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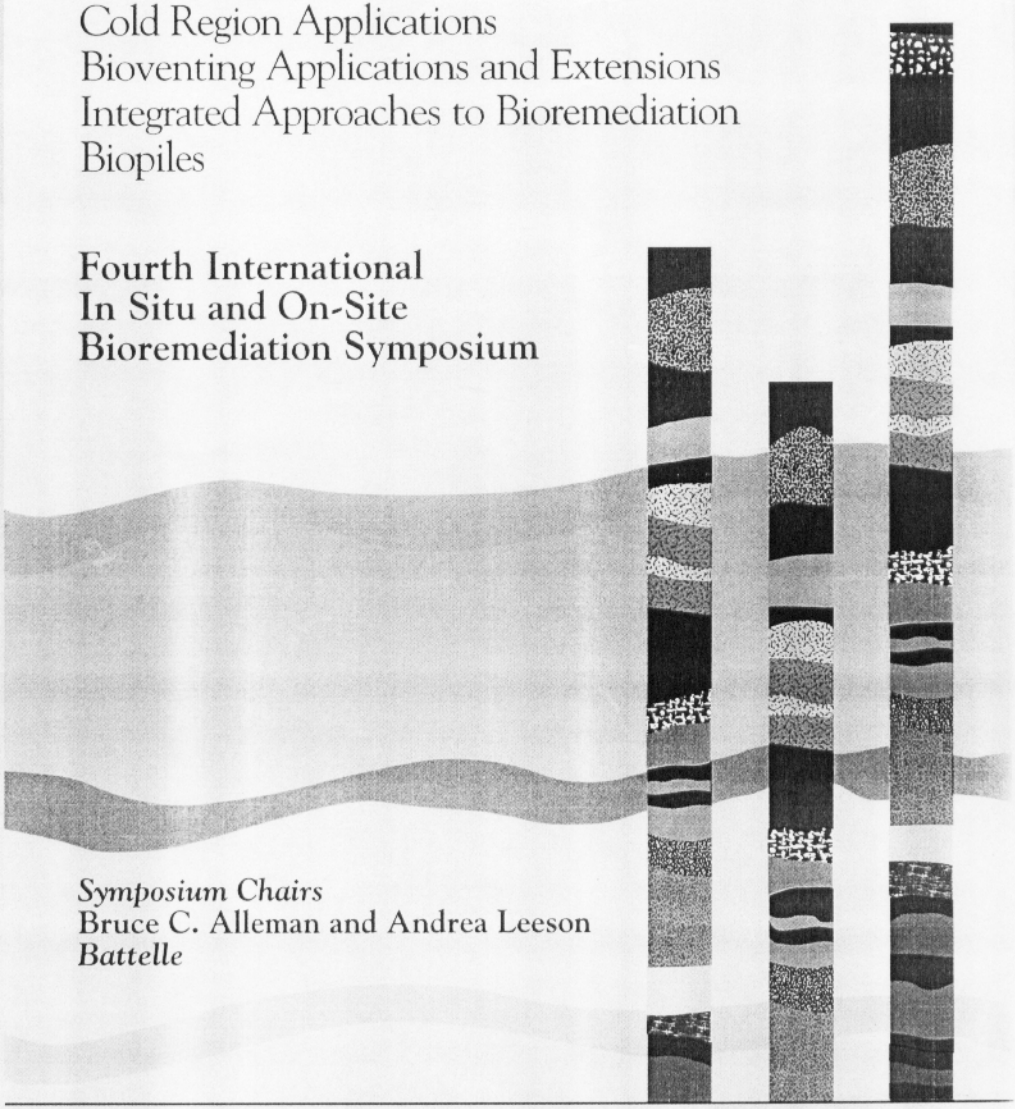

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EFFECT OF ETHANOL ON AEROBIC BTX DEGRADATION

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ABSTRACT: The effect of ethanol on the aerobic biodegradation of benzene, toluene, and *o*-xylene (BTX) was investigated using both aquifer microcosms and a pure culture. In aquifer microcosms, ethanol was utilized preferentially before the BTX. Inorganic nutrient addition sped up the degradation of all compounds without affecting the preferential utilization of ethanol. Interestingly, ethanol did not significantly affect the biodegradation activity of *Pseudomonas putida* F1 (PpF1), which utilized benzene, toluene, and ethanol simultaneously. The presence of 300 mg/L or less of ethanol did not slow benzene and toluene degradation by PpF1, and a slight inhibitory effect was observed at 500 mg/L ethanol. While ethanol is likely to exacerbate the biochemical oxygen demand and exert a diauxic effect that would inhibit in situ BTX degradation, the simultaneous utilization of ethanol and toluene as growth substrates for PpF1 represents a caveat against generalizations about the effect of fuel additives on BTX degradation patterns.

INTRODUCTION

Bioremediation, the use of microorganisms to degrade environmental pollutants, holds great promise as an approach to manage aquifer contamination by benzene, toluene, and xylenes (BTX). Indeed, this technique has been successfully used to clean up BTX contaminated groundwater from leaking underground fuel storage tanks at numerous sites (NRC, 1993). Several factors are known to influence the rate and extent of BTX biodegradation, including pollutant concentration, active biomass concentration, bioavailability, temperature, pH, availability of nutrients and electron acceptors, and microbial adaptation. While these factors have been recognized, limited attention has been placed on the effect that fuel additives have on in situ BTX degradation. Ethanol is widely used as an oxygenate at high concentrations (10 to 22%) to increase the octane of gasoline and to minimize air pollution from fuel combustion. Ethanol can also be used as an alternate fuel to better manage hydrocarbon resources, and as a reagent for in situ chemical extraction operations. Therefore, the presence of ethanol may become common in hydrocarbon plumes. A basic understanding of how ethanol affects BTX degradation kinetics is warranted for the selection, mathematical modeling, and monitoring of appropriate bioremediation systems.

MATERIALS AND METHODS

Microcosm Experiments. Aquifer microcosms were used to investigate the effects of ethanol on microbial acclimation to aerobic BTX degradation under both oxygen-limited and unlimited conditions. Microcosms were prepared with sandy aquifer material that had low organic content (0.2%) and no known previous exposure to BTX. Aquifer material was collected using sterilized tools from the phreatic surface at a depth of 4 ft, and stored at 4 °C until use. Samples were collected from a scarcely populated area near Jurerê Beach, in the north of Santa Catarina Island, Brazil. Groundwater samples were also collected from the same location for use in microcosm preparation.

The groundwater had the following characteristics: pH = 5.2; conductivity = 66.5 µS/cm; total acidity = 26 mg/l as $CaCO_3$; total alkalinity = 10 mg/l as $CaCO_3$; hardness 433 mg/l as $CaCO_3$; turbidity = 2 NTU; chloride = 10.7 mg/L; total phosphorus = 0.26 mg/L as P; TKN = 16.3 mg/L; ammonia = 11.1 mg/L as N.

Microcosms were prepared in 100-mL serum bottles with 20 g of drained aquifer material and 50 mL of groundwater. Various treatment sets were prepared in triplicate to investigate the effect of ethanol on BTX degradation patterns. Initial ethanol concentrations were 0, 20, 100 and 300 mg/L. BTX were fed at about 20 mg/L each using 10 µL gas-tight syringes, and the microcosms were sealed with Teflon-lined septa and aluminum crimps. Controls were poisoned with 1,000 mg/L sodium azide to discern biodegradation from volatilization losses. These experiments were also repeated with inorganic nutrient amendments, as described elsewhere (Corseuil and Weber, 1994), to investigate the reproducibility of the observed trends under different nutrient conditions. The toxicity of ethanol at higher concentrations (1,000 to 10,000 mg/L) was investigated in the absence of BTX using oxygen consumption as an indication of microbial activity. All microcosms were incubated quiescently in the dark at 20 °C.

Pure Culture Experiments. *Pseudomonas putida* F1 (PpF1) cells were grown on mineral salts basal medium (MSB) (Stanier et al., 1966) and pyruvate (1 g/L) to an OD_{600} of 0.1. To prepare the inoculum, cells were harvested, washed three times in MSB buffer, and resuspended to an OD_{600} of 0.2. The experiments were conducted in 250-mL serum bottles seeded with 0.2 mL of inoculum in 50 mL media and capped with Mini-nert valves. Toluene and benzene were fed at a concentration of 20 mg/L each, and their concentrations were monitored over time. Ethanol was added at concentrations of 0, 50, 100, and 300 mg/L. Each set of conditions was run in triplicate and incubated at 30°C on a rotary shaker (180 rpm).

Chemical Analyses. Benzene, toluene, *o*-xylene, and ethanol were analyzed in triplicate by direct aqueous injection into a Varian 2440 D gas chromatograph equipped with a flame-ionization detector. Separation was achieved isothermally at 160 °C using a 2-meter POROPAK Q 100/80 Mesh column. Dissolved oxygen was analyzed titrimetrically by the Winkler method (Standard Methods, 1992).

RESULTS AND DISCUSSION

Aquifer microcosms were used to investigate the effects of ethanol on aerobic BTX degradation under both oxygen-limited and unlimited conditions. In all cases, ethanol was preferentially utilized over all BTX compounds, and a lag period was observed during which ethanol was degraded before any measurable BTX degradation occurred. Figure 1 shows a typical example, in which most of the added ethanol (100 mg/L) was degraded before the onset of BTX degradation. Lag periods for BTX degradation increased with the initial ethanol concentration, and concentrations higher than 40,000 mg/L were toxic, as indicated by a complete stop in dissolved oxygen consumption. The preferential utilization of ethanol was also observed in microcosms not amended with inorganic nutrients, although the lags were longer. Figure 2 compares benzene degradation with and without nutrients added. In the absence of ethanol, 20 mg/L of benzene was degraded within 3 days in microcosms amended with inorganic nutrients and trace minerals, using the recipe of Corseuil and Weber (1994) (Figure 2(A)). The same amount of benzene lasted about 9 days without nutrient addition (Figure 2(B)). In both cases, microcosms amended with 300 mg/L ethanol had a greater biochemical oxygen demand (BOD) than available oxygen and became anoxic while degrading ethanol, and benzene was not degraded in the absence of molecular oxygen.

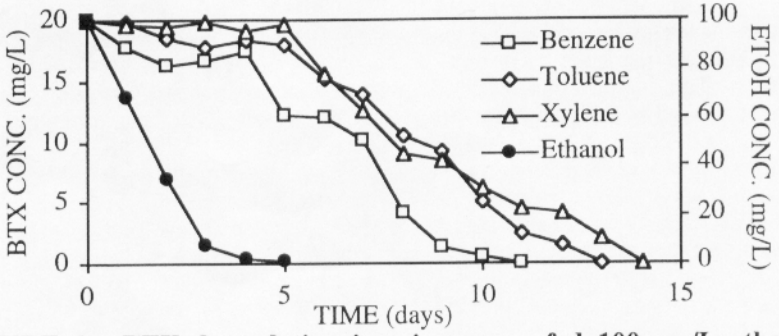

FIGURE 1. BTX degradation in microcosms fed 100 mg/L ethanol. BTX degradation did not begin until most of the ethanol had been degraded, regardless of whether the BTX were present together or individually.

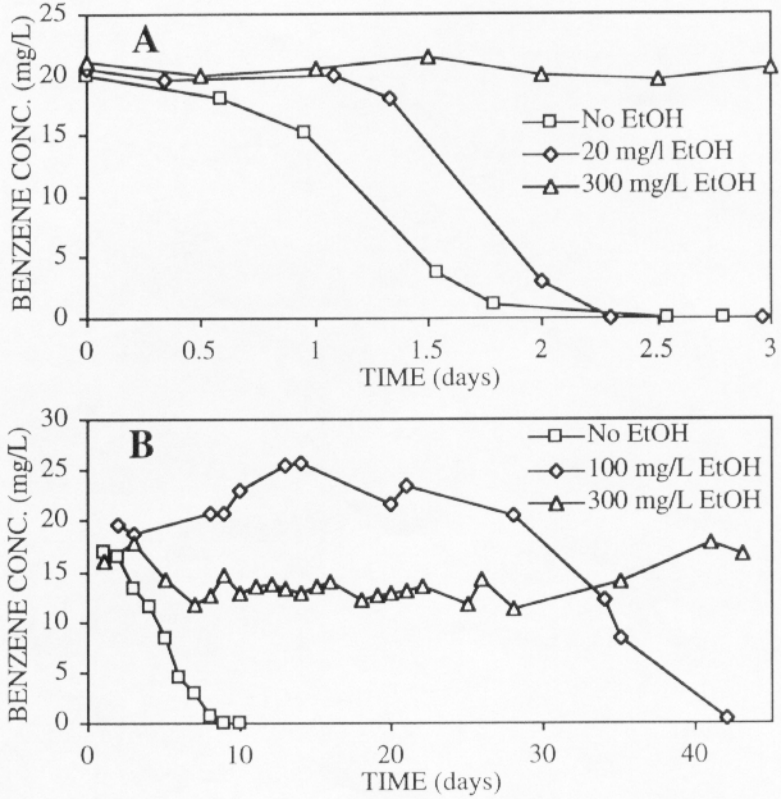

FIGURE 2. Effect of ethanol and nutrients on benzene degradation in microcosms. Microcosms amended with inorganic nutrients (**A**) exhibited shorter lags and degraded benzene sooner than microcosms prepared without nutrients (**B**). The lag time increased with increasing initial ethanol concentration. Microcosms fed 300 mg/L of ethanol had a greater biochemical oxygen demand than available oxygen, and became anoxic before the onset of benzene degradation.

The microcosm experiments illustrate that ethanol constitutes a significant additional oxygen demand to that exerted by other soluble components of gasoline, and is likely to decrease the extent of aerobic BTX degradation in oxygen limited aquifers. This is particularly important for the fate of benzene, which is the most toxic of the BTX and degrades very slowly if at all under anaerobic conditions (Alvarez and Vogel, 1995; Edwards et al, 1992; Grbić -Galić and Vogel, 1987). Figures 1 and 2 suggest that benzene could migrate without significant bioattenuation until most of the ethanol is biodegraded.

Characterizing a particular biocatalytic process in undefined systems is a difficult task due to potential confounding effects that influence microbial activities, including population interactions and shifts. Therefore, further studies of substrate interactions between ethanol and BTX were conducted in an axenic system with a much studied microbial strain, *Pseudomonas putida* F1. Initial ethanol concentrations were 0, 50, 100, and 300 mg/L. Considering that ethanol is completely soluble in water, and assuming a typical dilution ratio of about 1:100 near the source, these ethanol concentrations are within the expected range for groundwater contaminated with ethanol-gasoline mixtures.

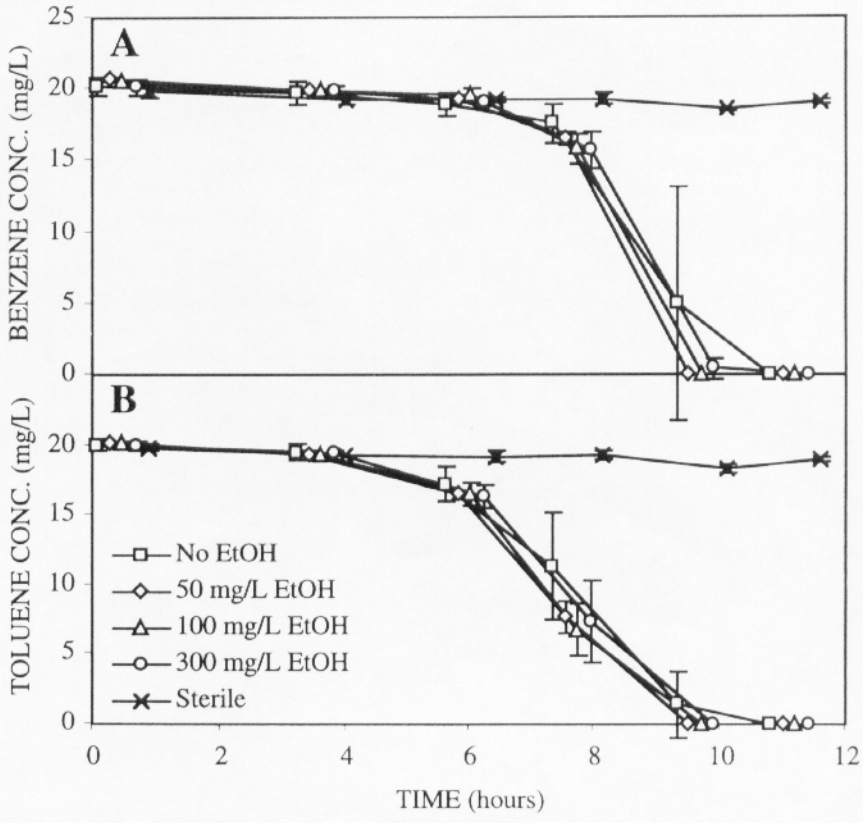

FIGURE 3. Concurrent biodegradation of benzene (A) and toluene (B) by PpF1 with varying initial ethanol concentrations. Benzene and toluene degradation was not affected by the addition of ethanol up to 300 mg/L. Oxygen was not limiting in any of these (larger) reactors.

Interestingly, the degradation of 20 mg/L each of benzene (Figure 3(A)) and toluene (Figure 3(B)) was not affected significantly by the presence of varying ethanol at the tested levels. The onset of toluene degradation preceded the onset of benzene degradation by about one hour, and both were fully degraded within 11 hours, regardless of the initial ethanol concentration. Sterile controls showed no loss during the same time period.

In a separate experiment with 500 mg/L of toluene, the rate of toluene degradation was slightly decreased with the addition of 500 mg/L of ethanol (Figure 4), although toluene and ethanol were used simultaneously as growth substrates by PpF1. In this experiment, microbial growth was monitored by optical density (600 nm), and correlated to dry weight biomass concentration using the following equation:

$$(\text{mg/L dry weight}) = 348\ (OD_{600}) - 3.5;\ r^2 = 0.999.$$

The observed inhibition on toluene degradation by 500 mg/L of ethanol should not be attributed to diauxy, which was shown not to be a factor in Figure 3. Competitive inhibition of catabolic enzymes can also be ruled out as the cause for this inhibitory effect since ethanol and toluene are degraded by PpF1 by different pathways. It remains to be determined whether the inhibitory effect was due to increased mass transfer limitations (resulting from increased bacterial density) or to the effect that higher ethanol concentrations may have on metabolic regulation.

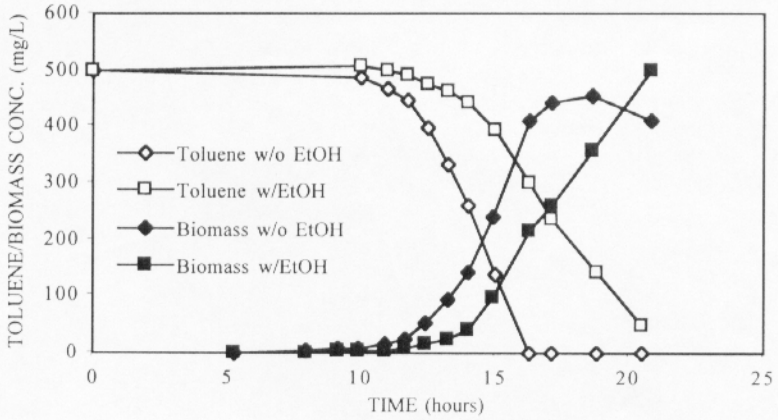

FIGURE 4. Aerobic growth of PpF1 on toluene in the presence and absence of ethanol (500 mg/L). Ethanol exerted a slight inhibitory effect on toluene degradation and PpF1 growth, but was used concurrently with toluene.

CONCLUSIONS

While ethanol is likely to exacerbate the biochemical oxygen demand and exert a diauxic effect that would inhibit in situ BTX degradation, the simultaneous utilization of ethanol and toluene as growth substrates for PpF1 reminds us that the metabolic diversity of microorganisms may prove wrong generalizations about the effect of fuel additives on BTX degradation patterns.

Further research is needed on the effect of ethanol on microbial community structure, enzyme induction, and biodegradation kinetics under different electron acceptor conditions to develop unifying principles that facilitate the management of aquifers contaminated with ethanol-gasoline mixtures.

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