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Effect of addition of *Pseudomonas aeruginosa* UG2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil

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SUMMARY

A laboratory study was undertaken to assess the effect of adding either *Pseudomonas aeruginosa* UG2 cells or the biosurfactants produced by this microorganism on the biodegradation of a hydrocarbon mixture in soil at 20 °C over a 2-month incubation period. The addition of 100 μg of UG2 biosurfactants per g soil significantly enhanced the degradation of tetradecane, hexadecene and pristane but not 2-methylnaphthalene, the most water-soluble of the hydrocarbons. Addition of UG2 cells at densities of 10^6, 10^7 and 10^8 per g soil did not have a significant effect on biodegradation of the hydrocarbon mixture.

INTRODUCTION

Hydrocarbons, such as oil products, petroleum products and halogenated compounds, form an important class of pollutants on a global scale. Through continuous input, spillage, improper handling or waste disposal, and natural seepage, these compounds are entering the soil and groundwater environments [23]. Once released, hydrocarbons tend to be dispersed over diffuse areas. The presence of hydrocarbons in the environment is of considerable public health and ecological concern due to their persistence, ability to be bioaccumulated and toxicity to a wide variety of biological systems [32,33]. Therefore, there is a need to clean up or remediate soils and groundwater which have been contaminated with both halogenated and non-halogenated hydrocarbons.

Traditionally, hydrocarbon-polluted soils have been decontaminated by physical or chemical methods such as thermal evaporation, extraction, steam or hot-air stripping, chemical oxidation, flooding, adsorption, detergent extraction, and immobilization [32]. Enhancement of biological degradation based on seeding with microorganisms and nutrient amendments has also been attempted. In situ bioremediation is an attractive and ecologically sound method of decontaminating hydrocarbon-polluted soils and groundwater, and has been claimed to be efficient, economical and versatile [23]. The success of bioremediation depends on a good understanding of the biochemical, physiological and ecological principles which govern microbial growth, activity and biological recalcitrance at introduction sites [9]. Many factors which affect the rate and extent of in situ hydrocarbon biodegradation have been identified. These include hydrocarbon concentration, solubility, presence of microorganisms bearing the catabolic enzymes, environmental conditions, nutrient availability, soil characteristics, and interactions [5,6,9,32].

Low aqueous solubility of hydrocarbons can affect their bioavailability, sorption characteristics and accessability. Many microorganisms produce extracellular surface-active agents when grown on hydrocarbon substrates [7,18,37]. The presence of these agents can increase the solubility of hydrophobic compounds by decreasing surface tension. By solubilizing the water-insoluble compounds, the bioemulsifying agents will increase mobility and surface area available for cell contact with hydrocarbons, and this may promote degradation [29].

Recently, we have isolated several *Pseudomonas* strains which exhibit high extracellular emulsifying activity [3,19]. One objective in producing bioemulsifying agents is to solubilize and disperse hydrocarbons. Initial studies re-

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vealed that the surface-active emulsifying agents produced by *P. aeruginosa* UG2 increased the partitioning of ^{14}C-labelled hexachlorobiphenyl into the aqueous phase of soil slurries by a factor of 30-fold over that of the water control [3]. The present study was undertaken to investigate if addition of UG2 inocula or the UG2 bioemulsifying agents might enhance biodegradation of a selected hydrocarbon mixture in soil.

MATERIALS AND METHODS

Chemicals. All chemicals used were of reagent grade and were obtained from commercial sources.

Microorganism. P. aeruginosa UG2, initially isolated in our laboratory [3], was maintained on tryptone soy extract agar (TSA, Difco Laboratories, Detroit, MI) plates and subcultured every 2 weeks.

Soil. The soil used was a silt loam (30% sand, 54% silt, 16% clay) of the Conestogo series (Brunisolic Grey Brown Luvisol) which contained 2.1% organic carbon and had a pH of 7.4 [31]. After collection, the soil was kept tightly sealed in the dark at 4 °C and was used within 1 month.

Growth of P. aeruginosa UG2 for surfactant production. A series of 500-ml baffled flasks were set up. Each flask contained 150 ml of medium which consisted of the following components per litre: glucose, 20 g; K_2HPO_4 0.65 g; KH_2PO_4, 0.17 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NaNO_3$ 0.5 g; NaCl, 0.5 g; $FeSO_4$ 0.01 mol; $CaCl_2$ 0.02 mol; and trace elements (Zn, Mn, B, Co, Cu and Mo) of 1 ml [11]. The pH of the medium was adjusted to 7.0 before autoclaving. $CaCl_2$ and $FeSO_4$ were prepared in concentrated 1 M solutions and autoclaved separately before being added to other medium components.

To each flask was added a loopful of cells from a TSA plate and the culture incubated in a 500-ml baffled flask at 28 °C with gyratory shaking at 200 rpm. Samples (1 ml) were removed at intervals, diluted with distilled water and the surface tension measured using a Fisher Tensiomat [15]. Biosurfactant production was also assessed qualitatively by using the drop-collapsing test [15].

Surfactant extraction. After 6 days of growth as above, cells were removed from the medium by centrifugation at $16000 \times g$ for 15 min (Sorval, GSA rotor). The pH of the medium was lowered to 2.0 using 1 N HCl. The biosurfactant was quantitatively extracted with diethyl ether using a modification of the method of Hisatsuka et al. [13]. Briefly, to the culture supernatant (about 900 ml) was added an equal volume of diethyl ether and the mixture placed in a separatory funnel and shaken vigorously for 5 min. The mixture was held stationary for 5 min to allow phase separation. The top diethyl ether layer containing the surfactant was removed and the bottom aqueous layer was re-extracted with diethyl ether until solutions from the bottom layer no longer exhibited drop collapsing ability. This usually required 5–8 extractions. The aqueous phase was further concentrated by drying under vacuum using a rotoevaporator at 50 °C to 20% of its original volume, and the diethyl ether extraction process repeated with this smaller volume of aqueous phase until all surfactant activity was removed, as ascertained by measurements of surface tension, emulsifying activity and the drop-collapsing ability using methods previously established in our laboratories [15,19]. One unit (U) of emulsifying activity was defined as the amount of emulsifier giving an absorbance of 1.0 at 540 nm. All of the extracts were pooled and the diethyl ether evaporated under N_2 gas. This material was further placed under vacuum for 1 h followed by heating at 80 °C for 15 min to remove other volatile substances in the extract.

Surface activity of the concentrated extract. The potency of the UG2 concentrated extract was assessed by measuring its critical micelle concentration (CMC) value. Various dilutions of the extract were prepared and their apparent surface tensions measured by a Tensiomat. The concentration of the UG2 extract at which the apparent surface tension increased dramatically was recorded as the CMC value.

Effect of UG2 biosurfactants or UG2 inoculum on degradation of a model hydrocarbon mixture. The degradation of a model hydrocarbon mixture in non-sterile soil was studied. The hydrocarbons selected were (a) tetradecane, a linear aliphatic alkane, (b) pristane, a branched aliphatic alkane, (c) hexadecene, a linear aliphatic alkene, and (d) 2-methylnaphthalene, an aromatic hydrocarbon. These compounds are components of crude oil [24] and their biodegradation by soil and aquatic bacteria has previously been reported [24,28]. A mixture of the hydrocarbons was prepared with the following concentrations (w/w): tetradecane 40%, pristane 25%, hexadecene 25%, and 2-methylnaphthalene 10%.

Degradation was studied in pre-sterilized screw-capped polypropylene centrifuge tubes (Falcon, 50-ml capacity). Each tube contained 12.5 g of soil which had a 25% moisture content (equivalent to water holding capacity). In the course of the experiment, the moisture content of each tube was maintained gravimetrically every 3rd–4th day using sterile distilled water. To each tube was added 0.1 g of the concentrated hydrocarbon mixture such that the initial amount of hydrocarbon per tube was 40, 25, 25, and 10 mg for tetradecane, pristane, hexadecene and 2-methylnaphthalene, respectively. Hydrocarbon mixture was spread dropwise as evenly as possible over the soil surface. After placing the screw caps on loosely, the tubes were incubated at 20 °C in the dark. At monthly intervals, tubes were removed and the complete content of each tube used for analysis.

To study the effect of adding UG2 inocula on hydrocarbon degradation, UG2 was grown in tryptone soy broth at 28 °C with gyratory shaking at 200 rpm as described above. Cells were harvested during mid-log phase (about 14 h) by centrifugation at 4000 × *g* and washed twice with sterile 0.85% (w/v) NaCl solution. Concentrated cell suspensions were prepared in 0.85% (w/v) NaCl solution before being added to the soil. To study the effect of adding the UG2 surfactants on hydrocarbon degradation, the biosurfactants were extracted as described above. The concentrated UG2 surfactants were dissolved in water by heating at 50 °C for 30 min with continuous stirring. A stock 0.1% (w/v) solution of the biosurfactant was prepared and diluted as specified with 0.85% (w/v) saline solution.

Tubes containing soils were amended with 1 ml of biosurfactant or cell at the indicated concentrations. All materials were added dropwise to ensure a uniform distribution over the soil surface. Sterile and non-sterile control treatments were run concurrently. In the sterile control, soil was previously autoclaved at 121 °C for 30 min. Non-sterile controls did not receive either the UG2 biosurfactant or inoculum. In both types of controls, 10^9 UG2 cells previously autoclaved at 121 °C for 15 min were added to each tube.

Hydrocarbon extraction from soil. Ten ml of hexane were added directly to each tube. The tube contents were subjected to the following sequence of treatments at 21 °C: mixed by vortexing for 1 min, placed in a horizontal position and shaken reciprocally along its vertical axis at 220 strokes/min (Eberbach shaker) for varying periods of time, and mixed by vortexing for 1 min. However, in the course of the study, this extraction method was found to be inadequate for soil samples which received hydrocarbons for more than 2 weeks. Therefore, several modifications of the above procedures were tested using both sterile and non-sterile control soil samples spiked with hydrocarbons for about 1 month. The modifications included using mixtures of hexane:methanol, hexane:ethanol, ethyl acetate:methanol, repeated extractions with solvents, using different combinations of solvents during repeat extractions, as well as prolonged shaking (Table 1). In most of these tests with extraction methods, only the pristane concentration was monitored. After extraction, the tubes were centrifuged at 3000 × *g* for 10 min at 21 °C. The supernatant was decanted and filtered through a 45-μm Millipore nylon filter. Moisture in the supernatant was removed by adding anhydrous sodium sulphate to a final concentration of 5% (w/v) prior to analysis.

Hydrocarbon analysis. Hydrocarbons in the extracts were analyzed using a Varian 2100 gas chromatograph equipped with a flame ionization detector. Separation was achieved with a 2 m × 2 mm stainless steel column containing 3% OV-17 on WHP Chromosorb (Chromatographic Specialties, Brockville, Ontario). The flow rate of the carrier gas, N_2, was 2 ml/min. The injector and detector temperatures were 250 and 300 °C, respectively. Immediately after sample injection, the column was subjected to a temperature gradient which increased from 70 to 200 °C at 6 °C/min. Ethyl octanoate, the internal standard, was added to the extract before injection.

TABLE 1

Effect of various solvents and ageing on pristane extraction from soil.

Sample age (days)	Solvent	Recovery (%)
0	10 ml hexane	100
12	10 ml hexane	104
24	10 ml hexane	54
28	20 ml hexane:methanol (2:1)	45
28	20 ml hexane:methanol (9:1)	52
28	(a) 20 ml hexane:ethanol (9:1)	36
	(b) reextracted two more times with 40 ml hexane:ethanol (9:1)	63
28	20 ml ethyl acetate:methanol (7:1)	50
28	(a) 20 ml hexane	57
	(b) reextracted one more time with 20 ml hexane	71
28	20 ml hexane[a]	101

Centrifuges tubes were placed on an Eberbach shaker and shaken reciprocally at 220 strokes/ml for 10 min.

[a] The centrifuge tube was shaken for 9 h on an Eberbach shaker.

Replication and statistical analysis. Three independent replicates were run for each degradation treatment. Values were expressed as means ± S.D. Statistically significant difference between mean values was evaluated by Student's *t*-test at 95% confidence level using Co-Stat (Cohort software, Berkeley, CA, U.S.A.).

RESULTS

Some characteristics of P. aeruginosa UG2 surfactants

Biosurfactants were produced by *P. aeruginosa* UG2 cultures mainly in stationary phase of growth. After 6 days of incubation in a non-hydrocarbon medium, about 5 ml (approximately 5 g) of crude surfactant could be recovered from 900 ml of culture supernatant. The concentrated material appeared yellow and had an oily consistency. The material readily caused drop collapse on an oily plate, suggesting that it possessed potent surfactant activity. The surfactant concentrate lowered the surface tension of water to 33.3 dyn/cm, achieved CMC at 18–19 μg/ml and exhibited an emulsifying activity of 314 U/ml.

Hydrocarbon extraction from soil

The extraction of hydrocarbons from soils was initially attempted using hexane with 10 min shaking on an Eberbach shaker. This procedure was found to be adequate for recovering about 99–100% of recently added (up to 2 weeks) hydrocarbons, however, it was insufficient for recovering all of the hydrocarbons which have been added to the soil for more than 2 weeks. For example, after 24 days, only about 50% of the added pristane could be recovered (Table 1).

Various modifications were tested for their ability to extract quantitatively the hydrocarbons in 4-week-old sterile control samples. The use of 10 ml hexane, 20 ml hexane:methanol mixtures (9:1 and 2:1, v/v), 20 ml hexane:ethanol (9:1, v/v), 3 extractions with 20 ml hexane: ethanol (9:1) or 20 ml ethyl acetate:methanol (7:3, v/v), and 2 extractions with 20 ml hexane, each followed by 10 min shaking did not result in satisfactory hydrocarbon recovery (Table 1). However, prolonged shaking with 20 ml hexane significantly improved hydrocarbon extraction. Improved extraction of all four hydrocarbons followed a similar trend and by 9 h, the hydrocarbons were completely extracted (Fig. 1). Since prolonged shaking with hexane yielded satisfactory extraction, prolonged shaking with other solvents was not tested. In all subsequent extractions, overnight (14–16 h) shaking was routinely carried out to ensure adequate extraction of the hydrocarbons.

Degradation of hydrocarbons

Considerable tetradecane degradation was observed in non-sterile controls and in all experimental treatments. In non-sterile controls, about 50% of tetradecane was degraded after 1 month incubation (Fig. 2). This value increased to about 71% after 2 months. Addition of UG2 surfactants or UG2 inocula generally improved tetradecane degradation over the non-sterile controls. However,

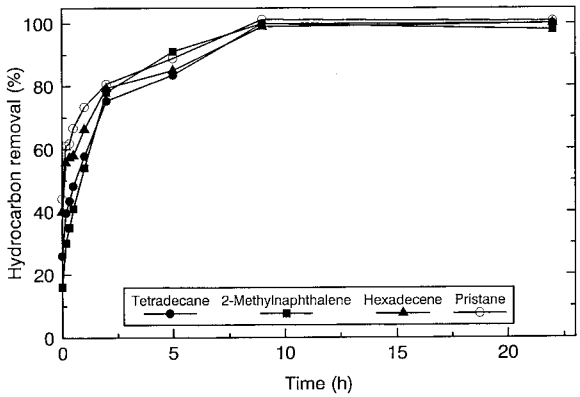

Fig. 1. Time course of extraction of soil bound hydrocarbons by hexane.

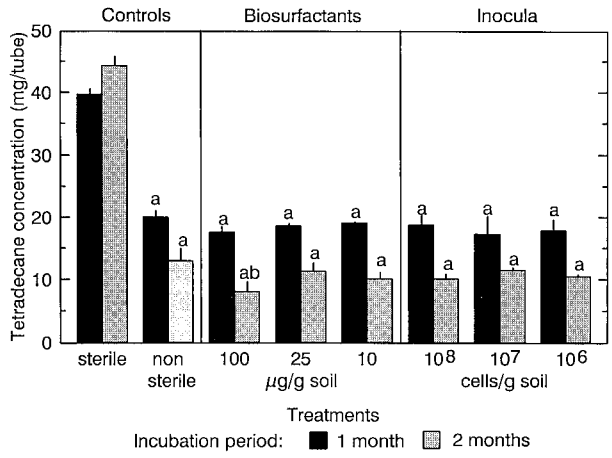

Fig. 2. Effect of *P. aeruginosa* UG2 inoculum or biosurfactant on tetradecane degradation in soil. Error bar represents 1 S.D. about the mean. Symbols: a, values were significantly different from sterile controls at $P<0.05$; b, values were significantly different from non-steride controls at $P<0.05$.

a statistically significant increase in degradation over the non-sterile control was observed only in the treatment which received 100 μg UG2 biosurfactants/g dry soil (82% degradation after 2 months) (Fig. 2). In sterile controls, degradation of tetradecane was not observed after 2 months of incubation.

The degradation trend of hexadecene resembled that of tetradecane except that lesser proportions of the initial hexadecene was degraded. For example, in non-sterile controls, about 32% and 60% of hexadecene was degraded after 1 and 2 months, respectively (Fig. 3). Similarly, statistically significant improvement in degradation was observed only in the treatment which received 100 μg

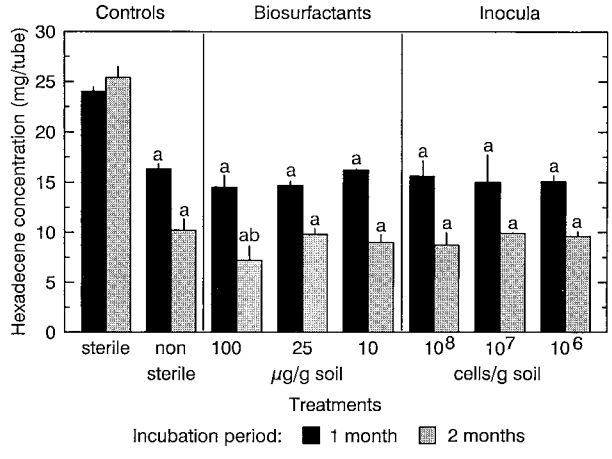

Fig. 3. Effect of *P. aeruginosa* UG2 inoculum or biosurfactant on hexadecene degradation in soil. Symbols as in Fig. 2.

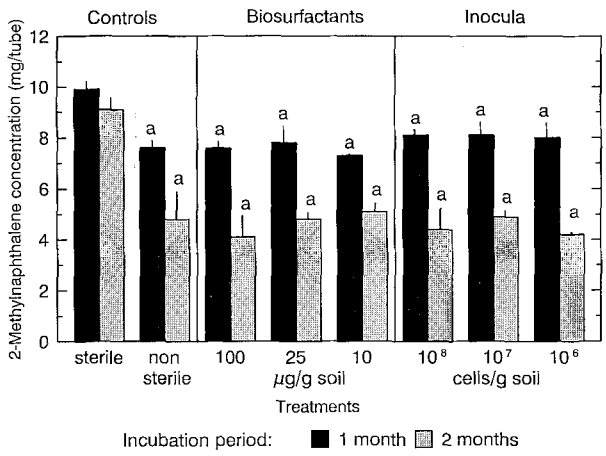

Fig. 4. Effect of *P. aeruginosa* UG2 inoculum or biosurfactant on 2-methylnaphthalene degradation in soil. Symbols as in Fig. 2.

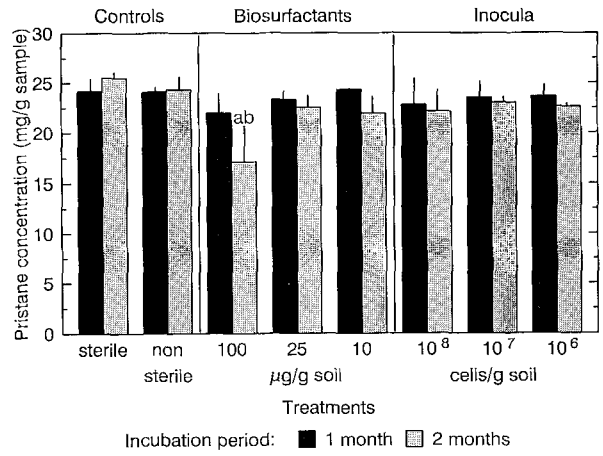

Fig. 5. Effect of *P. aeruginosa* UG2 inoculum or biosurfactant on pristane degradation in soil. Symbols as in Fig. 2.

UG2 biosurfactants/g dry soil (72% degradation after 2 months) (Fig. 3).

Compared to tetradecane or hexadecene, lesser amounts of 2-methylnaphthalene were degraded over 2 months in non-sterile controls and experimental treatments. In non-sterile controls, about 23% and 47% of 2-methylnaphthalene was degraded after 1 and 2 months, respectively (Fig. 4). No statistically significant difference was observed whether the soil received UG2 biosurfactants or inocula (Fig. 4). In sterile controls, about 9% of the added 2-methylnaphthalene was lost, possibly due to evaporation or volatilization.

Very little pristane was lost during the 2 months incubation period as compared to tetradecane, hexadecene or 2-methylnaphthalene. However, in all experimental treatments, there was a general trend towards increased degradation over the non-sterile controls. Statistically significantly increased pristane degradation was observed only in the treatment which received 100 µg UG2 biosurfactant (33% degradation after 2 months) (Fig. 5). In all other experimental treatments as well as in non-sterile controls, the loss of pristane was not statistically significantly different from the sterile control.

DISCUSSION

The crude biosurfactants produced by UG2 exhibited excellent surface activity, lowering the surface tension of water to 33 dyn/cm and achieving the CMC at 19 µg/ml. These values compare favourably to the surface tension reduction of 25–42 dyn/cm and CMC values of 10–200 µg/ml reported for biosurfactants produced by other *P. aeruginosa* strains [13,18]. The biosurfactants from UG2 were also stable at 80 °C and displayed excellent emulsifying activity [3,15; this study].

Our extraction experiments show that strong measures were required to recover hydrocarbons from soil after prolonged incubation. This may be due to the strong binding of hydrocarbons to soil particles and organic matter during ageing. How this ageing effect comes about is not presently understood. It is known, however, that this binding may be influenced by various physical and chemical factors, such as soil type, pH, temperature, moisture content, organic matter content and type of hydrocarbons [16,39].

The effect of hydrocarbon binding to soil on microbial degradation is not well understood. The effect may depend on the nature of the binding, the prevailing environmental conditions, as well as whether this binding seriously limits the availability of the hydrocarbons to active microbial degraders in the vicinity. Some authors have hypothesized that the effect could be minimal in situations where the binding is so loose as to allow easy diffusion and surface scavenging of the bound hydrocarbons by degraders [22]. However, there are reports that the biodegradation rate of some hydrophobic or more water-soluble hydrocarbons is limited by their rates of solubilization into the aqueous phase in both liquid-liquid or liquid-solid systems [1,22,26,35,36].

Several researchers have studied the effect of exogenous addition of surfactants on hydrocarbon biodegradation, and the effect seems variable. Atlas and Bartha [2] found the use of chemical surfactants had no effect on oil biodegradation in sea water. Lindley and Heydeman [18a] reported that the addition of phosphatidylcholine enhanced degradation of alkane mixtures up to 30% by the fungus *Cladosporium resinae*, with the increase being dependent on alkane size. In another study, Foght et al. [10] found the emulsifier Emulsan to inhibit alkane mineralization by pure and mixed bacterial cultures. This emul-

sifier stimulated aromatic mineralization by pure cultures, but inhibited aromatic degradation by mixed cultures. Oberbremer et al. [25] found that the biodegradation rate of a model oil mixture could be doubled by addition of sophorose lipid surfactants. While the results are variable, the positive effect reported in some of the studies show that exogenous application of bioemulsifiers might have merit and deserves closer examination as a means to enhance hydrocarbon biodegradation in situ.

The present study investigated the effect of adding either the *P. aeruginosa* UG2 biosurfactants or UG2 inoculum on biodegradation of a model hydrocarbon mixture in soil. The results showed that the addition of 100 μg of UG2 biosurfactants per g soil significantly enhanced the degradation of the aliphatic tetradecane, pristane and hexadecene, but not the aromatic 2-methylnaphthalene, the most water-soluble of the four compounds [24,30]. The positive effect observed with pristane is especially promising since this compound is normally difficult to degrade. The variability in the enhancement seen with UG2 biosurfactant may be related to the aqueous solubility rather than the chemical structure of the test compounds. In an earlier study of mixed hydrocarbon biodegradation by soil bacteria, Oberbremer and Muller-Hurtig [24] found that the more water-soluble naphthalene can be easily degraded while the less water-soluble hydrocarbons (including other aromatics) are only metabolized when the interfacial tension was lowered by biosurfactant production. These observations suggest that the requirement for surfactant solubilization may differ between different hydrocarbons. This requirement will likely depend on hydrocarbon bioavailability which is influenced by the solubility and the degree of binding of hydrocarbons to the soil matrix. Therefore, the use of surfactants in soil may be beneficial in situations where solubility clearly limits the bioavailability of the target hydrocarbons to an active, degrading microbial population. However, the potential benefits of in situ application of surfactants must also be weighed against the possibility of increased groundwater contamination caused by surfactant-mediated enhanced mobility of the hydrocarbons. In this regard, the use of a repeated but smaller surfactant dosage schedule should be investigated as a means to control contaminant mobility, in conjunction with careful monitoring of the rate and extent of hydrocarbon degradation.

Our study also showed that the addition of *P. aeruginosa* UG2 inocula had no effect on biodegradation of the four hydrocarbons. The reason(s) for this lack of effect is not clear. One possibility may be that the UG2 strain did not successfully establish itself in the new environment and was not capable of survival during the 2-month incubation period. This may be due to the combined toxicity of the four hydrocarbons in the mixture as well as the inability of UG2 to produce adequate amount of the biosurfactants when introduced into soil. Further studies are needed to assess the potential for advantageous use of biosurfactant producing organisms as this may provide one method of controlling the rate of biosurfactant production to suit the needs of the degraders.

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