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Surfactant inhibition of bacterial growth on solid anthracene

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Abstract

Surfactants have been proposed as a promising method to enhance bioremediation of hydrophobic compounds in contaminated soils. However, the results of effects of surfactants on bioremediation are not consistent. This study showed that Triton X-100 at low concentration (0.024 mM or 0.09 CMC) inhibited the rate of growth of either a *Mycobacterium* sp. or a *Pseudomonas* sp. on solid anthracene as sole carbon source. Recovery of microbial growth rate could be achieved by dilution of surfactants, while addition of more surfactant gave an immediate decrease in growth rate. No inhibition of growth by Triton X-100 was observed with growth on glucose. The surfactant sorbed onto the surfaces of both the cells and the anthracene particles, which could inhibit uptake of anthracene. The results were consistent with the hypothesis that inhibition of microbial adhesion of cells to anthracene was responsible for the inhibition of growth by Triton X-100.

Introduction

Bioremediation can be an effective method for removing and degrading hydrophobic contaminants from soil, particularly hydrocarbons (Skinner 1990; Baker & Herson 1994), but in some cases the rate and effectiveness of soil treatment may be limited by the low solubility and dissolution rate of the contaminants (Cerniglia 1992; Rouse et al. 1994). The physical state of hydrophobic organic compounds in the soil environment ranges from material sorbed in humic materials at low concentrations (Pignatello and Xing 1996) to non-aqueous phase liquids that coat the surfaces of soil particles and fill voids between soil particles when the total hydrocarbon concentration is of order 1% or higher (Karimi & Gray 2000). In the case of aged hydrocarbons from crude oil or creosote, the viscosity of the non-aqueous phase liquid (NAPL) in soil can range from free-flowing oily material to solid-like tars (Stelmack et al. 1998). In some cases, solid crystals of contaminants such as polynuclear aromatic hydrocarbons (PAHs) are observed in association with NAPL (e.g., Weissenfels et al. 1992). The availability of degradable components of NAPL to bacteria will have a major effect on the observed rate of biodegradation.

In many studies, surfactants were added to enhance bioavailability by providing components in readily accessible surfactant micelles or to disperse the organic phase and increase its interfacial area. Recent reviews of this research (Rouse et al. 1994; Volkering et al. 1998) found that the results were inconsistent. Addition of surfactants inhibited degradation in some cases and enhanced it in others, depending on the specifics of the target compound, bacterial species and choice of surfactant. Volkering et al. (1998) demonstrated that many of these results could be reconciled by considering the interactions between the bacteria, the surfactant and the organic phase. When bacteria attach directly to the surfaces of liquid or solid hydrophobic organic compounds to enhance uptake, then the addition of surfactants may inhibit degradation by dispersing the bacteria in the aqueous phase. In contrast, when the bacteria rely on dissolved substrate in the aqueous phase, addition of surfactant can enhance degradation by dispersing droplets of NAPL to increase interfacial area, solubilizing hydrophobic or-

ganic substrates in surfactant micelles or enhancing the dissolution of solid substrates (Grimberg et al. 1994; 1995; Volkering et al. 1998). In addition to the role of surfactants in altering the transport of substrate to the bacteria, they can also inhibit cell growth, change membrane permeability or serve as growth substrates for co-metabolism (Volkering et al. 1998).

Most studies of surfactants in biodegradation have focussed on concentrations above the critical micelle concentration (CMC) of the surfactant, yet in many environmental applications the surfactants will eventually be diluted to concentrations below the CMC regardless of the initial concentration. Consequently, it is important to understand the impact of surfactants on biodegradation even at low concentrations, below the CMC. In the case of high-viscosity NAPL or solid hydrophobic compounds, surfactants will not disperse the substrate and change interfacial area (Volkering et al. 1998), nor can micellar solubilization or enhanced rate of dissolution occur below the CMC (Grimberg et al. 1994). Low concentrations of surfactants can interfere with bacterial attachment, for example, Stelmack et al. (1999) found that both Triton X-100 and Dowfax 8390 (an anionic surfactant) inhibited the adhesion of *Mycobacterium* sp. and *Pseudomonas* sp. to NAPL and glass surfaces at a concentration of 50% of CMC. Both surfactants were also effective for removing previously adhered microorganisms from these surfaces.

Clearly, the growth of bacteria on hydrophobic contaminants in the presence of surfactants can depend on a number of binary and ternary interactions. Growth on solid substrates considers one extreme of the behavior of hydrophobic contaminants in the environment, and provides discrimination by preventing interactions such as dispersion of NAPL. Use of surfactants below their CMC provides further control by eliminating effects due to micelles. The model system selected in this study was growth of representative gram positive and gram negative bacteria on anthracene as sole carbon source, with addition of a non-ionic surfactant (Triton X-100) or an anionic surfactant (Dowfax 8390) at concentrations below CMC. Adsorption of surfactant to cell and anthracene surfaces was measured in order to relate the growth characteristics to surface interactions between the cells and the surfactant. This approach considers one extreme of the possible range of cell-substrate-surfactant interactions that are relevant to biodegradation in contaminated soils.

Materials and methods

Materials

A gram-positive *Mycobacterium* sp. (Tongpim & Pickard 1996) and a gram-negative *Pseudomonas* sp. (Stelmack et al. 1999) were used. The *Mycobacterium* sp. was close to, but not the same as, *Mycobacterium fortuitum* based on 16S rRNA analysis. The 16S rRNA sequence data are available in the EMBO (European Molecular Biology Organization) database under accession number Y15709 (*Mycobacterium* sp. strain S1). The *Pseudomonas* species was not characterized in as much detail (Stelmack et al. 1999). Both species were able to utilize anthracene as sole carbon source. Experiments on sorption of surfactant to gram-negative bacteria were carried out with a PAH degrading strain *Pseudomonas fluorescens* LP6a (Foght & Westlake 1996). This surrogate strain was similar to the anthracene-degrading strain, but was selected for convenience due its rapid growth on glucose. Sorption of surfactant to the *Mycobacterium* sp. was measured directly.

The microbial growth medium consisted of anthracene crystals (99% purity, Sigma Chemical Company, St. Louis, Missouri) at a concentration of 500 mg/L and mineral components: 1.33 g/L KH_2PO_4, 2.67 g/L K_2HPO_4, 1 g/L NH_4Cl, 2 g/L Na_2SO_4, 2 g/L KNO_3, 0.01 g/L $FeSO_4$. $7H_2O$, and 1ml/L trace metal solution (Fedorak and Grbic'-Galic', 1991). The medium was autoclaved for 20 minutes at 121 °C, then sterile $MgSO_4.7H_2O$ was added to the medium to a concentration of 2 g/L. Anhydrous D-glucose was obtained from BDH Inc., Toronto, Ontario for experiments with glucose as an alternative carbon source.

Two surfactants were selected; a nonionic alkyl phenoxyl ethoxylate (Triton X-100, MW 625, Rohm and Haas of Canada Ltd., West Hill, Ontario) and an anionic surfactant (Dowfax 8390, MW 642, Dow Chemical Company, Midland, Michigan) which has a hydrophilic group of diphenyl oxide disulfonate and a 16-carbon alkane hydrophobic tail.

Methods

Determination of critical micelle concentration

A series of standard solutions were prepared in the growth medium. The Triton X-100 concentrations ranged from 0.116 mM to 0.278 mM while the Dowfax 8390 concentrations ranged from 0.1 mM to 1.2 mM. The surface tension of each solution was measured by a surface tensiometer (Fisher Surface Tensiomat Model 21, Fisher Scientific, Hampton, New Hampshire). The log of surface tension was plotted against the surfactant concentration, and the critical micelle concentration was determined to be the inflection point (Rosen, 1989).

Microbial growth

Microbial strains were transferred from glycerol stocks at −70 °C to agar plates by streaking. The *Mycobacterium* sp. was grown on plates of plate count agar (Difco Laboratories, Detroit, Michigan) while the *Pseudomonas* species was grown on plates of trypticase soy agar (Becton Dickinson and Company, Cockeysville, Maryland). The incubation time was 7 days for the *Mycobacterium* sp. on plates of plate count agar and 3 days for the *Pseudomonas* sp. on plates of trypticase soy agar. The incubation temperature was 27 °C. After incubation, the plates were stored at 4 °C.

The microorganisms were transferred from plates to 500-ml Erlenmeyer flasks, containing 100 mL mineral salts medium and 50 mg anthracene. A 1.2-cm diameter steel coil was placed into each flask to prevent agglomeration of the microorganisms to the anthracene crystals. The flasks were incubated at 27 °C on a New Brunswick gyrotary shaker (New Brunswick, Edison, New Jersey) at 200 rpm for 14 days. For the *Mycobacterium* sp., the sample was taken from each flask 6 or 7 days after being transferred from plates to medium. For the *Pseudomonas* sp., the sample was taken from each flask 3 or 4 days after being transferred from plates to medium. The purity of both bacterial strains was checked by streaking samples on the respective plates.

Cultures were grown in quadruplicate. For the microbial growth experiments each flask contained 90 mL growth medium and was inoculated with 10 ml of liquid culture prepared by the above techniques. At the end of each experiment, the purity of the cultures was checked by streaking onto agar plates as described above. Contaminated flasks were excluded from the data analysis. The optical densities of microbial solutions were measured as absorbances by a UNICAM 8700 Series UV/VIS spectrometer (Unicam, Cambridge, UK) at a wavelength of 600 nm. Cell densities were proportional to OD_{600} up to an OD value of 0.4 and higher cell densities were diluted with 0.1 M phosphate buffer pH 7 until they fell within the linear range. Plate counts on the *Mycobacterium* sp. and *Pseudomonas* sp. gave a consistent ratio of 10^8 cfu/ml for each 0.1 unit of OD_{600}. The anthracene was present as thin flakes, approximately 0.7 mm in diameter, which settled much more rapidly than the cells. When taking samples from each flask for OD_{600} measurement, 10–15 minutes were allowed for the anthracene crystals to settle to avoid removing anthracene with the cells. When glucose was used as the single carbon source at a concentration of 500 mg/L, the other components of the growth medium were the same as growth on anthracene and all experimental methods were identical.

The inoculum was from cells growing on anthracene and the volume adjusted to give an initial OD_{600} from 0.05 to 0.10, in both control (no surfactant) and experimental flasks. Surfactants were added from a sterile concentrate prepared in growth medium. To determine the effect of changes in surfactant concentration on growth, cultures were diluted into fresh growth media at day 5 (Figure 4).

Sorption experiments

Different amounts of solid anthracene or previously harvested bacteria were added to 125-ml Erlenmeyer flasks, containing 25 ml growth medium and a known amount of Triton X-100. The control flasks contained exactly the same components as the above flasks except no anthracene was added to one series and no bacteria was added to another. The flasks were shaken at 200 rpm on a New Brunswick gyrotary shaker. After 1 day (for anthracene) or 1 hour (for the bacteria), the supernatant in the flasks was centrifuged at 33,000 × g for 1 hour (Sorvall RC-5B Refrigerated Superspeed Centrifuge, DuPont Instrument, Wilmington, Delaware), which eliminated the solid particles of anthracene or bacteria. The surface tensions of the centrifuged supernatant were measured, and used to determine the concentration of free surfactant by a standard curve. By comparing the concentration of surfactant in the supernatant and that of the control, the amount of Triton X-100 that was absorbed by the anthracene or bacteria was calculated. Sufficient anthracene particles or bacteria were added to ensure a

significant increase in the measured surface tension, indicating a decrease in free surfactant concentration.

Results

The critical micelle concentrations (CMCs) of the surfactants in the growth medium were determined from plots of ln(surface tension) versus concentration, at the point of inflection where surface tension became constant with increasing concentration. The CMC of Triton X-100 was 0.26 mM (160 mg/l), similar to previously reported values of 0.24 mM (Stelmack et al. 1999; Laha & Luthy 1992). The CMC of Dowfax 8390 was 0.48 mM (310 mg/l), lower than 0.8 mM as reported by Stelmack et al. (1999), and an order of magnitude lower than the value reported by Rouse et al. (1993). The wide variation in CMC values of Dowfax is due to the different interpretations of the plot of ln (surface tension) versus concentration, which gradually approaches a constant value over a range of concentration. The value of 0.48 mM was selected to ensure that no surfactant micelles were present in this study.

Microbial growth on anthracene

In the absence of surfactant, the *Mycobacterium* sp. grew to a maximum OD_{600} of 0.7 to 0.8 in 14 days, while the *Pseudomonas* sp. reached a density of 0.35 in 8 days. In all cases, the cells grew as a free suspension. Any adhesion of the cells to the surface of the anthracene particles would not give a significant error in OD_{600} measurement due to cell attachment on the low surface area of 50 mg of anthracene crystals. The data of Figure 1 show growth of the *Mycobacterium* sp. on anthracene with concentrations of Triton X-100 from 0.024 mM to 0.096 mM (0.09 CMC to 0.36 CMC). Equivalent experiments with Dowfax 8390 at 0.08 mM (0.17 CMC) and 0.4 mM (0.83 CMC) resulted in no growth. Equivalent results were observed with repeated series of experiments. The *Pseudomonas* sp. gave similar growth curves in the presence of Triton X-100 (data not shown). Linear growth has been reported previously for growth on solid PAH, where slow dissolution does not permit exponential growth (Volkering et al. 1995). While the data of Figure 1 show linear growth from day 2 to day 7 ($r^2 > 0.98$), the growth curves depart from linearity at short times and at longer times. The higher slope during the first 2 days may be due to rapid consumption of water-soluble substrate, while the slower growth after 7 days could be due to lower rates of dissolution of anthracene, due to consumption of particles.

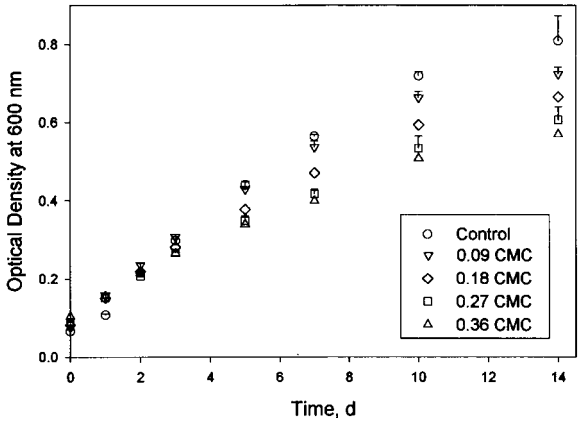

Figure 1. Growth of *Mycobacterium* sp. on anthracene with increasing concentrations of Triton X-100 from 0 (control) to 0.36 CMC (0.096 mM). The data are reported as the arithmetic average of 2 to 4 parallel experiments. The error bars show the standard deviation of the replicates when 3 or more measurements were recorded.

The data of Figure 1 showed that the presence of Triton X-100 inhibited the growth rate of the *Mycobacterium* sp. during the experimental period even when its concentration was as low as 0.09 CMC (0.012 mM). The data of Figure 2 show the linear growth rates for the two bacteria, calculated from the data for OD_{600} from day 2 to day 7 by linear regression. The data for the growth of *Mycobacterium* sp. show that the change in growth rate with increasing surfactant concentration was smaller at surfactant concentrations of about 0.18 CMC. This observation suggested that the system was becoming saturated with surfactant, so that further addition gave less suppression of growth rate. The *Pseudomonas* sp. was more sensitive to the surfactant, as the growth rate was reduced by a factor of three at only 0.09 CMC.

Microbial growth on glucose with surfactant

The data of Figure 3 show the growth of the *Mycobacterium* sp. on glucose with and without added surfactants. The data showed no significant difference between the growth curves on glucose with and without Triton X-100, therefore, Triton X-100 was not toxic to the *Mycobacterium* species. When Dowfax 8390 was added, the *Mycobacterium* species showed no growth, therefore, it was toxic at all concentrations tested below the CMC. The same experiment was repeated with *Pseudomonas* sp., and similar growth

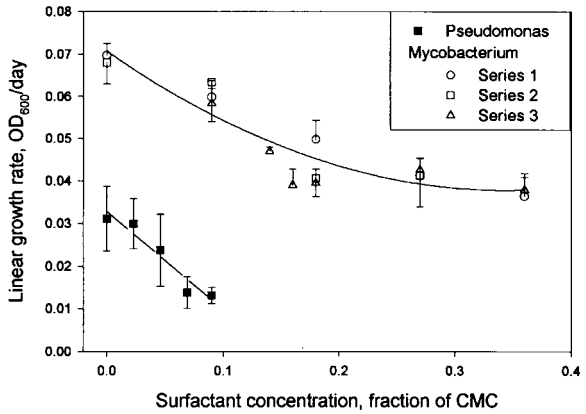

Figure 2. Linear growth rates for *Mycobacterium* sp. and *Pseudomonas* sp. on anthracene as a function of the concentration of Triton X-100. Growth rates were calculated by linear regression from data for day 2 to day 7, from 2 to 4 parallel experiments. The error bars give the 95% confidence interval for the growth rate. Experiments with *Mycobacterium* sp. were repeated with different batches of cells, shown as series 1 through 3. The dashed lines are least-squares regressions to the data; linear for *Pseudomonas* sp. and second-order for *Mycobacterium* sp.

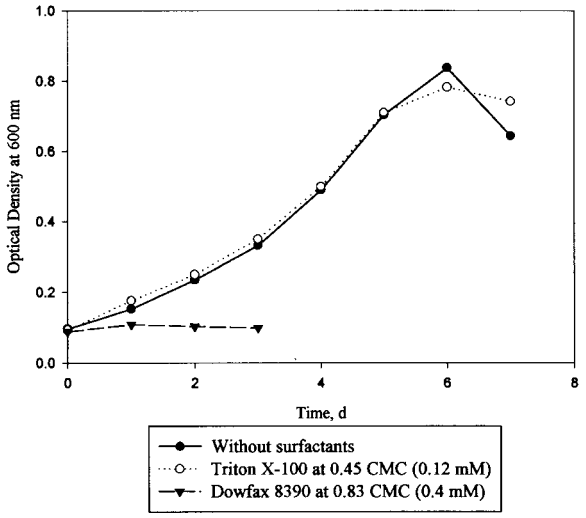

Figure 3. Growth of *Mycobacterium* sp. on glucose without surfactants (control), with 0.45 CMC of Triton X-100 and 0.83 CMC of Dowfax 8390. Data are mean values from 4 replicates.

curves were observed with and without added Triton X-100 (data not shown).

A similar experiment was carried out using Triton X-100 as the sole carbon source. Neither strain showed any growth on surfactant over a period of 7 days. Consequently, the surfactant was neither inhibitory to growth on glucose, nor was it a competitive substrate for growth in the presence of anthracene.

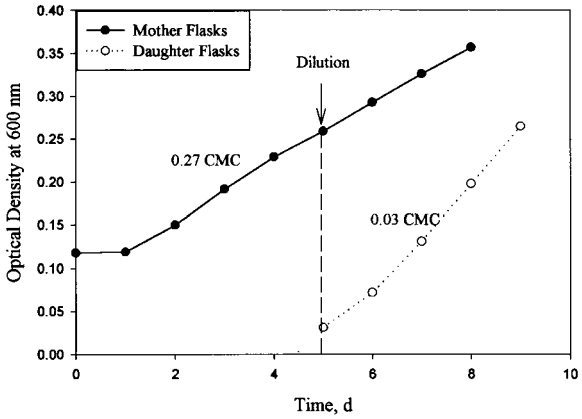

Figure 4. Effect of decreased surfactant concentration on growth of *Mycobacterium* sp. Triton X-100 concentration was decreased from 0.27 CMC to 0.03 CMC on day 5 by diluting the culture. Data are mean values from 2 parallel sets of flasks.

Effect of changes in surfactant concentration during growth

The data of Figure 4 show the effect of dilution of the Triton X-100 concentration from 0.27 CMC (0.072 mM) to 0.03 CMC (0.0072 mM) on the growth rate of *Mycobacterium* sp. on anthracene. The growth rate of the *Mycobacterium* sp. increased from 0.034 OD_{600}/day to 0.065 OD_{600}/day, consistent with the data obtained at a constant initial concentration (Figure 2).

Sorption of surfactants on anthracene and cells

Sorption of Triton X-100 to anthracene was determined at 5 different anthracene concentrations, from 16 to 32 g/L in order to obtain measurable changes in surface tension. The partitioning of surfactant between the anthracene and the growth medium was linear, with a coefficient of 0.34 ± 0.16 μmol surfactant/g anthracene (95% confidence interval). Since the amount of anthracene used in the bacterial growth experiments was only 50 mg, the amount of Triton X-100 sorbed onto the surface of anthracene would be 1.6×10^{-5} mmol, or 0.3% of the total surfactant at 0.18 CMC.

The partition coefficients for anthracene with *Mycobacterium* and *Pseudomonas* were measured by a similar method. The partition coefficients from triplicate determinations were 7.3×10^{-15} mmol/CFU for *Mycobacterium* and 1.1×10^{-15} mmol/CFU for *Pseudomonas* LP6a.

Discussion

The experimental data from this study showed that the rate of growth of the *Mycobacterium* sp. and the *Pseudomonas* sp. on anthracene were inhibited by Triton X-100 at concentrations well below CMC, and the inhibition was reversible. The growth on soluble carbon source (glucose) was not inhibited, nor did the cells grow on surfactant, therefore, the most likely mechanism of interaction was sorption of surfactant onto surfaces. The lack of toxicity of Triton X-100 was consistent with some previous studies, which indicated continued conversion of dissolved substrate in the presence of surfactant (Efroymson & Alexander 1991; Liu et al. 1995). Since sorption of surfactants to either microorganisms or hydrocarbons is a physical process, it should be completely reversible. In this study we observed that the growth rate could be completely and rapidly recovered by dilution with fresh medium. Laha and Luthy (1991; 1992) reported that phenanthrene mineralization by Triton X-100 was recovered by immediately by dilution of surfactant. The present data show reversible inhibition at much lower concentrations, well below the CMC of the surfactant.

Due to its low solubility, most of the anthracene present in this study was in solid phase. Several papers have suggested that growth of attached bacteria on the surface of solid PAH particles may be favored under some conditions (Tongpim & Pickard 1996; Volkering et al. 1998). In the case of liquid hydrocarbons, the addition of surfactant has been shown to prevent adsorption of bacteria at the oil water interface, and thereby suppress degradation of the oil (Aiba et al. 1969; Efroymson & Alexander 1991; Ortega-Calvo & Alexander 1994). Stelmack et al. (1999) observed that both Triton X-100 and Dowfax 8390 interfered with the adhesion of either the *Mycobacterium* sp. or *Pseudomonas* sp. to non-aqueous phase liquids (NAPLs) from contaminated soils. In the same study, they also reported that both surfactants were able to remove the previously bound *Mycobacterium* sp. or *Pseudomonas* sp. from the NAPL surfaces. The inhibition of growth due to surfactant in the present study could be due to changes in cell interaction with the solid PAH.

Neu (1996) suggested that surfactants could change microbial adhesion by adsorbing to the surfaces of microorganism, or to the surfaces of hydrocarbon, or both. This study showed that Triton X- 100 could bind to the *Mycobacterium* sp., to *Pseudomonas* and to the anthracene particles. At typical conditions for this study, the amount of Triton X-100 sorbed to cells was higher than the amount sorbed to anthracene. For example, with a concentration of 0.18 CMC (0.048 mM) and an inoculum of *Mycobacterium* sp. with an OD_{600} reading of 0.1 in 100 mL of medium, four times as much Triton X-100 would be bound to cells as to the anthracene particles. Growth of cells and consumption of anthracene would increase this ratio throughout each experiment. If we assume that the hydrophobic tail of Triton-X100 (0.6 nm $\times$ 0.3 nm) adsorbs to the anthracene and cell surfaces, then at 0.6 OD and 0.18 CMC approximately 20% of the surface of the *Mycobacterium* sp. (0.5 $\times$ 2 μm rod-shaped cells) would be covered by surfactant, while 67% of the anthracene surface (0.7 $\times$ 0.1 mm plates) would be covered. These levels of coverage would be sufficient to significantly alter the hydrophobicity or hydrophilicity of the surfaces, depending on the orientation of the surfactant. The partial saturation of inhibition that was observed for *Mycobacterium* sp. (Figure 2) suggested that the interaction with surfactant was saturable, consistent with a surface coverage mechanism. In general, the surfaces of *Pseudomonas* cells are less hydrophobic than *Mycobacterium*, therefore, if sorption of surfactant is to hydrophobic domains on the cell surface the former species would be more sensitive to surfactant. At a Triton X-100 concentration of 0.09 CMC (0.024 mM) the initial growth rate of the *Mycobacterium* sp. was 76% of its control, while the growth rate of the *Pseudomonas* sp. was only 50% of its control, at comparable OD levels in both cases.

Although physiological changes due to the addition of surfactant cannot be ruled out, the lack of any inhibition of growth on glucose suggests that the most likely role for surfactant was in altering physical interactions with the PAH. If transient adhesion of cells to the solid surface were important in anthracene uptake, then surfactant would suppress growth by inhibiting such contact. The adhesion measurements of Stelmack et al. (1999) showed that these bacteria would adhere to hydrophobic surfaces to a measurable extent, and that this adhesion was inhibited by surfactant. Given that none of the control cultures exhibited attached growth on solid anthracene under the conditions of these experiments, and the surface area of the anthracene particles was always much smaller than the area required to hold the cells, we can rule out attached growth on the PAH surface. Furthermore, Tongpim and Pickard (1996) achieved attached growth

of a biofilm with the same strain only under quiescent conditions, not in shake flasks.

The results of this study, combined with the work of Stelmack et al. (1999), suggest that the dispersion of bacteria from hydrophobic surfaces by surfactants can significantly reduce the rate of growth, even when the bacteria are not growing as a biofilm. As Volkering et al. (1998) point out, bacteria are not normally observed growing on the surfaces of solid substrates such as PAHs, whereas growth at the interface of hydrophobic liquids is more common. The physical properties of NAPL in soil can be expected to range from liquid to high-viscosity semi-solid due to factors such as weathering and prior biodegradation. Consequently, dispersion of bacteria from NAPL surfaces by sub- CMC concentrations of surfactant may inhibit growth, and concomitant bioremediation, for a range of NAPL properties.

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