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The effect of inorganic and organic supplements on the microbial degradation of phenanthrene and pyrene in soils

Lisa M. Carmichael[1] & Frederick K. Pfaender
Department of Environmental Sciences and Engineering, The University of North Carolina at Chapel Hill, NC 27599-7400, USA; ([1] Present Address: Department of Civil Engineering, North Carolina Agricultural and Technical State University, Greensboro, NC 27411, USA)

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Abstract

The effects of several bioremediation stimulants, including potential metabolism pathway inducers, inorganic/organic nutrients, and surfactants on the metabolism of phenanthrene and pyrene, as well as the population dynamics of PAH degrading microorganisms was examined in five soils with differing background PAH concentrations, exposure histories and physical properties. Most of the supplements either had no significant effect or decreased the mineralization of [^{14}C]-phenanthrene and [^{14}C]-pyrene in soil slurry microcosms. The effect of a particular supplement, however, was often not uniform within or across soils. Decreased mineralization of [^{14}C]-phenanthrene and [^{14}C]-pyrene was usually due to either preferential use of the supplement as carbon source and/or stimulation of non-PAH degrading microorganisms. Many of the supplements increased populations of heterotrophic microorganisms, as measured by plate counts, but did not increase populations of phenanthrene degrading microorganisms, as measured by the [^{14}C]-PAH mineralization MPN analysis or cellular incorporation of [^{14}C]-PAH. These results suggest that the PAH degrading community at each site may be unique in their response to materials added in an attempt to stimulate PAH degradation. The characteristics of the site, including exposure history, soil type, and temporal variation may all influence their response.

Introduction

Polynuclear aromatic hydrocarbons (PAH) are ubiquitous environmental contaminants associated with the combustion of fossil fuels. Heavily PAH contaminated soils are often associated with the use and disposal of petroleum containing materials and their derivatives (Verschueren 1983; Suess 1976). PAH are of environmental concern because many are mutagenic and/or carcinogenic (Pucknat 1981). While bioremediation has been suggested as a feasible technology for remediating PAH (US EPA 1992; Mueller et al. 1989) their sparingly solubility and tendency to form associations with organic matter greatly decrease the portion of PAH available to microorganisms (Mihelcec & Luthy 1993; Wodzinski & Coyle 1974). This means that successful remediation of PAH contaminated soils may require special strategies. The potential for *in situ* bioremediation of PAH may be further limited if conditions in soils are not optimal for the growth and metabolism of PAH degrading microorganisms. These difficulties may potentially be overcome by physically manipulating conditions in soils or by adding organic and inorganic supplements. In general, supplements are used either to increase the available concentration of the pollutant(s) of interest and/or to increase or stimulate populations of degrading microorganisms. Supplements can be divided into three general categories based on how they impact soil microbial processes:

1. surfactants or solubolizers to increase the available concentration of contaminants;
2. specific carbon sources or inducers to stimulate a particular group of microbes (i.e. PAH degraders),
3. organic and inorganic compounds to provide nutrients to increase the size and activity of the soil microbial community.

Supplements have been tested with a variety of pollutants, pure and mixed microbial cultures and environmental media with few overriding generalizations emerging from these studies. Additions of organic and inorganic nutrients, for example, have been found to increase microbial metabolism of some target pollutants (Manilal & Alexander 1991; Swindoll et al. 1988), decrease metabolism of other target pollutants (Manilal & Alexander 1991), shorten the adaptation period for certain compounds (Swindoll et al. 1988) or have no apparent effect on metabolism in other cases (Mihelcec & Luthy 1991). In much of this research, the effects of supplements on the population dynamics of the exposed microbial communities has not been addressed.

This study was designed to examine the influence of a variety of supplement types (surfactants, pathway intermediates, organic and inorganic nutrients) on the microbial metabolism of two model [^{14}C]-PAH, phenanthrene and pyrene, and on the population dynamics of microorganisms in several soils with different PAH exposure histories and soil characteristics. Several possible fates of [^{14}C]-PAH were monitored including, mineralization, production of non-mineral metabolites and the association of [^{14}C]-PAH with soils relative to an abiotic control.

Materials and methods

Chemicals

Radiolabeled [9-^{14}C]-phenanthrene (specific activity(SA): 10 mCi·mmol^{-1}), [4,5,9,10-^{14}C]-pyrene (SA: 32.3 mCi·mmol^{-1}) and [U-^{14}C]-salicylic acid (SA: 10.1 mCi·mmol^{-1}) were purchased from Sigma Chemical Company (St. Louis, MO; purity $>$ 98%). L-[U-^{14}C]-amino acids (SA: 7.137 mg·mCi^{-1}) were purchased from New England Nuclear Corp. (Boston, MA) and D-[U-^{14}C]-glucose (SA: 270 mCi·mmol^{-1}) was purchased from Amersham (Arlington Heights, IL). High purity solvents and inorganic chemicals used in all analysis were purchased from Mallinckrodt Specialty Chemical Company (Paris, KY) or EM Scientific (Gibbstown, NJ). NaN_3, used to inhibit microbial metabolism was purchased from Sigma Chemical Company. Other materials were purchased from a variety of sources: [^{12}C]-PAH (phenanthrene and pyrene) and pathway intermediates (salicylic acid, phthalic acid, cinnamic acid, gentisic acid, propionic acid; Aldrich Chemical Company; St Louis, MO); Triton X-100 (Fisher Scientific; Fairlawn, NJ) and Inipol EAP-22 (ELF Aquataine). Media for plate counts and nutrient broth were purchased from Difco Laboratories (Detroit, MI).

Soil samples

The characteristics of the five soils that were used are shown in Table 1; two soils are highly contaminated with PAH and were collected from the Reilly Tar and Chemical Company Superfund site (St.Louis Park, MN), two less contaminated subsurface soils were collected from the Dubose Oil Recycling Superfund site (Cantonment, FL) and a reference uncontaminated soil was collected from Bozeman, MT. All soil samples were stored in plastic containers at 4 °C in the dark until use.

The effect of supplements on the growth of microorganisms

The growth response of heterotrophic microorganisms in soils with supplements added was examined by using modified standard plate count and most probable number assay (MPN) methods. For plate counts (Standard Methods, 1992), individual supplements (10 mL of solution, at the same concentrations used in the metabolism experiments) were added to sterile tubes containing 1g of soil which were then incubated stationary for one week at 20 °C. After the 1 week incubation, a dilution series was done and plated in triplicate on R2A and nutrient agar. Two controls were also prepared, tubes incubated with no liquid addition (soil only) and tubes with distilled, deionized water only added to soil (no supplements).

The effect of the supplements on the size of the PAH degrading community was assessed with the [^{14}C]-phenanthrene MPN mineralization assay (Lehmicke et al. 1979). The MPN used 1 g of soil in an autoclaved 40 mL vial along with 10 mL of a supplement/water solution that was incubated for one week at 20 °C. After this time, a dilution series was done as with the standard MPN analysis and [^{14}C]-phenanthrene was added. The MPN assay was based on a comparison of mineraliza-

Table 1. Physical characteristics of exerimental soils

	SOIL				
	SWS	SSC	DCC	DCU	BOZ
Source	St. Louis Park, MN		Cantonment, FL		Bozeman, MT
Location	Sub-surface (4-8 ft)	Surface	Sub-surface (5-8 ft)		Surface
PAH (ppm)[a]	1571	269	11	2	0.082
f_{oc}[b]	0.025	0.029	0.0046	0.0026	0.024
CEC[c]	21.6	25.4	2.5	2.5	22
pH	8.3	7.8	6.3	6.3	6.0
%Nitrogen[d]	0.13 (0.006)	0.37 (0.03)	0.03 (0.001)	0.03 (0.001)	0.28 (0.02)
% Sand (2mm to 50mm)	90.0	58.0	90.0	87.0	7.0
% Silt (50 - 2μm)	8.0	34.0	4.0	6.0	70.0
% Clay ($\leq$2μm)	2.0	8.0	5.0	7.0	23.0

[a] Total PAH analyzed by Triangle Laboratories (Dublin, OH) (EPA SW846, method 8310).
[b] Fraction of organic carbon (non-PAH) analyzed by Huffman Laboratories (Golden, CO) (ASTM D5373).
[c] Cation exchange capacity; units: meq·100 cm^{-3}.
[d] Measured by combustion and measurement of N_2 gas, mean (n = 3) and standard deviation in parentheses.

tion in seven dilution levels from live (n = 5) and abiotic (n = 3; 0.50% (v/v) NaN_3 solution) microcosms over a 4.5 week incubation period. $^{14}CO_2$ was collected as described in the following section. Controls consisted of vials incubated with no liquid addition (soil only) and vials incubated only with distilled, deionized water and soil (no supplements). MPN values, based on the number of positive responses, were calculated with a microcomputer program (Clark & Owen 1983). A positive response was scored when the $^{14}CO_2$ evolution in the live microcosms was greater than the mean plus three times the standard deviation of the abiotic microcosms at the same dilution level.

The effect of supplements on the metabolism of [^{14}C]-phenanthrene and [^{14}C]-pyrene

Metabolism of [^{14}C]-PAH was monitored using methods derived from Dobbins & Pfaender (1988), Pfaender & Bartholomew (1982) and Carmichael & Pfaender (1997). All experiments were carried out in triplicate microcosms constructed with sterile, 40 mL vials closed by caps with Teflon lined septa. Each microcosm contained 1g of soil and 10 mL of sterilized distilled, deionized water containing the appropriate concentration of a single supplement which was sterilized prior to addition. Previous work (Aelion et al. pers. comm.) has shown that a 1:10 soil/water ratio produces optimal metabolic rates with a variety of organic pollutants. It does not, however, represent conditions in soils, where the soil:water ratio is much lower. Incubation was initiated when [^{14}C]-phenanthrene ($\approx$ 68 $\mu g \cdot L^{-1}$) or pyrene ($\approx$ 19.74 $\mu g \cdot L^{-1}$), in 50% ethanol, was pipetted into each vial containing the soil slurry (final ethanol concentration 4.5 $\mu L \cdot mL^{-1}$slurry). Metabolically inhibited controls containing 0.5% NaN_3(v/v) solution were included to monitor the abiotic fate and transformations of PAH. The amount of radiolabel added to each microcosm was measured by adding amounts identical to that added to the incubations directly to three scintillation vials containing 7 mL of Scinitisafe gel scintillation fluid (Fisher Scientific) followed by liquid scintillation counting (LSC) on a Packard Model 300CD or a Model, 1900 TR scintillation counter (Downers Grove, IL). All microcosms were incubated vertically in the dark at 20 °C for up to two months. Logistical constraints did not allow for the testing of every supplement with a particular soil and [^{14}C]-PAH at the same time. A set of vials without supplements and abiotic controls were completed for every experimental group tested, and results were normalized to these specific control incubations.

Each microcosm had a center well (Kontes, Vineland, NJ), containing a fluted 7cm strip of What-

man 1 chromatography paper saturated with 200 µL of 2N KOH. After incubation, microcosms were acidified to pH 2 with 20% (v/v) H_3PO_4 and placed on a rotary shaker at 50 rpm for at least 16 hours to transfer $^{14}CO_2$ from the gas phase and trap it on the base soaked filter paper. Following acidification, the filter paper in the $^{14}CO_2$ trap was removed and placed in a vial for LSC. $^{14}CO_2$ recovery efficiencies were estimated with triplicate [^{14}C]-$NaHCO_3$ amended control vials that were processed and analyzed in a manner identical to the microcosms containing [^{14}C]-PAH. Recovery efficiencies of [^{14}C]-$NaHCO_3$ were used to correct mineralization recoveries of [^{14}C]-PAH to 100%. $^{14}CO_2$ recovery efficiencies ranged between 60 to 100% across all soils (data not shown).

After the filter paper and the base trap were removed, the microcosms were closed with Teflon-lined caps and centrifuged at 270 × g for 30 minutes at 4 °C in a Sorval RC-5B centrifuge. After centrifugation, the water phase was decanted into a new 40 mL vial, 2.5 mL of hexane was added and mixed on a rotary shaker at 120 rpm for 30 minutes. The hexane and water were then allowed to separate and each was subjected to LSC. The solvent extractable material was assumed to represent unmetabolized PAH, but may also include non-polar metabolites. The label present in the aqueous phase was assumed to represent polar metabolites. Efficiency of the hexane extractions was verified with [^{14}C]-phenanthrene, [^{14}C]-naphthalene and [^{14}C]-salicylic acid and were shown to be over 98% (data not shown).

The soil pellet remaining after centrifugation, was extracted with a mixture of 5 mL of ethyl acetate and 10 mL of water. The vials were vortexed for 30 seconds and later centrifuged for 40 minutes at 270 × g. After centrifugation, 1 mL of the ethyl acetate was removed and placed in a vial for LSC, the remaining ethyl acetate was removed and discarded. The extracted soil slurry was agitated and 1 mL was removed and counted by LSC to account for [^{14}C]-PAH remaining in the soil. It was assumed that the radiolabel associated with the soils, in both the ethyl acetate extraction and the PAH remaining in soil after extraction, represents unmetabolized [^{14}C]-PAH, although this fraction could also include highly non-polar or immobilized metabolites.

Results and discussion

The influence of supplements on the growth of microorganisms in soil SWS

These experiments monitored the effect of several supplements (salicylic acid, phthalic acid, nutrient broth, Triton X-100, Inipol EAP-22, M9 buffer) on the growth of heterotrophic and PAH degrading microorganisms in SWS, a soil in which all of the supplements were tested. This soil has been exposed to high concentrations of PAH for an extended time (approximately 100 years). Each of these supplements can augment the pool of available organic or inorganic nutrients. When soil SWS was preexposed to Triton X-100 (1% v/v water solution), Inipol EAP-22 (1.66 $mL \cdot L^{-1}$) or the M9 buffer for one week there were more general heterotrophic microorganisms, as measured by plate counts on high (nutrient agar) and low (R2A agar) nutrient media, compared to soils incubated only with distilled, deionized water (Table 2). Salicylic acid, on the other hand decreased the number of heterotrophic microorganisms on both types of media.

None of the supplements types, however, produced a statistically significant increase in the population of phenanthrene degrading microorganisms over the same soils only exposed to distilled water (Table 2). Exposure of soil SWS to Triton X-100 and the M9 buffer, however, did result in larger numbers of phenanthrene degrading microorganisms than controls and salicylic acid a lower number than the controls. In other laboratories, Triton X-100 and Salicylic acid have both been associated with stimulating the growth of PAH degrading microorganisms (Tsomides et al. 1995; Volkering et al. 1995; Ogunseitan et al. 1991).

The influence of pathway intermediates on the mineralization of [^{14}C]-phenanthrene and [^{14}C]-pyrene

PAH metabolic pathway intermediates salicylic acid, phthalic acid, gentisic acid, and cinnamic acid (Heitkamp et al. 1988; Kiyohara & Nagao 1977; Kiyohara et al. 1976) were added in an attempt to stimulate the metabolic pathways responsible for the metabolism of [^{14}C]-phenanthrene and [^{14}C]-pyrene. Previous microcosm experiments without supplements had shown mineralization to be the primary biotic fate of [^{14}C]-phenanthrene and [^{14}C]-pyrene in these soils, which peaked after approximately two weeks

Table 2. The effect of supplements on the number of heterotrophic (plate counts) and [^{14}C]-phenanthrene degrading microorganisms (MPN assay) in SWS

Supplement	[CFU[a] (10^6· g soil^{-1})		Phenanthrene
	R2A Agar	Nutrient Agar	Degraders[b] ·g soil^{-1}
Distilled water	71 (1.1)	72 (1.3)	880 (282 - 2746)
Triton X-100 (1% v/v)	363 (14)	265 (41)	1444 (516 - 4041)
M9 Buffer	453 (57)	393 (37)	2664 (972 - 7301)
Inipol EAP-22 (8 mg·L^{-1})	532 (53)	645 (29)	548 (174 - 1728)
Nutrient broth (100 mg·L^{-1})	96 (15.6)	83 (13.5)	NT
Salicylic acid (100 mg·L^{-1})	38 (2.22)	24 (2.25)	240 (90 - 641)
Phthalic acid (100 mg·L^{-1})	NT	NT	543 (173 - 1701)

[a] mean CFU and (standard deviation).
[b] number of degraders and (95% confidence interval).
NT = Not tested

Table 3. Effect of pathway Intermediates (100 mg·L^{-1}) on the mineralization of (A) [^{14}C]-phenanthrene and (B) [^{14}C]-pyrene in soil microbial communities after 4 weeks

	SOIL	Control[a]	Pathway intermediates[b]		
			Salicylic acid	Phthalic acid	Gentisic acid
A)	SWS	59.1 (1.3)	0.99 (0.03)	1.03 (0.04)	0.92 (0.004)
	SSC	55.2 (3.3)	0.72 (0.09)	1.19 (0.10)	NT
	DCC	50.4 (2.7)	<0.001	NT	NT
	DCU	31.4 (0.9)	<0.001	NT	NT
	BOZ	2.5 (0.32)	1.22 (0.003)	NT	NT
B)	SWS	45.2 (4.35)	1.02 (0.05)	1.10 (0.06)	1.66 (0.05)
	SSC	40.2 (2.25)	1.02 (0.03)	0.99 (0.003)	0.95 (0.07)
	DCC	46.5 (1.05)	0.01 (<0.001)	0.03 (<0.001)	0.02 (<0.001)
	BOZ	0.5 (0.03)	1.09 (<0.001)	NT	2.23 (<0.001)

[a] % of added label mineralized with distilled, deionized water, mean (n=3) and (standard deviation).
[b] Fraction of control mineralization, mean (n =3) and (standard deviation).
NT = not tested

(Carmichael & Pfaender 1997). Table 3 describes the extent of mineralization for [^{14}C]-phenanthrene and [^{14}C]-pyrene after four weeks in soils exposed to pathway intermediates (100 mg·L^{-1}) compared to untreated microcosms. All data are described in terms of the metabolism of the [^{14}C]-PAH in the presence of the pathway intermediates compared to metabolism of [^{14}C]-PAH in the absence of the pathway intermediate, unless otherwise stated.

Salicylic acid (100 mg·L^{-1}), a known inducer of naphthalene and phenanthrene metabolism (Ogunseitan et al. 1991; Yen & Serdar 1988), did not influence the mineralization of [^{14}C]-phenanthrene or [^{14}C]-pyrene in soils SWS and BOZ (Table 3). In soil SSC, salicylic acid decreased [^{14}C]-phenanthrene mineralization by approximately 28% but had no effect on the mineralization of [^{14}C]-pyrene. The slight decrease in [^{14}C]-phenanthrene mineralization in soil SSC with salicylic acid was accompanied by a 10% increase in the production of water soluble metabolites (data not shown). Accumulation of metabolites in SSC, which was not noted with any of the other supplement or soil combinations, may have resulted from a blockage of the metabolic pathway responsible for [^{14}C]-phenanthrene metabolism by the added [^{12}C]-salicylic acid. Supplementing microorganisms with high concentrations of unlabeled metabolites of specific [^{14}C]-compounds is a technique that has been shown to cause an accumulation of metabolites up-stream of the spiked [^{12}C]-metabolite and is also known as isotope trapping (Edwards et al. 1994).

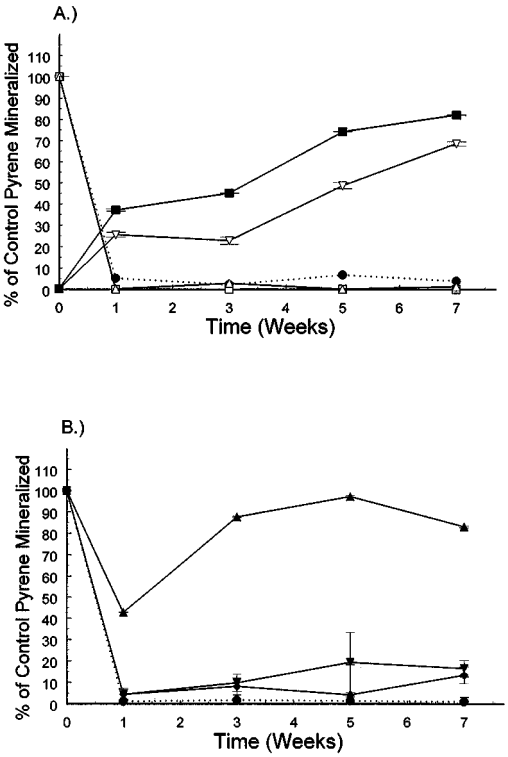

Figure 1. The effect of salicylic acid or cinnamic acid on the mineralization of [^{14}C]-pyrene in soil DCC over time (weeks). The data are presented as the percent of added [^{14}C]-pyrene mineralized compared to controls containing no salicylic or cinnamic acid. Pathway intermediates added at: (A) Salicylic acid: 100 mg·L^{-1} (...●...); 60 mg·L^{-1} (□); 40 mg·L^{-1} (△); 15 mg·L^{-1} (▽); and 5 mg·L^{-1} (■). (B) Cinnamic acid: 100 mg·L^{-1} (...●...); 60 mg·L^{-1}(▼); 40 mg·L^{-1}(◆); and 20 mg·L^{-1} (▲).
Mean value (n = 3) and standard deviation.

Table 4. Percent of added of [^{14}C]-salicylic acid, [^{14}C]-amino acids and [^{14}C]-glucose mineralized after 10 or 20 days of preexposure to water or the M9 buffer in soil SWS. Mean (n = 3) ± standard deviation in parenthesis

^{14}C-Substrate	Treatment	Days Pre-exposure	
		10 days	20 days
Amino acids	Water	30.0 (4.80)	40.0 (1.92)
	M9 Buffer	37.5 (0.56)	47.4 (0.99)
Salicylic acid	Water	37.1 (1.37)	35.7 (1.70)
	M9 Buffer	37.2 (1.70)	53.5 (0.45)
Glucose	Water	34.9 (3.87)	22.2 (0.86)
	M9 Buffer	66.5 (5.36)	32.4 (0.66)

In soils DCC and DCU, salicylic acid decreased the mineralization of [^{14}C]-phenanthrene and [^{14}C]-pyrene, by >99% (Table 3). Mineralization of [^{14}C]-pyrene in soil DCC was inhibited to a similar extent when salicylic acid was present at either 100, 60, or 40 mg·L^{-1} but only by 15 to 30% when salicylic acid was added below 15 mg·L^{-1} (Figure 1). The decreased mineralization of [^{14}C]-PAH in soils DCC and DCU was associated with an increased collection of the [^{14}C]-PAH in the solvent extract of the soil (data not shown). Label collected in the solvent extract of the soil is assumed to represent primarily unmetabolized [^{14}C]-PAH. Increased recovery of the [^{14}C]-PAH in the solvent extract of the soil was usually noted when mineralization decreased in the other supplement and soil combinations (data not shown). This shift suggests that the supplements are actually decreasing the metabolism of [^{14}C]-PAH rather than redirecting metabolism to other pathways or metabolic products.

Gentisic and phthalic acid (100 mg·L^{-1}) had little effect on the mineralization of [^{14}C]-phenanthrene or [^{14}C]-pyrene when tested in soils SWS, SSC, DCC and DCU, with the exception of a >99% decrease in the mineralization of [^{14}C]-pyrene when soil DCC was exposed to phthalic acid (Table 3). These supplements were tested predominantly with [^{14}C]-pyrene because [^{14}C]-phenanthrene was usually readily mineralized in the absence of supplements.

Cinnamic acid (100 mg·L^{-1}), an intermediate of pyrene metabolism by *Mycobacterium* sp. (Heitkamp et al. 1988), temporarily increased the mineralization of [^{14}C]-pyrene between two and six weeks in soil SWS (approximately 66%), increased [^{14}C]-pyrene mineralization in soil BOZ by greater than 100% and had no effect on [^{14}C]-pyrene mineralization in soil SSC (Table 3). In soil DCC, cinnamic acid decreased the mineralization of [^{14}C]-pyrene by >98% when added at 100 or 60 mg·L^{-1}, by 80% with 40 mg·L^{-1} cinnamic acid, and by 20% when cinnamic acid was added at 20 mg·L^{-1}compared to the percent of [^{14}C]-pyrene mineralized in the absence of cinnamic acid (Figure 1). Cinnamic acid more than doubled the mineralization of [^{14}C]-pyrene in soil BOZ, but the total amount of [^{14}C]-pyrene mineralized with cinnamic acid was still only slightly greater than 1% of the total added [^{14}C]-pyrene (data not shown). The transitory increase in [^{14}C]-pyrene mineralization in SWS, however, was more substantial. In order to test whether the stimulatory effect in soil SWS was the result of utilization of the hydrocarbon side chain of cinnamic acid, mineralization of [^{14}C]-phenanthrene and [^{14}C]-pyrene was monitored in the presence of a neutralized solution of propionic acid (100 mg·L^{-1};$C_2H_5CO_2H$) a surrogate for the hydrocarbon side chain. Propionic acid only slightly decreased the mineralization of [^{14}C]-

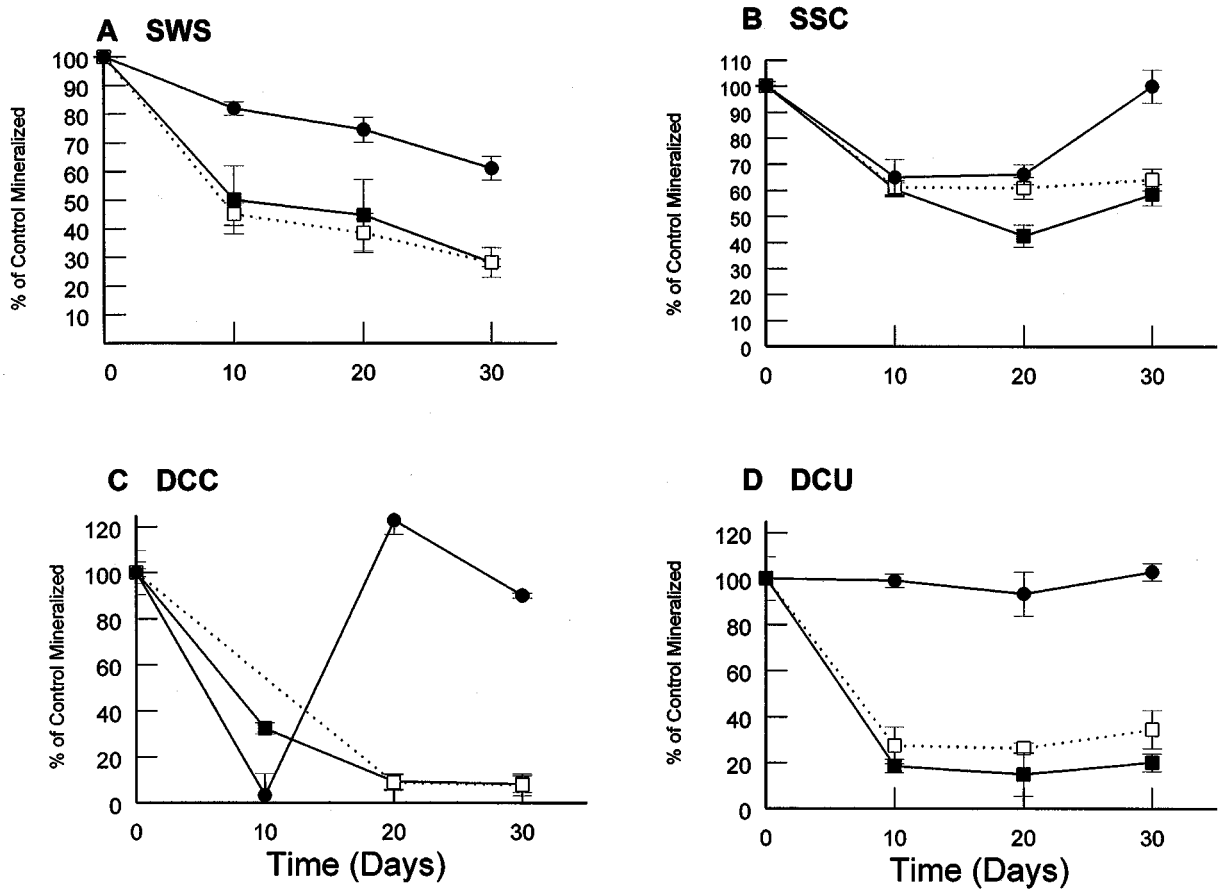

Figure 2. The influence of M9 buffer, nitrogen (as NH_4Cl; 2.5 g·L^{-1}) and phosphorous (as Na_2PO_4 [3 g·L^{-1}] and K_2HPO_4 [1.5 g·L^{-1}]) on the mineralization of [^{14}C]-pyrene. Data is presented as the percent of [^{14}C]-pyrene mineralized compared to an unamended control, in A) SWS, B) SSC, C) DCC and D) DCU over time (days). M9 buffer (■), N (□) and P (●).
Mean value (n = 3) and standard deviation.

phenanthrene (14% [±1.1] decrease) but decreased the mineralization of [^{14}C]-pyrene by approximately 50% (± 0.50). These results suggest that the hydrocarbon side chain itself is probably not stimulating [^{14}C]-pyrene metabolism in soil SWS.

The diverse effects seen with the pathway intermediates may be attributed to differences in the characteristics of soils and their native microbial communities. The pathway inducers had little effect on metabolism of either [^{14}C]-PAH in soils SWS and SSC. These soils were taken from a site with an extensive history of exposure to high concentrations of PAH which has resulted in an active community of PAH degrading microorganisms (Carmichael & Pfaender 1997). In soils DCC and DCU, most of the pathway intermediates greatly decreased the mineralization of [^{14}C]-phenanthrene or [^{14}C]-pyrene. Toxicity of the pathway intermediates in soils DCC and DCU was tested by assessing the mineralization of [^{14}C]-labeled pathway intermediates themselves and by comparing mineralization of a general [^{14}C]-carbon source (amino acids) in the presence or absence of the pathway intermediates. Salicylic acid, chosen as a model pathway intermediate, does not appear to be toxic to the microbial communities in DCC and DCU (or the other soils) because it was readily mineralized (33 to 50% of the added [^{14}C]-salicylic acid in four weeks; data not shown), even when [^{12}C]-phenanthrene or [^{12}C]-pyrene were also present (data not shown). In addition, the extent of [^{14}C]-amino acid mineralization with salicylic acid was not substantially different than in microcosms without salicylic acid in soils DCC, DCU, SWS, and SSC (data not shown). This data suggests that in soils DCC and DCU, it is likely that the path-

way intermediates were used preferentially as a carbon source over [^{14}C]-phenanthrene or [^{14}C]-pyrene. The decrease in the number of total heterotrophic microorganisms in SWS preexposed to salicylic acid, however, indicates that salicylic acid may negatively impact the growth of microorganisms in some cases (Table 2). In BOZ, an uncontaminated soil with a very small native PAH degrading community (Carmichael & Pfaender 1997), the pathway intermediates probably had little effect because initially there were few PAH degrading microorganisms in the soil.

The impact of inorganic nutrients on the mineralization of [^{14}C]-pyrene

Hydrocarbon wastes are often deficient in inorganic nutrients essential to microorganisms (Morgan & Watkinson 1989), and as a result, microbial metabolism of hydrocarbons in soils may be inorganic nutrient limited. Experiments were conducted to assess the impact of inorganic nutrients (M9 buffer: 3g of Na_2PO_4, 1.5g of KH_2PO_4, 2.5g of NH_4Cl and 0.12 g of $MgSO_4$ per L distilled, deionized water, pH = 7.3) on the mineralization of [^{14}C]-pyrene in the four contaminated soils (SWS, SSC, DCC and DCU). The M9 buffer decreased the mineralization of [^{14}C]-pyrene by approximately 50 to 90% in the soils compared to vials incubated without the buffer (Figure 2). In soil DCU, the mineralization of [^{14}C]-pyrene decreased to the same extent when the M9 buffer was added as a 75%, 50%, or 25% (v/v) M9 buffer/water solution (data not shown). Preexposure of soil SWS to the M9 buffer for 10 or 20 days before the addition of ^{14}C-substrates (salicylic acid, amino acids and glucose), however, increased their mineralization after one week (Table 4). The soils were preexposed to the M9 buffer before the ^{14}C-carbon sources were added because the mineralization of readily degradable carbon sources may saturate before the effects of the buffer occur. Increased mineralization of readily degradable substrates with the M9 buffer suggests that the M9 buffer is not toxic to the microorganisms but that it may stimulate the activity and growth (Table 2) of some heterotrophic microorganisms.

When the components of the buffer (nitrogen or phosphorus) were added individually at the same concentrations used in the complete buffer, nitrogen (NH_4Cl; 2.5 g·L^{-1}) decreased [^{14}C]-pyrene mineralization approximately as much as the complete M9 buffer in all of the soils, a decrease of 50 to 90% (Figure 2). Phosphrous (Na_2HPO_4 [3 g·L^{-1}] and KH_2PO_4 [1.5 g·L^{-1}]) had no effect on [^{14}C]-pyrene mineralization in soils SSC, DCC and DCU after four weeks while in soil SWS, phosphorous decreased the mineralization of [^{14}C]-pyrene by approximately 30%, compared to a 70% decrease for the complete M9 buffer or nitrogen alone. Together this suggests that nitrogen is primarily responsible for the decreases shown in Figure 2.

Different forms of nitrogen (NH_4Cl or $NaNO_3$), added separately or as a part of the M9 buffer, were tested for their effects on the mineralization of [^{14}C]-pyrene in soils DCC and DCU (Figure 3). In soil DCC, the mineralization of [^{14}C]-pyrene decreased by approximately 90% for the M9 Buffer with NH_4Cl or $NaNO_3$ and for the addition of NH_4Cl alone. If $NaNO_3$ was added separately, however, [^{14}C]-pyrene mineralization was generally unaffected for 20 days and then decreased by 40% after four weeks. In soil DCU, [^{14}C]-pyrene mineralization was decreased to the same extent by both forms of nitrogen, added either separately or in the M9 buffer.

In our soils, the addition of inorganic materials appears to produce an increase in the microbial metabolism of readily degradable carbon sources (see Table 4) in preference to the PAH. Inorganic ammendments shifting metabolism to non-PAH carbon sources could have a large impact on the metabolism of PAH, since most PAH are usually present in low available concentrations compared to other general carbon sources. Previous work has usually not found negative impacts of inorganic supplements on the metabolism of organic compounds. Additions of inorganic nutrients have been shown to produce no significant effects on metabolism (Thomas et al. 1989), shortened adaptation periods (Stefenson & Alexander 1995; Swindoll et al. 1988; Lewis et al. 1986), increase the total extent of mineralization (Efroymson & Alexander 1994), and in some cases decreases mineralization (Manilal & Alexander 1991). Specific forms of nitrogen (NH_4^+ vs. NO_3^-) have also been found to have varied effects on the metabolism of organic compounds (Aranha & Brown 1981; Swindoll et al. 1988). This difference could be attributed to differential availability of the two forms of nitrogen; NH_4^+ tends to adsorb to clay minerals and may be less available than NO_3^- (Schlessinger 1991). Nitrate is also unique because in addition to its use as a form of nitrogen for cellular maintenance, it may stimulate the metabolism of organic compounds when it is used as an alternative terminal electron acceptor (Leduc et al. 1992).

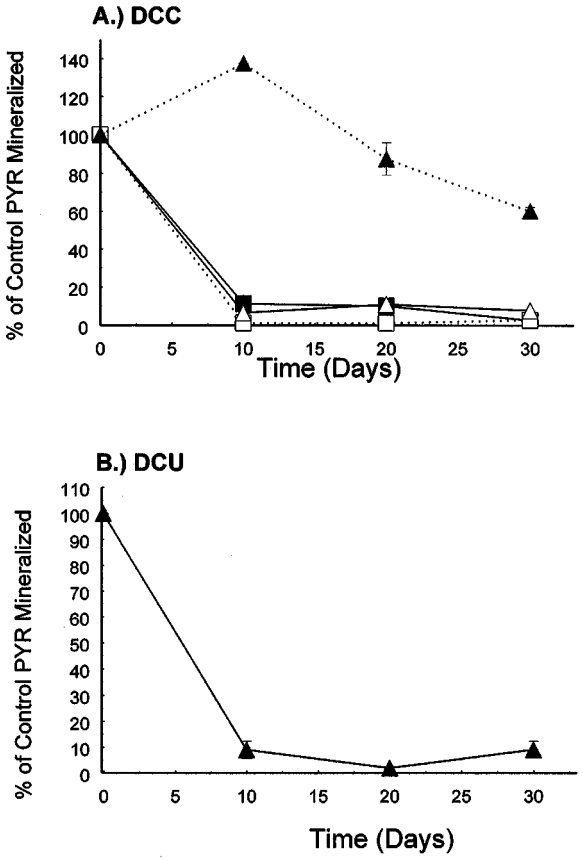

Figure 3. The effect of nitrogen (NH_4Cl or $NaNO_3$) on the mineralization of [^{14}C]-pyrene in A.)DCC and B.)DCU over time (days). Data is presented as the percent of [^{14}C]-pyrene mineralized compared to an unamended control. M9 Buffer with NH_4Cl(■), NH_4Cl(...■ ...) alone, M9 buffer with $NaNO_3$ (△) and NO_3 alone (...▲...)
Mean value (n = 3) and standard deviation.

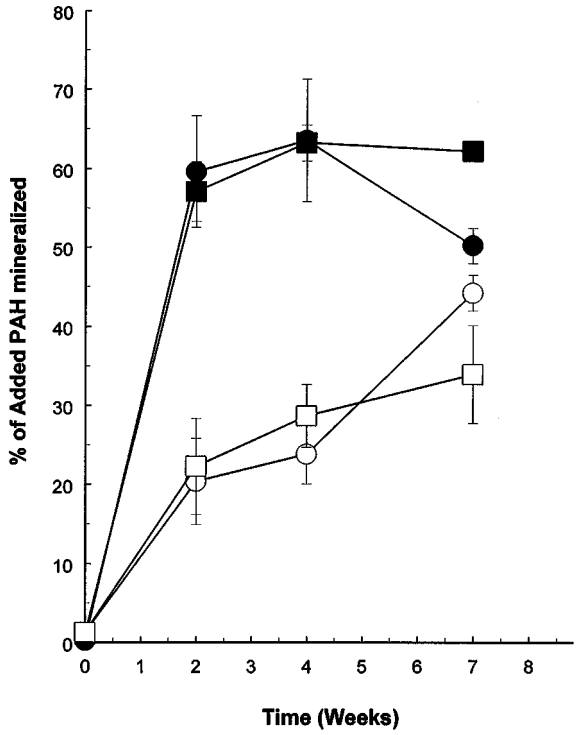

Figure 4. The influence of [^{12}C]-pyrene (0.12 mg·L^{-1}) on the mineralization of [^{14}C]-phenanthrene and [^{12}C]-phenanthrene (1.2 mg·L^{-1}) on the mineralization of [^{14}C]-pyrene in SWS over time (weeks). [^{14}C]-phenanthrene alone (■), [^{14}C]-phenanthrene with [^{12}C]-pyrene (□); [^{14}C]-pyrene alone (●), [^{14}C]-pyrene with [^{12}C]-phenanthrene (○).
Mean value (n = 3) and standard deviation.

The impact of non-inducer organic nutrients and other PAH on the mineralization of [^{14}C]-phenanthrene and [^{14}C]-pyrene

Organic compounds may be used as supplements to either support cometabolic transformations of PAH, which is thought to be important for the metabolism of PAH with three or more aromatic rings (Keck et al. 1989), or to generally stimulate microbial communities. Lower molecular weight PAH, PAH<three rings, have also been found to serve as cometabolic substrates for PAH metabolism (Bouchez et al. 1995). In these experiments, the effect of a widely used non-specific carbon source (nutrient broth; 100 mg·L^{-1}) and other [^{12}C]-PAH (either phenanthrene or pyrene) on the mineralization of [^{14}C]-phenanthrene and [^{14}C]-pyrene was monitored in several soils (SWS, SSC and BOZ).

Additions of nutrient broth (100 mg·L^{-1}), initially increased the mineralization of [^{14}C]-phenanthrene by approximately 20% in soil SWS (data not shown). After four weeks of exposure to nutrient broth, however, mineralization of [^{14}C]-phenanthrene had reached a plateau and then decreased by 10 to 25% after 7 weeks of incubation (46.97% [±1.25] of the added [^{14}C]-phenanthrene) compared to the non-nutrient broth amended controls (61.10% [±0.25] of the added [^{14}C]-phenanthrene). A similar pattern was seen in soil SSC, 35.25% [±2.25] of the added [^{14}C]-phenanthrene mineralized in the absence of nutrient broth compared to 28.02% [±1.85] of the added [^{14}C]-phenanthrene with nutrient broth. The inhibitory effect of nutrient broth (100 mg·L^{-1}) may have resulted from its preferential use as a carbon source over [^{14}C]-PAH. Because nutrient broth consistently decreased [^{14}C]-phenanthrene

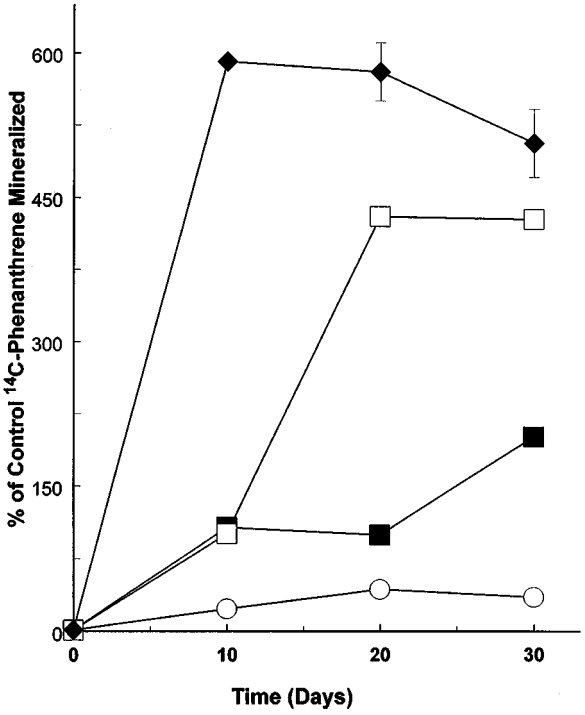

Figure 5. The influence of preexposure to multiple concentrations of [^{12}C]-phenanthrene for 10 days on the mineralization of [^{14}C]-phenanthrene in the BOZ soil over time (days). Data is presented as the percent of [^{14}C]-phenanthrene mineralized compared to an unamended control. [^{12}C]-Phenanthrene was added at: 3.33 mg·L^{-1} (○); 1.5 mg·L^{-1}(■); 0.80 mg·L^{-1}(□) and 0.58 mg·L^{-1} (◆). Mean value (n = 3) and standard deviation.

mineralization, it was not tested with the other soils or PAH.

When soil SWS was incubated with both [^{12}C]-pyrene (120 μg·L^{-1}) and [^{14}C]-phenanthrene (68 μg·L^{-1}) the extent of [^{14}C]-phenanthrene mineralization decreased by approximately 20% (Figure 4) and if the soil was exposed to [^{12}C]-phenanthrene (1200 μ·gL^{-1}) along with [^{14}C]-pyrene (20 μg·L^{-1}) the mineralization of [^{14}C]-pyrene also decreased, but by only 5% of the extent mineralized in the absence of the [^{12}C]-PAH. The decreased mineralization of [^{14}C]-PAH with [^{12}C]-PAH in soil SWS is thought to occur because the same degrading microorganisms are competing for both PAH substrates (Bouchez et al. 1995; Stringfellow & Aitken 1995). The larger decrease noted for [^{14}C]-phenanthrene may be attributed to a greater number of phenanthrene degraders found in the soils.

[^{12}C]-PAH were also added to soil BOZ, which has a limited ability to metabolize PAH and has few [^{14}C]-PAH degrading microorganisms (Carmichael & Pfaender 1997) to determine if preexposure of soil BOZ to [^{12}C]-PAH increased [^{14}C]-PAH metabolism. When BOZ was exposed to [^{12}C]-phenanthrene (3.3 mg·L^{-1}) for 10 days prior to the addition of the [^{14}C]-phenanthrene test substrate, subsequent mineralization of [^{14}C]-phenanthrene decreased by approximately 50% compared to an unexposed control (Figure 5). Preexposure at lower concentrations of phenanthrene (1.50 to 0.50 mg·L^{-1}), however, increased the extent of [^{14}C]-phenanthrene mineralization from 3% of the added [^{14}C]-phenanthrene with water alone up to 15% of the added [^{14}C]-phenanthrene with 0.58 mg·L^{-1}[^{12}C]-phenanthrene. In soil BOZ, exposure of the microbial communities to phenanthrene probably allowed for the development of a community of phenanthrene degrading microorganisms. The concentration effect of [^{12}C]-phenanthrene on the mineralization of [^{14}C]-phenanthrene in soil BOZ may be partially attributed to isotope dilution of [^{14}C]-phenanthrene with increased concentrations of [^{12}C]-phenanthrene, since the increase in mineralization observed is proportional to the amount of [^{12}C]-dilution of [^{14}C]-substrate. No effect was seen, however, on the mineralization of [^{14}C]-pyrene when the soils were preexposed to [^{12}C]-pyrene (data not shown), a compound that is an order of magnitude less soluble than phenanthrene.

The influence of surfactants on the mineralization of [^{14}C]-phenanthrene in soil SWS

In the environment, PAH are often associated with soil organic carbon in a form that may be largely unavailable to microorganisms (Mihelcec & Luthy 1993; Wodzynski & Coyle 1974). One proposed approach for increasing the metabolism of PAH has been to use surfactants to increase the available concentration. We tested the impact of a non-ionic surfactant, Triton X-100, and a compound with both surfactant and nutrient properties, Inipol EAP-22, on the mineralization of [^{14}C]-phenanthrene in soil SWS, the soil with the highest concentration of native PAH. Triton X-100 was added at four concentrations (1%, 0.75%, 0.50% and 0.25% v/v Triton X-100/water solution) above its critical micelle concentration (CMC) in a liquid phase system (≈.19 mg·mL^{-1}; Roch & Alexander 1995). When surfactants are added above the CMC, surfactant micelles form that can trap desorbed compounds. The CMC effect may be less important in soils, however, since the adsorption of surfactants to soil particles increases the concentration of surfactant necessary to

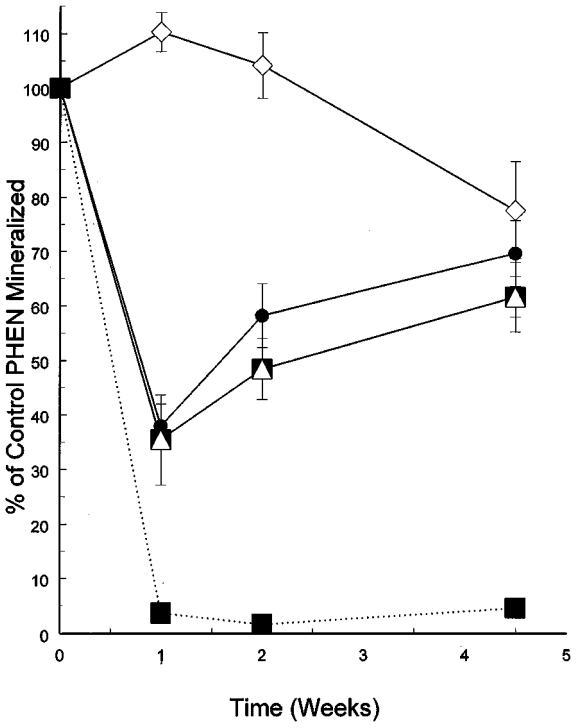

Figure 6. The effect of Triton X-100 (1, 0.75, 0.5, 0.25% v/v) and Inipol EAP-22 (1.66 mg·L^{-1}) on the mineralization of [^{14}C]-phenanthrene in SWS over time (weeks). Data is presented as the percent of [^{14}C]-phenanthrene mineralized compared to an unamended control. Triton X-100 (v/v): 1% (■), 0.75% (●), 0.50% (△), 0.25% (◇) and Inipol EAP-122 (...■...).
Mean value (n = 3) and standard deviation.

reach the CMC (Tsomides et al. 1995). Addition of Triton X-100 at 1%, 0.75% and 0.50% (v/v) decreased the mineralization of [^{14}C]-phenanthrene by 30 to 40% after 4 weeks of incubation (Figure 6) while additions of a 0.25% (v/v) Triton X-100 solution did not effect mineralization of [^{14}C]-phenanthrene until after eight weeks of incubation. Additions of Inipol EAP-22 (1.66 mL·L^{-1}), an oleophilic fertilizer, decreased mineralization of [^{14}C]-phenanthrene (Figure 6) by greater than 90% of the amount mineralized in the absence of supplements. Because these supplements decreased mineralization, they were not tested with [^{14}C]-pyrene and in the other soils.

Decreased mineralization of [^{14}C]-phenanthrene in soil SWS with Triton X-100 could be due to a multitude of causes, the sequestering of desorbed [^{12}C and ^{14}C]-PAH into micelles where they are thought to be unavailable to microorganisms (Laha & Luthy 1991); preferential use of Triton X-100 as a carbon source instead of the PAH (Mueller et al. 1990); toxic effects of surfactants (Robinson & Tanford 1975; Kwee et al. 1986), or that the surfactant increased water phase concentrations of [^{12}C]-PAH making more total PAH available for metabolism and therefore diluting the added radiolabeled materials. Although the CMC effect is most often cited for decreasing the metabolism of hydrophobic organic compounds (HOC), there are no consistent trends in the effect of Triton X-100 above or below the CMC (Roche & Alexander 1995; Laha & Luthy 1995; Liu et al. 1995; Tsomides et al. 1995). In addition, there are indications that decreased metabolism of HOC above the CMC is not due entirely to entrapment of the HOC in micelles but possibly due to toxic effects of the surfactant on surface proteins (Roche & Alexander 1995; Liu et al. 1995). Toxicity does not seem to be a problem in soil SWS, however, because Triton X-100 did not influence the mineralization of [^{14}C]-amino acids (data not shown) and it also stimulated the growth of total heterotrophic microorganisms (Table 2).

Conclusions

Many of the supplements examined in this work (pathway intermediates, inorganic and inorganic nutrients and surfactants) decreased or had little effect on the mineralization of [^{14}C]-phenanthrene and/or [^{14}C]-pyrene in the five soils tested. Most supplements increased populations of total heterotrophic microorganisms but they did not appear to stimulate populations of PAH degrading microorganisms. In most cases, supplement addition appeared to stimulate a segment of the microbial community that does not metabolize the PAHs. In addition to not stimulating the metabolism of PAH, the application of supplements may be disadvantageous because of regulatory concerns for addition of substances of unknown environmental impact, cost of supplements and/or concerns for uniform application of a supplement for in-situ remediation strategies. This research suggests that supplements should be chosen and applied with care if they are to be used as a part of a successful remediation strategy.

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