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Effect of nonionic surfactant addition on bacterial metabolism of naphthalene: Assessment of toxicity and overflow metabolism potential

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Abstract

Two factors potentially accounting for the variability of bioremediation outcomes when surfactant micelles are used to increase polycyclic aromatic hydrocarbon (PAH) bioavailability were investigated: (1) surfactant toxicity and (2) the link between microbial metabolism and the intended effect of surfactant addition, enhanced solubilization and mass transfer from a solid phase. The nonionic surfactant, octaethyleneglycol mono *n*-dodecyl ether, did not alter the metabolism of succinate and glucose by an isolate from a creosote-contaminated soil indicating that the surfactant is nontoxic. When the culture was supplied with solid naphthalene, growth was limited by the dissolution of solid naphthalene after the aqueous-phase naphthalene was depleted. Moreover, increasing dissolution rate by increasing interfacial surface area increased the microbial growth rate. However, increasing bioavailability further by increasing interfacial surface area, introducing convective mass transfer, and adding surfactant were all found to reduce growth rate and prompt incomplete metabolism of naphthalene to a compound whose UV absorption corresponds to 1,2-naphthaquinone. Lowering the surfactant concentration diminished the metabolic overflow and permitted sustained growth. The results suggest that different mismatches between solubilization/mass transfer and metabolic capacity may be among the factors responsible for variable bioremediation outcomes.

Keywords: Nonionic surfactant; Biodegradation; Overflow metabolism; *Pseudomonas*

1. Introduction

Polycyclic aromatic compounds are common environmental contaminants, many of which are suspected carcinogens and mutagens [1]. Polycyclic aromatic hydrocarbons (PAHs) are a subset of this class of compounds which have been

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demonstrated to be susceptible to biodegradation by soil microbes [2–14]. However, the low aqueous solubility and tendency for slow desorption from soil greatly limits the bioavailability of these compounds [5–16]. Low bioavailability is seen as a major obstacle in the microbial treatment of solid wastes and contaminated soils.

Surfactants have proven to be effective for stripping sorbed PAHs from contaminated soils thereby promoting mass transport from the solid to aqueous phase [14, 17–19]. In several laboratory investigations, adding surfactant was found to increase both the rate and extent of degradation [9, 14, 20–23]. However, counterexamples have also been found where the presence of surfactant micelles slowed degradation [7–9, 14]. Explanations for such results include: surfactant micelles may interfere with cellular processes, surfactant degradation may compete with PAH degradation, and the diversity of microbial populations may lead to different outcomes. Overall, surfactants are viewed as being potentially useful for aiding the bioremediation of PAH-contaminated sites if issues such as variable efficacy can be resolved.

We investigated two factors that potentially account for the variability of bioremediation outcomes when surfactant micelles are used to increase PAH bioavailability: (1) surfactant toxicity and (2) the link between microbial metabolism and the intended effect of surfactant addition, enhanced solubilization and mass transfer from a solid-phase. A *Pseudomonas* isolate, as opposed to a mixed microbial population, was used to suppress the effects of interspecies interactions. The isolate was provided with solid phase PAH. Thus, as in a field situation, biodegradation rate can be potentially limited by interphase mass transport. For fixed initial biodegradation capacity (microbial concentration), PAH bioavailability was modulated by altering interfacial surface area, introducing convection, and/or adding surfactant. Naphthalene was chosen as a representative PAH to facilitate comparisons to prior studies (e.g. Ref. [5]) and because it is frequently present in contaminated soils [24]. The nonionic surfactant, octaethyleneglycol mono *n*-dodecyl ether, was used because it has been extensively used in bioremediation trials and unlike ionic surfactants, nonionic surfactants interact less with soils [8, 17–19].

Our results show that after the aqueous-phase naphthalene was depleted, growth can be limited by solid naphthalene dissolution, as reported by others [5]. Moreover, increasing interphase mass transfer rate increased microbial growth rate. However, further enhancement of the mass transfer rate or adding surfactant resulted in metabolic by-product accumulation and reduced growth rate. These results suggest optimum surfactant doses may exist relative to the microbial population density present and extent of PAH bioavailability. Moreover, the variability of prior degradation studies may conceivably reflect, in part, different initial values of solubilization and biodegradation capacity.

2. Materials and methods

2.1. Materials

A pure *Pseudomonas* strain was obtained from the Environmental Microbiology Center at the Carnegie Mellon Research Institute. This strain was isolated from

a mixed naphthalene degrading population obtained from contaminated soil present at a creosote production facility. A seed culture was kept in a 20% glycerol solution at 0 °C. Fresh inoculum was grown for each trial by placing 1.0 ml of thi seed culture in 20 ml of fresh medium containing solid naphthalene. The culture was then grown to an optical density (at 550 nm) of 1.0 before being used as inoculum. Experiments were conducted using a minimal medium containing (per liter): 3.0 g K_2HPO_4, 1.5 g KH_2PO_4, 0.01 g NaCl, 0.1 g $MgSO_4$, 0.001 g $FeSO_4 \cdot 7H_2O$, and 1.25 g $(NH_4)_2SO_4$.

Solutions were prepared with deionized filtered water. The nonionic surfactant octaethyleneglycol mono *n*-dodecyl ether (polyethylene oxide block copolymer "$C_{12}E_8$" 99.9 + %purity) was purchased from Nikkol Chemical (BL-8SY). Naphthalene (BDH Micro-Analytical Reagents 99 + % purity) was chosen as a model PAH for this study.

Trials were inoculated with 1.0 ml culture in 50 ml of media. Naphthalene and surfactant were added several days prior to inoculation to allow the system to reach equilibrium. Experiments were conducted in sealed 125 ml Erlenmeyer flasks to minimize abiotic naphthalene losses.

2.2. *Instrumentation*

Absorbance measurements were obtained using a Hewlett-Packard 8451A Diode Array Spectrophotometer. Samples were placed in a 10 mm silica spectrophotometer cell and referenced to pure water. When absorbance exceeded a threshold value (1.0 for naphthalene and by-products, 0.4 for cell concentration) samples were diluted with the reference solution. The wavelengths monitored for cell concentration, naphthalene, and by-product concentrations were 550, 275, and 350 nm, respectively. Samples were filtered through a 0.20 μm PTFE filter to remove cells prior to measuring naphthalene and by-product concentrations. The absorbance at 550 nm for the filtered sample was subtracted from each unfiltered sample's absorbance to account for the contribution of extra-cellular material on cell concentration measurements. When naphthalene concentrations are reported from absorbance measurements, they are based on an extinction coefficient of 4.61×10^3 M^{-1} cm^{-1}.

3. Results and discussion

Fig. 1 shows cell growth on solid naphthalene (11.0 mg/50 ml; 6.25 times the aqueous saturation limit). Initially, the culture displayed exponential growth on the saturated aqueous phase. Thereafter, cell mass concentration became linear with time. The transition from exponential growth to pseudo-zero-order behavior corresponds well to other reports and model predictions [5, 6]. The behavior can be attributed to the high initial naphthalene concentration (aqueous solubility limit) resulting in rapid initial microbial growth. The increased cell mass depletes the aqueous-phase naphthalene and thereafter growth is restricted by mass transfer from the solid to liquid phase.

To observe the effect of increased mass transfer independently from the effects of surfactant addition, the dissolution rate was varied two ways. First, the rate of mass

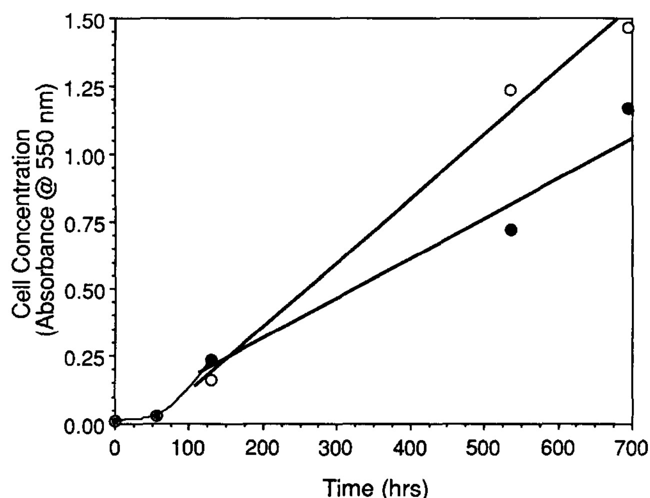

Fig. 1. Exponential growth on saturated aqueous phase naphthalene followed by dissolution rate-limited growth. Conditions correspond to low (●) and increased (○) mass transfer when crystals were finely divided.

transfer was increased by finely dividing the naphthalene crystals to increase interfacial surface area. As Fig. 1 shows, the growth rate in the linear regime increased, thus indicating that although the mass transfer rate was increased, it still limited the rate of biomass increase. The mass transfer rate was then increased further by intensely stirring a suspension containing finely divided naphthalene crystals. In this case (see Fig. 2), the growth in the linear regime was initially much higher (compared to the unstirred large crystals which are also shown), but then dropped off significantly. This reduction in the growth rate was accompanied by the appearance of a distinct yellow-tan coloration. As the coloration increased, it took on an orange hue.

The conditions providing for dissolution-limited growth were replicated to allow for the effect of surfactant addition to be determined. Fig. 3 shows the effect of surfactant addition (1.7 and 8.3 g/l; 44 and 217 times the critical micelle concentration) on growth. For 1.7 and 8.3 g/l surfactant, the total naphthalene initially solubilized in the aqueous and micellar pseudophases was 100 and 430 mg/l, respectively. The yellow/orange coloration appeared almost immediately and growth rates were significantly reduced. Moreover, the extent of growth rate reduction increased with surfactant concentration.

It is possible that surfactant toxicity was responsible for the reduction in growth rate that accompanied surfactant addition. Thus, a control experiment was performed where the culture was grown on glucose or succinate in the absence and presence of surfactant (5 g/l). The surfactant was found to have no effect on growth rate for both carbon sources (not shown).Thus, the deleterious effect of surfactant addition on growth rate would appear to be linked to altering the bioavailability of naphthalene and not surfactant toxicity.

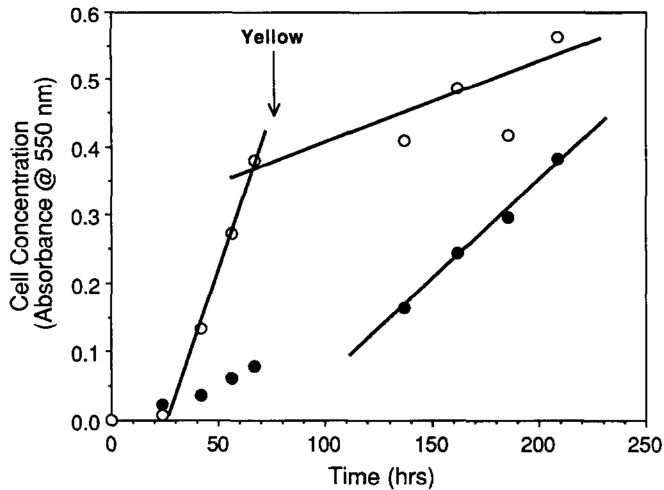

Fig. 2. Increasing mass transfer further by finely dividing the naphthalene crystals and intensely stirring the suspension leads to initially higher growth rates. Thereafter, a yellow/orange-colored by-product becomes visible at 75 h accompanied by reduced growth (arrow). Conditions correspond to low (●; unstirred large crystals) and greatly increased (○) mass transfer.

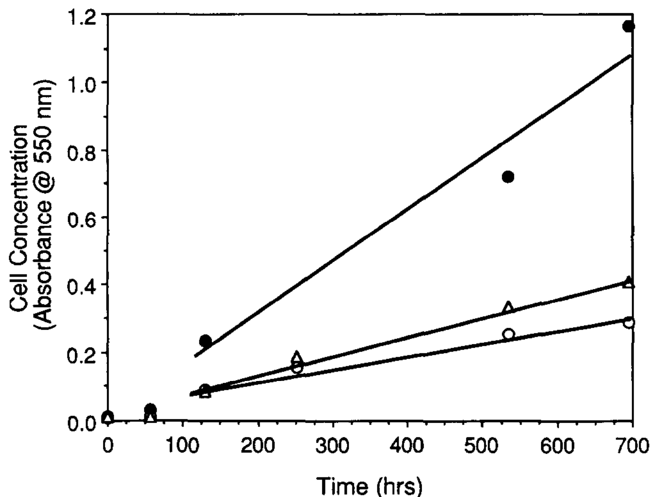

Fig. 3. Addition of surfactant results in almost immediate appearance of colored by-product and significantly inhibited growth. Conditions correspond to low mass transfer (●; data replotted from Fig. 1) and the addition of 1.7 (△) and 8.3 g/l (○) surfactant.

To ascertain the source of the coloration that develops when surfactant is added or mass transfer rate increased, the pathway for naphthalene metabolism was examined. Fig. 4 shows an early branch point in naphthalene metabolism by *Pseudomonas* [2]. Prior workers have observed that the intermediate, 1,2-dihydroxynaphthalene,

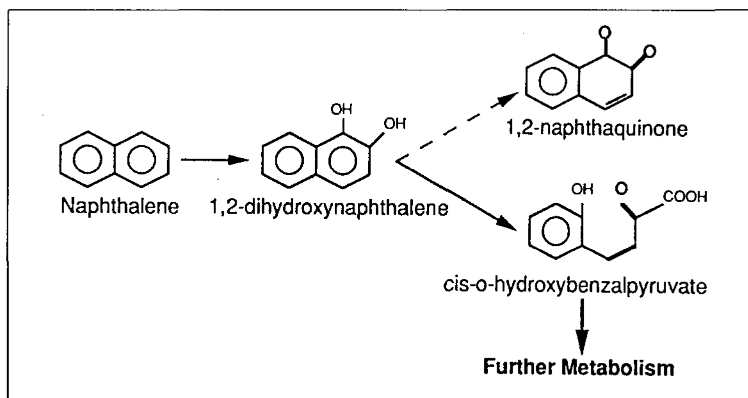

Fig. 4. Initial steps in metabolism of naphthalene by *Pseudomonas*. The 1,2-dihydroxynaphthalene intermediate is rapidly nonenzymatically converted to 1,2-naphthaquinone or enzymatically converted to *cis-o*-hydroxybenzalpyruvate.

is nonenzymatically oxidized to 1,2-naphthaquinone at a rate of approximately 20% per min [2]. Quinones also characteristically impart an orange color to an aqueous solution [3, 4]. Moreover, 1,2-naphthaquinone is not metabolized and its presence can interfere with naphthalene degradation [2–4]. The cleavage of the 1,2-dihydroxynaphthalene to the other branch point product, *cis-o*-hydroxybenzalpyruvate, is catalyzed via a Fe^{2+} ion-dependent enzyme. It has been established that sufficient enzyme to complete the cleavage in 1–2 min is required to avoid the irreversible formation of 1,2-naphthaquinone [2].

The absorption spectrum of the supernatant (see Fig. 5) is consistent with the presence of 1,2-naphthaquinone [26]. Light absorption occurs at wavelengths higher than those characteristic of naphthalene (major bands below 310 nm in most solvents) and corresponding to 1,2-naphthaquinone (absorbs at 250, 340, and 405 nm). The presence of a by-product in the culture medium plus the nontoxicity of the surfactant indicates that surfactant addition can result in overwhelming the cells' capability to metabolize naphthalene. Naphthalene utilization, in turn, can become uncoupled from cell mass increase. Moreover, based on prior reports, growth may also ultimately become inhibited as 1,2-naphthaquinone accumulates [4].

To explore further the relation between mass transfer, surfactant presence, and metabolism, the time dependence of naphthalene, by-product, and cell concentrations were determined. The surfactant dose was also lowered to 1 g/l to investigate whether by-product accumulation could be controlled better than in the aforementioned experiment (Fig. 3). In this case, the total naphthalene initially solubilized in the aqueous and micellar pseudophases was 65 mg/l.

Fig. 6 shows the outcome of experiments when large crystals with no stirring (low mass transfer) were used and surfactant was either present or absent. The initial total naphthalene concentration present in the aqueous and micellar pseudophases was 63 mg/ml when surfactant was present and 30 mg/l when surfactant was absent. The

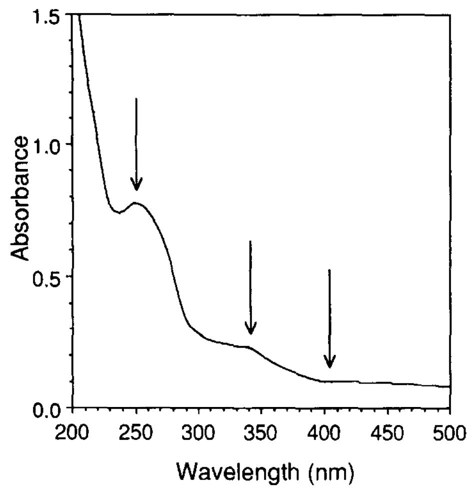

Fig. 5. Absorbance spectra of colored by-product. 1,2-naphthaquinone has absorbance peaks at 250, 340, and 405 nm, which correspond to the by-product's spectra as shown (arrows).

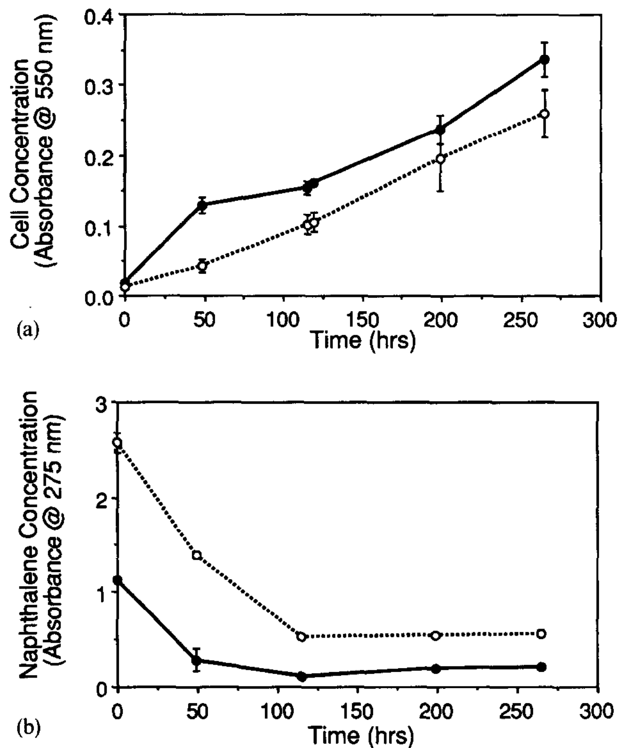

Fig. 6. Effect of surfactant addition on naphthalene degradation when low mass transfer from solid prevails and less surfactant is added. Large naphthalene crystals and no agitation constitutes the low mass transfer case (●). To the same system, 1.0 g/l surfactant was added to alter solubilization (○). The time dependence of (a) cell density, (b) total aqueous-phase naphthalene concentration, and (c) by-product formation are shown. By-product formation is linked to high aqueous-phase naphthalene concentration and heightened production is associated with surfactant addition.

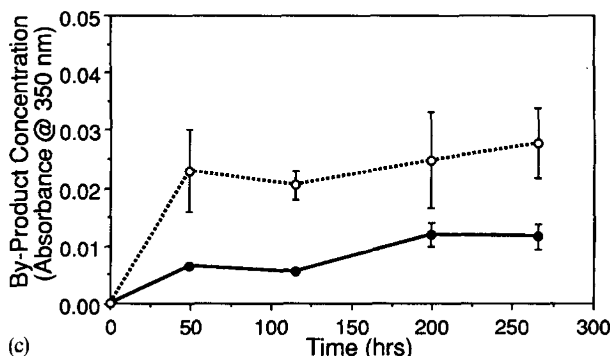

Fig. 6. Continued

error bars depict the standard deviations from the average values of several replicates. The standard deviations are small compared to the differences observed between the surfactant-present and surfactant-free trials indicating that the trends are reproducible and surfactant addition significantly affects naphthalene metabolism.

Growth was sustained when surfactant was absent or present (Fig. 6(a)). However, compared to the surfactant-free case, the presence of surfactant lowered the initial accumulation of cells (Fig. 6(a)). In both cases, the high initial naphthalene concentrations appeared to prompt significant by-product formation during the first 50 h (Fig. 6(c)). However, the initial by-product formation was greater when surfactant was present (Fig. 6(c)). After 50 h when the microbes had accumulated and lowered the total naphthalene concentration (Fig. 6(b)), the by-product production tapered off (Fig. 6(c)). Thus, it appears that enhancing solubilization by surfactant addition can overwhelm microbial metabolism, especially at the outset of biodegradation. However, the fact that lowering the surfactant dose ultimately allowed for both growth and degradation to be sustained suggests that solubilization-limited biodegradation may be manageable. Namely, better control of surfactant addition can maintain naphthalene mineralization while keeping by-product accumulation in check.

4. Conclusions and remarks

Our results showing the growth and biodegradation rates can be enhanced by increasing the rate of solid naphthalene dissolution are in accord with those from other studies [5]. However, increasing interfacial surface area or adding surfactant reduced the microbial growth rate. The reduction in growth rate cannot be attributed to surfactant toxicity because growth on succinate or glucose was unaffected by surfactant presence. Rather, incomplete metabolism of naphthalene to a by-product occurs which, based on prior reports in the microbiology literature and our UV

absorption measurements, appears to be 1,2-naphthaquinone. It is noteworthy that another naphthalene-degrading isolate (displays a different colony phenotype) also exhibited virtually the same response to surfactant addition (data not shown). Thus, the results do not appear to be unique to a particular isolate.

The incomplete metabolism of naphthalene is an example of "overflow metabolism" which occurs when carbon compound abundance is high and bottlenecks exist in metabolism. Such metabolism leads to the formation of incompletely metabolized by-products which can become inhibitory as they accumulate. The aerobic production of acetate from glucose is a classic example of overflow metabolism. An enzymatic limitation in the Krebs cycle has been proposed to lead to incomplete glucose oxidation and acetate overflow [25].

The increased susceptibility of naphthalene-degrading microbes to overflow metabolism when surfactant is present has several implications worthy of further investigation. When naphthalene bioavailability is enhanced, growth can become decoupled from naphthalene utilization due to by-product formation. Thus, monitoring residual naphthalene concentration or CO_2 evolution from labeled naphthalene may not always fully reflect the status or efficacy of a bioremediation process. Moreover, predictive mathematical models based on constant yield coefficient (g cell produced/ g naphthalene used) may not be able to capture bioremediation dynamics in all circumstances. Finally, if the by-product can accumulate to an inhibitory level, remediation time could be extended or the ultimate extent reduced. The likelihood of this deleterious effect would increase when "hot spots" are subjected surfactant-mediated bioremediation.

The results and implications merit consideration when identifying the sources of potential variability in prior laboratory experiments and field trials. When saturated soil systems are investigated, the relative amounts of water, soil, PAH, and surfactant will dictate how much PAH is available to the microbial population. For fixed PAH loading, increasing the surfactant dose relative to the soil could, for example, increase PAH bioavailability to the point where the microbial density present is incapable of completely transforming 1,2-dihydroxynaphthalene to *cis-o*-hydroxybenzalpyruvate. Consequently, different initial conditions may result in varying degrees of inhibition and/or coupling between growth and naphthalene use thereby altering the outcomes reported.

In summary, we examined the effect of nonionic surfactants on naphthalene biodegradation. Two factors were investigated: (1) surfactant toxicity and (2) the link between microbial metabolism and the intended effect of surfactant addition, enhanced solubilization and mass transfer. The results indicate that the retardation of PAH biodegradation by surfactant addition may not always be attributable to surfactant toxicity. Rather, increasing interfacial surface area or adding surfactant can increase solubilization and mass transfer to the extent that naphthalene is supplied in excess of what the cells' metabolic capacity can manage. The resulting metabolic overflow decouples growth from carbon source utilization and produces a by-product reported to be recalcitrant and inhibitory [4]. Thus, mismatching solubilization/mass transfer and metabolic capacities may be one of the factors contributing to the variability of past bioremediation trials.

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