0273-2289/92/3435—0753$02.60

Environmental Influences on Diethyl Phthalate Biodegradation Kinetics

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ABSTRACT

The effects of temperature, dissolved oxygen level, and diethyl phthalate (DEP) concentration on the rates of DEP biodegradation have been investigated in shake flask and fermenter experiments, using aerobic and facultatively aerobic microorganisms. The aerobic strain followed Monod growth kinetics, and was negatively affected by temperatures lower than 25°C and dissolved oxygen levels lower than 0.8 mg/L, whereas the specific DEP-degrading activity of the facultative strain was substrate inhibited under anaerobic conditions, higher at 15°C than 25°C under aerobic conditions, and unaffected by the dissolved oxygen level. Studies were also carried out in soil columns to identify additional factors that might be important for modeling DEP biodegradation.

Index Entries: Biodegradation; diethyl phthalate; soil columns; dissolved oxygen; temperature.

INTRODUCTION

In situ bioremediation is a promising method for the permanent removal of pollutants from soil and water environments. Despite its potential, this technology cannot be implemented reliably without adequate information on the types of microbially mediated reactions that can occur, their rates, and the influences of the physicochemical aspects of the environment on those rates. In the last five years, there has been a rapid

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Table 1
Some Environmental Factors Affecting *In Situ* Bioremediation

Temperature	(Xenobiotic) substrate concentration
Pressure	Water activity
pH	Salinity
Oxidation-reduction potential	Soil water content
Dissolved oxygen concentration	Soil organic content
Inorganic nutrient concentrations	Soil clay content
Levels of alternative carbon sources	Soil permeability

increase in reports of biodegradation of various xenobiotic chemicals (*1,2*). In many cases, the degradation pathways have been at least partially determined (*1,2*), and some papers have also reported degradation rate data (*3*). However, there have been relatively few publications on environmental effects (*4*).

It has long been known that factors, such as temperature and dissolved oxygen concentration, play a significant role in determining the level of microbial activity. In traditional bioprocesses, it is possible to control the cultivation conditions to their optimal levels, but such is not the case for *in situ* bioremediation. Thus, it becomes even more important to determine the extent of the effects of certain physical and chemical parameters (Table 1). In addition to typical characteristics, such as temperature, other factors (e.g., adsorption) arise when one considers soil environments.

Since it is usually tedious, time-consuming, and expensive to conduct biodegradation experiments in a soil/aquifer system, basic studies using liquid cultures (in shake flasks and fermenters) and soil columns have been common (*3,5*). Although the fundamental kinetics can be investigated using liquid cultures alone, soil column experiments provide valuable information on microbial activity in more complex environments, in which adsorption, flow, and other effects are important. Various mathematical models of *in situ* bioremediation have been developed and applied to the results of soil column experiments (e.g., *6*). These models invariably include terms for convection, dispersion, adsorption, and reaction of the contaminant, but the influences of environmental factors (especially on the reaction term) are included only rarely (*7*).

In this article, we describe the biodegradation of diethyl phthalate (DEP) by two microbial strains. DEP belongs to the family of phthalic acid esters, which are used primarily as plasticizers and are produced in very large quantities each year (*8*). DEP and several other phthalate esters are widely distributed in sediments, waters, and soils, are suspected teratogens and carcinogens, and have been listed as priority pollutants by the US Environmental Protection Agency (*9*). In an investigation of leachates from 58 landfills, DEP concentrations ranged from 0.004 to 300 mg/L (*10*). DEP biodegradation follows the general pathway (*11*):

diethyl phthalate → monoethyl phthalate → phthalic acid → protocatechuic acid

$$\rightarrow \rightarrow \text{small molecules} + CO_2 \quad (1)$$

Although many cultures have been found to degrade DEP in laboratories (*12,13*), this compound appears to be persistent (biodegrades very slowly) in nonlaboratory environments (*14*); in one study, DEP was not degraded at all (*15*), indicating that DEP biodegradation is significantly influenced by environmental parameters.

MATERIALS AND METHODS

Microorganisms

Strains A (aerobic) and B (facultatively aerobic) were isolated from waste-water treatment plant sludge (*16*). Both strains appear able to utilize DEP as the sole source of carbon (although the medium used in these experiments contained a low level of yeast extract) and to degrade DEP completely (*17*). These strains have not yet been identified.

Medium

The medium used in these experiments was a minimal medium, consisting of: 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.111 g $CaCl_2$, 0.247 g $MgSO_4 \cdot 7H_2O$, 69.2 mg $ZnSO_4 \cdot 7H_2O$, 270 mg $FeCl_3 \cdot 6H_2O$, 80 mg $MnSO_4$, 7.4 mg $CuCl_2$, 28.1 mg $CoSO_4 \cdot 7H_2O$, 3 mg H_3BO_3, and 10 mg yeast extract in 1 L of water. The concentration of DEP ranged from 50 to 400 mg/L; the exact levels used are presented along with the results of each experiment.

Experimental Apparatus

Liquid cultivations were carried out either in shake flasks (100-mL culture in 250-mL flasks) or in a 2-L fermenter (Biolab, B. Braun Biotech) equipped with temperature, oxygen, and pH control. Anaerobic shake flask cultivations were performed in a glove box.

For the soil column experiments, three identical columns (50 cm high, 5 cm ID) were run in parallel to allow the determination of adsorbed biomass during an experiment (which required the sacrifice of a column). Each column was provided with three sampling ports at 10, 25, and 40 cm above the inlet. The columns were packed with sand (20–40 mesh) that had been sterilized and then mixed with a DEP-degrading culture before packing. Sand was chosen to reduce the importance of adsorption in this transport-reaction system. The porosity of the sand-filled column was 0.4, and the total void vol was 314 cm^3. After packing, DEP-free medium was passed through the column for a short time. Each experiment began with a switch to medium containing 100 mg/L DEP. The fermenter was

used as the sterile medium reservoir, allowing the dissolved oxygen content and temperature at the inlet to be maintained at the desired values. Flow through the columns was upward and was supplied by a peristaltic pump at 3.8 mL/h.

Analytical Methods

DEP was assayed with a gas chromatograph and FID detector (HP 5890A), using a 2-m long, 4-mm ID glass column packed with OV-17. During the analyses, the column temperature was 240°C, and the N_2 carrier gas flow rate was 30 mL/min.

Suspended cell densities were estimated by measuring the optical density at 660 nm and using an experimentally derived correlation to obtain the concentration. In the soil column experiments, the amount of biomass adsorbed to the soil was estimated by the fluorescein diacetate method (*18*). For each assay, 5 g soil were suspended in 1.5 mL water. The amount of fluorescein released was proportional to the attached biomass and was determined by measuring the absorbance at 490 nm (*17*).

In the fermenter experiments, dissolved oxygen levels were measured with a calibrated galvanic oxygen sensor. In the soil column studies, the azide modification of the Winkler method was used to measure the dissolved oxygen concentration in samples removed at each port (*19*).

The retardation coefficient of the soil column was measured by pulsing in DEP and a conservative tracer (bromide) according to established procedures (*20*). This coefficient had a very low value of 1.06, indicating the DEP did not adsorb tightly to the sand.

RESULTS AND DISCUSSION

Fermenter and Shake Flask Cultivations

Effect of Initial DEP Concentration

These shake flask (batch) experiments were performed at 25°C to ascertain the basic kinetics of DEP biodegradation by strains A (under aerobic conditions) and B (under anaerobic conditions). For each strain, a range of initial DEP concentrations was used. Although the medium contained a low concentration of yeast extract, control experiments with both strains showed that no detectable growth occurred without DEP.

The results of the strain A experiments are presented in Fig. 1. It can be seen that the highest initial DEP concentration used, 400 mg/L, caused a longer lag time before degradation and growth began. In addition, the continued growth of the cells after the DEP was consumed indicates that an intermediate accumulated during the primary degradation of DEP. A compound, tentatively identified as phthalic acid by GC-MS, was found in these culture samples. Phthalic acid is known to be an intermediate in the DEP degradation pathway (*11*).

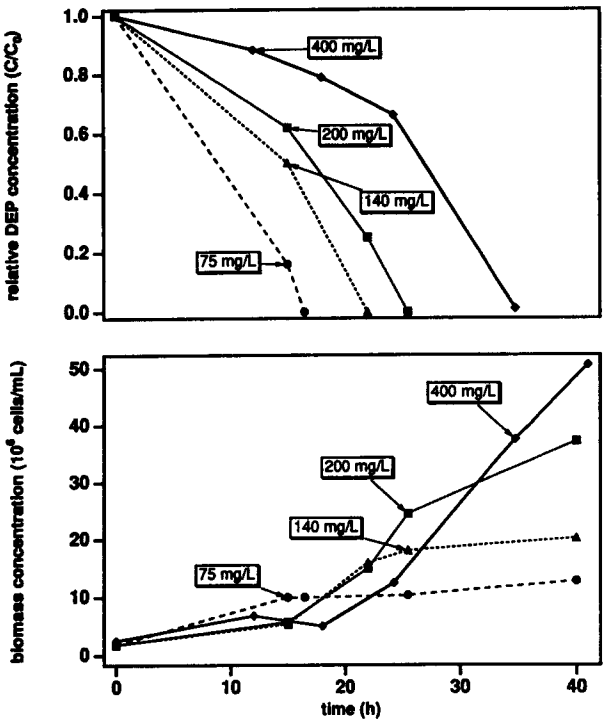

Fig. 1. Results of aerobic shake flask experiments using strain A; initial DEP concentrations are indicated for each data set. Top: relative DEP concentration (C/C_0). Bottom: biomass concentration.

Analysis of these data showed that DEP consumption by strain A followed Monod kinetics with a constant yield coefficient ($K_s = 28$ mg/L and $\mu_m = 0.4\ h^{-1}$). No substrate inhibition of the specific DEP degradation rate was observed, even at the highest initial DEP concentration. Apparently, the presence of the intermediate, phthalic acid, also did not inhibit these rates.

In Fig. 2, results are shown for anaerobic DEP degradation experiments involving strain B. Rates of degradation under these conditions are slower than those of both aerobic strain A (Fig. 1) and strain B under aerobic conditions (results not shown). As in the aerobic strain A cultures, phthalic acid was identified as an intermediate. In this case, however, the data were not represented by the Monod model with constant yield factor; specific degradation rates appeared to be attenuated by substrate inhibition.

Effects of Temperature

The effects of temperature, although well known in microbiology, have often been overlooked in models of bioremediation. Since soil/aquifier environments are usually cooler than "room temperature," it is important to determine biodegradation kinetics in the 5–20°C range. Figs. 3 and

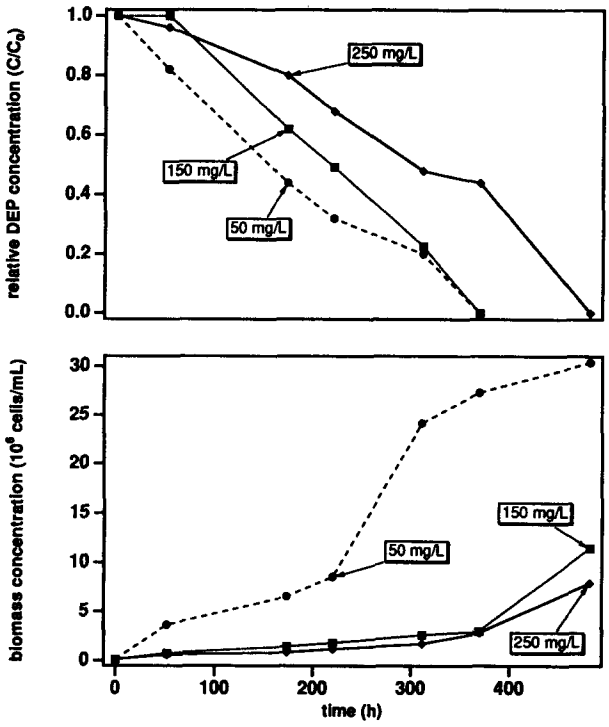

Fig. 2. Results of anaerobic shake flask experiments using strain B; initial DEP concentrations are indicated for each data set. Top: relative DEP concentration (C/C_0). Bottom: biomass concentration.

4 contain the results of aerobic, batch fermenter experiments with strains A and B in this temperature range.

The results for strain A (Fig. 3) shows that activity at 5°C was much lower than at either of the high temperatures, but was still significant. Growth at this temperature was preceded by a lengthy lag phase; lag phase duration is known to increase with decreasing temperature (*21*). Although the specific growth rates were highest at 25°C by a large margin, the specific rates of DEP degradation were approximately the same at 15 and 25°C, indicating that the enzyme responsible for DEP breakdown (dialkyl phthalate esterase) is less affected by low temperatures than are cell growth processes.

As can be seen in Fig. 4, aerobic growth and DEP degradation by strain B proceed well at 15°C. Analysis of these data reveals that the specific growth rates at 15 and 25°C were comparable and higher than those at 10°C. However, the specific DEP degradation rates were much higher at 10 and 15°C than at 25°C. Strain B thus appears to be slightly psychrophilic.

Effects of Dissolved Oxygen Level

The level of dissolved oxygen is an extremely important parameter for aerobic *in situ* bioremediation operations. In these situations, oxygen

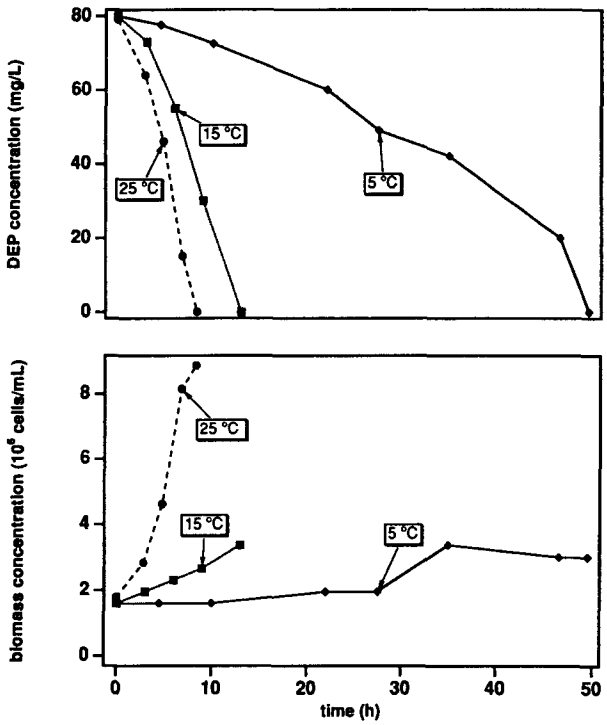

Fig. 3. Results of aerobic fermenter experiments using strain A at 5, 15, and 25°C; the initial DEP concentration was 80 mg/L in each case. Top: DEP concentration. Bottom: biomass concentration.

is usually the rate-limiting nutrient. Unfortunately, it is often the most difficult compound to supply to a subsurface site owing to its low solubility in water. Interestingly, almost all models of *in situ* bioremediation assume that the carbon-containing xenobiotic is the rate-limiting nutrient. The exception to this is BIOPLUME II, which assumes that oxygen will be the limiting substrate (7). The results of batch fermenter experiments with strains A and B over a wide range of dissolved oxygen levels are shown in Figs. 5 and 6.

As can be seen in Fig. 5, neither the growth nor the DEP-degrading rates of aerobic strain A were oxygen limited at dissolved oxygen levels above 0.85 mg/L. However, dissolved oxygen concentrations below this significantly reduced the rates of growth and DEP consumption. Modeling of these data with Monod kinetics (using values at 40 mg/L DEP) and viewing oxygen as the limiting substrate yielded a K_{O2} of 0.5 mg/L oxygen.

Although the specific growth rate of facultative strain B was lower when exposed to an oxygen level of 0.085 mg/L than a concentration of 8.5 mg/L, the overall rate of DEP removal was nearly the same, making the specific rate of DEP removal higher at the lower oxygen level (Fig. 6). This means that the yield coefficient relating growth and substrate consumption rates was different, a fact that indicates that the cells of the

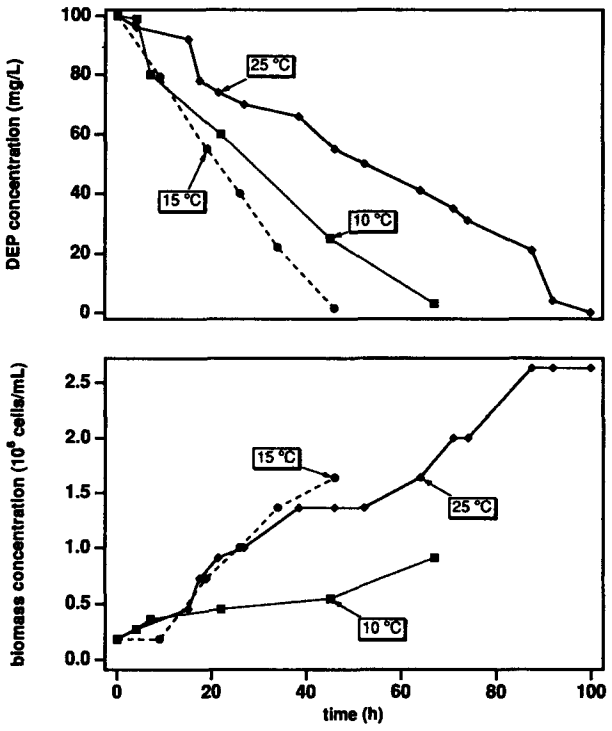

Fig. 4. Results of aerobic fermenter expèriments using strain B at 10, 15, and 25°C; the initial DEP concentration was 100 mg/L in each case. Top: DEP concentration. Bottom: biomass concentration.

facultative strain changed their metabolism in response to the lowered oxygen concentrations.

Soil Column Experiments

Strain A

In this experiment, three columns were packed with wet sand that had been exposed to an active culture of strain A. As described in Materials and Methods, the fermenter was used to supply medium to the column at controlled temperature and dissolved oxygen levels. In this case, the temperature was 25°C, the inlet dissolved oxygen concentration was 0.85 mg/L, and the inlet DEP concentration was 100 mg/L of liquid.

As can be seen in the top plot of Fig. 7, the concentration of DEP in the column achieved a steady profile after a few days, and all of the DEP in the feed was consistently removed in the lower half of the column. Decreases in the dissolved oxygen concentration in this portion of the col-

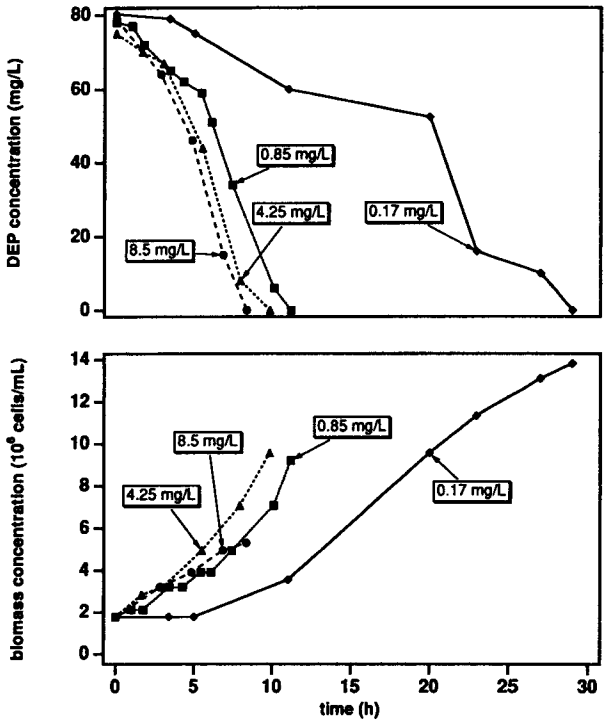

Fig. 5. Results of fermenter experiments using strain A under conditions of different dissolved oxygen concentrations (as labeled); the initial DEP concentration was 80 mg/L in each case. Top: DEP concentration. Bottom: biomass concentration.

umn are the result of the activity of the microbes consuming the DEP. However, as the bottom plot indicates, strain A did not adsorb well to the sand; in fact, the amount of viable, adsorbed biomass decreased by a factor of 2–3 during the 2-wk long experiment. Retention of adsorbed cells was slightly higher in the lower parts of the columns in which substrate was present. It is apparent that nonadsorbed cells played a large role in removing DEP in this experiment.

It is worth considering how such a phenomenon might be accounted for by typical *in situ* bioremediation models. The general form of a one-dimensional contaminant transport-reaction model is

$$R\,(\partial c / \partial t) = D\,(\partial^2 c / \partial z^2) - u_p\,(\partial c / \partial z) - (r_d / \Theta) \qquad (2)$$

in which c is the concentration of the solute, Θ is the volumetric water content, D is the hydrodynamic dispersion, u_p is the pore water velocity, R is the retardation coefficient ($r = 1 + \rho K_A/\Theta$), ρ is the bulk density of the soil, K_A is the coefficient of the linear adsorption isotherm, and r_d is a general degradation term.

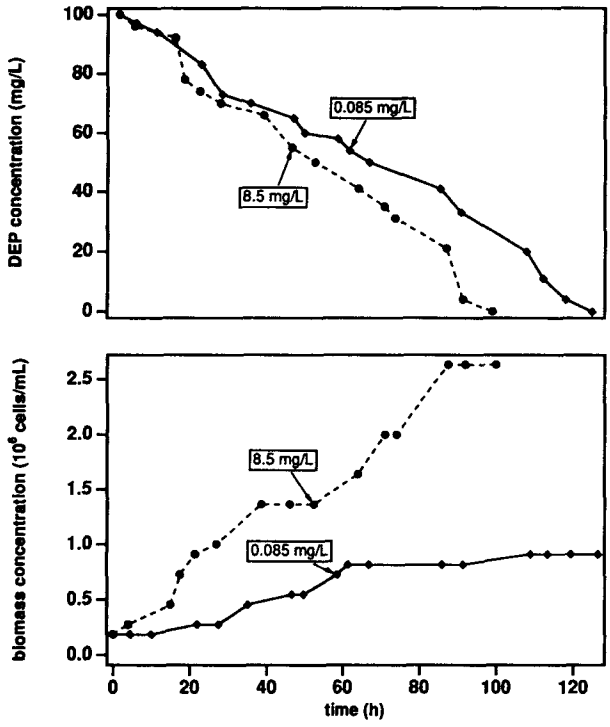

Fig. 6. Results of fermenter experiments using strain B under conditions of different dissolved oxygen concentrations (as labeled); the initial DEP concentration was 100 mg/L in each case. Top: DEP concentration. Bottom: biomass concentration.

Biodegradation is included in the r_d term, as are abiotic transformations of the contaminant being modeled. In this case, a control experiment indicated that DEP was not removed by abiotic mechanisms. For simplicity, biodegradation is often modeled as first order in the carbon source concentration, biomass concentration is not taken into consideration, temperature effects are ignored, and the possibility of rate limitation owing to low oxygen concentration is excluded. Our results with strain A show that:

1. Monod kinetics would be more appropriate;
2. Temperature effects are important;
3. Oxygen can indeed become the limiting substrate at levels < 0.5 mg/L; and
4. An additional transport equation for the microorganisms is necessary.

Strain B

A similar soil column experiment was carried out using the facultative strain B and sand. Again, the inlet DEP concentration was 100 mg/L, the

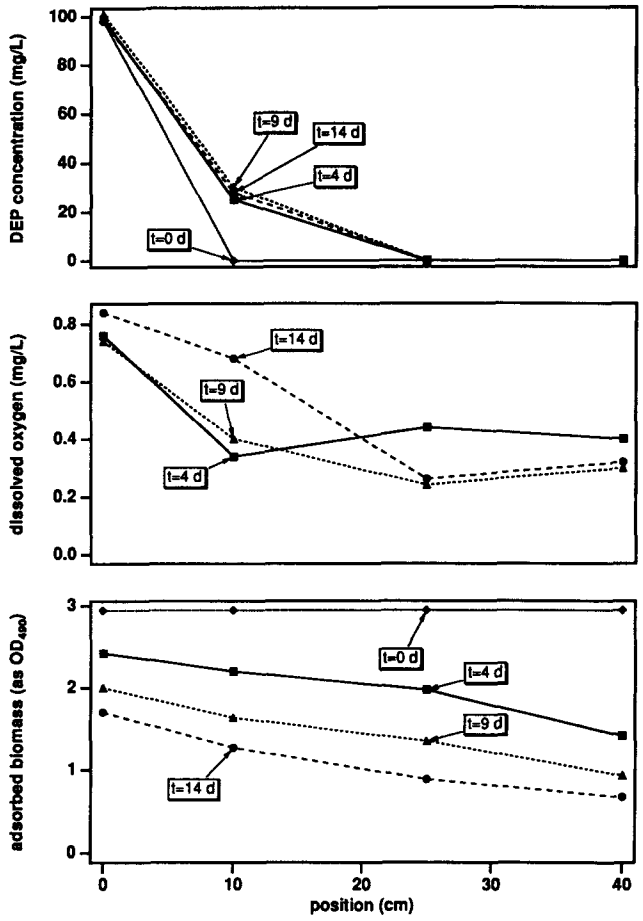

Fig. 7. Results of the soil column experiment using strain A. At zero time, the inlet DEP concentration underwent a step change from 0 to 100 mg/L; the inlet dissolved oxygen level was 0.85 mg/L, and the temperature was 25°C. Top: DEP concentration. Middle: dissolved oxygen concentration. Bottom: viable, soil-adsorbed biomass concentration (as equivalent OD_{490} values).

inlet dissolved oxygen level was 0.85 mg/L, and the temperature was 25°C. The results of this experiment are shown in Fig. 8.

In this case, it is apparent from the top plot that the overall rate of DEP removal increased with time, and more than 20 d were required before the outlet DEP concentration was reduced to zero. The higher levels of metabolic activity later in the experiment are also apparent from the profiles of dissolved oxygen concentration (middle plot). The results shown in the bottom plot reveal that the increasing rates of DEP removal are the result of increased levels of viable, adsorbed biomass. It is apparent that strain B adsorbs better to the sand particles than strain A.

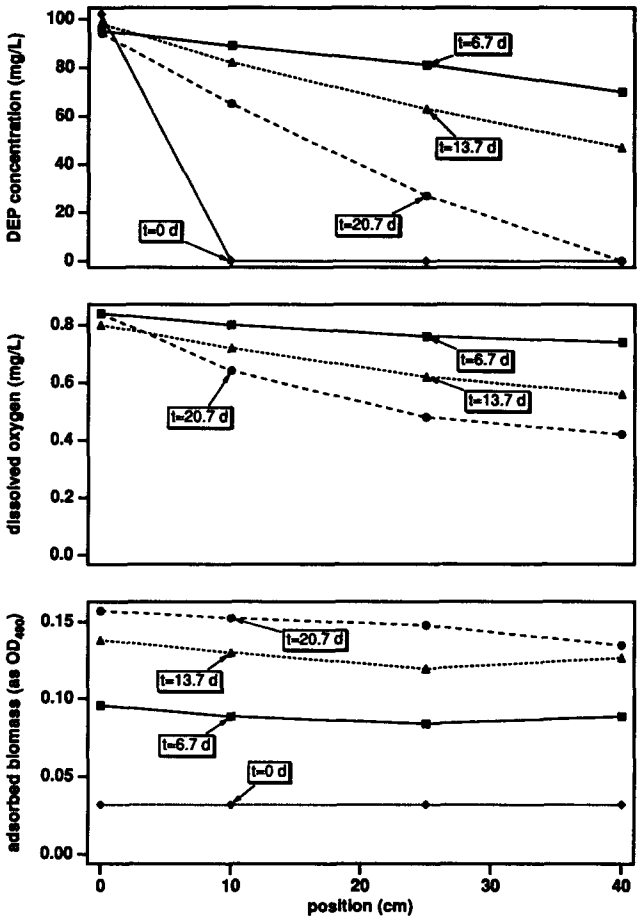

Fig. 8. Results of the soil column experiment using strain B. At zero time, the inlet DEP concentration underwent a step change from 0 to 100 mg/L; the inlet dissolved oxygen level was 0.85 mg/L, and the temperature was 25°C. Top: DEP concentration. Middle: dissolved oxygen concentration. Bottom: viable, soil-adsorbed biomass concentration (as equivalent OD_{490} values).

From the results obtained with strain B, it is clear that most *in situ* bioremediation models would be unable to describe its DEP consumption kinetics. As outlined above, the typical model would need to be modified to include:

1. Substrate inhibition kinetics;
2. Temperature effects (though not as important in this case); and
3. An additional equation for the growth of the adsorbed microorganisms.

ACKNOWLEDGMENTS

This work was supported by the Colorado Agricultural Experiment Station. The authors are grateful to Feisal Abedeen for his help in the laboratory.

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