International Journal of Environmental Bioremediation & Biodegradation, 2015, Vol. 3, No. 2, 40-47
Available online at http://pubs.sciepub.com/ijebb/3/2/1

DOI:10.12691/ijebb-3-2-1

The Effect of Hydroxycinnamic Acids on the Microbial Mineralisation of Phenanthrene in Soil

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Received January 21, 2015; Revised April 15, 2015; Accepted May 17, 2015

Abstract The effect of hydroxycinnamic acids (caffeic, ferulic and *p*-coumaric acids) on the microbial mineralisation of phenanthrene in soil slurry by the indigenous microbial community has been investigated. The rate and extent of ^{14}C–phenanthrenemineralisation in artificially spiked soils were monitored in the absence of hydroxycinnamic acids and presence of hydroxycinnamic acids applied at three different concentrations (50, 100 and 200 µg kg^{-1}) either as single compounds or as a mixture of hydroxycinnamic acids (caffeic, ferulic and *p*-coumaric acids at a 1:1:1 ratio). The highest extent of ^{14}C–phenanthrene mineralisation ($P< 0.001$) was obtained in soils amended with 50 µg kg^{-1} ferulic acid (52.9% ± 0.45) compared to that obtained in unamended soils (37.2% ± 0.23). In addition, mineralisation of ^{14}C–phenanthrene was monitored in pre–incubated artificially spiked soils at various time intervals (0, 16, 32 and 48 d) following amendment with hydroxycinnamic acids at a concentration of 100 µg kg^{-1}. After 16 d of pre-exposure, artificially spiked soils amended with 100 µg kg^{-1} ferulic acids had the highest extents of ^{14}C–phenanthrene mineralisationcompared to those obtained soils with other treatment conditions. The results obtained showed enhanced mineralisation of ^{14}C–phenanthrene in freshly spiked soils amended with hydroxycinnamic acids and the extents of ^{14}C–phenanthrene mineralisation range in the order of $50 \geq 100 > 200$ µg kg^{-1}. Depending on its concentrationin soil, hydroxycinnamic acids can either stimulate or inhibit mineralisation of phenanthrene by indigenous soil microbial community. Therefore, effective understanding of phytochemical–microbe–organic contaminant interactions is essential for further development of phytotechnologies for remediation of PAH–contaminated soils.

Keywords: *microbial mineralization, phenanthrene, hydroxycinnamic acids, caffeic acid, ferulic acid, p-coumaric acid, soil*

Cite This Article: Aniefiok E. Ite, Nicola F. Hanney, and Kirk T. Semple, "The Effect of Hydroxycinnamic Acids on the Microbial Mineralisation of Phenanthrene in Soil." *International Journal of Environmental Bioremediation & Biodegradation*, vol. 3, no. 2 (2015): 40-47. doi: 10.12691/ijebb-3-2-1.

1. Introduction

Roots secrete an enormous number of chemical compounds into the surrounding soils (rhizosphere) where the secreted chemicals mediate multi-partite interactions and alter their immediate environments around plant roots [1,2]. Despite the technical difficulties inherent in the study of plant roots [3,4], increasing evidence suggests that the type, total and relative amounts of plant–secreted chemicals depend on the age of the plants [5,6,7], physiological status [8] and environmental conditions [9,10]. According to Cunningham *et al.* [11], plants and their associated indigenous microflora are used to transform or degrade contaminantsin the soil via the process of phytoremediation. Phytoremediation studies have demonstrated enhanced biodegradation of organic contaminants in the rhizosphere of certain plants [12,13,14]. For example, Yoshitomi and Shann [13] attributed enhanced polycyclic aromatic hydrocarbon (PAH) degradation in the rhizosphere to the stimulation effect of plant root exudates. It is widely known that exudates initiate and modulate dialogue between roots and indigenous soil microflora. Root exudates have been shown to support the growth of xenobiotic degrading microbial species [15], which subsequently enhance the microbial degradation of the target organic contaminants [16]. In the study, Narasimhan*et al.* [17] identified 125 secondary metabolites in the root exudates from the *Arabidopis*family, 76 % of which belonged to aromatic acid ora class ofphenylpropanoid compounds.Aromatic root components such as hydroxycinnamic acids, which are common in poplar plants (*populus*), are known components of plant root exudates [10].

Hydroxycinnamic acids (hydroxycinnamates), hydroxy derivatives of cinnamic acid, belong to a class of natural occurring polyphenols having a $C_6 – C_3$ structure (Figure 1). Hydroxycinnamic acidsare precursors in either the production of lignin in vascular plants [18] orformation of lignin-carbohydrate bridges in grasses [19]. Hydroxycinnamic acids such as caffeic (3,4-

dihydroxycinnamic) acid [20], ferulic (3-methoxy-4-hydroxycinnamic) acidand *p*-coumaric (4-hydroxycinnamic) acid are abundant in soil, where they are incorporated to the humic substances [21]. Caffeic, ferulic and *p*-coumaric acids are all present in cruciferae family of plants and the genera include *Brassica*, *Matthiola*and *Raphanus [4]*. Phenolic contents of roots can range from 0.02 – 0.40 % (g phenolic g^{-1} root) for root exudates and 2.2 – 4.7 % for ethanol extracted roots [6,22]. Plant secreted phenolic compounds are the key signalling components in many plant–microbe interactions [23] and secondary plant metabolites that are structurally analogous to xenobiotic compounds have been found to stimulate microbial degradation of organic contaminants [6,24]. According toRentz*et al.* [25], phenolic compounds are thought to be potential inducers of PAH degradation in soil. It is known that root tissues of *Raphanussativus* (radish) contain caffeic, ferulic and *p*-coumaric acids [4] and radish has been found to be effective in promoting the biodegradation of pyrene in soil [26].

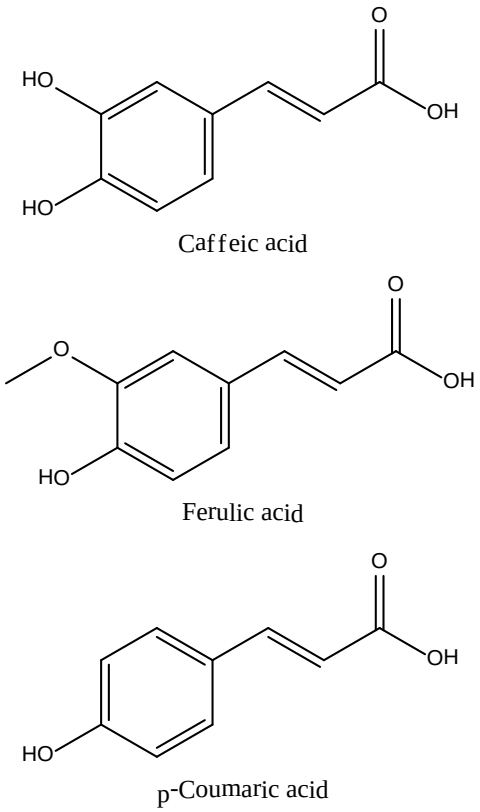

Figure 1. Chemical structures of some of the hydroxycinnamic acids used in this study

The hypotheses for this study were: (i) the presence of hydroxycinnamic acids above a threshold concentration in contaminated soil may stimulate microbial activity and prolific microbial growth (population increase) and (ii) the addition of appropriate concentration of hydroxycinnamic acids or a mixture of hydroxycinnamic acids may increase bioavailability of organic contaminant, intrinsic microbial catabolic activity and enhancement of PAHmineralisation in soil by active indigenous microbial degrader population. To address these hypotheses, the following aims were considered: (i) assessment of the microbial mineralisation of ^{14}C–labelled phenanthrene in soil, (ii) monitoring of microbial mineralisation of PAH in artificially spiked soils in the absence and presence of hydroxycinnamic acids at three different concentrations (50, 100 and 200 µg kg^{-1}) either as single compounds or as a mixture of hydroxycinnamic acids (caffeic, ferulic and *p*-coumaric acids at a 1:1:1 ratio) and (iii) monitoring of microbial activity following pre–exposure of indigenous soil microflora to phenanthrene and subsequent amendment of artificially spiked/pre–incubated soils with 100 µg kg^{-1} hydroxycinnamic acids.

2. Materials and Methods

2.1. Materials

Phenanthrene (>96%) and [9–^{14}C] phenanthrene (specific activity = 50 mCi $mmol^{-1}$, radiochemical purity 99.6%) were obtained from American Radiolabeled Chemicals (ARC) UK. Sigma–Aldrich UK supplied caffeic acid (98%), ferulic acid (99%) and *p*-coumaric acid (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 for 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, UK. The Goldstar liquid scintillation cocktail and 7 ml glass scintillation vials were supplied by Meridian UK.

2.2. Soil Sampling and Analysis

A DystricCambisol soil 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 sieving through a 2 mm mesh to remove stones and residual plant materials. The field moisture content was determined in triplicate by oven drying at 105 °C for 24 h [27]. Soil texture was determined using sedimentation. The soil pH was determined using a calibrated pH meter, ratio 10 g soil:25 ml dH_2O. The total extractable organic carbon content was determined using loss on ignition method (450 ^{0}C for 24 h) and a Carlo Erba CHNS–OEA 1108 CN–Elemental analyzer was used to determine the total carbon and nitrogen contents. 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).

2.3. Soil Spiking with Target Hydrocarbon

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 phenanthrene using acetone as the carrier solvent to give a final ^{12}C–hydrocarbon concentration of 20 mg kg^{-1} (dry weight). Each soil contaminatedmixture was then blended following the method developed by Doick*et al.* [28]. Controls consisting 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 control were incubated in darkness at 21.5 ± 0.5°C and 45% relative humidity for 0 to 48 d. The pre–incubated artificially spiked soilswere sampled at various time intervals (0, 16, 32 and 48 d) for both respirometric assays and microbial analysis.

2.4. Mineralisation of ^{14}C–phenanthrene in Soil

The extent of mineralisation of ^{14}C–phenanthrene in the artificially spiked soils was measured (in triplicate) through the evolution of $^{14}CO_2$ producedusing respirometric assays [29]. The mineralisation assay was performed inrespirometers: the setup 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 [28]. Each of the respirometers was spiked with phenanthrene standards prepared in toluene to deliver ^{12}C–phenanthrene 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 ^{14}C–phenanthrene mineralisation.

Unamended respirometers were prepared as outlined above, with 10 ± 0.1 g phenanthrene spiked soil (wet weight) and 30 ml of autoclaved MBS solution. To investigate techniques to optimise the microbial mineralisation of the target organic contaminant, respirometers were also prepared as above but with the following treatments: (i) fresh artificially spiked soils amended with 50, 100 or 200 µg kg^{-1} hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric acids) and a mixture of hydroxycinnamic acids (caffeic, ferulic, and *p*-coumaric acids at a 1:1:1 ratio) using toluene as the carrier solvent, (ii) artificially spiked soils (pre-incubated) sampled at various time intervals (0, 16, 32 and 48 d) and amended with 100 µg kg^{-1} hydroxycinnamic acids and a mixture of hydroxycinnamic acidsprior to mineralisation assays and (iii) artificially spiked soils withno hydroxycinnamic acids amendment used as controlsto assess any increase in rates and extents ofmicrobial mineralisationof the target organic contaminant.

Respirometers containing only 10 ± 0.1 g rehydrated soil (wet weight) and 30 ml of autoclaved MBS solution were also prepared as analytical blanks. 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 over the 14 d mineralisation assay sampling period. 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 (emission of light as a result of a chemical reaction). The trapped ^{14}C-activity was counted using a Packard Canberra Tri-Carb 2300TR liquid scintillation counter and quantified using standard counting protocols and automatic quench correction [30]. The lag phases, rates and extents of ^{14}C–phenanthrene 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 Phenanthrene–degrading Bacteria

The number of total heterotrophic bacteria (THB) and indigenous phenanthrene–degrading bacteria were evaluated following standard aseptic plate count techniques [31]. 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% phenanthrene as the sole carbon source for phenanthrene–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 phenanthrene–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

Data collection from mineralisation assays was analysed at various time intervals and statistically verified using *t*–tests after normality and equal variance tests (Tukey test, $P \leq 0.05$) following blank correctionusing statistical software –SigmaStat®, Version 3.5 (Systat Software Inc.). The mineralisation profiles in soils with different treatment conditions are presented using graphing software package – SigmaPlot®, Version 12.5 (Systat Software Inc.).

3. Results

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–phenanthrene was measured in fresh artificially spiked soils (Figure 2 and Table 2) and pre–incubated artificially spiked soils (Figure 3 and Table 3) in the absence and presence of hydroxycinnamic acids. The lag phases (time taken to achieve 5% mineralisation) in freshly spiked soils varied between each target concentration of hydroxycinnamic acids (108.02 – 123.54 h in systems amended with 50 µg kg^{-1}; 124.81 – 141.80 h in systems amended with 100 µg kg^{-1} and 123.43 – 138.06 h in systems amended with 200 µg kg^{-1}) and a lag phase of 127.61 ± 2.48 h wasobtained in the system with phenanthrene only (Table 2). The shortest lag phase was obtained in freshly spiked soils amended with 50 µg kg^{-1} caffeic acid compared to unamended soil and other treatment conditions ($P < 0.05$). Apart from amendment with a mixture of hydroxycinnamic acids, soils amended with 50 µg kg^{-1} hydroxycinnamic acids exhibited shorter lag phases compared tosoils amended with< 100 µg kg^{-1} hydroxycinnamic acids and unamended soil ($P< 0.05$). The longest lag phase of 141.80 h was obtained in freshly spiked soil amended with 100 µg kg^{-1}*p*–coumaric acid compared to those in other systems amended with 100 µg kg^{-1} hydroxycinnamic acids and unamended soil ($P< 0.05$). In the pre–incubated spiked soils amended with 100 µg kg^{-1} hydroxycinnamic acids, the lag phases ranged from 124.58 – 146.00 h at 0 d; 55.64 – 62.53 h after 16 d; 28.95 – 31.29 h after 32 d; 34.70 – 37.77 h after 48 d and the lag phase in phenanthrene only systems ranged from 35.72 h

after 48 d – 139.73 h at 0 d (Table 3). Generally, shorter lag phases were observed in pre–incubated spiked soils amended with 100 µg kg^{-1} hydroxycinnamic acids and soils amended with a mixture of hydroxycinnamic acids after 32 d comparedto those in other amended pre–incubated spikedsoils (0 – 16 d) (P<0.001) and unamended soil (P< 0.05).

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})	$1.07 \times 10^5 \pm 3.33 \times 10^3$
	Phenanthrene degraders (CFU g^{-1})	$1.20 \times 10^5 \pm 5.77 \times 10^3$

Table 2. Mineralisation of ^{14}C–phenanthrene in fresh artificially spiked soils amended with hydroxycinnamic acids at concentrations of 50 µg kg^{-1}, 100 µg kg^{-1}, 200 µg kg^{-1} and unamended soil (control). Values are the mean (*n=3*) ± SEM

Conditions	Soil Treatment	Mineralisation of ^{14}C–phenanthrene		
		Lag phase (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
50 µg kg^{-1}	Caffeic acid	108.02 ± 3.03	0.69 ± 0.11	47.92 ± 1.67
	Ferulic acid	112.70 ± 4.77	0.96 ± 0.09	52.93 ± 0.45
	p-coumaric acid	116.99 ± 1.71	0.78 ± 0.09	47.70 ± 2.21
	1:1:1 mixture of the 3 hydroxycinnamic acids	123.54 ± 0.65	0.51 ± 0.04	37.47 ± 2.19
100 µg kg^{-1}	Caffeic acid	126.82 ± 3.20	0.58 ± 0.15	45.62 ± 2.36
	Ferulic acid	124.81 ± 4.03	0.58 ± 0.27	42.76 ± 1.91
	p-coumaric acid	141.80 ± 4.37	0.63 ± 0.16	44.94 ± 1.75
	1:1:1 mixture of the 3 hydroxycinnamic acids	125.41 ± 1.57	0.77 ± 0.05	46.05 ± 1.25
200 µg kg^{-1}	Caffeic acid	138.06 ± 5.16	0.70 ± 0.21	43.70 ± 2.81
	Ferulic acid	133.50 ± 6.40	0.74 ± 0.05	46.77 ± 1.00
	p-Coumaric acid	134.83 ± 5.15	0.55 ± 0.02	44.94 ± 2.67
	1:1:1 mixture of the 3 hydroxycinnamic acids	123.43 ± 1.44	0.55 ± 0.05	38.50 ± 2.15
Control	Phenanthrene only	127.61 ± 2.48	0.54 ± 0.06	37.20 ± 0.23

Table 3. Mineralisation of ^{14}C–phenanthrene in pre-incubated artificially spiked soils(0, 16, 32 and 48 d) amended with 100 µg kg^{-1} hydroxycinnamic acids. Values are the mean (*n=3*) ± SEM

Time	Soil Treatment	Mineralisation of ^{14}C–phenanthrene		
		Lag phase (h)	Maximum rate (% h^{-1})	Cumulative extent (%)
0 d	Caffeic acid	142.71 ± 2.50	0.79 ± 0.08	45.37 ± 2.25
	Ferulic acid	146.00 ± 2.69	0.73 ± 0.13	41.67 ± 3.22
	p-Coumaric acid	129.03 ± 2.24	0.85 ± 0.09	41.49 ± 1.35
	1:1:1 mixture of the 3 hydroxycinnamic acids	124.58 ± 1.69	0.77 ± 0.14	39.05 ± 3.10
	Phenanthrene only	139.73 ± 2.56	0.65 ± 0.02	37.68 ± 1.22
16 d	Caffeic acid	55.64 ± 0.62	0.52 ± 0.05	34.05 ± 0.97
	Ferulic acid	55.56 ± 1.43	0.84 ± 0.06	50.05 ± 3.49
	p-Coumaric acid	62.53 ± 3.70	0.58 ± 0.09	38.88 ± 0.92
	1:1:1 mixture of the 3 hydroxycinnamic acids	57.27 ± 1.58	0.58 ± 0.06	41.57 ± 0.07
	Phenanthrene only	56.55 ± 1.23	0.69 ± 0.02	39.43 ± 0.39
32 d	Caffeic acid	29.37 ± 1.51	0.60 ± 0.11	42.01 ± 1.97
	Ferulic acid	29.60 ± 0.90	0.59 ± 0.05	42.00 ± 0.71
	p-Coumaric acid	31.29 ± 1.19	0.55 ± 0.03	45.30 ± 1.22
	1:1:1 mixture of the 3 hydroxycinnamic acids	28.95 ± 1.01	0.61 ± 0.09	42.62 ± 1.73
	Phenanthrene only	56.55 ± 1.23	0.69 ± 0.02	39.43 ± 0.39
48 d	Caffeic acid	34.70 ± 2.26	0.66 ± 0.04	41.58 ± 0.43
	Ferulic acid	37.77 ± 0.81	0.53 ± 0.12	40.59 ± 2.65
	p-Coumaric acid	37.66 ± 0.68	0.58 ± 0.05	38.51 ± 1.34
	1:1:1 mixture of the 3 hydroxycinnamic acids	36.03 ± 1.60	0.58 ± 0.06	41.23 ± 1.11
	Phenanthrene only	35.72 ± 3.17	0.51 ± 0.04	40.95 ± 1.93

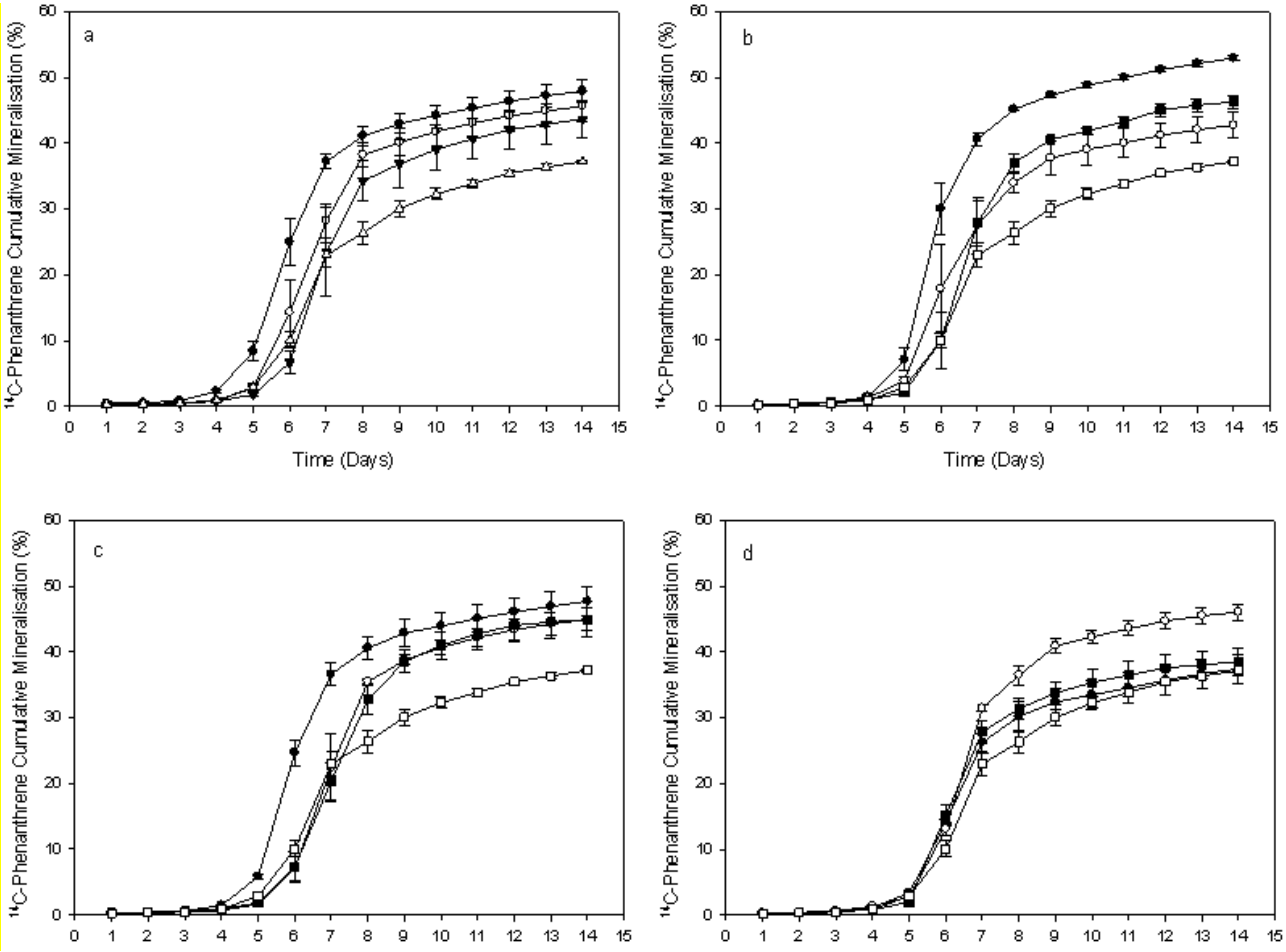

Figure 2. Mineralisation of ^{14}C–phenanthrene in fresh artificially spiked soils amended with (a) caffeic acid, (b) ferulic acid, (c) *p*-coumaric acid, (d) a mixture of hydroxycinnamic acids (caffeic acid, ferulic acid and *p*-coumaric acid at a 1:1:1 ratio) at concentrations of 50 µg kg^{-1} (●), 100 µg kg^{-1} (○), 200 µg kg^{-1} (■) and unamended soil (□). Error bars, where visible, represent standard error of mean (n = 3)

The rates of ^{14}C–phenanthrene mineralisation in fresh artificially spiked soils varied from 0.51 % $^{14}CO_2$ – 0.96 % $^{14}CO_2$ h^{-1} in systems amended with 50 µg kg^{-1}; 0.58 % $^{14}CO_2$ – 0.77 % $^{14}CO_2$ h^{-1} in systems amended with 100 µg kg^{-1}; 0.55 % $^{14}CO_2$ – 0.70 % $^{14}CO_2$ h^{-1} in systems amended with 200 µg kg^{-1} hydroxycinnamic acids, and 0.54 % $^{14}CO_2$ h^{-1} in systems with no amendment (Table 2). Fresh artificially spiked soils amended with 50 µg kg^{-1} ferulic acid exhibited the fastest rates of ^{14}C–phenanthrene mineralisation (P<0.05) comparedto rates in unamended soils. Statistical analyses of the results showed that the lag phases and overall extents of ^{14}C–phenanthrene mineralisation in soils were enhanced by addition of 50 µg kg^{-1} caffeic acid (P<0.05). In addition, soils amended with 100 µg kg^{-1} mixture of hydroxycinnamic acids (caffeic, ferulic and *p*-coumaric acids at a 1:1:1 ratio) exhibited the fastest rates of ^{14}C–phenanthrene mineralisation compared to rates in soils amended with 100 µg kg^{-1} hydroxycinnamic acids and unamended soil (P<0.05). Furthermore, the rates of ^{14}C–phenanthrene mineralisation in pre–incubated spiked soils amended with 100 µg kg^{-1} hydroxycinnamic acids varied from 0.73 % – 0.85 % $^{14}CO_2$ h^{-1} at 0 d; 0.52 %–0.84 % $^{14}CO_2$ h^{-1} after 16 d; 0.55 % – 0.61 % $^{14}CO_2$ h^{-1} after 32 d; 0.53 % – 0.66 % $^{14}CO_2$ h^{-1} after 48 d, and 0.51 % – 0.69 % $^{14}CO_2$ h^{-1} in systems with no amendment (Table 3). The overall fastest rates of ^{14}C–phenanthrene mineralisation in pre–incubatedspiked soils were obtained in systems amended with 100 µg kg^{-1} *p*–coumaric acid (0.85 % $^{14}CO_2$ h^{-1}) at 0 d and 100 µg kg^{-1} ferulic acid (0.84 % $^{14}CO_2$ h^{-1}) after 16 d compared to rates in the unamended soils (P<0.05).

The extents of ^{14}C–phenanthrene mineralisation in fresh artificially spiked soils ranged from 37.47 ± 2.19 to 52.93 ± 0.45 % in systems amended with 50 µg kg^{-1}; 42.76 ± 1.91 to 46.05 ± 1.25 % in systems amended with 100 µg kg^{-1}; 38.50 ± 2.15 to 46.77 ± 1.00 % in systems amended with 200 µg kg^{-1} hydroxycinnamic acids, and 37.20 ± 0.23 % in systems with no amendment (Figure 2 and Table 2). Freshly spiked soils amended with 50µg kg^{-1} ferulic acid had the highest extent of ^{14}C–phenanthrene mineralisation (52.93 ± 0.45 %) compared to those inthe unamended soils (P< 0.05). In addition, the extents of ^{14}C–phenanthrene mineralisation in spiked soils amended with 50 µg kg^{-1}hydroxycinnamic acids were statistically higher compared tothose in soils with no amendment (P< 0.05). However, systems amended with caffeic acid and*p*–coumaric acid exhibited similar mineralisation patterns at all concentrations. The extents of ^{14}C–phenanthrene mineralisationincreased in soils amended with caffeic acid and*p*–coumaric acid at concentrations of 50 µg kg^{-1}compared to those in systems amended with higher concentrations (≥ 100 µg kg^{-1}). Although enhanced extents of ^{14}C–phenanthrene mineralisation were obtained in soils amended with 50 µg kg^{-1}hydroxycinnamic acids, addition of 50 µg kg^{-1}mixture of hydroxycinnamic acids (caffeic, ferulic and *p*-coumaric acids at a 1:1:1 ratio) failed to enhance ^{14}C–phenanthrene mineralisation in freshly spiked soils.

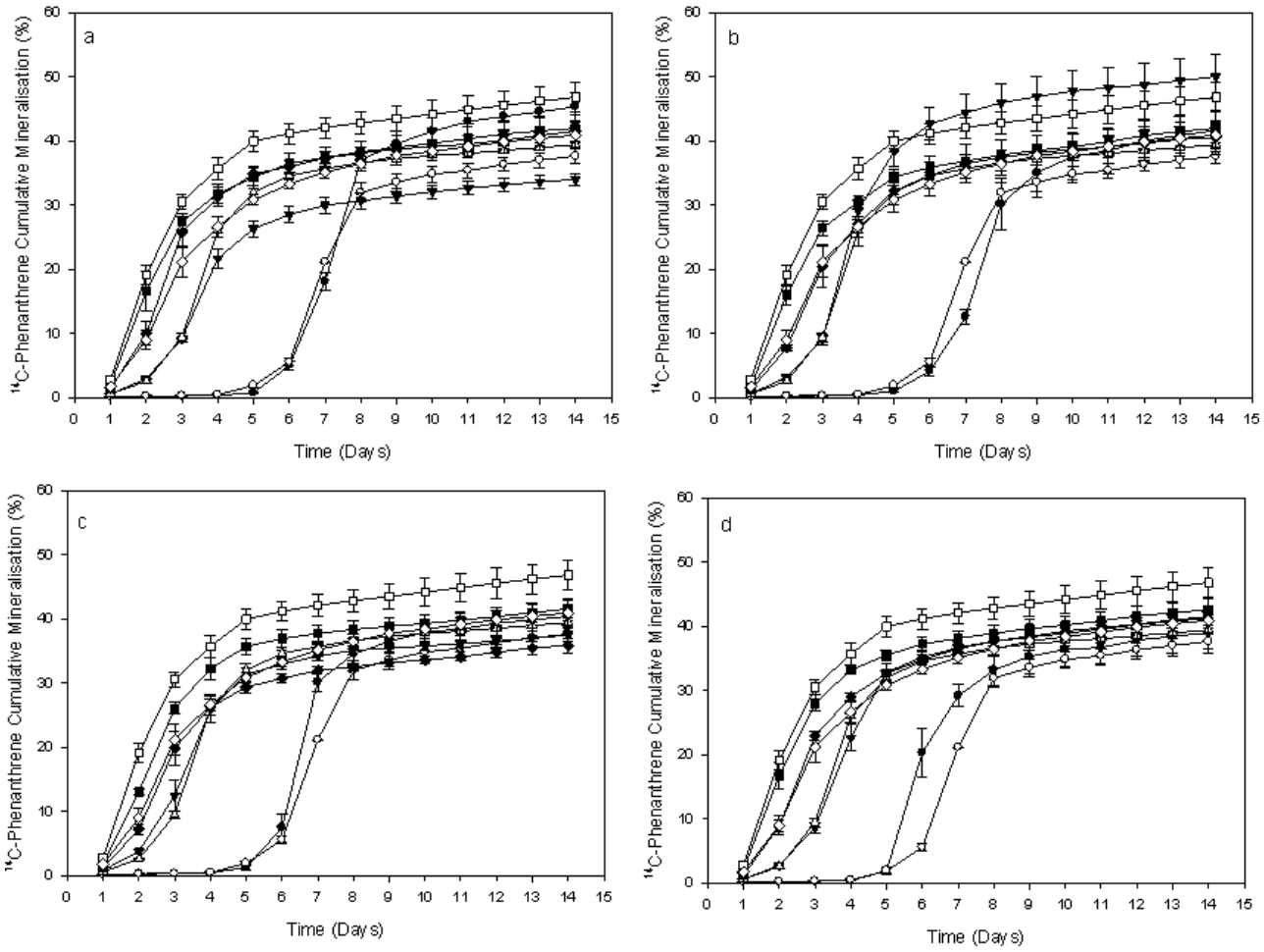

Figure 3. Mineralisation of ^{14}C–phenanthrene in artificially spiked soils amended with 100 µg kg^{-1} (a) caffeic acid, (b) ferulic acid, (c) *p*-coumaric acid, (d) a mixture of hydroxycinnamic acids (caffeic acid, ferulic acid and *p*-coumaric acid at a 1:1:1 ratio) at 0 d (T1 [● = amended; ○ = unamended]), 16 d (T2 [▼= amended; △= unamended]), 32 d (T3 [■ = amended; □ = unamended]) and 48 d (T4 [◆= amended; ◇= unamended]) time points. Error bars, where visible, represent standard error of mean (n = 3)

The extents of ^{14}C–phenanthrene mineralisation in pre–incubated spiked soils amended with 100 µg kg^{-1} hydroxycinnamic acids ranged from 39.05 ± 3.10 to 45.37 ± 2.25 % at 0 d; 34.05 ± 0.97 to 50.05 ± 3.49 % after 16 d; 42.00 ± 0.71 to 45.30 ± 1.22 % after 32 d; 38.51 ± 1.34 to 41.58 ± 0.43 % after 48 d, and 37.68 ± 1.22 % to 40.95 ± 1.93 % in unamended soil (Figure 3 and Table 3). The extents of ^{14}C–phenanthrene mineralisation increased in pre–incubated spiked soils amended with 100 µg kg^{-1} caffeic acid at 0 d and systems containing ferulic acid after 16 d were significantly increased compared to those in other treatment conditions (P< 0.05) (Figure 3 and Table 3). In general, soil–organic contaminant pre–exposure does not increase the extents of ^{14}C–phenanthrene mineralisation in systems amended with hydroxycinnamic acids. The results obtained showed that the lower concentration (≤ 100 µg kg^{-1}) of hydroxycinnamic acids and mixture of hydroxycinnamic acids (caffeic, ferulic and *p*-coumaric acids at a 1:1:1 ratio) stimulated indigenous microbial activity and further enhanced phenanthrenemineralisation in freshly contaminated soil.

4. Discussion

The extents of ^{14}C–phenanthrene mineralisation in freshly spiked soils indicate that the indigenous microflora are capable of degrading phenanthrene. The mineralisation of ^{14}C–phenanthrene in freshly spiked and pre–incubated spiked soils amended with hydroxycinnamic acids followed the standard 3–stage mineralisation curve [32]. The results obtained also showed that addition of 50 µg kg^{-1} hydroxycinnamic acids and 100 µg kg^{-1} mixture of hydroxycinnamic acids stimulated microbial degradation of phenanthrene in freshly spiked soils. In a previous related study, Zhou and Wu [33] observed that theshorter lag phases were consistent with induction of catabolic enzymes and adaptation to PAHs by the microbial population. Several studies have shown that phenolic acids in soils have complex chemistry and influence indigenous microbial community as well as microbial activity in the rhizosphere [34,35,36,37,38]. In this study, the hydroxycinnamic acids may have promoted the bacterial populations involved inphenanthrene degradation and amendment of ferulic acid may have changed the indigenous microbial community structure in soil.

In some related studies [39,40,41], it has been found that secondary plant metabolites are often effective at very low concentrations. It is possible that the addition of hydroxycinnamic acids at higher concentrations might have increased the pH slightly to become acidic and/or toxic to the indigenous phenanthrene degraders in soil. Although many secondary plant metabolites can be toxic to microorganisms [42], soil microflora exposed to high

concentrations of hydroxycinnamic acids may require longer recovery time prior to microbial mineralisation. According to Semple *et al.* [43], it takes time for the degraders to undergo morphological, physiological and behavioural adaptations when responding to environmental stress. The rate of ^{14}C–phenanthrene mineralisation (0.96 ± 0.09 $^{14}CO_2$ % h^{-1}) in soil amended with 50 µg kg^{-1} ferulic acid was significantly faster compared to that inthe unamended soil systems ($P< 0.05$). The fastest rates and increased extents of ^{14}C–phenanthrene mineralisation were obtained in spiked soils amended with 100 µg kg^{-1} ferulic acid after 16 d soil–phenanthrene pre–exposure. Sparling *et al.* [36] reported that *p*–hydroxybenzoic, vanillic,ferulic and caffeic acids had no overall toxic effect on thesoil microbial biomass when added at the rate of 5 mg g^{-1}soil. In addition, there were increases intotal biomass and in the rate of production of respiratory CO_2, indicating that the biomass was able to metabolisethe acids [36].

From previous study,it has been found that root exudates including phenolic acids significantly enhanced phenanthrene biodegradation in rhizosphere soils [44], either by increasing contaminant bioavailability and/or selective enrichment of PAH degrading population size and activity.In this study, amendment of soils with hydroxycinnamic acids may have resulted in availability of alternative carbon sources for microbial growth [45,46]. However, the inconsistencies in the results obtained for some of the treatment conditions in thepresent study are similar to other reported studies [47,48] and the differences might be attributed to the methodsof addition ofhydroxycinnamic acids to the soils.According to Blum and Shafer [34], lower concentrations (< 100 mg kg^{-1}) of phenolic acids can act as substrates for soil microbes. The concentrations of ferulic acid and *p*-coumaric acid (200 – 340 mg kg^{-1}) obtained in the soil [49] are similar to those reported to inhibit bacterial and fungal activity [34]. In this study, there were subtle differences in the number of phenanthrene degraders (CFU g^{-1}) obtained in the pre–incubated spiked soil amended with hydroxycinnamic acids compared to those in the unamended soils (control) at various time points. The cinnamic acid derivatives (ferulic and *p*-coumaric acids) are readily metabolized by microorganisms, sometimes without detectable population changes, when adequate mineral nutrients are present [34]. The microbial cell numbers in ferulic acid amended soil and diversity indices of microbialcommunity were lower in soils amended with ferulicacidthan those in the unamended soil for bacterial community [33]. The microbial data have not been reported in this study due the similar cell counts in hydroxycinnamic acid–amended soils and unamended soils, and lack of statistical differences in microbial cell numberbetween various treatment conditions.

5. Conclusion

The results obtained from this study have shown that addition of ≤ 100 µg kg^{-1} hydroxycinnamic acids enhanced the catabolic capabilities of indigenous soil microorganisms and subsequent degradation of phenanthrene in freshly contaminated soil. Depending on its concentrations, addition of hydroxycinnamic acids and other secondary compounds may either stimulate or inhibit microbial degradation of PAH 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. With an increased understanding it should be possible to identify more precisely the classes of compounds that are most effective at stimulating microbial degradation of specific contaminants. Therefore, further study would focus on identification of inducers that are effective against a broad range of contaminants or prepare cocktails that are tailor-made for contaminated sites with mixed contaminants. The use of artificial plant–secreted chemicals to stimulate microbial catabolic activity and subsequent degradation of organic contaminants offers the opportunity to develop sustainable systems and/or approaches for remediation of contaminated sites.

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