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Cyclodextrin-Enhanced Biodegradation of Phenanthrene

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The effectiveness of in situ bioremediation in many systems may be constrained by low contaminant bioavailability due to limited aqueous solubility or a large magnitude of sorption. The objective of this research was to evaluate the effect of hydroxypropyl-β-cyclodextrin (HPCD) on phenanthrene solubilization and biodegradation. Results showed that analytical-grade HPCD can significantly increase the apparent solubility of phenanthrene. The increase in apparent solubility had a major impact on the biodegradation rate of phenanthrene. For example, in the presence of 10^5 mg L^{-1} HPCD, the substrate utilization rate increased from 0.17 mg h^{-1} to 0.93 mg h^{-1} while the apparent solubility was increased from 1.3 mg L^{-1} to 161.3 mg L^{-1}. As a result, only 0.3% of the phenanthrene remained at the end of a 48 h incubation for the highest concentration of HPCD tested (10^5 mg L^{-1}). In contrast, 45.2% of the phenanthrene remained in the absence of HPCD. Technical-grade HPCD, which contains the biodegradable impurity propylene glycol, also increased the substrate utilization rate, although to a lesser extent than the analytical-grade HPCD. On the basis of these results, it appears that HPCD can significantly increase the bioavailability, and thereby enhance the biodegradation, of phenanthrene.

Introduction

There is tremendous interest in using in situ bioremediation for the cleanup of contaminated soil and groundwater. However, biodegradation rates in the subsurface are often constrained by a limited oxygen supply and by factors related to bioavailability, such as solubility, dissolution rate, and sorption (*1*−*4*). Recent research has examined the possibility of enhancing the bioavailability of low solubility and highly sorptive compounds by adding a "solubilization" agent, such as a surfactant, to the system (*5*−*13*). We have been investigating cyclodextrin as an alternative agent for enhancing solubilization (*14*).

Cyclodextrins are cyclic, nonreducing maltooligosaccharides produced from the enzymatic degradation of starch and related compounds by certain bacteria that contain the cyclodextrin glycosyltransferases (*15*). The most pertinent property of cyclodextrins is that they have a hydrophilic shell and a toroidal-shaped, apolar (hydrophobic) cavity. Thus, cyclodextrins have the ability to form water-soluble inclusion complexes by incorporating suitably sized low-polarity molecules in their cavities. Cyclodextrins are widely used in pharmaceutical, food, and cosmetic industries (*16*). Through research for these applications, it has been shown that cyclodextrins can aid the microbial transformation of water-soluble compounds, such as vanillin (*17*), and low solubility compounds, such as cholesterol (*18*) and steroids (*19*).

Recently, cyclodextrins have been used in environmental applications to improve the remediation of contaminated soil and groundwater. For example, it has been demonstrated that cyclodextrins have the ability to increase the apparent water solubilities of low-polarity organic compounds such as trichloroethene, naphthalene, anthracene, chlorobenzene, and DDT (*20*). They can also reduce the sorption and facilitate the transport of these compounds through soil (*21*). A recent field study demonstrated that a cyclodextrin solution removed significant amounts of multicomponent, immiscible-organic liquid contamination from an aquifer (*22*). It has been shown that β-cyclodextrin can decrease the microbial toxicity of some pesticides and aromatics for wastewater treatment and bioreactor applications (*23, 24*). The bioavailability and biodegradation of such compounds in the presence of cyclodextrins has not yet been investigated for in situ environmental applications. The objective of this study was to evaluate the influence of hydroxypropyl-β-cyclodextrin (HPCD) on the biodegradation of a selected nonionic, low-polarity organic chemical (phenanthrene).

Materials and Methods

Materials. Analytical-grade hydroxypropyl-β-cyclodextrin (HPCD) (purity >99%) and technical-grade HPCD [with 3.2% (w/w) propylene glycol] were supplied by Cerestar USA, Inc. (Hammond, IN). Phenanthrene (purity >98%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). [9-^{14}C]-Phenanthrene (specific activity, 13.3 mCi/mmol, reported purity >98%) was purchased from Sigma Chemical Co. (St. Louis, MO). The actual purity of [9-^{14}C]phenanthrene, determined by HPLC, was 96.3%. The mineral salts medium (MSM), specifically Bushnell-HAAS (Difco Laboratories, Detroit, MI), consisted of 0.2 g of $MgSO_4$, 0.02 g of $CaCl_2$, 1 g of KH_2PO_4, 1 g of $(NH_4)_2(HPO_4)$, 1 g of KNO_3, and 0.05 g of $FeCl_3$/L of distilled water. Agar (Bacto-Agar) and R_2A agar employed in bacterial cultivation were also purchased from Difco Laboratories (Detroit, MI). Propylene glycol was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). ScintiVerse BD scintillation cocktail was purchased from Fisher Scientific Chemical (Fair Lawn, NJ). Chloroform, used to dissolve or extract phenanthrene, was purchased from Mallinckrodt Baker, Inc. (Paris, KY). Methanol (HPLC grade), used to dilute the extracts of phenanthrene, was purchased from J. T. Baker, a division of Mallinckrodt Baker Inc. (Phillipsburg, NJ). Acetonitrile UV, employed as a carrier for HPLC analysis, was purchased from Burdick & Jackson Inc. (Muskegon, MI).

Solubility of Phenanthrene. The solubility of phenanthrene in HPCD solutions was determined by use of ^{14}C-labeled phenanthrene. Two milliliters of a mixture of phenanthrene and [^{14}C]phenanthrene in chloroform was added to 250 mL flasks and allowed to evaporate, which produced a 12.5 mg coating of phenanthrene (specific activity, 0.082 mCi/mmol) on the bottom of the flasks. A 25 mL aliquot of aqueous HPCD solution was then added to each flask. Samples were prepared in triplicate for seven HPCD concentrations (including zero) and placed on an orbit shaker (Lab-Line Instruments, Inc., model 3527, Melrose Park, IL) at 200 rpm for 3 days at room temperature. A 50 μL aliquot of each sample was added to 5 mL of ScintiVerse BD, and the radioactivity was determined using a liquid scintillation

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counter (Packard Tri-Carb Co., Model 1600 TR, Meriden, CT).

Phenanthrene-Degrading Isolate. A phenanthrene-degrading isolate was obtained from an estuary sediment sample. In the enrichment procedure, 10 g of estuary sediment and 50 mL of MSM were added to flasks coated with phenanthrene. The enrichment culture was shaken on an orbit shaker at 200 rpm at room temperature for 1 month, and then was transferred to fresh flasks coated with phenanthrene and containing Bushnell-HAAS MSM. The culture was incubated for 2 weeks. After several such transfers, the incubation period was decreased gradually from 2 weeks to 3 days until a single phenanthrene degrading isolate was obtained. The isolate was maintained on Bushnell-HAAS agar plates spray-coated with phenanthrene.

The phenanthrene degrader used in this study was characterized as a Gram-negative rod, which carries a plasmid of approximately 83 kb. The isolate was identified by 16S rDNA using polymerase chain reaction. Polymerase chain reaction with the primers *forward* 5′ AGA GTT TGA TCC TGG CTC AG 3′ and *reverse* 5′ ACG GTT ACC TTG TTA CGA CTT 3′ for Eubacterial 16S rDNA (synthesized by IDT, Inc., Coralville, IW) were used to amplify a 1.5 kb fragment from isolate DNA. Reaction conditions were as follows. Primer mix of forward and reverse 1 pM, 200μM dNTPs (Pharmacia Biotec, Uppasala, Sweden), 1× Pfu buffer, 2.5 units of Pfu DNA polymerase enzyme (Stratagene Inc., La Jolla, CA), and 10−50 ng of template DNA. Cycling was done in the GenAmp PCR System 9600 (Perkin-Elmer Cetus Corp., Norwalk, CT): one cycle of 95 °C for 1 min denaturation, followed by 30 cycles of 94 °C 15 s denaturation, 55 °C 15 s annealing, 72 °C 30 s extension, finishing with a 72 °C 2 min final extension.

The PCR products were then cloned and sequenced. Either the Invitrogen pCR 2.1 (Invitrogen, San Diego, CA) or Stratagene pCR-Script SK(+) (Stratagene, La Jolla, CA) vector was used to clone the 1.5 kb ribosomal sequence following manufacturers' directions. Selection of white colonies from LB, IPTG, X-gal, and ampicillan plates were used to grow 25 mL cultures in SOC. Plasmids were extracted using the QiaFilter midiprep system (Qiagen Inc., Chatsworth, CA). Sequencing was performed using the ABI 377 automated sequencer (LSME, Tucson, AZ). Whole, 1.5 kb sequences were aligned and assembled on GCG (Genetics Computer Group Inc., Madison, WI). FASTA was used to search GenEMBL for sequence similarity. This procedure resulted in two possible isolate identities. *Burkholderia sp.* isolate CRE 7 (accession U37340) showed 95.4% similarity, and *Burkholderia cepacia* (accession X87275) showed 94.6% similarity, to the 16S rDNA sequence of the phenanthrene degrader.

Biodegradation Studies. The biodegradation of phenanthrene was quantified in two ways: (1) direct measurement of phenanthrene loss, used to determine substrate utilization, and (2) cell counts, used to evaluate cell growth. For the substrate utilization experiments, which were conducted in triplicate, 12.5 mg of phenanthrene was dissolved in chloroform and added to 250 mL flasks. The solvent was allowed to evaporate overnight, leaving a thin coating of phenanthrene covering the sides and bottom of the flasks. Purified and analytical-grade HPCD in Bushnell-HAAS broth (25 mL) were added in various concentrations. One set of samples was inoculated with 0.5 mL aliquots of the phenanthrene degraders from late-log precultures to achieve a final cell density of approximately 10^7 cfu mL^{-1}. A second set of samples was inoculated with diluted late-log precultures of phenanthrene degraders to a final cell density of 10^4 cfu mL^{-1}. Samples were incubated at 200 rpm on a gyratory shaker at room temperature.

Periodically, a set of triplicate samples was sacrificed, and the contents of each flask were serially extracted three times with 50 mL of chloroform. Samples from the first 2 days had higher levels of phenanthrene. Therefore, the first extract was diluted 100-fold with HPLC-grade methanol. The second and third extracts were concentrated with a Rotavapor Evaporation System (Büchi Co., Switzerland) to 1 mL, after which the volume was adjusted to 5 mL with HPLC-grade methanol. The amount of phenanthrene in both the first extract and in the combined second and third extracts was quantified by high-performance liquid chromatography (Waters, Milford, MA). The total remaining phenanthrene was the sum of these determinations. For samples sacrificed after 2 days, all extracts were combined for analysis as described above.

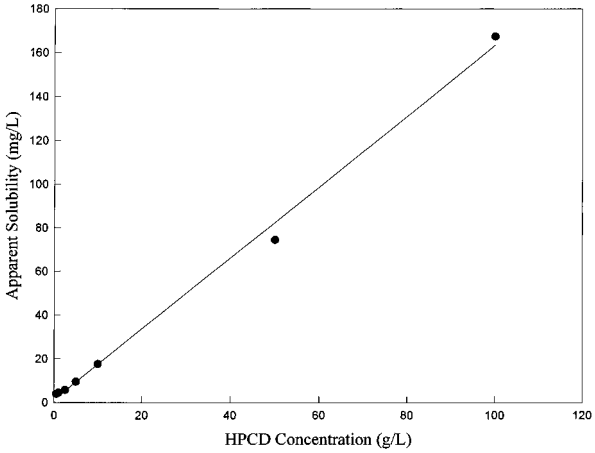

FIGURE 1. The relationship between the apparent solubility of phenanthrene and the concentration of hydroxypropyl-β-cyclodextrin (HPCD).

HPLC analysis was performed isocratically using a mobile phase of 5% water (HPLC grade) and 95% acetonitrile UV (HPLC grade) and a reversed-phase column, Adsorbosphere UHS C18 5U (Alltech, Deerfield, IL), with i.d. 4.6 mm and length 150 mm. The flow rate was 1 mL/min and the wavelength used for detection of phenanthrene was 254 nm.

Cell growth was measured by viable plate counts on R_2A agar. The culture was serially diluted and triplicate plates were inoculated at each dilution ratio. Phenanthrene degraders were enumerated after incubation for 2 days at room temperature.

Cell growth on propylene glycol, which is a major impurity in technical-grade HPCD, was determined by protein content analysis based on the Lowry method (*25*). In this experiment, flasks containing propylene glycol as the only carbon source were inoculated with 10^7 cfu mL^{-1} late-log precultures of phenanthrene degraders. The concentrations of propylene glycol were 160 and 3200 mg L^{-1}, which were equivalent to the amount that would be present in 5000 and 10^5 mg L^{-1} technical-grade HPCD, respectively. Analysis of protein content was carried out by UV−visible spectrophotometry (Hitachi, Model U-2000) at a wavelength of 650 nm.

Results and Discussion

Solubility. The biodegradation of PAHs is limited by their low bioavailability resulting from extremely low water solubility and high sorption. It is hypothesized that cyclodextrins can increase the apparent solubility of phenanthrene by forming inclusion complexes, thereby enhancing the bioavailability of phenanthrene. Apparent solubility is defined as the amount of phenanthrene in solution in the presence of HPCD. This hypothesis is supported by results from this study, which showed a linear relationship between the apparent solubility of phenanthrene in HPCD solution and the concentration of HPCD from 0 to 10^5 mg L^{-1} (Figure 1).

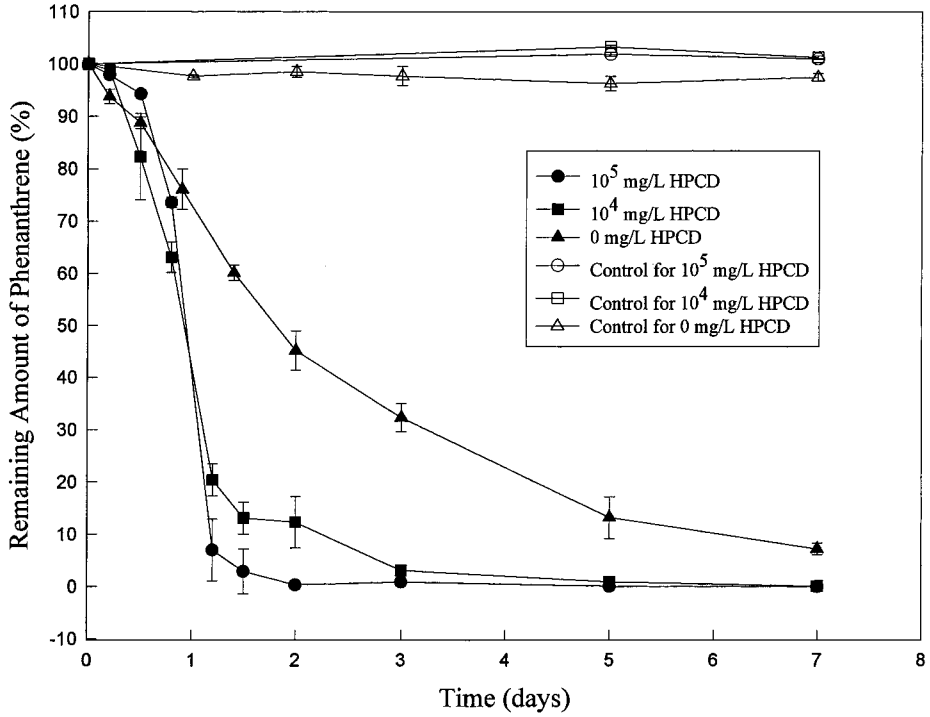

FIGURE 2. Effect of HPCD (analytical grade) on phenanthrene disappearance with time. (Inoculum density was 10^7 cfu/mL.)

TABLE 1. Substrate Utilization Rate and Remaining Amount of Phenanthrene

HPCD concn (mg/L)	substrate utilization rate (mg/day)	phenanthrene remaining (%)		
		(after 2 days)	(after 5 days)	(after 7 days)
10^5	22.35	0.3	0.1	0
10^4	14.35	12.3	0.9	0.1
0	4.07	45.2	13.2	7.2

These results indicate that HPCD significantly increased the apparent solubility of phenanthrene. For example, the apparent solubility of phenanthrene in a 10^5 mg L^{-1} HPCD solution was 161.3 mg L^{-1}, an increase of 124 times compared to the measured aqueous solubility of 1.3 mg L^{-1}. The larger apparent solubility means more substrate is directly available in solution for supporting bacterial activity at equilibrium conditions. In addition to the magnitude of solubility, the rate of dissolution may also influence the rate at which bacteria utilize substrate (*2*, *3*). All systems essentially approached equilibrium within 30 min, including 10^5 mg L^{-1} HPCD (data not shown).

Biodegradation. (1) Substrate Disappearance. The impact of analytical-grade HPCD on biodegradation of phenanthrene is shown in Figure 2. Samples containing higher concentrations of HPCD exhibited more rapid biodegradation of phenanthrene. Thus, more mass of phenanthrene was biodegraded at the end of the experiment. The amount of phenanthrene remaining was used to evaluate the extent of biodegradation (Table 1). For example, in the presence of 10^5 mg L^{-1} HPCD, the phenanthrene remaining after 2 days was 0.3% (w/w) of the initial amount. In contrast, 12.3% (w/w) and 45.2% (w/w) of the phenanthrene remained after 2 days in the samples with 10^4 mg L^{-1} HPCD and without HPCD, respectively. At the end of the 7 day experiment, phenanthrene was completely degraded in the samples with 10^5 mg L^{-1} HPCD. However, 7.2% (w/w) of the phenanthrene remained in the samples without HPCD. These results clearly indicate that HPCD can significantly enhance the rate of phenanthrene biodegradation.

Although phenanthrene degradation was more rapid in the presence of HPCD, the systems with 10^5 mg L^{-1} HPCD exhibited a longer lag phase (about 12 h) than the systems with 10^4 mg L^{-1} HPCD or without HPCD. A longer lag phase suggests that either the phenanthrene degraders required some time to adapt to the presence of HPCD or they required time to adapt to the high bioavailable levels of phenanthrene. However, once adapted, the degraders benefitted from the presence of HPCD as indicated by the increased growth rate discussed below.

The effect of inoculum density on phenanthrene degradation in the presence of analytical-grade HPCD is shown in Figure 3. Clearly, the lower inoculum density caused the lag phase to increase from 0.5 days (10^7 cfu mL^{-1}) to 1.3 days (10^4 cfu mL^{-1}) for samples with 10^5 mg L^{-1} HPCD. However, the extent and rate of phenanthrene disappearance were essentially identical for the two inoculum densities.

For control samples that contained phenanthrene and HPCD but no degraders, the added phenanthrene was completely recovered, even after 7 days. For control samples that contained phenanthrene and MSM but no degraders, 100 and 97.5% of the added phenanthrene was recovered after 1 and 7 days, respectively. Also, 100% of added phenanthrene was recovered after chloroform evaporation. The results of these control tests confirm that, first, HPCD did not have any adverse effect on the efficacy of chloroform extraction, second, no mass loss of phenanthrene occurred during overnight evaporation of chloroform, and third, all mass loss was caused by biodegradation due to inoculation with the phenanthrene degraders. This third result was confirmed by the results of preliminary $^{14}CO_2$ evolution experiments, which showed that [^{14}C]phenanthrene was mineralized to $^{14}CO_2$ in the presence of the degraders (data not shown).

(2) Cell Growth. The effect of HPCD on phenanthrene biodegradation was confirmed by determining viable counts as a measure of cell growth (Figure 4). In this system, the lag phase before significant cell growth occurred was approximately 5 h, which is sufficient for phenanthrene solubilization to reach equilibrium. Therefore, the bioavail-

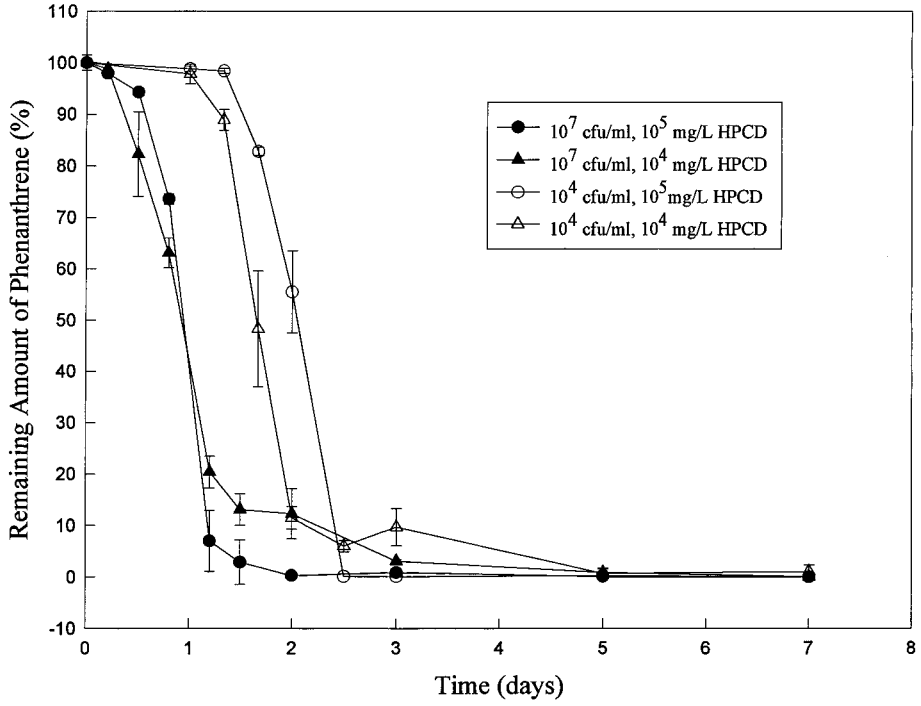

FIGURE 3. Effect of inoculum density on biodegradation of phenanthrene in the presence of analytical-grade HPCD.

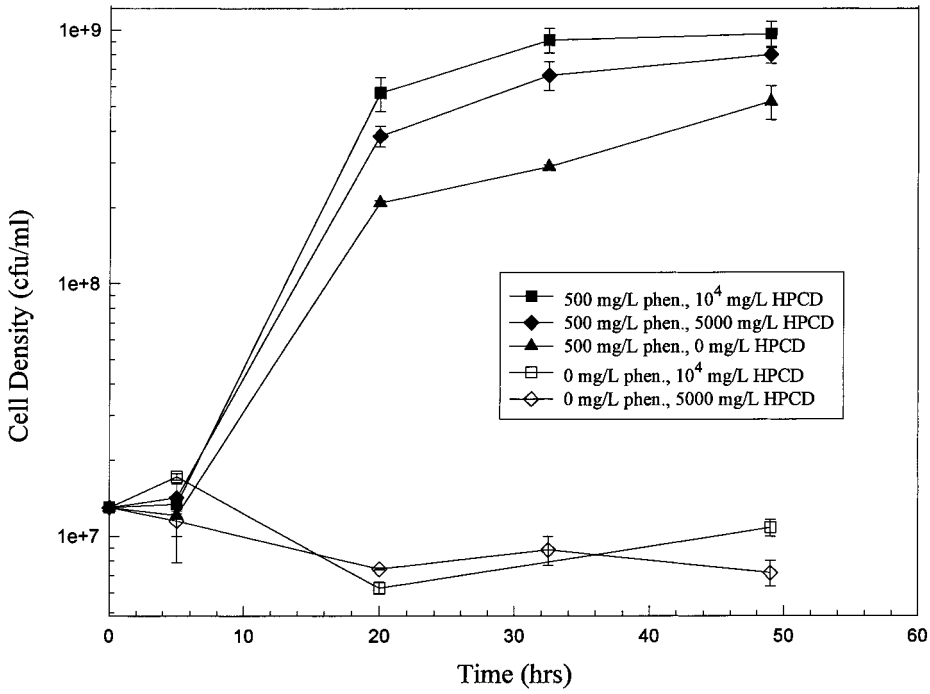

FIGURE 4. The effect of analytical-grade HPCD on phenanthrene biodegradation as measured by viable counts. (Inoculum density was 10^7 cfu/mL.)

ability of phenanthrene in the aqueous solution should be directly correlated to HPCD concentration. The results presented in Figure 4 also show that HPCD did not inhibit cell growth during biodegradation of phenanthrene. Finally, the results demonstrate that HPCD was not used as a carbon or energy source during the experiment. This is consistent with the fact that the genus *Pseudomonas*, some species of which were recently reclassified as *Burkholderia*, produces very low amounts of amylolytic enzymes, which are required to hydrolyze HPCD (*26*).

(3) Substrate Utilization Rate. The substrate utilization rate was calculated for phenanthrene degradation in the presence and absence of HPCD. This was done by evaluating the slope of the substrate utilization curves during exponential growth (Figure 2). The calculated substrate utilization rates, which are the maximum slopes of the curves in Figure 2, are presented in Table 1. The substrate utilization rate for samples with 10^5 mg L^{-1} HPCD was 1.6 times greater than the utilization rate in the presence of the 10^4 mg L^{-1} HPCD, and was 5.5 times larger than for 0 mg L^{-1} HPCD. Because a higher substrate utilization rate requires more bioavailable substrate, the substrate utilization rate should be directly related to the dissolution rate, which is dependent on HPCD concentration.

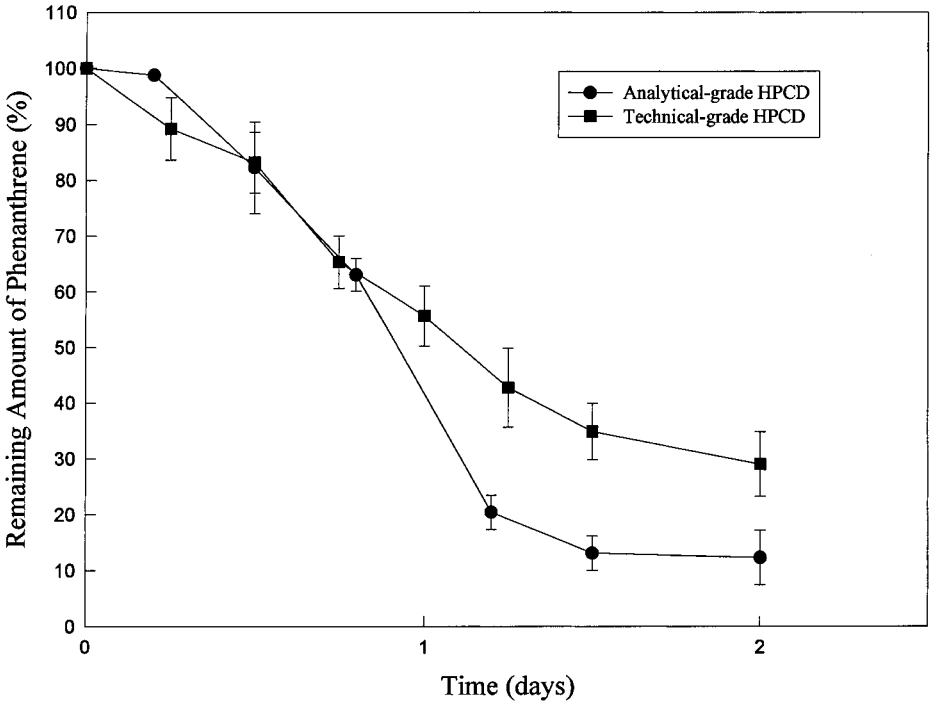

FIGURE 5. Comparison of the impact of technical-grade HPCD (10^4 mg L^{-1}) and analytical-grade HPCD (10^4 mg L^{-1}) on the biodegradation of phenanthrene. (Inoculum density was 10^7 cfu/mL.)

For samples with HPCD, there are three phases of phenanthrene (solid, dissolved, and complexed) when excess phenanthrene is present, and two phases of phenanthrene (dissolved and complexed) when phenanthrene is not in excess. It is not clear whether bacteria can biodegrade complexed phenanthrene directly. Addressing this issue requires knowledge of the dissociation rate of phenanthrene–HPCD complexes. If dissociation is instantaneous, as is generally assumed, the enhanced rate of phenanthrene biodegradation in the presence of HPCD could be explained by the biodegradation of only dissolved phenanthrene. If the dissociation process is not instantaneous, the enhanced rate of phenanthrene biodegradation could indicate that both dissolved and complexed phenanthrene are used directly.

(4) Influence of Impurities in HPCD. The use of analytical-grade HPCD for field applications would generally be prohibitively expensive. It is much more likely that technical-grade forms would be used. In such cases, it is important to evaluate the potential impact of HPCD impurities on contaminant fate. Therefore, an experiment was conducted to examine the impact of propylene glycol, which was the predominant impurity [3.2% (w/w)] in technical-grade HPCD, on phenanthrene degradation.

The biodegradability of propylene glycol by the phenanthrene degrader used in these experiments was evaluated by determining protein as a measure of cell growth. Propylene glycol was added as sole carbon source in amounts equivalent to that found in 5000 and 10^5 mg L^{-1} technical-grade HPCD. Results showed that propylene glycol was degraded and therefore could possibly compete with phenanthrene as a carbon source (data not shown).

The impact of technical-grade HPCD on the biodegradation of phenanthrene is shown in Figure 5, wherein is presented the degradation of phenanthrene in the presence of both 10^4 mg L^{-1} technical-grade and analytical-grade HPCD. An inoculum of 10^7 cfu mL^{-1} was used for these experiments. Phenanthrene degradation was somewhat slower in the presence of technical-grade HPCD as compared to the analytical grade. For example, after 2 days, 12.3% (w/w) phenanthrene remained in samples with analytical-grade HPCD, whereas 29.0% (w/w) remained in samples with technical-grade HPCD. Substrate utilization rates were calculated to be 6.4 mg day^{-1} for technical-grade HPCD and 14.4 mg day^{-1} for analytical-grade HPCD. These results indicate that impurities (e.g., propylene glycol) in the technical-grade HPCD competed with phenanthrene as a carbon source. However, the substrate utilization rate in the presence of technical-grade HPCD was still higher than the utilization rate in the absence of HPCD, which was 4.1 mg day^{-1}. While this might in some cases be a drawback, e.g., presence of high microbial numbers, it should be noted that in situations where there are low numbers of microbes present, e.g., a subsurface environment, the HPCD impurity may help build up a phenanthrene degrading population.

The impact of surfactants, a widely used solubilization agent, on the biodegradation of organic compounds is under investigation. In aqueous systems, some surfactants appear to enhance biodegradation of phenanthrene, such as monorhamnolipid (*13*), an anionic biosurfactant. Conversely, the biodegradation of phenanthrene was inhibited by some anionic synthetic surfactants, such as SDS (sodium dodecyl sulfate) (*9*), and by some nonionic synthetic surfactants, such as Tergitol NP-10 (*6*), Triton N101 (*11*), Triton X-100 (*6, 12*), and Brij 30 (*6, 12*). It is difficult, however, to compare these results directly given the variety of experimental designs and initial/control conditions used.

The results of this study indicate that HPCD can enhance the solubility and dissolution rate of phenanthrene, which increases its bioavailability for degradation by bacteria. Our previous research has shown that HPCD can also enhance the desorption and dissolution of similar compounds (*21, 22*). Thus, cyclodextrins hold promise for use as an agent to increase the performance of in situ bioremediation systems.

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