoids		$6.06\times10^{7} \pm 0.00\times10^{0}$	$7.03\times10^{7} \pm 0.00\times10^{0}$	$7.99\times10^{8} \pm 3.33\times10^{2}$	$5.05\times10^{8} \pm 3.23\times10^{2}$
Pyrene	Unamended soil	$3.56\times10^{6} \pm 1.17\times10^{1}$				
	Flavone		$8.99\times10^{6} \pm 3.33\times10^{6}$	$5.96\times10^{7} \pm 3.33\times10^{4}$	$4.21\times10^{8} \pm 0.00\times10^{0}$	$5.09\times10^{6} \pm 0.00\times10^{0}$
	Morin hydrate		$8.99\times10^{5} \pm 3.33\times10^{3}$	$3.99\times10^{6} \pm 3.23\times10^{5}$	$3.50\times10^{6} \pm 5.77\times10^{2}$	$2.66\times10^{6} \pm 3.33\times10^{6}$
	3-Hydroxyflavone		$7.49\times10^{7} \pm 3.33\times10^{4}$	$5.96\times10^{7} \pm 3.33\times10^{2}$	$6.06\times10^{7} \pm 1.00\times10^{1}$	$5.15\times10^{7} \pm 3.00\times10^{3}$
	1:1:1 mixture of the three flavonoids		$6.06\times10^{7} \pm 0.00\times10^{0}$	$4.49\times10^{7} \pm 5.77\times10^{2}$	$5.99\times10^{7} \pm 3.33\times10^{3}$	$4.07\times10^{7} \pm 6.67\times10^{3}$
(B) Pre–incubated artificially spiked soils (28 d)						
Naphthalene	Unamended soil	$8.73\times10^{7} \pm 5.77\times10^{2}$				
	Flavone		$7.29\times10^{7} \pm 2.23\times10^{4}$	$1.96\times10^{9} \pm 0.00\times10^{0}$	$1.98\times10^{9} \pm 0.00\times10^{0}$	$1.91\times10^{9} \pm 5.77\times10^{4}$
	Morin hydrate		$6.89\times10^{6} \pm 2.13\times10^{4}$	$8.99\times10^{7} \pm 3.33\times10^{4}$	$8.70\times10^{7} \pm 5.77\times10^{3}$	$5.95\times10^{7} \pm 1.00\times10^{1}$
	3-Hydroxyflavone		$7.97\times10^{7} \pm 3.13\times10^{4}$	$1.98\times10^{9} \pm 0.00\times10^{0}$	$1.96\times10^{9} \pm 0.00\times10^{0}$	$1.69\times10^{9} \pm 1.77\times10^{2}$
	1:1:1 mixture of the three flavonoids		$7.66\times10^{7} \pm 1.00\times10^{1}$	$1.86\times10^{8} \pm 8.82\times10^{4}$	$1.99\times10^{8} \pm 3.33\times10^{4}$	$1.26\times10^{9} \pm 1.00\times10^{0}$
Phenanthrene	Unamended soil	$7.46\times10^{7} \pm 0.00\times10^{2}$				
	Flavone		$8.29\times10^{7} \pm 2.23\times10^{4}$	$1.96\times10^{9} \pm 0.00\times10^{0}$	$1.91\times10^{9} \pm 0.00\times10^{0}$	$7.86\times10^{8} \pm 1.53\times10^{4}$
	Morin hydrate		$6.89\times10^{6} \pm 2.13\times10^{4}$	$7.69\times10^{7} \pm 3.33\times10^{4}$	$7.90\times10^{7} \pm 5.77\times10^{4}$	$9.99\times10^{7} \pm 1.00\times10^{4}$
	3-Hydroxyflavone		$7.97\times10^{7} \pm 3.13\times10^{4}$	$1.06\times10^{9} \pm 0.00\times10^{0}$	$2.66\times10^{9} \pm 0.00\times10^{0}$	$1.69\times10^{9} \pm 0.00\times10^{0}$
	1:1:1 mixture of the three flavonoids		$7.66\times10^{7} \pm 1.00\times10^{1}$	$1.03\times10^{9} \pm 0.00\times10^{0}$	$1.89\times10^{9} \pm 3.33\times10^{2}$	$1.05\times10^{9} \pm 3.23\times10^{4}$
Pyrene	Unamended soil	$5.56\times10^{7} \pm 1.00\times10^{1}$				
	Flavone		$7.29\times10^{7} \pm 2.23\times10^{4}$	$6.96\times10^{8} \pm 3.33\times10^{4}$	$5.21\times10^{8} \pm 0.00\times10^{0}$	$6.09\times10^{7} \pm 1.00\times10^{4}$
	Morin hydrate		$6.89\times10^{6} \pm 2.13\times10^{4}$	$4.99\times10^{6} \pm 3.23\times10^{5}$	$4.94\times10^{7} \pm 5.77\times10^{2}$	$4.66\times10^{7} \pm 3.33\times10^{4}$
	3-Hydroxyflavone		$7.97\times10^{7} \pm 3.13\times10^{4}$	$6.96\times10^{8} \pm 3.33\times10^{2}$	$7.06\times10^{8} \pm 0.00\times10^{0}$	$6.15\times10^{8} \pm 3.00\times10^{2}$
	1:1:1 mixture of the three flavonoids		$7.66\times10^{7} \pm 1.00\times10^{1}$	$5.49\times10^{8} \pm 5.77\times10^{2}$	$6.99\times10^{8} \pm 2.33\times10^{2}$	$5.07\times10^{8} \pm 6.67\times10^{3}$

3.2. Numbers of Total Heterotrophic and PAH–degrading Bacteria

Amendment of artificially spiked soils with flavonoids greatly stimulated the growth of total heterotrophic bacteria and PAH–degrading microbial communities compared to the unamended soils (controls) as presented in Table 4. The numbers of total indigenous microbial communities in fresh artificially spiked soils amended with flavonoids ranged from 10^{5} – 10^{7} colony forming units per gram soil dry weight (CFU g^{-1}) for heterotrophic bacteria and 10^{6} – 10^{9} CFU g^{-1} for PAH–degrading microbial communities. The microbial cell number of heterotrophic bacteria and PAH–degrading microbial communitieswere in the range of 10^{6} CFU g^{-1} in artificially spiked soils in the absence of flavonoids amendment. In artificially spiked soils in the presence of flavonoids amendment, the numbers of CFU g^{-1} of total heterotrophic bacteria and PAH–degrading microbial communities greatly increased after 28 d pre–incubation compared to those obtained in the fresh artificially spiked soils (Table 4). The numbers of naphthalene and phenanthrene degraders were significantly ($P < 0.05$) increased in the pre–incubated artificially spiked soilscompared to the number of pyrene degrader sin fresh artificially spiked soils. The greatest numbers of PAH–degrading microbial communities in the range of 10^{9} CFU g^{-1} were measured in systems amended with 50 – 100 µg kg^{-1}3-hydroxyflavone, flavone and the mixture of flavonoids (flavone, morin hydrate and 3-hydroxyflavone at a 1:1:1 ratio)following 28 d soil–contaminant pre–exposure. In direct comparison, there were significant ($P < 0.001$) increases in the numbers of PAH–degrading microbial communitiesin artificially spikedsoils amended with 100 µg kg^{-1} 3-hydroxyflavone compared to unamended soils. In this study, the highest numbers of PAH–degrading microbial communities were measured in artificially spiked soils amended with 100 µg kg^{-1} 3-hydroxyflavone.

4. Discussion

The extents of ^{14}C–PAH mineralisation in fresh artificially spiked soils indicate that the indigenous microbial communities were capable of degrading naphthalene, phenanthrene and pyrene. PAHs are environmental contaminants (of either natural or anthropogenic origin) that are ubiquitously detectable in soils from both non-polluted and polluted areas [45]. Although PAHs biodegradation is feasible in previously uncontaminated soils, soil–contaminants pre–exposure of the indigenous microbial communities has been linked to enhanced microbial activity and growth–linked mineralisation of PAHs in soils [11,16,21,46]. The sigmoidal nature of ^{14}C–naphthalene, ^{14}C–phenanthrene and ^{14}C–pyrene mineralisation curves observed in fresh and pre–incubated artificially spiked soils show that microbial acclimation is required prior to enhanced rates of mineralisation [47]. In this study, shorter lag phases and enhanced microbial activities can be attributed to increase in microbial populations, induction of catabolic enzymes and microbial adaptation following 28 d pre–incubationof the PAHs spiked soils [13]. From the results obtained, naphthalene exhibited the shortest ($P < 0.001$) lag phases compared to phenanthrene and pyrene under the same treatment conditions. The observed rapidmicrobial mineralisationin fresh artificially spiked soils could be attributed to selective enrichment in the numbers of microbial community members that have the ability to degrade naphthalene[8]. However, microbial degradation for different petroleum–derived contaminants are known to vary as a result of physicochemical and biological properties of the soil [48].

Enhanced mineralisation of PAHs observed in artificially spiked soils amended with 50 – 100 µg kg^{-1} flavone, 3-hydroxyflavone and a mixture of the three flavonoids (flavone, morin hydrate and 3-hydroxyflavone at a 1:1:1 ratio) can be attributed to selective increase in numbers of PAH–degrading populations and stimulation of appropriate enzymatic pathways [49,50]. Several flavonoids released from plants can regulate activities of rhizosphere associated microbes at micromolar and nanomolar concentrations [36]. According to Reilley *et al.* [9], the addition of organic acids, typically found in root exudates, enhanced the rates of pyrene mineralisation in rhizosphere soil. Siciliano and Germida [51] have shown enhanced losses of PAHs in planted soils and suggested that the mechanism by which this occurs is via plant root exudates stimulating the microbial communities involved in the dissipation of aromatic compounds. It is known that the degradation of flavonoids by rhizospheric bacteria leads to the formation of intermediates, including resorcinol, phloroglucinolphenylacetic acid, substituted cinnamic acids and protocatechuic acid [52]. Although these organic compounds are likely to be mineralised through the β-ketoadipate pathway [53], protocatechuate is an intermediate in the degradation of PAHs in some microorganisms [54]. In a study, Da Silva *et al.*[55] reported that phenolic-rich root extracts obviously stimulated phenanthrene mineralisation as well as total oxygenase activity. Leigh *et al.*[56] demonstrated that seasonal fine root death releases several flavones which act as substrates for polychlorinated biphenyl (PCB) degrading bacteria. The release of organic compounds, such as flavonoids, into the root zone or rhizosphere increases the concentration of available organic carbon and also stimulates the activity of soil heterotrophic bacteria, which are largely prevailing over soil autotrophic microorganisms [57].

The inhibition of pyrene mineralisation and decreases in number of PAH–degrading microbial communities following amendment of artificially spiked soils with higher concentrations of morin hydrate (≥ 100 µg kg^{-1}) could be attributed to formation of toxic intermediates/metabolites and/or preferential utilisation of the soluble flavonoids. However, interpretation of mineralisation kinetics may be hampered when compounds with high toxicity to bacteria are involved as mineralisation rates may be reduced as a result of toxic inhibition [58]. In a further possible explanation, the solubility of flavonoids in solvents depends on their existing forms and chemical structures. Flavone is insoluble in water [59] and 3-hydroxyflavone is insoluble in water at room temperature [60]. However, morin hydrate is soluble in water (0.25 mg ml^{-1}, 20 °C; 0.94 mg ml^{-1},100 °C) and it is also soluble in methanol (50 mg ml^{-1}) [61]. Qiu*et al.*[62] reported that the addition of flavone and morin at concentrations greater than 10 mumol l^{-1} hindered ^{14}C-B[a]P mineralisation in root zone soil slurries. Flavonoids and other phenolics have been found to inhibit a range of root pathogens and pests, ranging from bacteria to fungi and insects [63]. This has been attributed to their role as antimicrobial toxins [64] and anti- or pro-oxidants [65]. In addition, metabolism of flavonoids by rhizosphere associated microbial communities could alter the relative abundance of some indigenous microbial species, activity of microbial populations and contaminant availability in the soil [39]. This study showed that the rates and extents of ^{14}C–PAHs mineralisation in soils were influenced by the available concentrations of flavonoids.

In the pre–incubated artificially spiked soils, enhanced rates and extents of ^{14}C–PAHs mineralisation could be attributed to acclimatisation of microbial communitiesand subsequent increase in the numbers of microorganisms capable of degrading PAHs. Furthermore, soil chemical changes related to the presence of flavonoids and products of their microbial turnover could affect microbial activity, population diversity, availability of nutrientsand solubility of contaminants in the soil slurries. Rhodes *et al.*[66] found that pre–incubated spiked woodland soils exhibited a higher level of [UL-^{14}C] 2,4-dichlorophenol degradation, which was subsequently enhanced by the addition of monoterpenes ($P < 0.001$), with the VOC mix and *α*–pinene amendments showing increased [UL-^{14}C] 2,4-dichlorophenol catabolism. The apparent high levels of PAHs mineralisation in the pre–incubated soils could be attributed to selective enrichment and relative abundance of indigenous PAH–degrading microbial species compared to fresh artificially spiked soils. The results of this study suggested that contaminated soils amended with appropriate concentrations of flavonoids can promote growth of indigenous microbial communities capable of degrading PAHs and subsequently stimulate growth–linked mineralisation of ^{14}C–PAHs in soils. Overall, the results of this study agree with the findings of Johnson *et al.* [10] and there is an indirect evidence for soil microbial response to plant–secreted chemicals during the biodegradation of petroleum contaminated soil.

5. Conclusion

The results obtained in this study have shown that amendments of contaminated soils with some flavonoids at appropriate concentrations (50 – 100 μg kg^{-1}) enhancedthe catabolic capabilities of indigenous soil microbial communities and subsequentgrowth–linked mineralisation of ^{14}C–PAHs.In this study, soils amended with 100 μg kg^{-1} 3–hydroxyflavone exhibited the highest extents of ^{14}C–PAH mineralisation, while amendment with morin hydrate at higher concentrations (≥ 100 μg kg^{-1}) inhibited ^{14}C–pyrene mineralisation. This study provide further understanding into the role of flavonoids on the development of microbial mineralisation of ^{14}C–PAHsin fresh and pre–incubated artificially spiked soils.Depending on its concentrations, addition of flavonoids and other secondary compounds may either stimulate or inhibit microbial degradation of PAHs in soil.In practice, the effects of single compounds or mixed secondary plant metabolites on microbial mineralisation may likely become complicated due to apparent solubility and bioavailability of mixed contaminants in soils.Therefore, researchers should be cautious in attempts to manipulate flavonoids rhizosphere signals during phytoremediation processes due to the overlapping functions of many flavonoids as either stimulators or inhibitors of microbial activity. Further research is recommended in order to better understand the diversity and functions of phytochemicals in bioremediation of petroleum–contaminated soils.

References

[1] Ite, A. E., U. J. Ibok, M. U. Ite, and S. W. Petters, "Petroleum Exploration and Production: Past and Present Environmental Issues in the Nigeria's Niger Delta," *American Journal of Environmental Protection,* 1 (4). 78-90, 2013.

[2] Ite, A. E., and K. T. Semple, "14 Biodegradation of petroleum hydrocarbons in contaminated soils," *Microbial Biotechnology: Energy and Environment,* R. Arora, ed., pp. 250-278: CAB International, 2012.

[3] Ambrosoli, R., L. Petruzzelli, J. Luis Minati, and F. Ajmone Marsan, "Anaerobic PAH degradation in soil by a mixed bacterial consortium under denitrifying conditions," *Chemosphere,* 60 (9). 1231-1236, 2005.

[4] Mahro, B., G. Schaefer, and M. Kästner, "Pathways of Microbial Degradation of Polycyclic Aromatic Hydrocarbons in Soil," *Bioremediation of chlorinated and polycyclic aromatic hydrocarbon compounds,* R. E. Hinchee, ed., pp. 203-217: CRC Press, 1994.

[5] Simarro, R., N. González, L. Bautista, R. Sanz, and M. Molina, "Optimisation of Key Abiotic Factors of PAH (Naphthalene, Phenanthrene and Anthracene) Biodegradation Process by a Bacterial Consortium," *Water, Air, & Soil Pollution,* 217 (1). 365-374, 2011.

[6] Semple, K. T., J. Stokes, and B. J. Reid, "Temporal chances in the bioavailability of PAHs in soils," *Bioremediation Technologies for Polycyclic Aromatic Hydrocarbon Compounds*247-252, 1999.

[7] Cerniglia, C. E., "Biodegradation of polycyclic aromatic hydrocarbons," *Current Opinion in Biotechnology*, 4 (3). 331-338, 1993.

[8] Smith, M. J., G. Lethbridge, and R. G. Burns, "Bioavailability and biodegradation of polycyclic aromatic hydrocarbons in soils," *FEMS Microbiology Letters,* 152 (1). 141-147, 1997.

[9] Reilley, K. A., M. K. Banks, and A. P. Schwab, "Dissipation of polycyclic aromatic hydrocarbons in the rhizosphere," *Journal of Environmental Quality,* 25 (2). 212-219, 1996.

[10] Johnson, D. L., D. R. Anderson, and S. P. McGrath, "Soil microbial response during the phytoremediation of a PAH contaminated soil," *Soil Biology and Biochemistry,* 37 (12). 2334-2336, 2005.

[11] Couling, N. R., M. G. Towell, and K. T. Semple, "Biodegradation of PAHs in soil: Influence of chemical structure, concentration and multiple amendment," *Environmental Pollution,* 158 (11). 3411-3420, 2010.

[12] Macleod, C. J. A., and K. T. Semple, "The influence of single and multiple applications of pyrene on the evolution of pyrene catabolism in soil," *Environmental Pollution,* 139 (3). 455-460, 2006.

[13] Macleod, C. J., and K. T. Semple, "The adaptation of two similar soils to pyrene catabolism," *Environmental Pollution,* 119 (3). 357-364, 2002.

[14] Wei, X. Y., L. Z. Sang, J. N. Chen, Y. X. Zhu, and Y. Zhang, "The effects of LMWOAs on biodegradation of multi-component PAHs in aqueous solution using dual-wavelength fluorimetry," *Environmental Pollution,* 157 (11). 3150-3157, 2009.

[15] Harayama, S., "Polycyclic aromatic hydrocarbon bioremediation design," *Current Opinion in Biotechnology,* 8 (3). 268-273, 1997.

[16] MacLeod, C. J. A., and K. T. Semple, "Influence of Contact Time on Extractability and Degradation of Pyrene in Soils," *Environmental Science & Technology,* 34 (23). 4952-4957, 2000.

[17] Azaizeh, H., P. M. L. Castro, and P. Kidd, "Biodegradation of Organic Xenobiotic Pollutants in the Rhizosphere " *Organic Xenobiotics and Plants: From Mode of Action to Ecophysiology,* Plant Ecophysiology P. Schröder and C. D. Collins, eds., pp. 191-215: Springer Netherlands, 2011.

[18] Crowley, D. E., E. Luepromechai, and A. Singer, "Metabolism of Xenobiotics in the Rhizosphere," *Pesticide Biotransformation in Plants and Microorganisms: similarities and divergences,* ACS Symposium Series 777, J. C. Hall, R. E. Hoagland and R. M. Zablotowicz, eds., pp. 333-352: American Chemical Society, 2000.

[19] Suresh, B., and G. A. Ravishankar, "Phytoremediation - a novel and promising approach for environmental clean-up," *Critical Reviews in Biotechnology,* 24 (2-3). 97-124, 2004.

[20] Chaudhry, Q., M. Blom-Zandstra, S. Gupta, and E. J. Joner, "Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment," *Environmental Science and Pollution Research,* 12 (1). 34-48, 2005.

[21] Ite, A. E., N. F. Hanney, and K. T. Semple, "The Effect of Hydroxycinnamic Acids on the Microbial Mineralisation of Phenanthrene in Soil," *International Journal of Environmental Bioremediation & Biodegradation,* 3 (2). 40-47, 2015.

[22] Fester, T., J. Giebler, L. Y. Wick, D. Schlosser, and M. Kästner, "Plant–microbe interactions as drivers of ecosystem functions relevant for the biodegradation of organic contaminants," *Current Opinion in Biotechnology,* 27 (0). 168-175, 2014.

[23] Binet, P., J. M. Portal, and C. Leyval, "Dissipation of 3-6-ring polycyclic aromatic hydrocarbons in the rhizosphere of ryegrass," *Soil Biology & Biochemistry,* 32 (14). 2011-2017, 2000.

[24] Yoshitomi, K. J., and J. R. Shann, "Corn (*Zea mays L.*) root exudates and their impact on 14C-pyrene mineralization," *Soil Biology and Biochemistry,* 33 (12–13). 1769-1776, 2001.

[25] Shann, J. R., and J. J. Boyle, "Influence of Plant Species on In Situ Rhizosphere Degradation," *Bioremediation through Rhizosphere Technology,* ACS Symposium Series T. A. Anderson and J. R. Coats, eds., pp. 70-81, Washington DC: American Chemical Society, 1994.

[26] Fletcher, J. S., and R. S. Hegde, "Release of phenols by perennial plant roots and their potential importance in bioremediation," *Chemosphere,* 31 (4). 3009-3016, 1995.

[27] Gilbert, E. S., and D. E. Crowley, "Plant compounds that induce polychlorinated biphenyl biodegradation by Arthrobacter sp. strain B1B," *Applied and Environmental Microbiology,* 63 (5). 1933-1938, 1997.

[28] Focht, D. D., "Strategies for the improvement of aerobic metabolism of polychlorinated biphenyls," *Current Opinion in Biotechnology,* 6 (3). 341-346, 1995.

[29] Badri, D. V., V. M. Loyola-Vargas, C. D. Broeckling, and J. M. Vivanco, "Root Secretion of Phytochemicals in Arabidopsis Is Predominantly Not Influenced by Diurnal Rhythms," *Molecular Plant,* 3 (3). 491-498, 2010.

[30] Badri, D. V., and J. M. Vivanco, "Regulation and function of root exudates," *Plant, Cell & Environment,* 32 (6). 666-681, 2009.

[31] Narasimhan, K., C. Basheer, V. B. Bajic, and S. Swarup, "Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal

of polychlorinated biphenyls," *Plant Physiology*, 132 (1). 146-153, 2003.

[32] Aoki, T., T. Akashi, and S.-i. Ayabe, "Flavonoids of Leguminous Plants: Structure, Biological Activity, and Biosynthesis," *Journal of Plant Research*, 113 (4). 475-488, 2000.

[33] Cesco, S., G. Neumann, N. Tomasi, R. Pinton, and L. Weisskopf, "Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition," *Plant and Soil*, 329 (1). 1-25, 2010.

[34] Azimova, S. S., and V. I. Vinogradova, *Natural Compounds: Flavonoids*: Springer New York, 2012.

[35] Hassan, S., and U. Mathesius, "The role of flavonoids in root–rhizosphere signalling: opportunities and challenges for improving plant–microbe interactions," *Journal of Experimental Botany*, 2012.

[36] Phillips, D. A., and S. M. Tsai, "Flavonoids as plant signals to rhizosphere microbes," *Mycorrhiza*, 1 (2). 55-58, 1992.

[37] Shaw, L. J., and J. E. Hooker, "The fate and toxicity of the flavonoids naringenin and formononetin in soil," *Soil Biology and Biochemistry*, 40 (2). 528-536, 2008.

[38] Rao, J. R., and J. E. Cooper, "Rhizobia catabolize nod gene-inducing flavonoids via C-ring fission mechanisms," *Journal of Bacteriology*, 176 (17). 5409-5413, 1994.

[39] Shaw, L. J., P. Morris, and J. E. Hooker, "Perception and modification of plant flavonoid signals by rhizosphere microorganisms," *Environmental Microbiology*, 8 (11). 1867-1880, 2006.

[40] Rowell, D. L., *Soil science : methods and applications*, Harlow, Essex; New York: Longman Scientific & Technical ; Wiley, 1994.

[41] Doick, K. J., P. H. Lee, and K. T. Semple, "Assessment of spiking procedures for the introduction of a phenanthrene-LNAPL mixture into field-wet soil," *Environmental Pollution*, 126 (3). 399-406, 2003.

[42] Reid, B. J., C. J. MacLeod, P. H. Lee, A. W. Morriss, J. D. Stokes, and K. T. Semple, "A simple ^{14}C-respirometric method for assessing microbial catabolic potential and contaminant bioavailability," *FEMS Microbiology Letters*, 196 (2). 141-146, 2001.

[43] Macleod, C. J. A., and K. T. Semple, "The adaptation of two similar soils to pyrene catabolism," *Environmental Pollution*, 119 (3). 357-364, 2002.

[44] Lorch, H. J., G. Benckieser, and J. C. G. Ottow, "Basic methods for counting microorganisms in soil and water," *Methods in Applied Soil Microbiology and Biochemistry*, K. Alef and P. Nannipieri, eds., pp. 146-161, New York: Academic Press, 1995.

[45] Nam, J. J., G. O. Thomas, F. M. Jaward, E. Steinnes, O. Gustafsson, and K. C. Jones, "PAHs in background soils from Western Europe: Influence of atmospheric deposition and soil organic matter," *Chemosphere*, 70 (9). 1596-1602, 2008.

[46] Lee, P. H., K. J. Doick, and K. T. Semple, "The development of phenanthrene catabolism in soil amended with transformer oil," *FEMS Microbiology Letters*, 228 (2). 217-223, 2003.

[47] Grosser, R. J., D. Warshawsky, and J. R. Vestal, "Indigenous and enhanced mineralization of pyrene, benzo[a]pyrene, and carbazole in soils," *Applied and Environmental Microbiology*, 57 (12). 3462-3469, 1991.

[48] Grosser, R. J., J. R. Vestal, and D. Warshawsky, "Mineralization of polycyclic and N-heterocyclic aromatic compounds in hydrocarbon-contaminated soils," *Environmental Toxicology and Chemistry*, 14 (3). 375-382, 1995.

[49] Bais, H. P., T. L. Weir, L. G. Perry, S. Gilroy, and J. M. Vivanco, "The role of root exudates in rhizosphere interactions with plants and other organisms," *Annual Review of Plant Biology*, 57 (1). 233-266, 2006.

[50] Singer, A. C., D. Smith, W. A. Jury, K. Hathuc, and D. E. Crowley, "Impact of the plant rhizosphere and augmentation on remediation of polychlorinated biphenyl contaminated soil," *Environmental Toxicology and Chemistry*, 22 (9). 1998-2004, 2003.

[51] Siciliano, S. D., and J. J. Germida, "Biolog analysis and fatty acid methyl ester profiles indicate that pseudomonad inoculants that promote phytoremediation alter the root-associated microbial community of *Bromus biebersteinii*," *Soil Biology and Biochemistry*, 30 (13). 1717-1723, 1998.

[52] Shaw, L. J., and R. G. Burns, "Enhanced mineralization of [U-(14)C]2,4-dichlorophenoxyacetic acid in soil from the rhizosphere of *Trifolium pratense*," *Applied and Environmental Microbiology*, 70 (8). 4766-4774, 2004.

[53] Parke, D., D. A. D'Argenio, and L. N. Ornston, "Bacteria Are Not What They Eat: That Is Why They Are So Diverse," *Journal of Bacteriology*, 182 (2). 257-263, 2000.

[54] Kim, S. J., O. Kweon, R. C. Jones, R. D. Edmondson, and C. E. Cerniglia, "Genomic analysis of polycyclic aromatic hydrocarbon degradation in *Mycobacterium vanbaalenii* PYR-1," *Biodegradation*, 19 (6). 859-881, 2008.

[55] Da Silva, M. L. B., R. Kamath, and P. J. J. Alvarez, "Effect of simulated rhizodeposition on the relative abundance of polynuclear aromatic hydrocarbon catabolic genes in a contaminated soil," *Environmental Toxicology and Chemistry*, 25 (2). 386-391, 2006.

[56] Leigh, M. B., J. S. Fletcher, X. Fu, and F. J. Schmitz, "Root turnover: an important source of microbial substrates in rhizosphere remediation of recalcitrant contaminants," *Environmental Science & Technology*, 36 (7). 1579-1583, 2002.

[57] Toal, M. E., C. Yeomans, K. Killham, and A. A. Meharg, "A review of rhizosphere carbon flow modelling," *Plant and Soil*, 222 (1). 263-281, 2000.

[58] Shaw, Beaton, Glover, Killham, and Meharg, "Development and characterization of a lux-modified 2,4-dichlorophenol-degrading Burkholderia sp. RASC," *Environmental Microbiology*, 1 (5). 393-399, 1999.

[59] Eagleson, M., *Concise Encyclopedia of Chemistry*, Berlin; New York: Walter de Gruyter, 1994.

[60] Santos, J. P., M. E. D. Zaniquelli, W. F. De Giovani, and S. E. Galembeck, "Aluminum ion complex formation with 3-hydroxyflavone in Langmuir and Langmuir–Blodgett films," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 198–200 (0). 569-576, 2002.

[61] O'Neil, M. J., *The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals* Whitehouse Station, NJ, USA: Chapman Hall Electronic Publishing Division, 2001.

[62] Qiu, X., B. E. Reed, and R. C. Viadero, "Effects of Flavonoids on 14C[7,10]-Benzo[a]pyrene Degradation in Root Zone Soil," *Environmental Engineering Science*, 21 (5). 637-646, 2004.

[63] Makoi, J., and P. A. Ndakidemi, "Biological, ecological and agronomic significance of plant phenolic compounds in rhizosphere of the symbiotic legumes," *African Journal of Biotechnology*, 6 (12). 1358-1368, 2007.

[64] Cushnie, T. P. T., and A. J. Lamb, "Recent advances in understanding the antibacterial properties of flavonoids," *International Journal of Antimicrobial Agents*, 38 (2). 99-107, 2011.

[65] Jia, Z., B. Zou, X. Wang, J. Qiu, H. Ma, Z. Gou, S. Song, and H. Dong, "Quercetin-induced H_2O_2 mediates the pathogen resistance against Pseudomonas syringae pv. Tomato DC3000 in Arabidopsis thaliana," *Biochemical and Biophysical Research Communications*, 396 (2). 522-527, 2010.

[66] Rhodes, A. H., S. M. Owen, and K. T. Semple, "Biodegradation of 2,4-dichlorophenol in the presence of volatile organic compounds in soils under different vegetation types," *FEMS Microbiology Letters*, 269 (2). 323-330, 2007.