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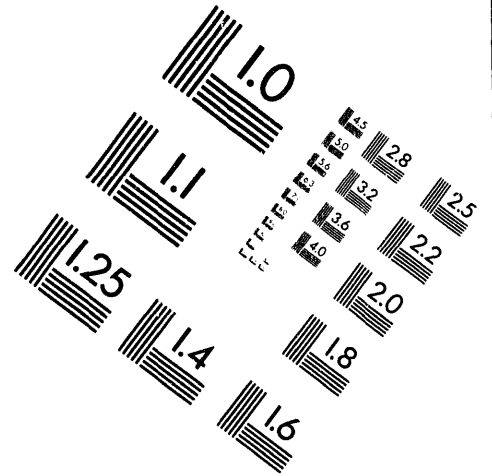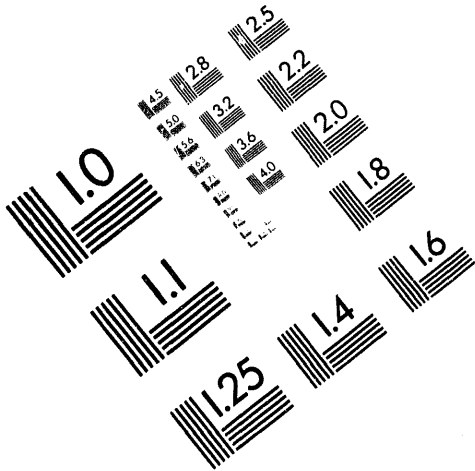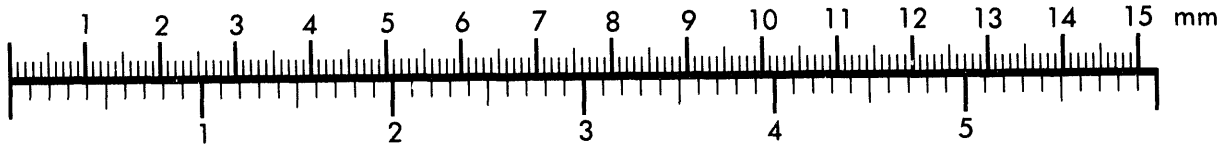
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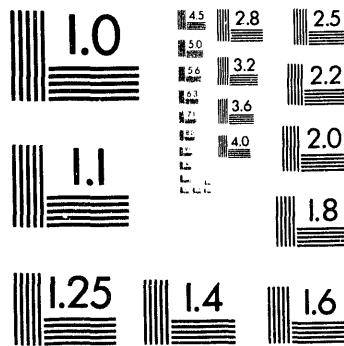
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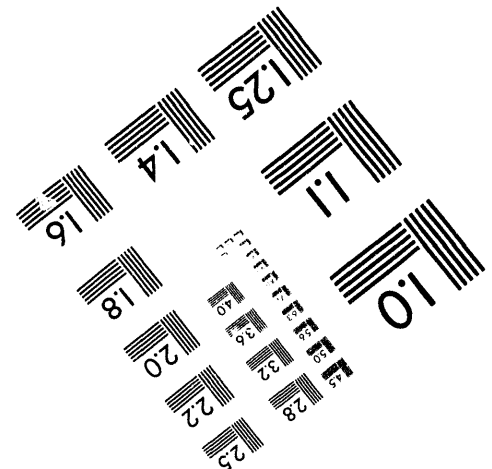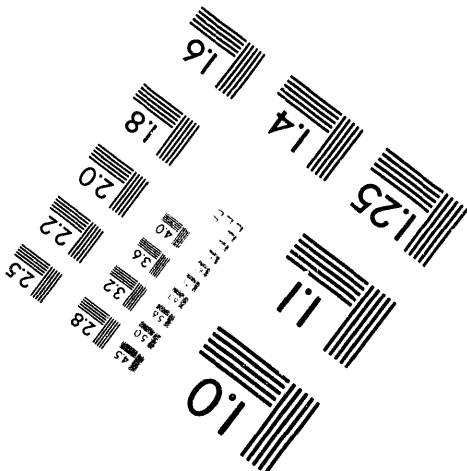
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Effect of the Bioemulsifier Emulsan on Naphthalene Mineralization from Coal Tar in Aqueous Systems

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ABSTRACT

Coal tar in aerobic aqueous systems was treated with purified emulsan, the anionic heteropolysaccharide bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1; with inocula of various concentrations of stationary phase RAG-1 cells; or with cell-free broth from stationary phase RAG-1 cultures. Naphthalene mineralization by a mixed PAH-degrading population was measured by recovering $^{14}CO_2$ evolved during biotransformation of the $[^{14}C]$naphthalene-labeled coal tar. There was no evidence of naphthalene mineralization by RAG-1 cells alone. The addition of emulsan, RAG-1 inocula, or cell-free broth to systems containing the PAH-degrading population did not significantly affect naphthalene mineralization in any of the systems tested. Coal tar in these experiments was present either as a free dense nonaqueous phase liquid (DNAPL), or as DNAPL imbibed into microporous silica particles. Emulsification of the tar was not observed in either case. The presence or absence of microporous silica did not affect the extent or rate of naphthalene mineralization, nor did the concentration of RAG-1 inocula or the amount of broth added. The addition of cell-free broth, emulsan, or RAG-1 cells late in the experiments did not yield significantly different results compared to initial addition of these substances. Thus, emulsan and related fractions from RAG-1 cultures were ineffective in altering naphthalene mineralization in this study.

KEYWORDS — Emulsan   Coal tar   Naphthalene

Mineralization   Bioemulsifier

INTRODUCTION

Coal tar is a dense nonaqueous phase liquid (DNAPL) by-product of coal carbonization. During carbonization, coal is exposed to high temperatures and may be reacted variously to produce a combustible gas, hydrocarbon liquids, coke, or tar [1]. Coal tar is a complex mixture, with over 400 compounds having been identified in various tars [2]. However, most coal tars consist primarily of highly hydrophobic aromatic compounds ranging in size from single-ring benzene structures to complex, multi-ring polycyclic aromatic (PAH) compounds [3].

The accidental release of coal tar into the environment and the subsequent penetration of tar into the subsurface have resulted in soil and groundwater contamination by PAH compounds and other coal tar constituents at certain former manufactured gas plant sites. PAH compounds have low aqueous solubilities, which may affect the rate of their biodegradation in soil/water systems [4], and they exhibit the ability to persist and bioaccumulate in nature [5]. Aerobic biodegradation of 2- and 3-ring PAH compounds has been widely observed, although the transformation rate for 2-ring substrates, such as naphthalene, is typically greater than that for 3-ring compounds, i.e., anthracene or phenanthrene [6]. The PAH-degrading organisms used in this study have tentatively been identified as a consortium of *Pseudomonas* species. Pseudomonads are widespread in soil and water and possess enzyme systems capable of transforming a broad range of natural organic compounds and xenobiotics, even those with very different structures [7].

One of the most important factors affecting biodegradation is the availability of substrate to microorganisms. Three modes of hydrocarbon transport to bacterial cells have been postulated [8]. The first is the interaction of bacteria with hydrocarbon dissolved in the aqueous phase. The second is the interaction of cells with solubilized or pseudosolubilized hydrocarbon droplets much smaller than the cells, in which case hydrocarbon microdroplets attach to cells rather than vice versa. The third is direct contact between cells and large hydrocarbon droplets, with uptake thought to occur through diffusion or active transport by the cells. Substrate availability is especially significant for bioremediation of highly hydrophobic mixtures such as coal tar, which may preferentially partition into soil or other hydrophobic phases present, or remain underground

as a dense nonaqueous phase liquid (DNAPL) [9]. Substrate availability from coal tar is affected by the release of solute from the coal tar phase, and by solute partitioning into the soil phase, which attenuates the transport of hydrophobic organic compounds through the subsurface. These processes may limit the fraction of substrate present in the bulk aqueous phase, where microbial uptake is thought to predominate [10,11]. This in turn may decrease the biodegradation rate compared to tar-free or soil-free systems with the same quantity of contaminant.

Biosurfactants and bioemulsifiers

Biosurfactants and bioemulsifiers are two classes of compounds that could prove useful in counteracting substrate insolubility or sorption, and enhancing hydrocarbon biodegradation in the aqueous phase. The addition of surfactants to soil-water systems has been proposed as a means of desorbing hydrophobic contaminants from soil and solubilizing them through incorporation into surfactant micelles [12]. One disadvantage of adding synthetic surfactants in situ may be possible toxicity or inhibition of microorganisms responsible for biodegradation, or use of the surfactant as substrate in preference to the target hydrocarbon. However, the production of biosurfactants and bioemulsifiers by hydrocarbon-utilizing bacteria is well documented [8], and toxicity or inhibition by these substances may be less of a concern [13]. The increase in surface area of the hydrocarbon-water interface resulting from hydrocarbon emulsification by these bioproducts could enhance the availability of hydrophobic contaminants to bacteria and promote biodegradation in the aqueous medium.

Biosurfactants are classed as glycolipids, phospholipids, neutral lipids, fatty acids, polysaccharides and lipopeptides [14]. They function by lowering the interfacial tension between water and hydrocarbon compounds, thus permitting emulsification of the hydrocarbons into the water phase [13]. Bioemulsifiers, on the other hand, are usually large biopolymers such as polysaccharides that do not reduce interfacial tension appreciably [13,14] but instead bind tightly to the water-oil interface and prevent oil droplet coalescence [13]. Emulsan, a nontoxic bioemulsifier

produced by the gram-negative bacteria *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012), was used in this study.

Emulsan is the extracellular form of a polyanionic, cell-associated heteropolysaccharide (molecular weight $\sim 10^6$) produced by the RAG-1 organism [15,16]. Although emulsan exhibits specificity regarding the hydrocarbons it emulsifies, it is excreted into the growth medium even when RAG-1 is grown on water-soluble substrates such as ethanol or acetate [15]. The bioemulsifier's amphipathic nature is due to its hydrophilic polysaccharide backbone with covalently-linked fatty acid ester side chains, which comprise approximately 15% of the polymer by weight [15]. The polysaccharide portion of the molecule contains the principal sites responsible for its emulsifying activity and is composed of two major sugars, N-acetyl-D-galactosamine and an N-acetyl hexosamine uronic acid [16]. D-glucose is a minor sugar component, comprising 5.2% of the molecule by weight [17]. Emulsan also contains 15 to 20% (wt/wt) noncovalently bound protein [16], which is not necessary for emulsification but does enhance the biopolymer's activity. An approximate structure has been shown for emulsan [13].

The production of emulsan by RAG-1 and the bioemulsifier's role in RAG-1 growth and adherence are both growth-associated [18]. Emulsan initially accumulates on the RAG-1 cell surface as a cell-associated minicapsule and is released as an active emulsifier during conditions of unbalanced growth or as the cells approach stationary phase [16,19]. Cell-associated emulsan capsules create a hydrophilic environment at the RAG-1 cell surface and preclude adherence to hydrocarbons, while cell-free emulsan orients itself at the hydrocarbon-water interface to form a hydrophilic film around oil droplets, effecting emulsification [20]. The stability and physical characteristics (i.e. droplet size and emulsan film thickness) of emulsions stabilized by emulsan depend upon the relative, not absolute, concentrations of emulsan and hydrocarbon [21], indicating that the bioemulsifier interacts directly with hydrocarbon rather than simply altering surface tension [18].

Emulsan is optimally active when a mixture of aromatic and aliphatic hydrocarbons is present [22]. For example, hexadecane stimulates emulsification of many PAH compounds that by

themselves are not emulsified by emulsan, while aromatic compounds including 1-methylnaphthalene, 2-methylnaphthalene, toluene, and *p*-xylene induce emulsification of aliphatic compounds. Furthermore, 1-methylnaphthalene was found to enhance slightly the emulsification of naphthalene, phenanthrene, and biphenyl by emulsan, despite the fact that none of these compounds alone is emulsified [22]. Because 1-methylnaphthalene was a major identifiable component of the coal tar used in this research (Table 1), the potential existed for it to affect naphthalene emulsification and mineralization in the systems being studied.

Emulsan's role in hydrocarbon biodegradation by species other than RAG-1 is unclear but is probably substrate- and species-specific. Several studies have examined the bioemulsifier's effect on hydrocarbon biodegradation by other microorganisms. One study [23] found that marine microcosms containing fuel oil and 0.015% emulsan showed increased microbial respiration and increased numbers of hydrocarbon-degrading bacteria compared to systems without emulsan, although increased respiration may have resulted in part from emulsan degradation. Another study [20] used 0.005% emulsan with both pure and mixed bacterial cultures. Emulsan was found to inhibit alkane mineralization (n-hexadecane and pristane) by six pure cultures. Aromatic mineralization (phenanthrene and carbazole) by two pure cultures of PAH-degrading organisms was either slightly stimulated or unaffected by emulsan, while aromatic mineralization in crude oil by a mixed population was inhibited. Clearly, emulsan's effect on hydrocarbon biodegradation under laboratory conditions depends upon the microorganisms and the hydrocarbon substrate being considered.

Practical use of emulsan in science and industry is apparently still limited. However, it has been suggested [20] that emulsan's ability to stabilize emulsions of hydrocarbons in water could prove useful in the petroleum industry, where it could be used to form heavy oil-water emulsions for viscosity reduction during pipeline transport. Emulsan may also find a place in microbiological studies as an antiadherence agent for desorbing bacteria that adhere vigorously to hydrophobic surfaces [24].

This study assesses whether the bioemulsifier emulsan or related bioproducts affect naphthalene mineralization from coal tar in aerobic aqueous systems. Batch systems containing coal tar either as a free-phase DNAPL, or as DNAPL imbibed in microporous silica were studied. Purified emulsan, RAG-1 organisms, or cell-free RAG-1 culture medium was added to these systems. The results of this work intend to show whether emulsan or related substances are able to significantly enhance naphthalene mineralization by PAH-degrading soil bacteria under laboratory conditions.

MATERIALS AND METHODS

Organisms and culture conditions

The naphthalene-degrading culture used in this study consisted of a mixed population of PAH-degrading organisms that had been isolated from contaminated soils and wastes [9]. The bioemulsifier-producing strain used was *Acinetobacter calcoaceticus* RAG-1, which produces emulsan, an extracellular amphipathic heteropolysaccharide bioemulsifier [16].

The PAH-degrading organisms were grown aerobically in autoclaved BOD dilution water [25] with crystalline naphthalene (purity >98%, Fisher Scientific, Fair Lawn, NJ) as the sole carbon source. The BOD dilution water was amended with fifty times the suggested NH_4Cl concentration to prevent NH_3-N-limited growth conditions. Cultures were kept at room temperature on an orbital shaker (Lab Line Orbital Shaker, Model 3590, VWR Scientific Co.) and shaken continuously at 50 rpm.

PAH-degrading organisms were enumerated by spreading 0.2 mL aliquots of 10^{-4} to 10^{-7} culture dilutions on duplicate agar plates. The plates were prepared using high-nitrogen BOD dilution water with 1.5% (w/w) agar (Difco Bacto-Agar, Difco Laboratories, Detroit, MI). After the agar was spread with culture, the petri dishes were inverted and crystalline naphthalene was placed on the inside of the lid. Vapors from the solid naphthalene entered the agar, providing a carbon source for the organisms. After four days or longer, colonies were identifiable on the agar surface. The PAH-degrading bacteria typically grew to ~10^7 CFU/mL.

The bioemulsifier-producing RAG-1 organisms were grown on an ethanol-minimal salts medium containing (per liter of water): $K_2HPO_4 \cdot 3H_2O$, 22.2 g; KH_2PO_4, 7.26 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 4 g; ethanol, 20 g [15]. The cultures were maintained in flasks at room temperature and were mixed on electromagnetic stir plates (15-point stirrer, Cole Palmer Instruments, Chicago, IL) set to stir for sixty seconds every five minutes. These organisms were enumerated by spreading 0.2 mL aliquots of 10^{-5} to 10^{-9} culture dilutions on nutrient agar (BBL Nutrient Agar, Becton Dickinson Microbiology Systems, Cockeysville, MD). Cream-colored colonies appeared within 3-4 days, and the RAG-1 bacteria typically grew to ~10^9 CFU/mL in liquid medium. The medium changed color from clear to opaque yellow during the two weeks following inoculation, and vigorous shaking of the culture resulted in the formation of froth.

Mineralization tests

Aerobic biodegradation of naphthalene was studied in closed systems using radiolabeling techniques. The systems were contained in 250-mL biometer flasks (Bellco Glass, Inc., Vineland, NJ). The flask side of the apparatus held bacteria in a liquid medium, while the glass side tube contained 5 mL of 2 M NaOH solution, which was used to capture CO_2 evolved by the PAH-degrading organisms. Both the flask and side tube openings were sealed with Neoprene stoppers, which were removed only for sampling and weekly aeration with oxygen.

Mineralization tests were performed using coal tar as the sole carbon source. The coal tar used was obtained from the subsurface at the site of a former manufactured gas plant site in Stroudsburg, PA [26]. A partial composition of Stroudsburg coal tar is shown in Table 1. Prior to use, the coal tar was radiolabeled by adding a measured volume of naphthalene-1-^{14}C (Sigma Chemical Co., St. Louis, MO) stock solution. Approximately 0.9 - 2 µCi of [^{14}C]naphthalene, corresponding to ~2×10^6 - 4×10^6 dpm, was added per milliliter of coal tar.

Radiolabeled coal tar was added to biometer flasks either as a separate liquid phase or imbibed in microporous silicon dioxide, which was used to simulate microporous soil particle aggregates. The silica had a median particle diameter of 236µm, a pore diameter of ~200 Å and a porosity of 0.78 (IMPAQ RG 20150Si granular chromatographic silica, PQ Chromatography

Products, Valley Forge, PA). The silica was pretreated by heating in a furnace at 800°F for two hours to cross-link surface hydroxide groups and slightly increase the material's hydrophobicity, thus enhancing its ability to imbibe and hold the coal tar. After cooling, five-gram portions of silica were weighed and placed in 50 mL Pyrex centrifuge tubes. One milliliter of radiolabeled coal tar was added to each tube, and the tubes were capped and mixed until the coal tar was evenly distributed among the silica granules. The tubes were placed on a rotator for 24 hours to ensure thorough mixing, then put aside for at least a week to allow the coal tar to be imbibed by the silica.

After a week or more, 5 gram samples of tarred silica were placed in biometer flasks with 50 mL of sterile nitrogen-enriched BOD dilution water. The pH was adjusted to 5.5-6.0 and readjusted the following day if necessary. Systems containing coal tar without silica were treated similarly and contained 1 mL of labeled tar in 50 mL of BOD dilution water. All biometer tests were performed at least in duplicate.

At the beginning of each experiment, biometer flasks were inoculated with PAH-degrading organisms, bioemulsifier-producing RAG-1 bacteria, or a combination of both. The inocula consisted of 2 mL aliquots of the respective cultures. PAH-degrading inocula contained ~10^7 CFU/mL, while RAG-1 inocula were used at approximately 10^7, 10^8, 10^9, and 10^{10} CFU/mL. Variations in the RAG-1 inoculum concentration were achieved either by diluting the original culture, or by centrifuging the culture and resuspending pelleted cells in a smaller volume of sterile BOD dilution water. Control flasks were not inoculated and were used to verify that naphthalene mineralization or volatilization did not occur in the absence of added microorganisms.

Some biometer flasks also initially received purified bioemulsifier (emulsan) or cell-free supernatant from bioemulsifier-producing cultures to assess whether substances produced by RAG-1 bacteria, but no longer associated with the cells, could affect mineralization by PAH-degrading organisms. Emulsan was supplied in powder form by Dr. Carol Litchfield (Keystone Environmental, Monroeville, PA) and used as received [23]. It was added to biometer flasks to give a final concentration of 0.015% by weight water [23]. Cell-free supernatant was obtained by centrifuging RAG-1 cultures for 20 minutes at 6500 x g to pellet the cells. The supernatant was

separated from the pellet, expressed through a 0.45 μm Millipore filter, and added to biometer flasks in 10 mL aliquots.

The behavior of [14C]naphthalene was assumed to represent that of the unlabeled compound in biometer flasks. Naphthalene mineralization was tracked by measuring $^{14}CO_2$ that was released by PAH-degrading organisms and captured in the NaOH solution of the biometer flasks. During sampling, 0.5 mL of NaOH was removed in triplicate from each biometer and added to 10 mL of scintillation cocktail (Optifluor, Packard Instrument Co., Downers Grove, IL) in scintillation vials. After dark overnight storage to minimize chemiluminescence, the samples were counted for ^{14}C in a Beckman LS 5000 TD liquid scintillation counter with the H# quench monitoring technique and automatic quench compensation [4]. Sodium hydroxide was replaced in the side arm of each biometer after every sampling interval.

RESULTS

Substrate uptake

Figure 1 [27] illustrates some of the processes envisioned to lead to microbial uptake and biodegradation of a PAH compound released from the coal tar DNAPL in the porous silica medium. Visual observations and scanning electron microscopy suggest that most of the DNAPL is present as a liquid within microporous aggregates, as illustrated. Once dissolution of the PAH compound occurs at the pore water-DNAPL interface, the PAH compound may diffuse into the bulk aqueous phase, where it becomes acccessible for degradation by microbes.

Mineralization of [14C]naphthalene from coal tar in aqueous systems with silica

All mineralization data have been corrected for background dpm. The dpm obtained from abiotic controls were consistently at background levels, indicating that the volatility of radiolabeled naphthalene did not significantly contribute to the measured activity.

Systems containing coal tar imbibed in silica did not contain a visible nonaqueous liquid phase at any time; instead, the coal tar remained held in the microporous silica for the duration of

these studies. However, the aqueous phase in the biometer flasks turned a translucent gray color during the course of individual experiments lasting sixty days.

Two sets of experiments with [^{14}C]naphthalene-labeled coal tar and silica were performed with slightly different results. In general, the mineralization achieved after 60 days in the first set of experiments was somewhat higher than in the second set of experiments under similar conditions (~30% cumulative mineralization vs. ~20%). This difference is most likely attributable to variations in the initial viability of PAH-degrading culture used for the inocula. Data from the two sets of experiments are shown separately, although the trends exhibited by the data sets corroborate each other.

Abiotic systems were used to establish that naphthalene mineralization was not occurring in the absence of added organisms (Figs. 2A and 2B). Furthermore, it was found that the bioemulsifier-producing *Acinetobacter calcoaceticus* RAG-1 bacterium did not contribute to ^{14}CO$_2$ evolution, indicating that PAH-degrading organisms were solely responsible for naphthalene mineralization (Figs. 2A and 2B). Control flasks inoculated only with PAH-degrading organisms yielded about 23% mineralization of labeled naphthalene after 10 days, 29% after 38 days, and 30% after 60 days in the first set of experiments. By day 60 mineralization was clearly increasing at a slow rate (Fig. 2A). The second set of experiments resulted in about 11%, 18%, and 21% cumulative mineralization after 13, 38, and 60 days, respectively (Fig. 2B). These data are used as references for assessing the effects on naphthalene mineralization from addition of emulsan, RAG-1 organisms, or cell-free culture broth.

Naphthalene mineralization in coal tar-silica systems did not change significantly upon the addition of cell-free culture broth from stationary-phase RAG-1 cultures, as shown in Figure 3A. The initial addition of 10 mL of this broth in the first set of experiments resulted in a response similar to that of the corresponding control flasks. Mineralization reached about 13% after 10 days, 25% after 38 days, and 29% by 60 days (Fig. 3A). In the second set of tests, mineralization was approximately 8%, 18%, and 22% complete after 13, 38, and 60 days, respectively (Fig. 3B). Although mineralization in these systems was slower during the first two weeks than in the

respective control flasks without broth, the average absolute difference from the controls after 60 days was less than 1% for both experiments. Systems receiving 10 mL of the broth after 45 days were similarly unaffected by the addition; in the first set of experiments, average mineralization reached 29% after 60 days (Fig. 3A). Doubling the volume of broth added after 45 days produced no apparent effect, yielding 27% mineralization by day 60 (Fig. 3A).

Mineralization in coal tar-silica systems was also apparently unaffected by the addition of 0.015% emulsan, as shown in Figure 3B. The bioemulsifier was added either initially or after 45 days in the second set of experiments. Initial addition of emulsan resulted in an average of about 20% mineralization after 60 days, which was not significantly different from the control. The addition of emulsan at day 45 appeared to cause a slight increase in the mineralization curve, although after 60 days mineralization was essentially the same as in the control.

The initial addition of various concentrations of RAG-1 cells to coal tar-silica systems was investigated and found to produce no significant effect in either set of experiments. Figures 4A and 4B show the results of adding 2 mL inocula of 10^{10}, 10^9, 10^8, or 10^7 CFU/mL of stationary-phase RAG-1 culture to biometer flasks containing PAH-degrading organisms. Cumulative mineralization at the tenth day in the first set of experiments varied from a low value of about 17% to a high value of 24% for 10^9 and 10^7 CFU/mL RAG-1 inocula, respectively. The control flask containing only PAH-degrading organisms showed an intermediate value of 23%. Mineralization at day 60 was highest in the 10^7 CFU/mL RAG-1 system (36%) and lowest in the control (30%). The remaining RAG-1 systems exhibited 60-day mineralization that was only slightly higher in absolute terms than the control, with no apparent relationship between extent of mineralization and the number of cells added. The lack of a relationship between the addition of RAG-1 and an effect on naphthalene mineralization was also demonstrated in the second set of experiments (Fig. 4B), in which 60-day mineralization was slightly higher in the 10^9 CFU/mL RAG-1 system (23%), followed by the control (21%) and the 10^7 RAG-1 system (19%).

Addition of stationary-phase RAG-1 cells 45 days after the start of an experiment showed results very similar to those for initial RAG-1 addition. No clear relationship between the

concentration of cells added and naphthalene degradation was found, and no sudden increase in mineralization following RAG-1 addition was observed. Figure 5 shows the results of adding 2 mL of 10^{10}, 10^9, 10^8, and 10^7 CFU/mL RAG-1 inocula at day 45 in the first set of experiments. Mineralization after 60 days was approximately 28% complete in all except the 10^{10} case, which showed slightly higher mineralization (~33%) even before inocu'ation with RAG-1. Control flasks showed ~30% mineralization at day 60; thus, late addition of RAG-1 cells was ineffective in altering naphthalene mineralization in the first set of experiments. The addition of RAG-1 cells at 45 days was also tested in the second set of experiments (Fig. 4B). A slight increase in mineralization followed the addition of a 10^9 CFU/mL inoculum of RAG-1 cells, although cumulative mineralization at day 60 (22%) was effectively indistinguishable from that attained in the control flasks (21%).

Mineralization of [^{14}C]naphthalene from free-DNAPL coal tar in aqueous systems

This set of experiments evaluated systems without silica that contained coal tar as a free dense nonaqueous phase liquid (DNAPL). The coal tar DNAPL coalesced into several globules at the bottom of biometer flasks. A certain amount of tar also formed thin layers at the surface of some biometer flasks over time, as described below. Inoculation with PAH-degrading microbes in systems containing silica was performed at the same time, and from the same culture used in the second set of experiments with silica, which accounts for the ~20% mineralization achieved in the control flasks described here.

Abiotic systems with free coal tar showed that naphthalene mineralization did not occur in the absence of added organisms, and biometer flasks inoculated only with RAG-1 bacteria also showed no mineralization. This demonstrated again that PAH-degrading bacteria were solely responsible for naphthalene mineralization. Mineralization in control flasks containing only PAH-degrading organisms reached 18% after 59 days (Fig. 6). This is comparable to the controls in

systems with coal tar imbibed in silica, which reached 21% mineralization by day 59 but did not plateau as quickly as in the systems with free-DNAPL tar.

Figure 7 shows the result of initially adding 0.015% emulsan to systems with free-DNAPL coal tar. The shape of the mineralization curve is very similar to that of the control, although mineralization is somewhat higher by day 59 compared to that in Figure 6 (22%). Nonetheless, the corresponding mineralization in systems with coal tar imbibed into silica was 20%, so neither the absence of silica nor the presence of emulsan seems to influence naphthalene degradation significantly.

The addition of RAG-1 cells was found to have little or no effect on mineralization over two months in systems containing free-DNAPL coal tar. RAG-1 inocula consisting of 2 mL of 10^7 or 10^9 CFU/mL of stationary-phase culture were added initially. After 59 days, mineralization had reached about 19% in the former case and 23% in the latter, which is not very different from the control or from corresponding tests with silica (Figs. 4B, 6, 8). The addition of a 10^9 CFU/mL RAG-1 inoculum at day 45 resulted in a 5% increase in mineralization immediately after the addition, giving 26% naphthalene mineralization after 59 days. Unlike all other tests, the data shown for this case (Fig. 8) were obtained from only one biometer.

The tar in systems without silica remained a separate phase, with little or no emulsification being visually observed. After being added to the aqueous medium in biometer flasks, the tar settled as blobs at the bottom of the flasks. The tar blobs remained intact and resistant to flow during oscillatory shaking, and they frequently became attached to the flask bottom by a thin neck, which prevented the coal tar from freely moving about in the medium.

Several systems with free-phase coal tar DNAPL retained a clear, colorless, aqueous phase during the entire study. In these systems coal tar did not adhere to the flask in a ring along the flask wall, nor was it present as a film at the surface. Other biometer flasks developed a ring-like accumulation of coal tar on the flask wall over time, and in these instances the aqueous medium turned a translucent gray color after several weeks. Surface layers of tar were present in varying degrees, from being absent to forming a thin oily sheen to a brown film that nearly covered the

interface between air and water. However, surface films or tar rings on glass did not appear to be related to the contents of flasks. For example, of the biometer flasks with clear aqueous medium and no tar rings or films, one contained only PAH-degrading organisms and the other held RAG-1 bacteria as well. Three other flasks contained a tar ring, grayish medium and a sparse surface film: one contained PAH-degrading organisms, the second held PAH-degrading organisms and RAG-1, and the third had PAH-degrading bacteria and 0.015% emulsan. The differences in the appearance of tar in these systems is due to unknown factors.

DISCUSSION

The results presented here indicate that the bioemulsifier emulsan and two related fractions of *Acinetobacter calcoaceticus* RAG-1 cultures were ineffective in altering naphthalene mineralization from coal tar in the aqueous systems studied. Cell-free culture broth from RAG-1 cultures, stationary-phase RAG-1 inocula of various concentrations, or 0.015% emulsan was added to systems containing $[^{14}C]$naphthalene-labeled coal tar and PAH-degrading bacteria that had been isolated from contaminated soils and wastes. Coal tar in these systems was either imbibed in microporous silica or present as a free dense nonaqueous phase liquid (DNAPL). Naphthalene mineralization was determined from $^{14}CO_2$ evolution and was compared to control systems receiving PAH-degrading bacteria but none of the abovementioned additions. Napthalene mineralization in these studies was neither enhanced nor inhibited by the presence of emulsan, RAG-1 cells, or RAG-1 cell-free broth.

Two sets of experiments were performed under similar conditions with slightly different results. The first set of experiments resulted in approximately 30% mineralization of naphthalene after 60 days, while the second set of tests yielded about 20% mineralization over 60 days. This difference was probably a result of variations in the PAH-degrading culture used for the inocula. Nevertheless, the trends shown in both experiments corroborate each other.

The addition of cell-free broth, emulsan, or RAG-1 inocula late in the experiments did not give significantly different results from initial additions of these substances. The concentration of

RAG-1 inocula was not correlated to any change in naphthalene mineralization, nor was the response dependent on whether coal tar was free-phase DNAPL or imbibed within microporous silica. Coal tar imbibed into microporous silica remained entrapped within the silica throughout the experiments and was never present as a free phase liquid. Tar in systems without silica existed as a separate liquid phase without observable emulsification, despite the fact that the tar contained 1-methylnaphthalene, which has been shown to enhance slightly the emulsification of naphthalene and other aromatic compounds by emulsan [22]. In some cases free-phase tar formed ring-like accumulations on flask walls or films of various thickness at the water-air interface, but these phenomena were apparently unrelated to the contents of the systems.

The very slow rate of naphthalene mineralization observed after 40 days in most systems is not explained by these tests. Perhaps mass transport of naphthalene from coal tar into the aqueous medium limits biodegradation. Free-phase coal tar in experimental systems without silica existed as large globs at the bottom of flasks, with relatively little interfacial area for mass transfer to occur over. In systems with silica, the sorption of tar onto the pore walls and outer surfaces of silica particles may retard mass transport and biodegradation [10]. Another possible explanation is the formation of a semi-rigid skin or film of coal tar that may impede mass transfer across the DNAPL-water interface [3]. A third explanation for the decline in naphthalene mineralization could be the divergent metabolic pathways of the PAH-degrading pseudomonads used in this study. Although the broad-based enzymes of *Pseudomonas* species allow these organisms to degrade many natural organic compounds and xenobiotics, "dead-end" intermediates can form when enzymes for subsequent degradation steps are not available [7]. Perhaps the PAH-degrading bacteria transformed naphthalene to intermediates that could not be mineralized. However, this is not reported for naphthalene [10], and further, the recovery of dpm from the aqueous phase of systems at the end of this and related studies indicates that if such compounds were formed, they were only sparingly soluble in water. Aqueous phase dpm values were not significantly greater than those expected for naphthalene alone. Whether or not the above explanations are sufficient to account for the decline in mineralization is unknown. Work is in progress to assess the rate of mass transfer of

naphthalene to the aqueous phase from coal tar imbibed into microporous silica for both freshly-prepared and aged samples.

It is unclear why emulsan, cell-free RAG-1 broth or living RAG-1 cells had no effect on naphthalene emulsification and mineralization. Emulsan was found to inhibit aromatic mineralization from crude oil by a mixed PAH-degrading culture in a previous study [20], but because inhibition was not observed from emulsan or cell-free culture broth in these experiments, interactions between emulsan and PAH-degrading bacteria may be species-specific. The intact RAG-1 cells incorporated into systems with coal tar were in the stationary growth phase, and they should have lost most of their cell-associated emulsan prior to being added. The absence of cell-associated emulsan would have given the cells a higher affinity for adherence and growth on hydrocarbons [19]. However, RAG-1 cells spread on agar plates with naphthalene as the sole carbon source did not grow, so *Acinetobacter calcoaceticus* may not have been capable of producing emulsan in these experiments. Finally, the coal tar used in these experiments contained no identifiable aliphatic compounds, and thus conditions for PAH emulsification by emulsan were probably not optimum.

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TABLE 1. Ten major identifiable components of Stroudsburg, PA coal tar [26]

	Molecular Weight	Weight Percent
methylchrysenes	242	4.4
trialkylated naphthalenes	170	4.3
methylpyrene	216	3.9
1-methylnaphthalene	142	3.80
2-methylnaphthalene	142	3.75
dimethylphenanthrene	206	2.3
naphthalene	128	2.16
phenanthrene	178	2.12
2,6-dimethylnaphthalene	156	1.99
2-ethylnaphthalene	156	1.84
total[a]		30.56

[a]The total weight percent of all identifiable compounds was about 48% [26].

FIGURE LEGENDS

Figure 1. The processes through which dissolution, mass transfer and biodegradation of PAH compounds from coal tar dense nonaqueous phase liquid (DNAPL) imbibed in microporous media is envisioned to occur [27].

Figure 2. Naphthalene mineralization in aqueous systems containing coal tar imbibed in microporous silica in first set (A) and second set (B) experiments. Data are reported as the percent of ^{14}C-labeled naphthalene mineralized. In this and subsequent figures, inocula of 2 mL were used and PAH-degrading inocula were taken from a 10^7 CFU/mL culture. Neither the abiotic control nor those systems containing only bioemulsifier-producing RAG-1 bacteria exhibited naphthalene mineralization, indicating that PAH-degrading bacteria were solely responsible for $^{14}CO_2$ evolution. Data points are the average of several tests, and error bars represent one standard deviation.

Figure 3. Naphthalene mineralization in first set (A) and second set (B) experimental systems containing coal tar imbibed in microporous silica, with PAH-degrading inocula, and cell-free RAG-1 culture broth or 0.015% emulsan. Cell-free broth was obtained from stationary-phase RAG-1 cultures. The control systems contained PAH-degrading bacteria but neither broth nor emulsan.

Figure 4. Naphthalene mineralization in first set (A) and second set (B) experimental systems containing coal tar imbibed in microporous silica with PAH-degrading inocula, and additions of RAG-1 inocula at concentrations from 10^7 to 10^{10} CFU/mL.

Figure 5. Naphthalene mineralization in first set experimental systems containing PAH-degrading bacteria and coal tar imbibed in microporous silica. RAG-1 inocula at concentrations from 10^7 to 10^{10} were added at 45 days.

Figure 6. Naphthalene mineralization in second set experimental systems containing coal tar as a free dense nonaqueous phase liquid (DNAPL). Neither the abiotic control nor those systems containing only RAG-1 bacteria exhibited naphthalene mineralization, indicating that PAH-degrading organisms were solely responsible for $^{14}CO_2$ evolution.

Figure 7. Naphthalene mineralization in second set experimental systems with free-phase coal tar and 0.015% added emulsan. The controls contained PAH-degrading inocula only.

Figure 8. Naphthalene mineralization in second set experimental systems with free-phase coal tar and additions of RAG-1 cells at the times shown.

PAH dissolution at
NAPL interface

Pore sorption
and diffusion

Bulk Phase Transfer

Bulk Phase PAH

BACTERIUM

PAH Degradation
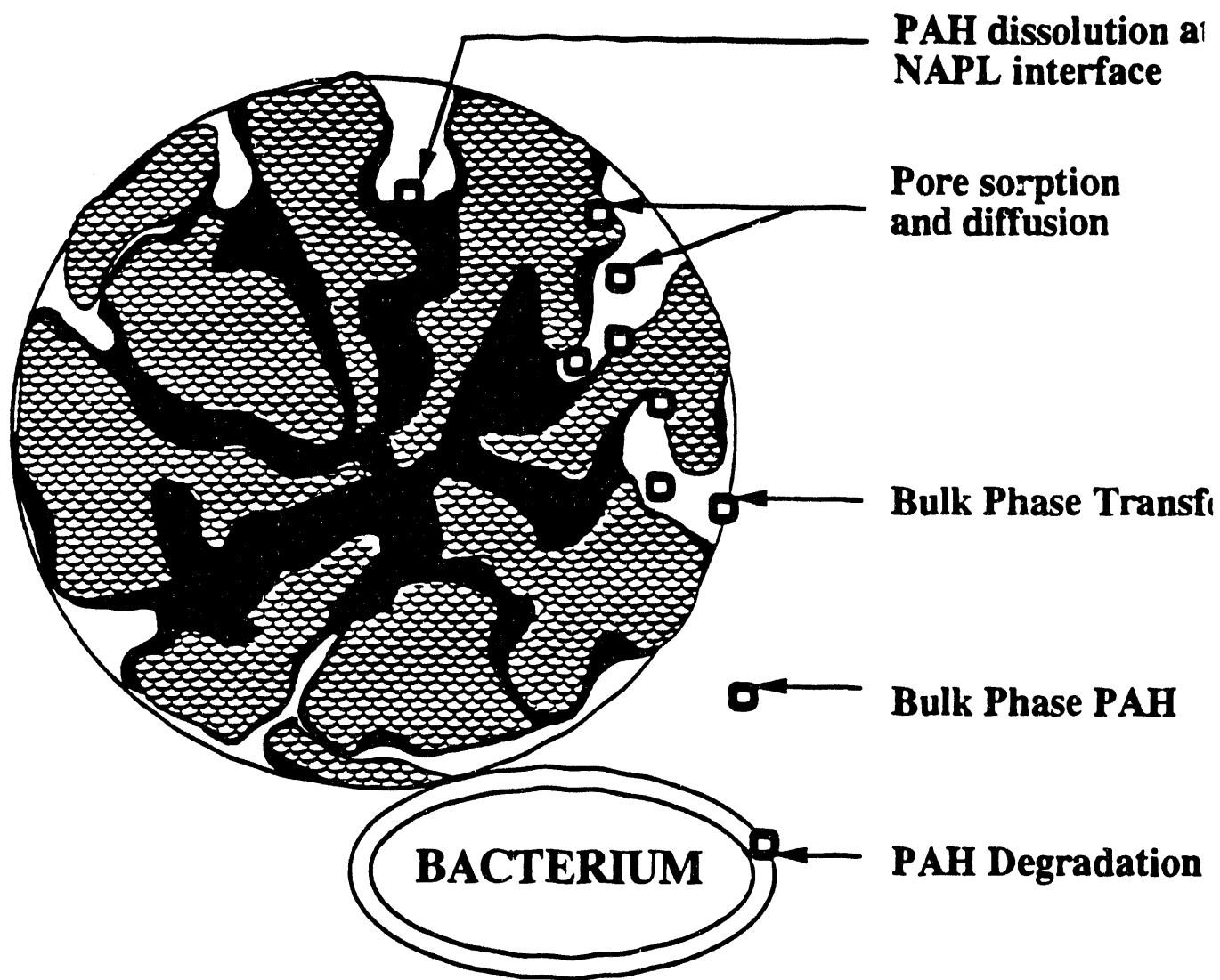
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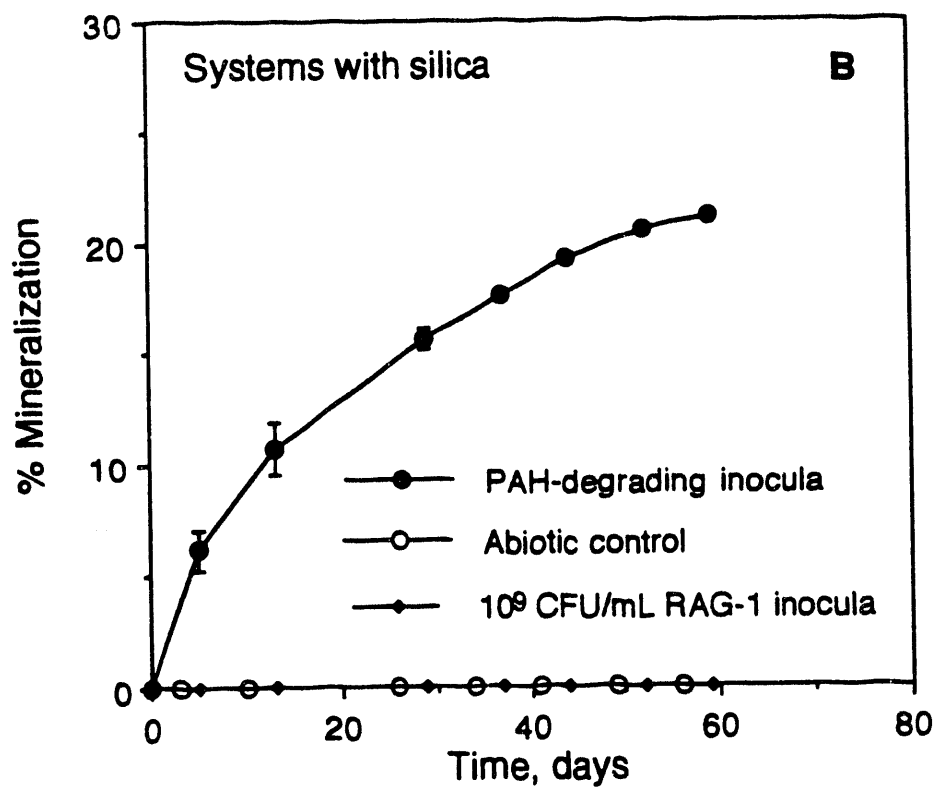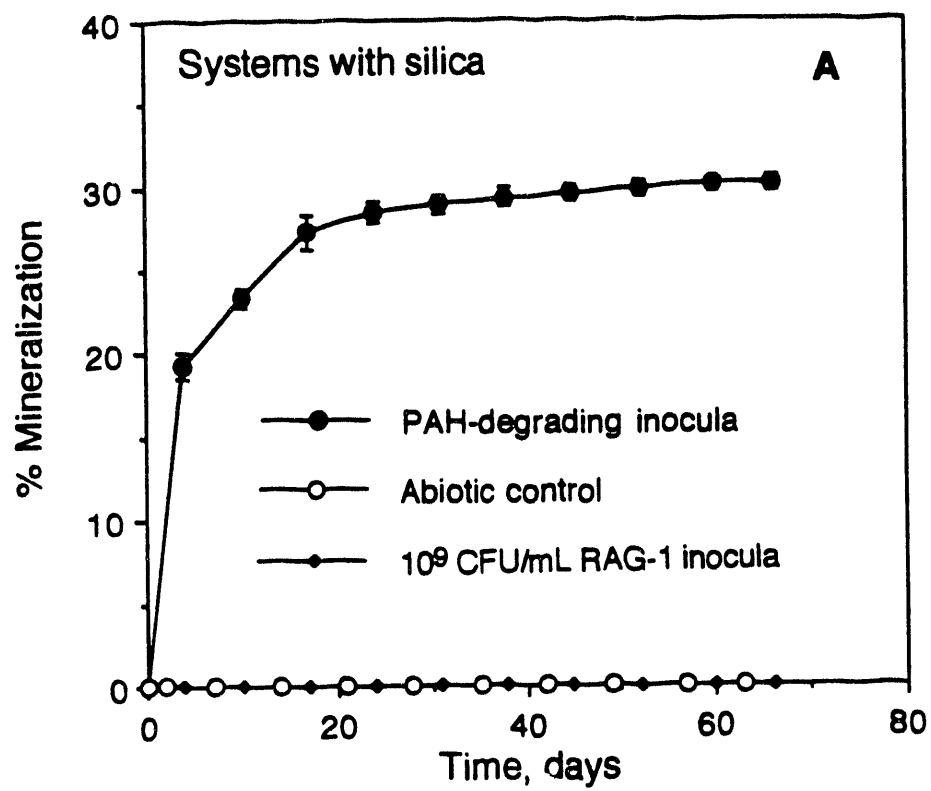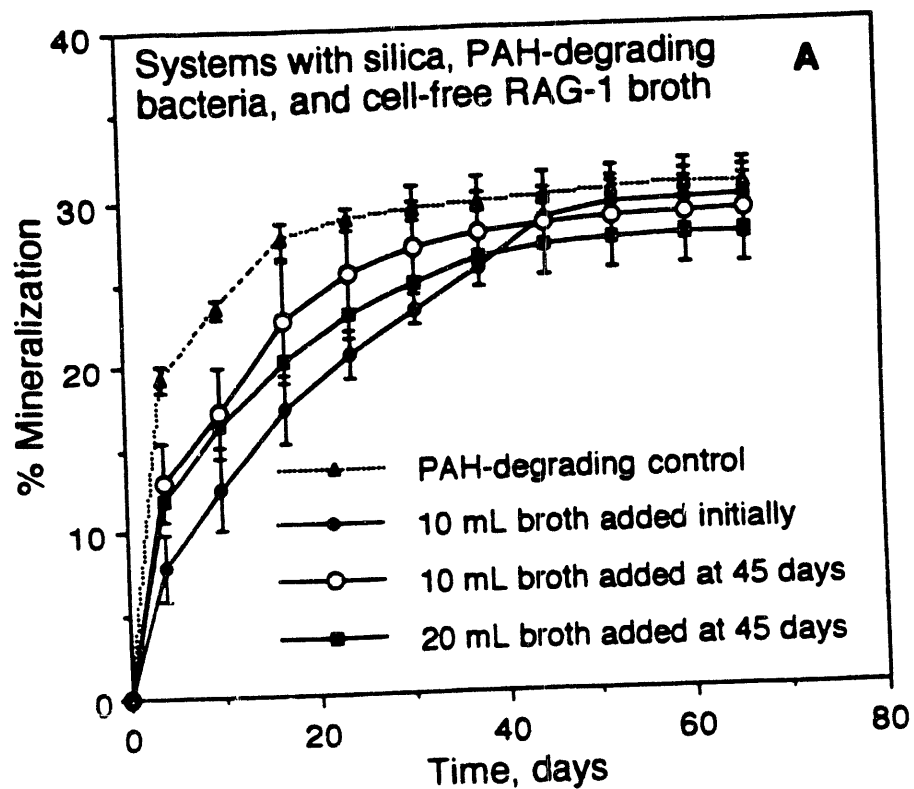
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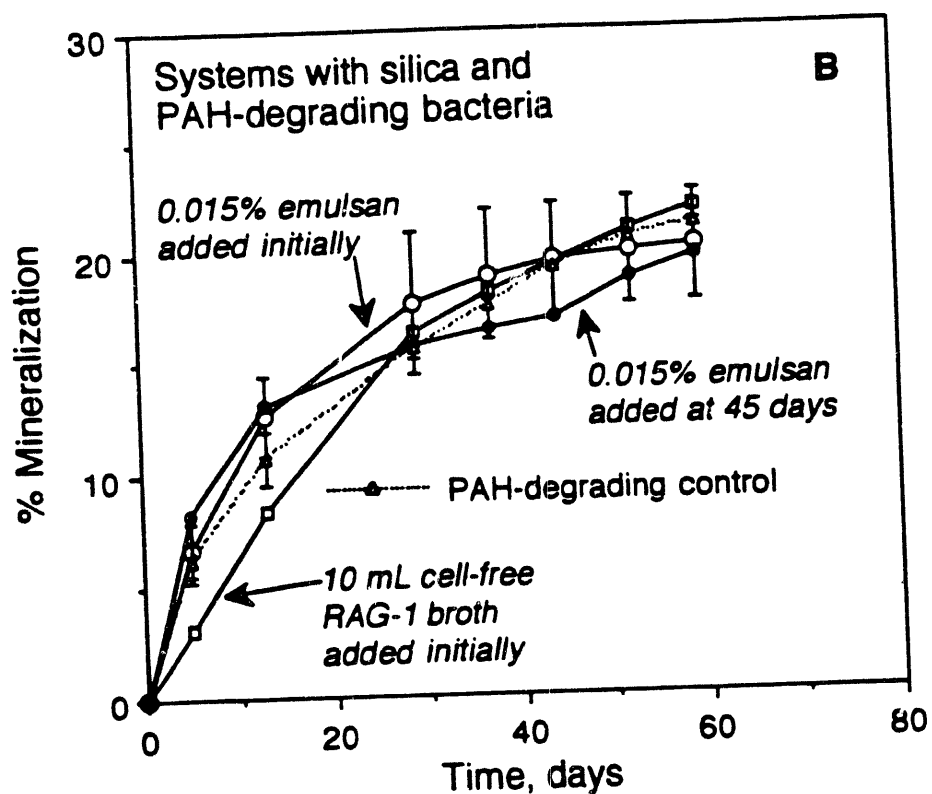
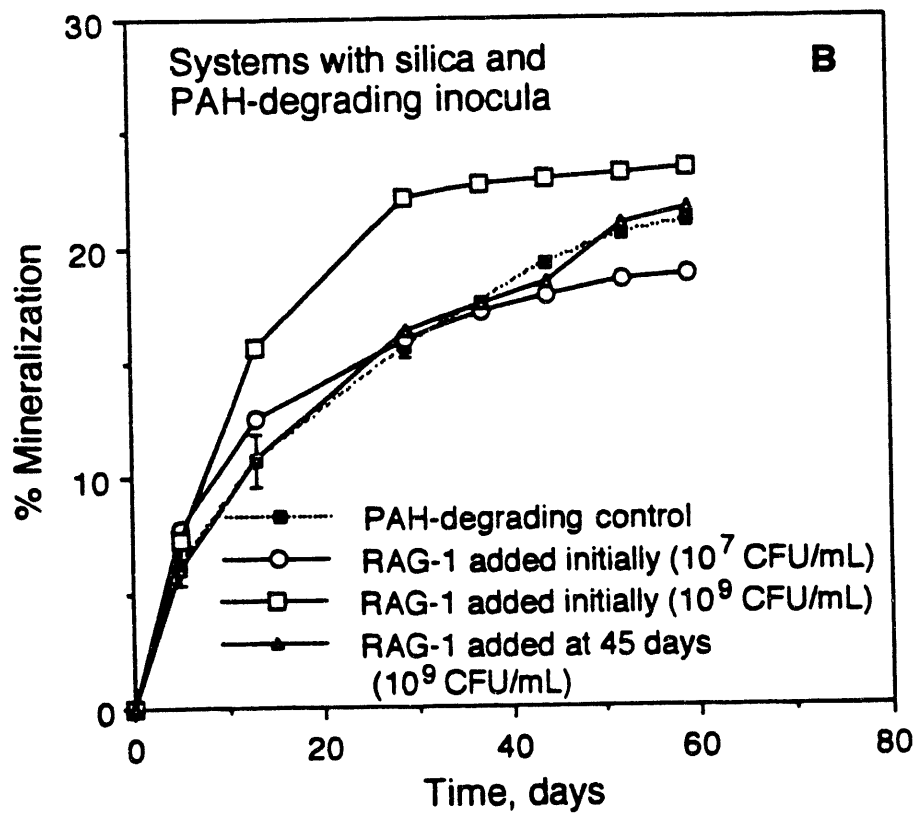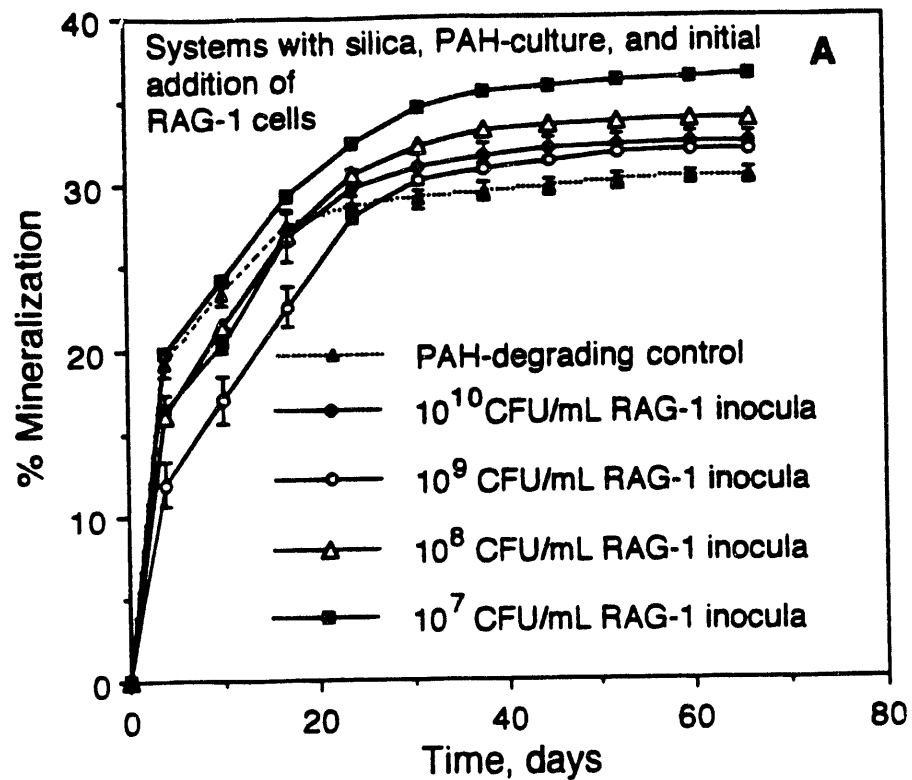Figure 3. Karen L. Skubal and Richard G. Luthy
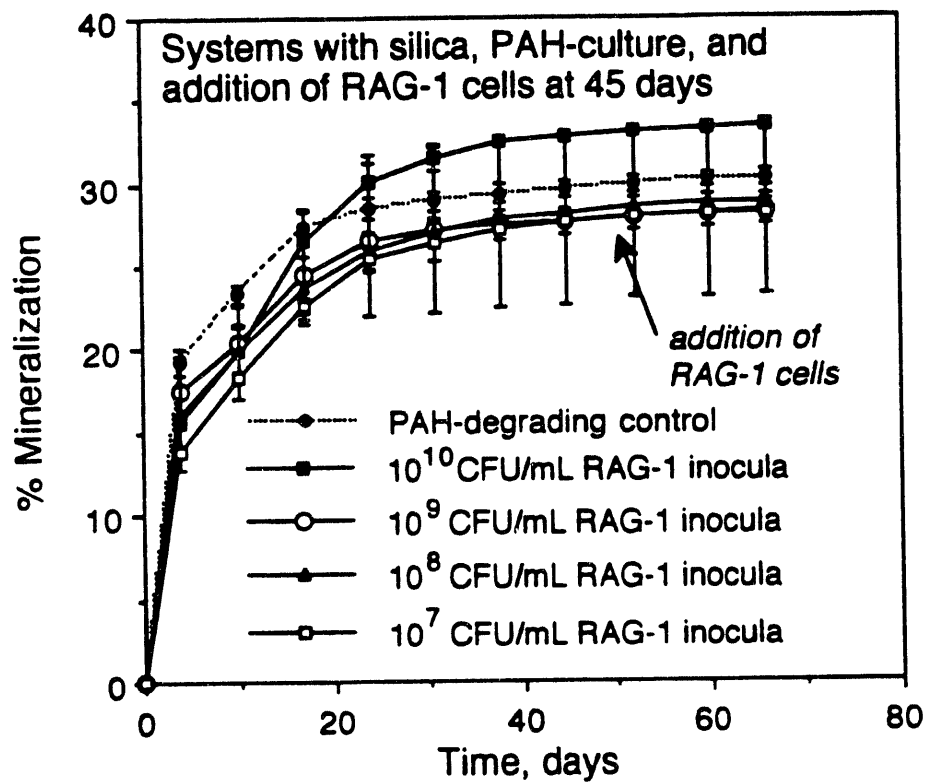
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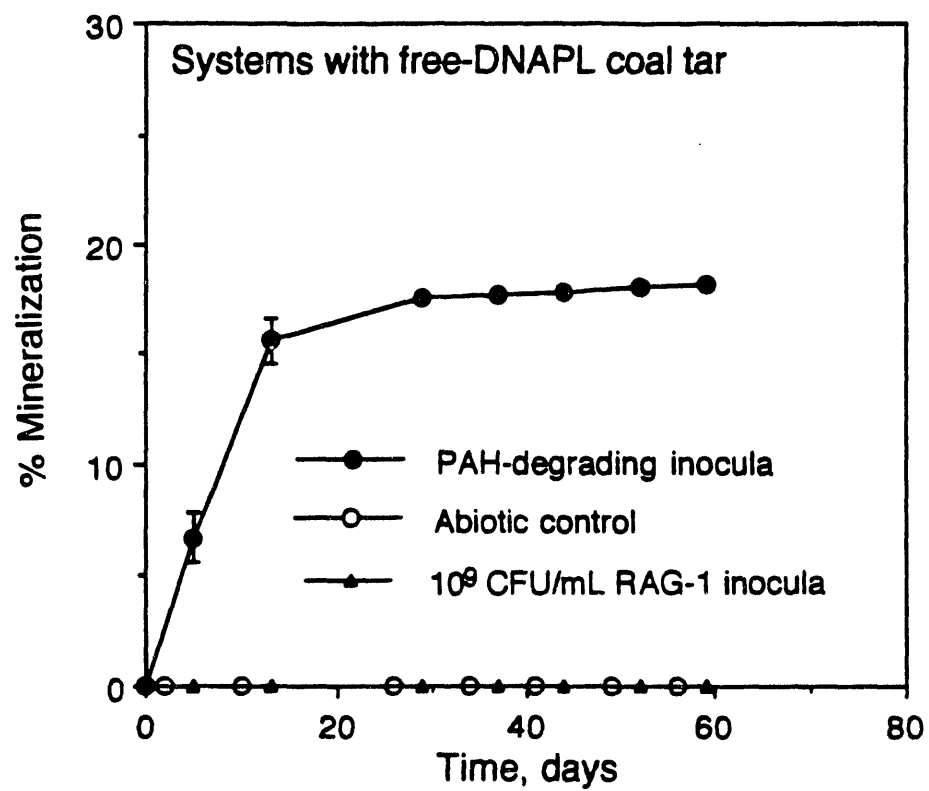Figure 5. Karen L. Skubal and Richard G. Luthy
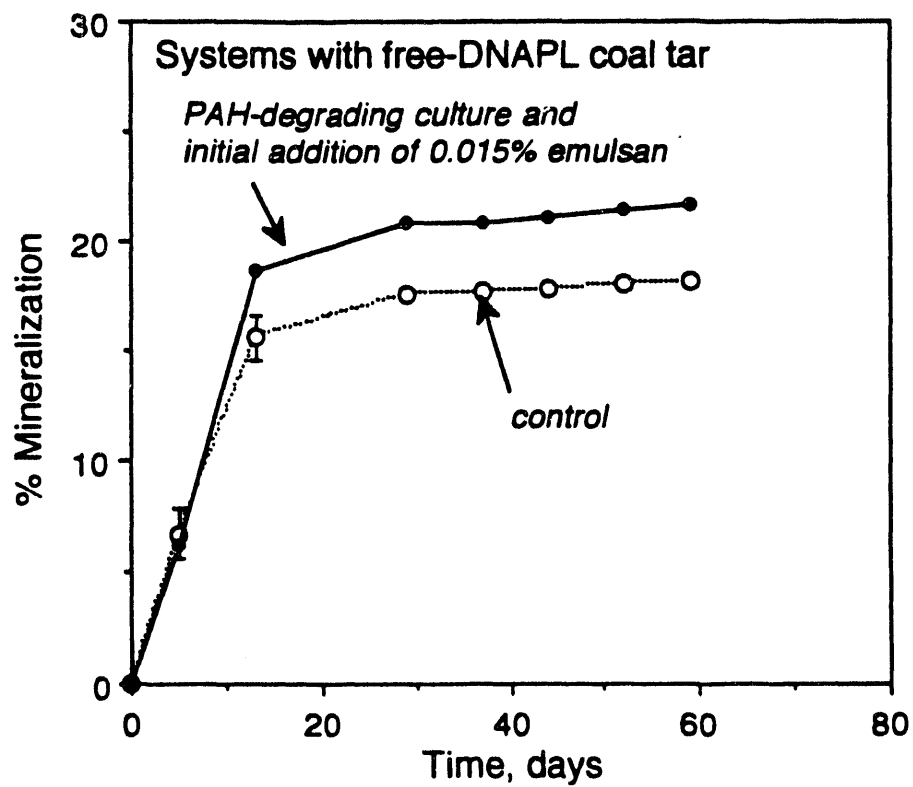
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Figure 7. Karen L. Skubal and Richard G. Luthy

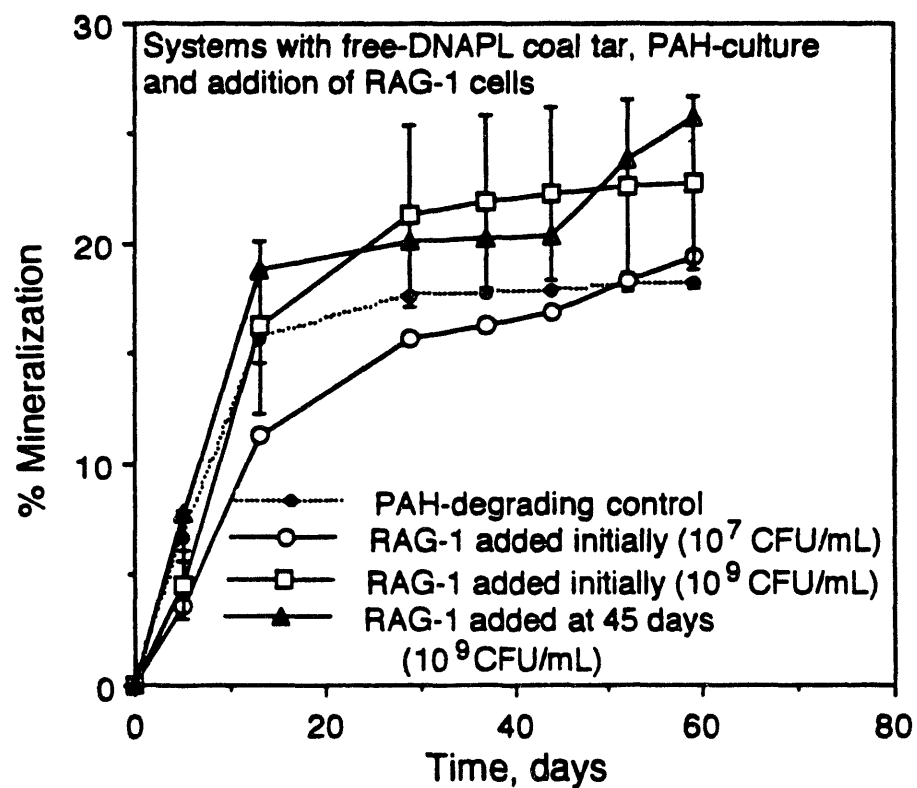Figure 8. Karen L. Skubal and Richard G. Luthy

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