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Effects of surfactant addition on the biomineralization and microbial toxicity of phenanthrene

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Abstract

Surfactants are known to increase the apparent aqueous solubility of polycyclic aromatic hydrocarbons and may thereby enhance their bioavailability. In this study the effects of four surfactants on the mineralization of phenanthrene by *Pseudomonas aeruginosa* in liquid culture and in soil-water suspensions was studied in batch reactors over a 15-week study period. In the absence of surfactant, liquid cultures mineralized approximately 50% of the phenanthrene added within seven weeks following a one-week lag period and an initial mineralization rate of 0.04 mg/d. Mineralization in soil-water suspensions proceeded without any measurable lag period. The initial mineralization rate was lower (0.006 mg/d), but mineralization continued to >70% over the fifteen week period. In general, the addition of very low concentrations of surfactant ($\leq$0.001%) to liquid cultures did not impact mineralization significantly. At higher surfactant concentrations ($\geq$CMC) all surfactants were seen to be inhibitory. In soil-water systems, the rate of phenanthrene mineralization was decreased even at surfactant doses that did not produce significant solubilization. In summary, none of the surfactants enhanced the mineralization of phenanthrene by *P. aeruginosa* in liquid culture or in soil-water suspensions. In order to rank surfactant toxicity, microbial toxicity tests were performed measuring the light output of bioluminescent bacteria as affected by the presence of surfactants. Additional toxicity testing indicated that the presence of solubilized phenanthrene increased the toxicity of the surfactant by a 100-fold suggesting that the toxicity of solubilized substrate needs also to be considered in the application of surfactant-amended remediation.

Background

Hydrophobic organic compounds (HOCs) constitute a particularly vexatious class of environmental contaminants. When HOCs occur in subsurface environments, their hydrophobicity and limited aqueous solubilities result in either excessive sorption onto soil or existence as nonaqueous phase liquids (NAPLs). This renders HOCs less amenable to treatment by conventional remediation technologies (Lee et al. 1988). Polycyclic aromatic hydrocarbons (PAHs) are a class of HOCs that have received much attention in recent years. They consist of two or more fused benzene rings in linear, angular, or cluster arrangements, and include compounds such as phenanthrene, fluoranthene, acenaphthene and fluorene. PAHs are listed as a class of priority pollutants with several identified as toxic, carcinogenic or mutagenic.

Surfactants possess both polar and nonpolar regions on the same molecule, and at solution strength greater than the critical micelle concentration (CMC) surfactant molecules aggregate to form micelles (Cross 1986). Micelles have a nonpolar hydrocarbon-like core surrounded by the polar entities of the surfactant molecules. Micelles are highly dynamic colloids with characteristic sizes in the low nanometer range (Mukerjee and Mysels 1971). HOCs may be solubilized in water by incorporation into the hydrophobic core of surfactant micelles and desorbed from soil or dissolved from NAPL through the re-

duction of interfacial tension produced by surfactant action (Rosen 1989; Lee et al. 1988; Mueller et al. 1989). For micellar solubilization of HOCs, the surfactant's concentration must be greater than its CMC. Solubilization is a function of the surfactant nature, solute, temperature, ionic strength, concentrations, surfactant-soil interactions, and time of contact between the contaminant and soil (Gadelle et al. 1995; Guha and Jaffé 1996a).

The addition of surfactants has been suggested as a mechanism to enhance the bioavailability of HOCs (Lee et al. 1988; Mueller et al. 1989). Research into the applications of surfactants for HOC bioremediation have yielded inconclusive results, some of the more recent of which are summarized below. Margesin and Schinner (1999) investigated the effects of varying concentrations of the anionic surfactant sodium dodecyl sulfate (SDS) on the biodegradation of diesel oil by cold-adapted microorganisms in liquid culture and soil-water systems. Low SDS concentrations ($<$100 mg/L) significantly enhanced oil biodegradation in liquid culture, while higher surfactant concentrations (500 to 1000 mg/L) inhibited hydrocarbon biodegradation. All the SDS concentrations used were below CMC. Oil biodegradation in soil was inhibited at all SDS concentrations tested. Mihelcic et al. (1995) observed that a sub-CMC concentration of 0.0012% (v/v) or $\sim$12 mg/L of the nonionic ethoxylate surfactant Triton X-100 neither enhanced nor inhibited the biodegradation rate of naphthalene by a *Pseudomonas fluorescens* species, whereas a concentration of 1.2% or 12,000 mg/L enhanced biodegradation.

In another study, Willumsen and coworkers (1998) examined two *Mycobacterium* and two *Sphingomonas* strains for their sensitivity toward nonionic surfactants. The effects of supra-CMC Triton X- 100 and Tween 80 on the mineralization of ^{14}C-glucose and ^{14}C-fluoranthene was measured in shaker flasks. Tween 80 had no toxic effect on any of the tested strains; it inhibited fluoranthene mineralization by the hydrophobic *Mycobacterium* spp. slightly, but more than doubled that by the less hydrophobic *Sphingomonas* strains. Triton X-100 inhibited fluoranthene mineralization by all strains. Triton X-100 also inhibited glucose mineralization by the *Sphingomonas* strains, indicating non- specific toxic effects caused by micelle-to-cell interactions. These strains, however, recovered from initial Triton X-100 toxicity within 500 h of exposure. The ratio of surfactant concentration to initial cell density was seen to critically affect the bacterial response to surfactants. Overall these results indicate that fluoranthene solubilized in surfactant micelles is only partially available for mineralization by the bacteria tested (Willumsen et al. 1998). In contrasting the effects of the same surfactant Triton X-100 on the biotransformation of naphthalene and phenanthrene by two dioxygenase-expressing bacteria *Pseudomonas* sp. strain 9816/11 and *Sphingomonas yanoikuyae* B8/36, Allen and coworkers (1999) report that the surfactant increased the oxidation rate of PAHs with strain 9816/11. However, the surfactant inhibited the biotransformation of both naphthalene and phenanthrene with strain B8/36 under the same conditions (Allen et al. 1999). The observation that a nonionic surfactant could have such contrasting effects on PAH oxidation by different bacteria may explain why previous research on the application of surfactants to PAH bioremediation has yielded inconclusive results.

Guha and Jaffé (1996a) report that the rate of degradation of phenanthrene in the presence of surfactant was dependent on the type of surfactant used. Their studies used four different surfactants and concluded that phenanthrene present in the micellar phase is not directly bioavailable. In fact, for systems containing a fixed amount of phenanthrene, the amount of bioavailable micellar-phase phenanthrene decreased as the micelle concentration increased (Guha and Jaffé 1996b). Cell surface hydrophobicity as well as other microbial membrane processes may also play a role in the transfer of phenanthrene from micelles to the cells. Interferences with the activities of enzymes or other membrane proteins involved in phenanthrene degradation too may impact biodegradation. The role of bacterial adhesion to soil contaminants was investigated by Stelmack and coworkers (1999). They report that surfactants at 0.5 $\times$ CMC concentration inhibited the adhesion of two bacterial strains (*Pseudomonas* and *Mycobacterium*) to NAPL, although the surfactant was not toxic to the bacteria. In addition, these researchers observed that low concentrations of surfactant also inhibited the growth of both species on anthracene indicating decreased uptake of solid carbon source (Stelmack et al. 1999).

Results from a study using wastewater from a contaminated site showed that biodegradation was significantly enhanced by amendment with surfactants and nutrients (Zhang et al. 1998). The enhancement, based on TOC reduction, was almost 50% higher for wastewater amended with surfactant compared to wastewater alone. Similarly, the addition of the organic solvent hydroxypropyl-beta-cyclodextrin (HPCD) was seen to significantly increase the ap-

parent solubility of phenanthrene and enhance its utilization rate by five times, suggesting that HPCD substantially increases the HOC bioavailability (Wang et al. 1998). In a more comprehensive examination of surfactant-amended biodegradation of higher molecular weight PAHs, Boonchan et al. (1998) report on the effects of a range of synthetic surfactants on the utilization of pyrene, fluoranthene, and benzo[a]pyrene by the PAH-degrader *Stenotrophomonas maltophilia* VUN 10,010. Anionic and cationic surfactants were seen to be highly toxic to this strain, while the Tween series of surfactants were used as growth substrate. Five nonionic surfactants (including alkylethoxylate and alkylphenol ethoxylates) were less toxic and not utilized by strain VUN 10,010. Four of these surfactants enhanced the rate of pyrene degradation whereas Igepal CA-630 inhibited pyrene degradation and microbial growth. The addition of Tergitol NP-10 to VUN 10,010 cultures substantially improved degradation of PAHs individually as well as in mixtures (Boonchan et al. 1998). In contrast, an earlier study reports Tergitol NP-10 to have either no effect or even an inhibitory effect at higher concentrations on the mineralization of phenanthrene in soil-water systems (Laha and Luthy 1991).

The foregoing discussion on the research performed to date on surfactant-amended HOC bioremediation suggests that the inconclusive results observed may be attributed to specific effects of the HOC-degrading microorganism, the nature of the HOC substrate, the type of surfactant, and HOC-surfactant-microbe interactions. The presence or absence of a sorbent such as soil also plays a significant role in determining the efficacy of surfactant addition, i.e., whether desorption or dissolution kinetics control biodegradation. Although there has been a significant improvement in understanding these processes, reliable prediction of surfactant-enhanced biodegradation is still in its infancy. This study examines the biomineralization and toxic effects of phenanthrene in aqueous and soil-water systems as affected by the presence of four surfactants. The primary goals of the present work are to assess the effects of using a high organic carbon soil that promotes increased phenanthrene sorption, to examine the effects of a wide range of surfactant types on the phenanthrene-degrading activity of a selected micro-organism, and to explore the microbial toxicity of individual surfactants and surfactant- solubilized phenanthrene utilizing a commonly-used toxicity assay. The phenanthrene-degrading microorganisms were isolated from contaminated sediments; the microorganisms used for the toxicity tests are proprietary bioluminescent bacteria (Microbics Corp., CA).

Materials and methods

This study proposed to examine the effects of using a wide range of surfactant types, specifically, nonionic (polyethoxylate), cationic, and anionic surfactants. Representative surfactants were selected to fit these three categories. Tween 20 or polyoxyethylene sorbitan monolaurate was selected as a representative nonionic since it belongs to the sorbitan polyethoxylate family of surfactants known to be generally nontoxic and to have applications in the food processing and pharmaceutical industries. Sodium dodecyl sulfonate (SDS) was selected as a representative anionic surfactant owing to its widespread use in earlier studies. It was preferred over sodium dodecyl benzene sulfonate that has also been used in several previous studies, because it lacks the generally problematic benzene ring in its structure. The most commonly used cationic surfactants are the ammonium bromides, and tetradecyl trimethyl ammonium bromide (TTAB) was selected as representative of this group of surfactants. A commercial emulsifier Citrikleen had been used in our laboratory to wash glassware stained with heavy oils and coal tar. Due to its remarkable effectiveness at removing these NAPLs from glassware, Citrikleen was included in the list of surfactants studied. The selected surfactants and their properties are presented in Table 1. The model HOC used in this study is the three-ring PAH phenanthrene ($C_{14}H_{10}$, molecular weight 178). Its aqueous solubility is 1.29 mg/L at 25 °C and its log octanol-water partition coefficient (log K_{ow}) is reported as 4.54 (Dzombak and Luthy 1984).

Soil samples

The soil used in batch experiments was collected as grab samples from the top 10 to 15 cm of several locations near the Taylor Slough channel (approximate coordinates: N 25° 30′, W 80° 28′) of the Everglades National Park, FL. Much of the earlier work on surfactant-amended remediation used soil with low to moderate levels of organic carbon (generally around 1% or less). The Everglades soil has a high organic carbon fraction (>6%) and is expected to provide greater sorption of phenanthrene onto soil. We were interested in examining these effects on the subsequent

Table 1. Properties of selected surfactants in aqueous systems

Surfactant	Nature	Molecular Weight	Reported Aqueous CMC[1]	Aqueous CMC from surface tension measurements[2]		Surfactant dose for onset of solubilization[3]		SR[4]	MSR[4]	Log K_m[5]
			%	%	mg/L	%	mg/L			
Polyoxyethylene sorbitan monolaurate (Tween 20)	nonionic	1225	0.01	0.01	100	0.01	100	0.014	0.105	6.2
Tetradecyl trimethyl ammonium bromide (TTAB)	cationic	336	0.15	0.1	1,000	0.1	1,000	0.011	0.021	5.8
Sodium dodecyl sulfate (SDS)	anionic	288	0.24	0.16	1,600	0.17	1,700	0.008	0.013	5.2
Citrikleen	commercial emulsifier	n/a	n/a	0.2	2,000	0.05	500	0.004	–	–

[1]Manufacturer-reported CMC values, specific gravities of liquid surfactants assumed unity.
[2]CMC values determined in our laboratory using surface tension measurements (Bramwell 1997).
[3]Solubilization tests performed in our laboratory (Bramwell 1997).
[4]SR is the solubilization ratio on a mass basis from solubilization data, MSR is the molar solubilization ratio on a molar basis (Bramwell 1997).
[5]Micelle-water partition coefficients (K_m) were calculated using MSR values as per Edwards et al. 1991 (Bramwell 1997).

biodegradation of phenanthrene. Taylor Slough being within the boundaries of the National Park, the soil may be assumed relatively uncontaminated. Grab samples were mixed to yield a composite soil sample that was air-dried and passed through U.S. Standard No. 10 (size 2 mm) sieve prior to use. The pH of soil-distilled water suspension was 8.2, while the pH of soil-tap water was between 7.2 and 7.9. The moisture content of the freshly collected soil was gravimetrically determined to be approximately 63%; however this value varies significantly with the seasons. The soil organic carbon content ranged from 6 to 7% as determined by the Walkley-Black procedure (ASA 1965).

Chemicals

Radiolabeled (^{14}C) phenanthrene was purchased from Sigma Chemical Co., St. Louis, MO. Its specific activity was 8.3 mCi/mmol and the radiochemical purity exceeded 98%. Non-labeled phenanthrene was obtained from Aldrich Chemical Co., WI (purity > 96%) and was used as received. The inorganic reagents used to prepare mineral salts media were analytical grade. The surfactants Tween 20, SDS, and TTAB were obtained from Sigma Chemical Co., St. Louis, MO and were analytical grade with purities exceeding 95% for TTAB and SDS. The commercial emulsifier Citrikleen was obtained from Penetone Corp., Tenefly, NJ. The surfactants were used as received without further purification.

At room temperature (20 ± 1 °C) Tween 20 and Citrikleen exist as liquids with specific gravities of 1.1 and 0.95 respectively. Their aqueous concentrations are reported on a volume/volume basis (v/v %) and are converted to mg/L neglecting corrections for density. SDS and TTAB exist as solid substances with their concentrations reported either on a percent weight-to-volume basis (w/v %) or mg/L. Surfactant solutions used in all tests were prepared in biochemical oxygen demand (BOD) dilution water (APHA 1989). The pH of this solution was 7. BOD dilution water is a mineral salts medium containing the following chemicals: KH_2PO_4, K_2HPO_4, $Na_2HPO_4.7H_2O$, NH_4Cl, $MgSO_4.7H_2O$, $CaCl_2$ and $FeCl_3.6H_2O$. All experiments were conducted at room temperature using sterilized BOD dilution water.

Characterization of PAH-degrading inoculum

PAH-degrading microorganisms were isolated via enrichment culture from contaminated Hudson River sediments. The initial liquid growth medium contained 200 mg/L phenanthrene as the sole carbon and energy source. The liquid enrichments were streaked on phenanthrene-spread plates to facilitate isolation of individual colonies. Individual colonies were characterized and transferred to successive growth media

for inoculation purposes. A staining procedure was used to identify the selected isolate as gram- negative bacteria. BIOLOG (Biolog Inc., Hayward, CA) plates were used to further characterize the isolate as *Pseudomonas aeruginosa*.

In order to promote rapid bacterial growth for inoculation purposes, a little glucose (∼100 mg/L) was added to subsequent liquid growth media. After several weeks of incubation, enumeration of the inoculum was performed by plating 0.2-mL aliquots from appropriate dilutions of the PAH-degrading culture onto phenanthrene-spread plates. The plates were prepared by spreading 0.2-mL portions of a 4 mg/mL phenanthrene-acetone mixture onto agar medium. The acetone was allowed to evaporate overnight before adding the diluted phenanthrene-degrading inoculum that was spread evenly across the agar. Each plate was incubated at 30 °C. Following incubation the plates were checked for clear circular zones indicating growth of phenanthrene-degrading bacterial colonies. These zones were counted and yielded an average of 1.2×10^{10} colony forming units (CFU) per mL of inoculum.

Mineralization tests

Phenanthrene biodegradation tests were performed in liquid culture and in soil-water suspensions using 125-mL Erlenmeyer flasks. Aqueous systems received 50 mL BOD dilution water and 1 mg phenanthrene with ∼110,000 dpm ^{14}C-phenanthrene. Soil-water suspensions received an additional 5 g of soil. The soil-to-water ratio of 1 g : 10 mL used in each soil-water mineralization test was the same used for previous solubilization tests (Bramwell 1997). Each reactor was spiked with a mixture of labeled and unlabeled phenanthrene dissolved in methanol. Methanol was allowed to evaporate overnight, before adding the various surfactant solutions at concentrations below, above and at CMC. Soil used in the mineralization tests was not sterilized prior to inoculation in order to preserve its natural quality. Some surfactants solutions including Citrikleen and SDS were seen to form precipitates in aqueous and soil-water systems hence requiring greater surfactant dosages for solubilization and suggesting possible clogging problems in proposed remediation applications.

Reactors were inoculated with 2 mL of the phenanthrene- degrading culture, tightly sealed and fitted with a center well containing fluted filter paper saturated with 0.2 mL of a 2 M NaOH solution. The caustic solution served as a trap for $^{14}CO_2$ produced during mineralization of phenanthrene. For each experimental run, biomineralization tests were performed in duplicate. However, most experiments were repeated at least twice ensuring a minimum of four readings for each data point. Reported results represent an average of the values obtained; variations between readings were within 5% of the reported means. [^{14}C]-Phenanthrene losses through volatilization or other abiotic processes were assessed through the use of abiotic controls prepared by adding 200 mg/L mercuric chloride ($HgCl_2$) as a biocide. The evolution of $^{14}CO_2$ was monitored to examine the mineralization of phenanthrene. At each sampling, the saturated filter paper was removed and placed into a scintillation vial containing 10 mL Ecoscint liquid scintillation cocktail (National Diagnostics, Atlanta, GA). Prior to analyzing samples for ^{14}C they were stored overnight in the dark to minimize chemiluminescence. A Beckman 3801 liquid scintillation counter (LSC) was programmed to use the H# quench-monitoring technique with automatic quench compensation (AQC) and random coincidence monitoring (RCM) in the analysis of samples. LSC data were reported as disintegrations per minute (dpm) which when related to the initial dpm present in each reactor yields percent mineralization.

After sampling a reactor, the filter paper in the center well was immediately replaced with another fluted/folded filter paper saturated with fresh 2 M NaOH solution. To obtain phenanthrene mineralization as a function of time, the ^{14}C count obtained for successive sampling events was summed to yield the cumulative percent mineralization with respect to time. Acidification of reactor contents at the conclusion of the experiment indicated that $^{14}CO_2$ release after acidification was not significant, hence it was not necessary to account for [^{14}C]-carbonate in samples.

Toxicity tests

The toxicity of different aqueous surfactant solutions was evaluated using a Microtox Toxicity Model 500 Analyzer in basic test mode (Microbics/Azur Corporation, Carlsbad, CA). The test procedure exposed bioluminescent organisms to different concentrations of selected surfactants with or without phenanthrene, and measured the effects of these substances on the light output of the organisms. The Microbics toxicity bioassay uses freeze-dried luminescent bacteria that were rehydrated with reconstitution solution to

provide a ready-to-use suspension of test organisms. The Microtox system measures the light output of the luminescent bacteria after they have been challenged by a possibly toxic sample and compares that light output to the light output of a control (reagent blank) containing no toxic substances. The difference in light output between the sample and the control is attributed to the effect of the sample on the microorganisms. The results are reported as EC_{50} values or the effective concentration (given in mg/L) of the toxic substance at which the light output was decreased by 50% (Microbics Corporation 1992). Light output measurements for samples were made 5 minutes after introducing sample to luminescent bacteria.

Mass balance

Following completion of the mineralization tests, the contents of each flask were analyzed for residual ^{14}C to ascertain that mass balance constraints were satisfied. For extraction purposes, 20 mL of hexane were added to each reactor, which was then sealed and shaken for 30 minutes. Samples of both the hexane and aqueous phases were analyzed for ^{14}C in the LSC. The mass balance performed on the batch reactors for aqueous systems showed up to 100% recovery. Systems containing soil proved to be more difficult to extract: only about 50% of the added phenanthrene was accounted for in soil-water systems. Because recovery efficiencies were excellent for aqueous systems, it is not anticipated that poor mass balances for soil-water systems result from unknown ^{14}C losses, rather they are attributable to inadequate solvent extraction procedures, i.e., the inability to effectively remove residual phenanthrene from soil. The contents of reactors used in earlier abiotic solubilization tests were also analyzed for residual phenanthrene before disposal in order to examine mass balance (Bramwell 1997). Again, the mass balance performed for systems without soil accounted for almost 100% of phenanthrene added (using both chromatographic and radiolabeled techniques), whereas recovery for systems containing soil was between 40 and 73%, further indicating that the current discrepancy in biotic systems may also be accounted by the poor extraction efficiency from soil.

Results and discussion

Phenanthrene mineralization in liquid cultures

An earlier examination of surfactant solubilization of phenanthrene indicated that the apparent increased aqueous concentration of phenanthrene was a micellar phenomenon, occurring at surfactant doses above the CMC. Figure 1 indicates the solubilization of phenanthrene by Tween 20 in aqueous systems receiving 1 mg and 10 mg phenanthrene respectively. Percent solubilization observed for 1 mg phenanthrene in 100 mL solution was much more significant at the surfactant doses tested, with systems receiving 800 mg/L (or 0.08%) Tween 20 showing nearly complete phenanthrene solubilization, in contrast to ~10% solubilization in systems containing 10 mg phenanthrene. (Both apparent phenanthrene solubilities correspond to approximately 10 mg/L, but are easier to monitor via radiolabeled techniques using the lower phenanthrene dose.) Therefore it was preferred to use 1 mg phenanthrene in each reactor for subsequent experiments. The CMC of Tween 20 is reported as 100 mg/L or ~0.01%; this CMC was confirmed in laboratory measurements of surface tension (Bramwell 1997).

At Tween 20 concentrations below CMC phenanthrene solubilization was observed to be minimal, whereas phenanthrene solubilization at supra-CMC Tween 20 increased linearly indicating that surfactant solubilization is a micellar phenomenon. The slope of the linear part of the solubilization curve determines the molar solubilization ratio (MSR) on a molar basis or the solubilization ratio (SR) on a mass basis. The micellar phase/aqueous phase partition coefficient (K_m), representing the mole fraction of HOC in the micellar phase to its mole fraction in the aqueous phase, may be calculated using MSR values (Edwards et al. 1991). Table 1 presents reported CMC values for the selected surfactants, the CMC values determined in our laboratory from surface tension measurements, and the surfactant doses required for the onset of phenanthrene solubilization (determined from laboratory solubilization tests reported in Bramwell 1997). Table 1 also lists MSR, SR, and log K_m values for the selected surfactants: these values provide indication of the solubilizing capacity of surfactants. From the data presented in Table 1 it appears that Tween 20 is most effective at solubilizing phenanthrene since it demonstrates the lowest CMC, and the maximum SR and K_m values among the selected surfactants.

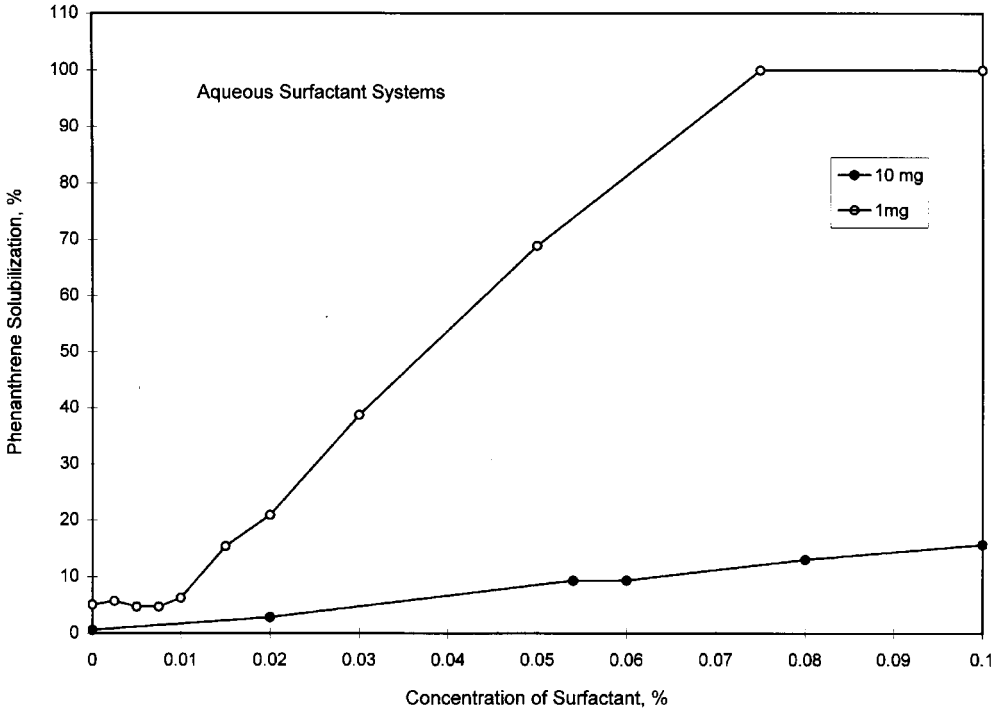

Figure 1. Solubilization of 1 mg and 10 mg phenanthrene in Tween 20.

Biological mineralization experiments were designed considering the micellar phase of each surfactant. Mineralization of phenanthrene was monitored by measuring $^{14}CO_2$ produced. The data is presented in graphical form indicating cumulative mineralization as a function of time. Figure 2 shows the mineralization of [^{14}C]-phenanthrene in both aqueous and soil-aqueous systems in the absence of surfactant; results from abiotic controls are also indicated. There was no $^{14}CO_2$ evolution measured for the abiotic controls during the fifteen week study period indicating negligible volatilization losses of phenanthrene and confirming that abiotic losses of phenanthrene are probably insignificant. The aqueous system was able to mineralize ~50% of the 1 mg phenanthrene added, while almost 70% phenanthrene was mineralized in the presence of soil. In all aqueous systems a lag period of about a week was observed, followed by a period of fairly rapid mineralization (maximum mineralization rate measured was ~0.04 mg/d phenanthrene). In contrast, soil-aqueous systems showed no significant lag period before mineralization commenced, but the initial mineralization rate (estimated at 0.006 mg/d) was lower.

The microbial mineralization of phenanthrene in aqueous systems containing the surfactants Tween 20 and Citrikleen are shown in Figures 3 and 4, respectively. All experimental systems were tested with surfactant solutions above, below and at CMC. The sub-CMC concentrations selected for Tween 20 are 1 mg/L (or 0.0001 %) and 10 mg/L (or 0.001%), representing 0.01 × CMC and 0.1 × CMC, respectively. At these concentrations, up to 50% phenanthrene mineralization was achieved, identical to liquid cultures with no surfactant present. For liquid cultures receiving Tween 20 at CMC (100 mg/L or 0.01%), the total phenanthrene mineralization achieved was reduced to 25%. From the solubility enhancement data obtained earlier (Figure 1) it is evident that less than 10% of the added phenanthrene was solubilized at such surfactant concentrations, yielding aqueous pseudo-phase phenanthrene concentrations of <1 mg/L. Excess phenanthrene, representing 90% of the added PAH, was present as a solid-phase. When the concentration of Tween 20 was increased to 10 × CMC (i.e., 1000 mg/L or 0.1%), no $^{14}CO_2$ evolution was observed. These results suggest that low concentrations of Tween 20 have no effect on the degradation of phenanthrene by the test microorganisms, whereas higher concentrations (≥CMC) appear inhibitory. At 1000 mg/L (10 × CMC) Tween 20 there was no detectable production of $^{14}CO_2$ even though

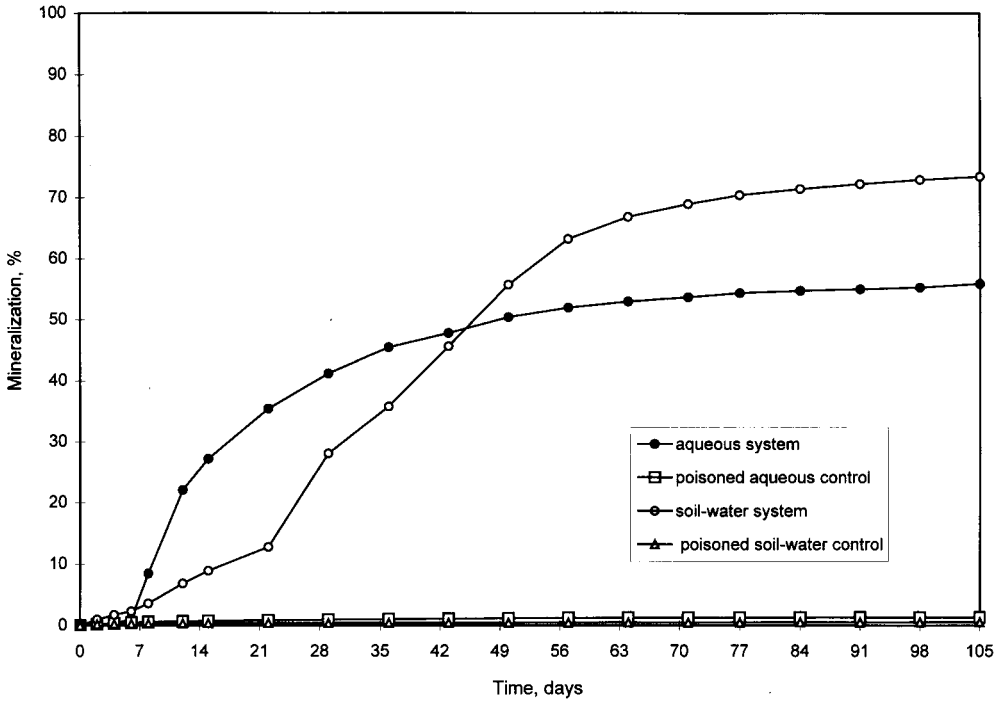

Figure 2. Microbial mineralization of phenanthrene in the absence of surfactant.

the aqueous pseudo-phase concentration of phenanthrene was measured at about 10 mg/L, i.e., ten time its aqueous solubility.

Citrikleen is a commercial emulsifier derived from citrus and has been identified as a biodegradable product and therefore a suitable candidate for site remediation applications. The CMC of Citrikleen in aqueous systems was determined to be ~2,000 mg/L or 0.2% (Bramwell 1997). Several Citrikleen concentrations were tested for their effect on the microbial degradation of phenanthrene. Figure 4 indicates that phenanthrene mineralization decreased as the Citrikleen concentration increased, as in the case of Tween 20. Phenanthrene mineralization of 50% was achieved for aqueous systems without surfactant as well as systems receiving 1 mg/L or 0.0001% (0.0005 × CMC) and 10 mg/L or 0.001% (0.005 × CMC) Citrikleen. A 10% reduction in mineralization was observed in systems containing 250 mg/L or 0.025% (0.125 × CMC) Citrikleen, with phenanthrene concentration estimated at ~2 mg/L and 90% of phenanthrene present as undissolved solid phase. No significant amount of phenanthrene was mineralized at a Citrikleen concentration of 5000 mg/L or 0.5% (2.5 × CMC) even though the phenanthrene solubilized was >14 mg/L (Bramwell 1997).

Results obtained for phenanthrene mineralization in the presence of anionic surfactant SDS are presented elsewhere (Bramwell 1997). At low SDS concentrations of 1 and 10 mg/L (corresponding to 0.0007 × CMC and 0.007 × CMC, respectively), phenanthrene mineralization was essentially similar to that obtained in the absence of surfactant. The lag period appeared to increase to two weeks for 10 mg/L SDS, but the phenanthrene mineralization measured in ten weeks was approximately 50% for both liquid cultures. The CMC of SDS is ~1600 mg/L or 0.16%, and at SDS concentration close to and above CMC microbial mineralization of phenanthrene was completely inhibited (Bramwell 1997). Below CMC, phenanthrene concentrations were estimated between 0.4 and 0.5 mg/L, at CMC the aqueous pseudo-phase phenanthrene concentration was 0.7 mg/L, while above CMC as much as 10 mg/L phenanthrene is solubilized (Bramwell 1997). This again indicates that increases in the micellar phenanthrene concentration need not translate into increased bioavailability of HOC.

The cationic surfactant TTAB is used in herbicides and germicides and owing to its known biocidal properties may not generally be appropriate for bioenhancement purposes. However, TTAB was also investigated in order to compare the results obtained

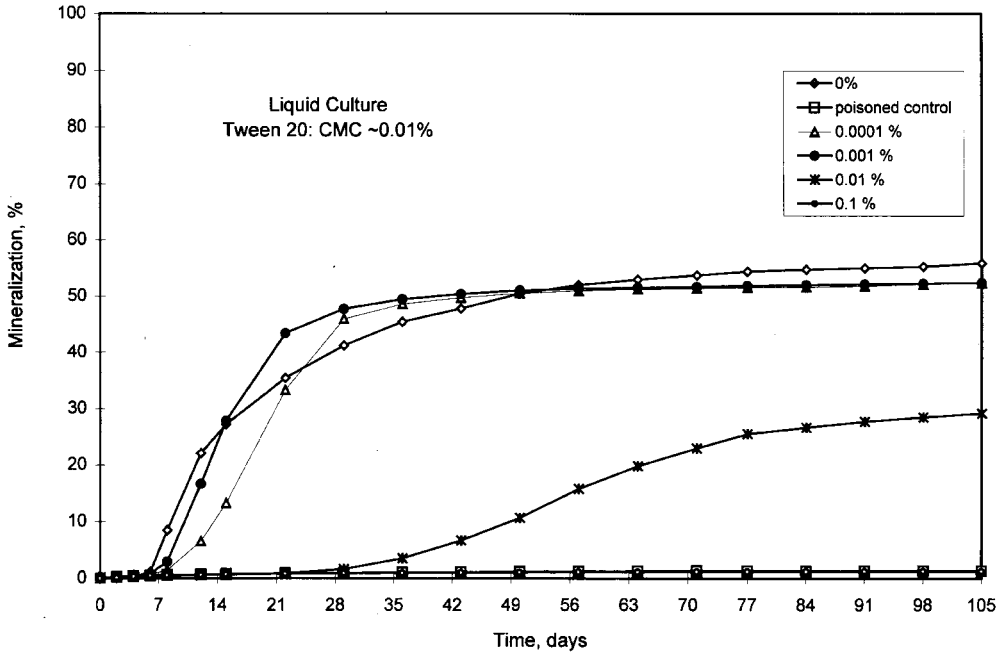

Figure 3. Mineralization of phenanthrene in aqueous systems receiving Tween 20.

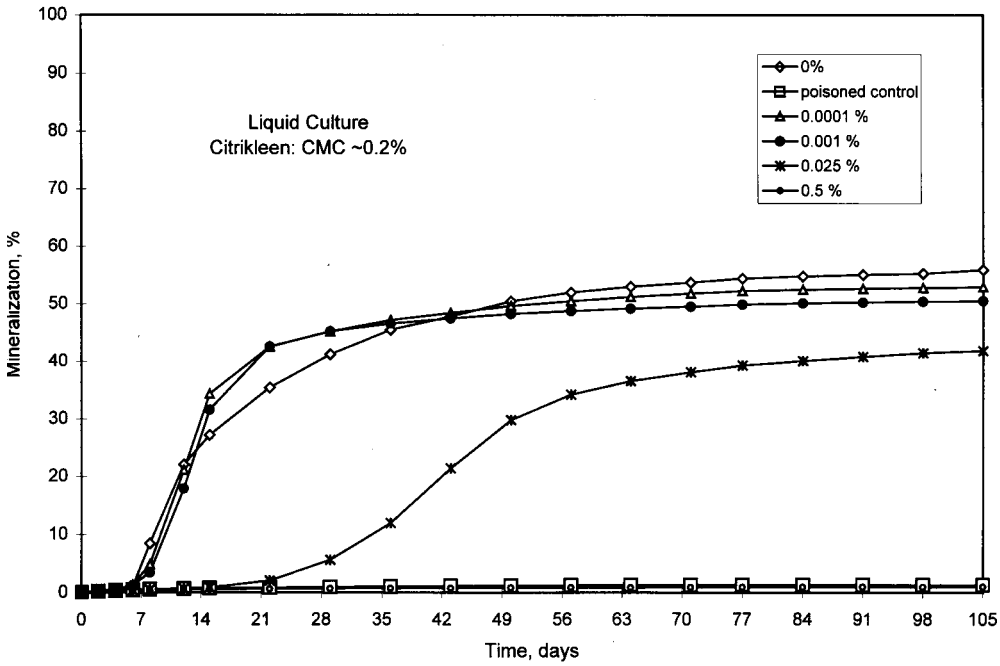

Figure 4. Mineralization of phenanthrene in aqueous systems receiving citrikleen.

for the different surfactant types and also because of the lack of existing research involving cationic surfactants. TTAB appeared to have an inhibitory effect on phenanthrene mineralization at all concentrations tested (Bramwell 1997). Even at 1 mg/L TTAB (corresponding to 0.001 × CMC), only 15% of added phenanthrene was mineralized relative to >50% in the absence of surfactant. Solubilization data indicate that at this surfactant concentration less than 1 mg/L phenanthrene is in aqueous phase (Bramwell 1997). At TTAB doses of ≥10 mg/L (including 0.01 × CMC, 0.1 × CMC, and 1.0 × CMC), no phenanthrene was mineralized over the 15-week study (Bramwell 1997). TTAB appears, therefore, to have a more significant inhibitory/toxic effect than the other surfactants tested in this study, not dependent on micelle-microbe interactions.

Mineralization of phenanthrene in aqueous systems of Tween 20 and Citrikleen appear least inhibitory; however, no benefit was observed in adding surfactant to aqueous systems containing phenanthrene. The inhibitory effect of higher surfactant doses on phenanthrene mineralization may be due to various phenomena, including: micro-organism-specific toxic effects of the surfactant or phenanthrene, toxic effects of micellized phenanthrene, lower bioavailability of micellar phenanthrene, the preferential use of surfactant as substrate in lieu of phenanthrene, or interference of the surfactant micelles with microbial metabolic processes (Laha and Luthy 1991; Laha et al. 1995). The toxic effect of phenanthrene and surfactant-phenanthrene combinations is addressed later in this paper.

Phenanthrene mineralization in soil-water suspensions

The effects of soil on the mineralization of [^{14}C]-phenanthrene by the PAH-degrading micro-organisms were examined in batch reactors. Organic substrate sorption onto soil may serve to decrease the total amount available for microbial degradation, as well as result in the entrapment of substrate in soil micropores for long periods of time (Mihelcic and Luthy 1991). Table 2 presents the apparent CMC values obtained for each surfactant in soil-aqueous systems through surface tension measurements (Bramwell 1997). These values are compared with aqueous CMC values as well as the surfactant dosages required to initiate phenanthrene solubilization in soil-water suspensions. For example, the CMC reported for Tween 20 in aqueous solutions was ∼100 mg/L or 0.01%. However, in the presence of soil, a Tween 20 dose of 16,000 mg/L or 1.6% was required to achieve micelle formation, suggesting significant surfactant losses (99%) by sorption onto soil or precipitation. Overall, soil-water suspensions required considerably larger surfactant doses in order to initiate solubilization. Data presented in Table 2 also suggest some phenanthrene desorption may be facilitated by surfactant monomers, or that micelles form below the apparent CMC measured. For example, only 5000 mg/L or 0.5% Tween 20 resulted in enhanced phenanthrene solubilization whereas 16000 mg/L Tween 20 was required to create micelles.

The microbial mineralization of phenanthrene in soil-aqueous systems receiving Tween 20 is shown in Figure 5. More than 70% of phenanthrene is mineralized when a dose of 500 mg/L or 0.05% (v/v) Tween 20 is applied, similar to mineralization results obtained for the soil-water systems receiving no surfactant. At this dose (corresponding to ∼0.03 × apparent CMC) there is no increased phenanthrene solubilization, and 95% of the added phenanthrene is sorbed on the soil/surfactant matrix. At a ten-fold greater surfactant dose of 5000 mg/L or 0.5% (∼0.3 × CMC) the amount of phenanthrene mineralized was reduced to approximately 20%. At 10,000 mg/L or 1% Tween 20 only 14% phenanthrene mineralization was achieved. Overall Tween 20 doses of 5000 mg/L and greater resulted in significant inhibition of microbial mineralization, with less than 20% phenanthrene mineralized over 15 weeks.

The mineralization of phenanthrene in soil-water suspensions receiving Citrikleen doses of 250 mg/L or 0.025% (0.02 × CMC), 2500 mg/L or 0.25% (0.2 × CMC), and 10,000 mg/L or 1% (0.8 × CMC) are shown in Figure 6. The CMC of Citrikleen in soil-aqueous systems was determined to be about 13,000 mg/L or 1.3% (v/v), although only 5000 mg/L or 0.5% was required to initiate phenanthrene solubilization (Table 2). Phenanthrene mineralization in 250 mg/L Citrikleen reached about 50% in 15 weeks, less than the mineralization achieved in the absence of surfactants. Solubilization data indicate that less than 1% phenanthrene is solubilized at this surfactant dose (Bramwell 1997). In comparison, about 10% phenanthrene is solubilized and 40% phenanthrene mineralized at 250 mg/L Citrikleen in liquid culture. When the Citrikleen dose was increased to 2500 and 10,000 mg/L, no phenanthrene was mineralized over the 15-week period.

Table 2. Measured CMCs for surfactants in soil-water suspensions

Surfactant	Aqueous CMC from surface tension measurements		Apparent CMC in soil-water systems from surface tension measurements		Minimum surfactant dose for phenanthrene solubilization in soil-water systems*	
	%	mg/L	%	mg/L	%	mg/L
Tween 20	0.01	100	1.6	16,000	0.5	5,000
TTAB	0.10	1,000	1.3	13,000	0.5	5,000
SDS	0.16	1,600	0.6	6,000	0.8	8,000
Citrikleen	0.20	2,000	1.3	13,000	0.5	5,000

Soil to water ratio used was 1 g soil : 10 mL aqueous solution.
* This represents the surfactant dose beyond which surfactant-enhanced solubilization is apparent (Bramwell 1997).

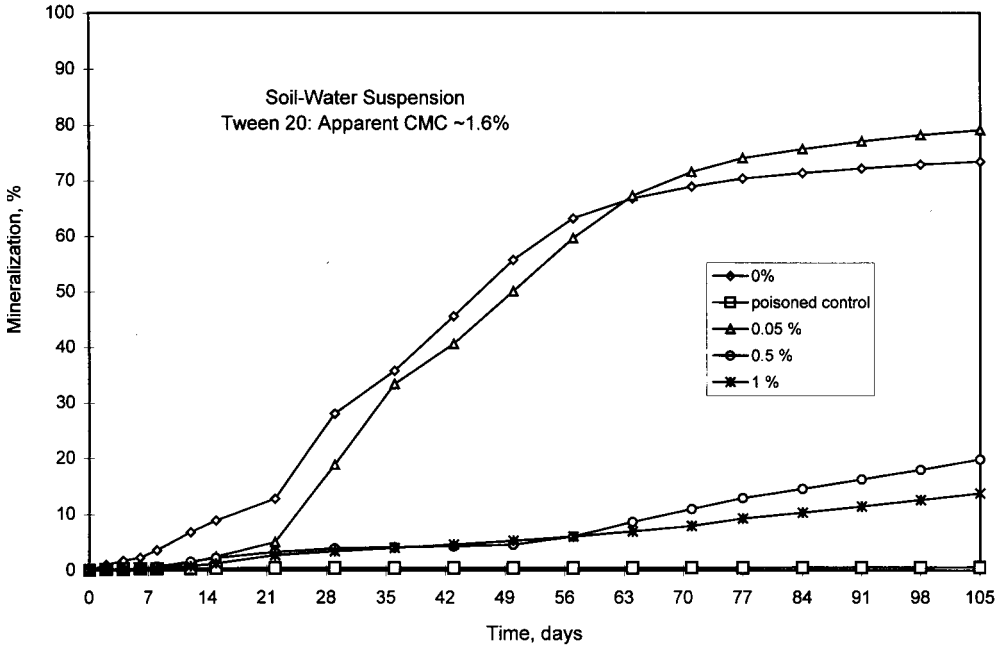

Figure 5. Mineralization of phenanthrene in soil-water systems receiving Tween 20.

Pseudo-aqueous phase phenanthrene concentrations are estimated at ∼1 mg/L for all the soil-water systems considered, indicating no significant surfactant-enhanced solubilization. In general, a greater final extent of microbial mineralization was observed in non-inhibitory soil-water systems relative to aqueous systems, although the maximum reaction rates were higher in the absence of soil. SDS and TTAB used in soil-aqueous systems were found to completely inhibit the microbial mineralization of phenanthrene at all the doses tested (Bramwell 1997). This is not surprising since these surfactants have been used as biocides.

The rate of phenanthrene mineralization appeared to be slower in the presence of soil, perhaps due to decreased substrate availability (Jahan et al. 1997). For example, phenanthrene mineralization in the aqueous system containing 10 mg/L or 0.001% (0.01 × CMC) Citrikleen was very close to its maximum after ∼56 days, in contrast to the corresponding soil-aqueous system with 250 mg/L or 0.025% (∼0.02 × CMC) Citrikleen which appears to peak after 105 days (Figure 6). Low surfactant doses had less severe impacts on phenanthrene biodegradation but did not enhance the rate or extent of phenanthrene mineralization. Microbial inhibition was observed at surfactant doses significantly below the apparent CMC in soil-water systems.

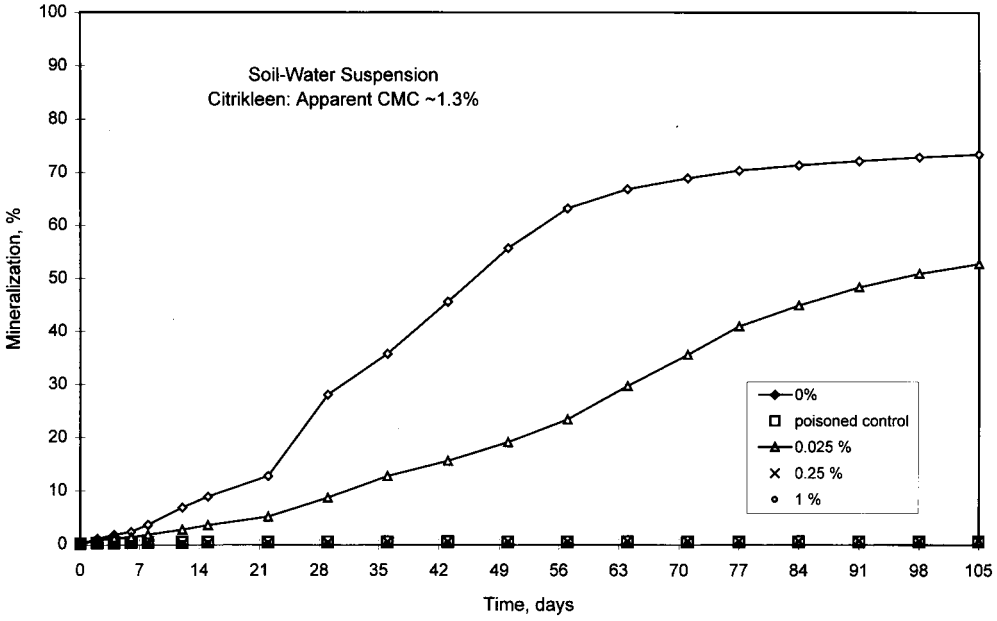

Figure 6. Mineralization of phenanthrene in soil-water systems receiving citrikleen.

Toxicity of surfactant and phenanthrene

The inhibition of microbial mineralization at certain surfactant doses could have been due to surfactant toxicity at high concentrations, or the toxicity of the solubilized phenanthrene present (Laha and Luthy 1992). To evaluate surfactant and micellized-phenanthrene toxicity to the microorganisms, toxicity analyses were performed using a Microtox Toxicity Model 500 Analyzer.

In general, nonionic surfactants are less toxic than ionic surfactants and gram-negative bacteria are less sensitive than gram-positive bacteria (Volkering et al. 1995). However, it is obvious that the presence of surfactants at the concentrations used in this study had a negative impact on bacterial activity. Table 3 indicates the EC_{50} values obtained for the various surfactant solutions tested using the Microbics toxicity assay. The EC_{50} value is the effective concentration in mg/L at which there is a 50% decrease in the light output of test bioluminescent micro-organisms exposed to samples. EC_{50} values obtained for Tween 20, TTAB, SDS, and Citrikleen are ~150, 1.4, 1.0 and 5 mg/L respectively, suggesting Tween 20 is the least toxic, followed by Citrikleen, TTAB and SDS. This corroborates the results obtained from the biodegradation tests where Tween 20 systems appear to have the least adverse effects on phenanthrene mineralization. The relatively high toxicity observed for Citrikleen (EC_{50} = 5 mg/L) suggests that the bioluminescent bacteria used for toxicity testing may have been more sensitive to this surfactant than the PAH-degrading micro-organisms since Citrikleen did not inhibit biomineralization of phenanthrene in liquid culture at concentrations up to 250 mg/L.

Based on these results Tween 20 was selected for additional toxicity testing. An excess amount of phenanthrene (corresponding to maximum dose of 12 mg/L) was added to 100 mg/L Tween 20 solutions and the EC_{50} value was determined to be 4.5 mg/L Tween 20. This represents a 50-fold increase in the toxicity of Tween 20 in systems containing solubilized phenanthrene since without the excess phenanthrene, the EC_{50} measured for Tween 20 was approximately 150 mg/L. This is possibly due to toxicity of solubilized phenanthrene, and it is suggested that toxicity of solubilized phenanthrene may play a significant role in microbial inhibition, and needs to be addressed when attempting to enhance substrate bioavailability through increased substrate solubilization.

Conclusions

The primary conclusion from this study is that surfactant amendment did not enhance the mineralization of phenanthrene by *P. aeruginosa* in liquid culture or in soil-water suspensions. This may be because

Table 3. Toxicity data for aqueous surfactant systems

Surfactant	Surfactant concentration used in toxicity test mg/L	EC_{50} values after 5 minutes mg/L	Measured CMC in aqueous solutions mg/L
Tween 20	100	146	100
	1,000	215	
	10,000	774	
TTAB	1	n/a	1,000
	10	2.0	
	100	1.4	
SDS	10	1.8	1,600
	100	1.0	
Citrikleen	10	6.0	2,000
	100	5.0	
	1,000	1.1	
	10,000	1.3	

the phenanthrene was readily accessible for microbial degradation. In the absence of surfactant, approximately 50% of the phenanthrene added to liquid culture was mineralized in ten weeks, compared to ∼70% in soil-water suspensions. Considering that mineralization only measures that portion of the substrate converted to CO_2, and that biomass and other end products may also be produced, it is possible that these results suggest a more complete biotransformation of phenanthrene. Each 50-mL liquid culture was inoculated with 2.4×10^{10} CFU of previously isolated PAH-degrading microorganisms. Soil-water systems receiving 5 g soil and 50 mL MSM were similarly inoculated. However, indigenous soil microorganisms probably provided additional seed resulting in the shorter lag period and greater extent of mineralization observed in the soil-water systems. The slower mineralization rate observed for the soil- water systems suggests that phenanthrene mineralization was mass transfer limited in the presence of soil.

Among the surfactants examined Tween 20 appears the best candidate for use in surfactant soil washing applications for HOCs like phenanthrene because of its superior solubilizing capacity. Tween 20 is a relatively nontoxic surfactant used as a food additive and would probably be the least harmful to the environment. Tween 20 also appears least inhibitory to the microbial mineralization of phenanthrene – phenanthrene was mineralized by *P. aeruginosa* at concentrations up to 100 mg/L in liquid culture. In soil-water suspensions, the rate of phenanthrene mineralization was significantly decreased at Tween 20 doses of ≥5000 mg/L. Soil washing applications generally recommend the use of surfactant solutions of concentrations in excess of 1% or 10,000 mg/L. The results obtained in this study indicate that such surfactant solutions may inhibit concomitant HOC biodegradation. In a study by Willumsen et al. 1998, it is suggested that microorganisms were able to recover from the toxic/inhibitory effects of surfactant after three weeks of exposure. In this study, we too observed that phenanthrene mineralization commenced after a lag period of three or four weeks for liquid cultures receiving 100 mg/L Tween 20 or 250 mg/L Citrikleen. It is not clear whether the lag period is a result of microbial adaptation to higher surfactant doses or surfactant biodegradation in the intervening period, since the surfactant concentration was not measured at the conclusion of the biodegradation tests. Citrikleen, which is reported to be biodegradable and is produced from citrus wastes, was seen to form precipitates in aqueous and soil-water systems hence requiring greater surfactant dosage for solubilization and suggesting possible clogging problems in proposed remediation applications. Citrikleen was also observed to produce inhibitory effects on phenanthrene biodegradation at much lower doses. The ionic surfactants SDS and TTAB appear least suitable for surfactant-amended subsurface remediation. They were seen to be inhibitory to the

microbial mineralization of phenanthrene in soil-water systems at all concentrations used.

The rapid release of $^{14}CO_2$ in microbial biodegradation tests indicates that the bacterial culture used was well acclimated to phenanthrene as the primary substrate. However, it is still possible that surfactants may be biodegraded in preference to phenanthrene. A comparison of solubilization, surface tension, and mineralization data suggests that the inhibitory effect on microbial degradation of phenanthrene may be related to the CMC of the surfactant in liquid culture. However, in soil-water suspension phenanthrene mineralization was adversely affected at surfactant doses well below those required for phenanthrene solubilization and micelle formation. No single physical or chemical property of the surfactant was found to be an accurate indicator of its performance in possible *in situ* bioremediation applications. Selection of surfactants will depend on a large number of factors, with the primary emphasis on their hydrocarbon-solubilizing power, sorptive properties, toxicity to micro-organisms or the environment, and their ultimate biodegradability. Information regarding specific micro-organisms involved in HOC degradation will also be essential since surfactant effects are seen to be microbe-specific. Additional studies are required to identify the exact mechanisms by which surfactant-microorganism interactions occur as well as surfactant biodegradation.

Work performed to date suggests that surfactant effects on biotransformation of HOCs vary enormously for different bacteria (Allen et al. 1999; Willumsen et al. 1998; Boonchan et al. 1998). Surfactant dose was also seen to have a critical effect on phenanthrene biodegradation, with enhancement observed at lower concentrations while higher concentrations inhibited biodegradation (Margesin and Schinner 1999; Guha and Jaffé 1996a; Aronstein et al. 1991). In the present study the bacterial inoculum used has been tentatively identified as *P. aeruginosa*. This is the microorganism responsible for phenanthrene mineralization in liquid culture. However, indigenous soil microorganisms are also likely to have played a role in phenanthrene degradation in soil- water suspensions. Because the soil-water systems used are more likely to reflect actual site remediation conditions and inoculated micro-organisms may not survive very well in subsurface environments, it will be necessary to characterize soil microorganisms prior to the application of surfactant-amended remediation. The increased toxicity of solubilized HOC appears to contribute to the ineffectiveness of surfactant amended remediation in this study.

In general, before the widespread application of surfactants to site remediation it will be necessary to develop a predictive framework for surfactant-amended biodegradation. The primary contribution of this study is corroboration that PAHs like phenanthrene are readily amenable to treatment via bioremediation. However, surfactant amended-bioremediation appears infeasible for the conditions used in this study.

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