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Biodegradation of Monoaromatic Hydrocarbons in Groundwater under Denitrifying Conditions

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Indigenous subsurface bacteria can degrade a variety of fuel hydrocarbons, including the monoaromatic compounds benzene, toluene, and xylene (BTX), under favorable conditions (Karlson and Frankenberger 1989). Enhanced in-situ biodegradation has received increasing attention for aquifer remediation. The success of this approach depends largely on the ability to distribute sufficient quantities of an electron acceptor (such as oxygen) to satisfy the oxidation demand of fuel hydrocarbons in both the contaminated soils and groundwaters. Due to the low solubility of oxygen in water, hydrogen peroxide (H_2O_2) is the oxygen source most widely used. Unfortunately, the rapid decomposition of H_2O_2 in the subsurface may often occur due to catalysis by transition metals such as iron, (Britton 1985) or by bacterial catalase activity (Spain et al. 1989).

The use of denitrification, whereby nitrate (or nitrite) is used as an alternate electron acceptor to oxygen, represents an innovative remediation approach, since nitrate is very soluble in water and can be easily distributed throughout an aquifer. However, to date, there have only been a few field-scale trials of in-situ bioremediation using denitrification (Berry-Spark et al. 1986; Sheehan et al. 1988. Currently, there is very little information available on the factors that may control denitrification in the subsurface environment. This report evaluates the effect of nutrients and organic amendments on BTX loss in gasoline-contaminated groundwater incubated anoxically under denitrifying conditions.

METHODS AND MATERIALS

Groundwaters were obtained from a gasoline-contaminated site in San Diego, California, at monitoring well #1 (MWI)

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with a stainless steel bailer after purging the well of three well volumes in order to obtain a representative sample. The water was distributed to 120-mL glass bottles fitted with gas-tight teflon septa. Transfer of the groundwater into these bottles was carried out so as to minimize the introduction of oxygen, and indeed oxygen measurements using the Winkler method showed levels below 1 mg L^{-1} after transfer. At these low O_2 levels, denitrification will proceed if an alternate electron acceptor such as nitrate is available. According to stoichiometry, small amounts of oxygen (1 mg L^{-1}) present at the start of incubation will be used up rapidly after the consumption of less than 1 mg L^{-1} of BTX. The samples were incubated in 120-mL bottles at room temperature ($21^0C \pm 3^0C$)in a N_2-gas-filled atmosphere. Samples received nutrient amendments by syringe. Nutrients used were KNO_3 and/or K_2HPO_4. Unamended or mercury (Hg)-killed samples served as the control. Trace metals were added as sodium molybdate and Ferric-EDTA.

BTX disappearance was followed by peak attenuation of benzene, toluene, and total xylene isomers on a gas chromatograph (Varian Model 3300) equipped with purge and trap concentrator (Valco, Model ATOC-1), a flame-ionization detector (FID), and a 2m 5% SP-1200 (Supelco) stainless steel packed column. The operating conditions were: purge, 60 mL min^{-1} for 12 min; sample size, 5 ml; He, 40 mL min^{-1}; H_2, 100 mL min^{-1}; air, 250 mL min^{-1}; column temperature, 50-90°C, 6^0C min^{-1}; detector temperature, 150°C; integrator, Varian 4290. Peak areas and retention times were compared to a reference standard (Supelco #4-8852M).

Data shown represents mean of duplicate or triplicate assays. However, there is some variability in BTX levels between replicate samples, most probably due to the several millimeters of free gasoline product floating on the water table and incorporated into our samples. The presence of minuscule colloidal suspensions of liquid hydrocarbon could account for the variability observed.

Tests to determine statistical significance were done using the Student t test. Total denitrifiers were enumerated by a most probably number technique on nitrate broth (Difco Laboratories, Detroit, Michigan).

RESULTS AND DISCUSSION

In-situ levels of BTX compounds in groundwaters varied temporally in the aquifer during the study. Levels at MW1 (the most contaminated well) were in the range of 9-14 mg L^{-1} for benzene, 23-81 mg L^{-1} for toluene, and 13-171 mg L^{-1} for total xylene isomers.

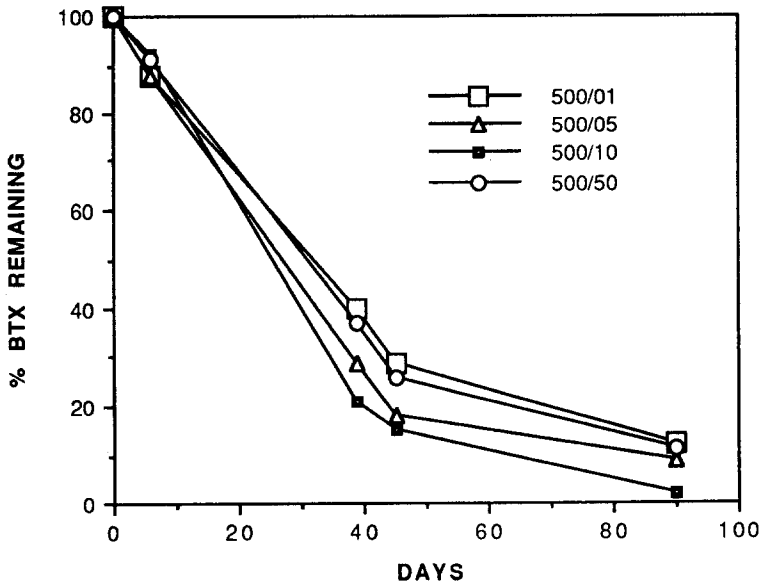

Figure 1. Percent loss of BTX (benzene, toluene and xylene) in groundwater incubated anoxically with 500 mg L^{-1} NO_3^--N and varying amounts of phosphorus, ranging from 1-50 mg L^{-1} PO_4^{-3}-P. All values are calculated as compared to the unamended control.

In order to examine the effect of phosphorus on BTX biodegradation under denitrifying conditions, an experiment was performed with groundwaters incubated for 51 days anoxically with 500 mg L^{-1} NO_3^--N, and varying levels of phosphorus, from 1-50 mg L^{-1} (Fig. 1). The results show that rates increased only slightly with increasing P levels from 1 mg L^{-1} - 10 mg L^{-1}, and then decreased somewhat at the highest level of P(50 mg L^{-1}). Although water chemistry analyses of groundwaters from the contaminated site show phosphorous levels to be very low (< 0.1 mg L^{-1}), apparently final concentrations of between L-10 mg L^{-1} are enough to satisfy assimilatory demand during denitrification. At the enrichment combination of 500 mg L^{-1} of NO_3^--N and 10 mg L^{-1} of PO_4^{-3}-P where the rate of BTX loss was highest, the percent BTX removals of 93%, 97%, and 64%, respectively (as compared to unamended controls).

Since the range of concentrations where P appears to be limiting denitrification seems to be below 1 mg L^{-1}, we next conducted anoxic incubations with 500 mg L^{-1} NO_3^--N and varying P levels from 0-1 mg L^{-1} (Fig. 2). In the incubations with nitrate (500 mg L^{-1} N) enrichment but no phosphorus, BTX levels decreased by 95% as compared to

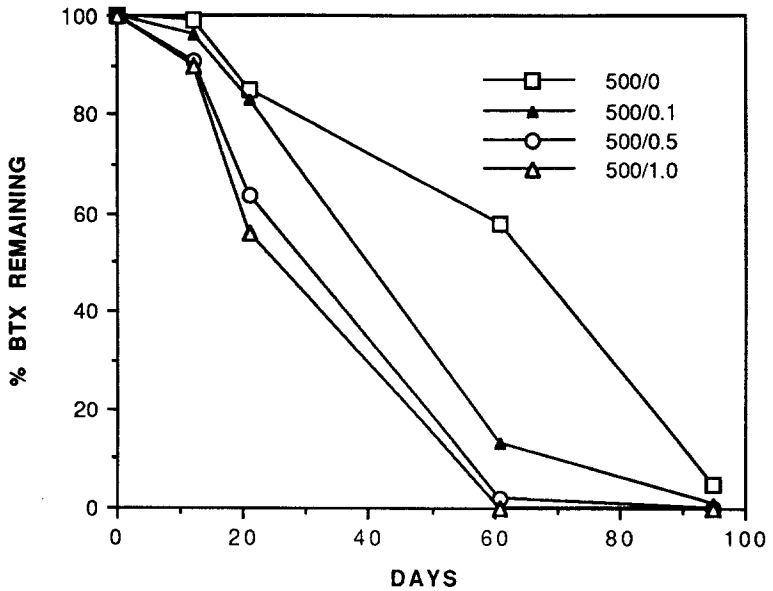

Figure 2. Percent loss of BTX (benzene, toluene and xylene) in groundwater incubated anoxically with 500 mg L^{-1} NO_3^--N and varying amounts of phosphorus, ranging from 0-1.0 mg L^{-1} PO_4^{-3}-P. All values are calculated as compared to the unamended control.

the unamended control after 95 days, but highest initial rates of BTX loss were observed in treatments with both N and P (either 0.5 or 1.0 mg L^{-1} P) enrichment where BTX removal was complete (>98%) after only 61 days. Ghiorse and Balkwill (1983) and White et al. (1983) concluded that bacteria in the subsurface environment are adapted to long-term nutrient limitation since they contained storage deposits indicative of oligotrophy. Swindoll et al. (1988) showed directly that adding phosphorus and nitrogen increased bacterial numbers by aerobic mineralization of toluene in aquifer material. Our results show that although phosphorus enrichment leads to more rapid BTX loss under denitrifying conditions, apparently there is sufficient phosphorous in the unenriched waters to satisfy the nutrient demand of denitrification, since after about 3 months, the level of BTX in groundwater enriched with nitrate but no phosphorus, is similar to that in samples enriched with nitrate alone (Figs. 1 and 2).

Organic amendments such as acetate and methanol significantly decreased BTX loss rates compared to the unamended (without organics) control (Table 1). Major et al. (1988) added organic substrates to incubations of

subsurface samples under aerobic and anaerobic conditions and found no significant enhancement. However, they didn't add nitrate to test the loss of BTX via denitrification. Slater and Capone (1987) found that addition of glucose stimulated denitrification in the lower layer of a nearshore aquifer. We found no similar significant stimulation of BTX loss after organic enrichment of gasoline-contaminated groundwaters, but instead observed the opposite effect, as if the added organics were competitors with BTX for the available electron acceptor.

Table 1. Effect of acetate and methanol addition on BTX loss under denitrifying conditions

Treatment	Percent BTX Remaining (as compared to unamended control)	
	25 days	37 days
N + P (500 mg L^{-1}N and 10 mg L^{-1}P)	71%	13%
N + P + Acetate (1.5 g L^{-1})	67%	23%
N + P + Acetate (1.0 g L^{-1})	67%	22%
N + P + Acetate (2.0 g L^{-1})	72%	24%
N + P + Methanol (1.0 g L^{-1})	68%	20%

Both iron (Fe) and molybdenum (Mo) are known to be required for the dissimilatory nitrate reductase step of denitrification. The effect of these trace element additions are shown in Table 2. Both Fe and Mo, either singly or in combination, did significantly ($P<0.05$) increase the rate of BTX loss after 31 days, but not after 53 days.

In order to measure the response of the bacterial population to nutrient enrichment, we next measured BTX loss (Fig. 3) as well as numbers of total denitrifying bacteria (Fig. 4). BTX loss in the Hg^{+2}-killed sample represents disappearance due to purely physical processes such as volatilization. The relatively small difference between BTX loss in the non-amended versus the Hg-killed treatments points to the fact that most of the loss in unenriched controls is due to physical processes. After 49 days of incubation, about 92% of BTX was lost in the treatment with 500 mg L^{-1} NO_3^--N and 10 mg L^{-1} P, when compared to the initial BTX level. The treatment with

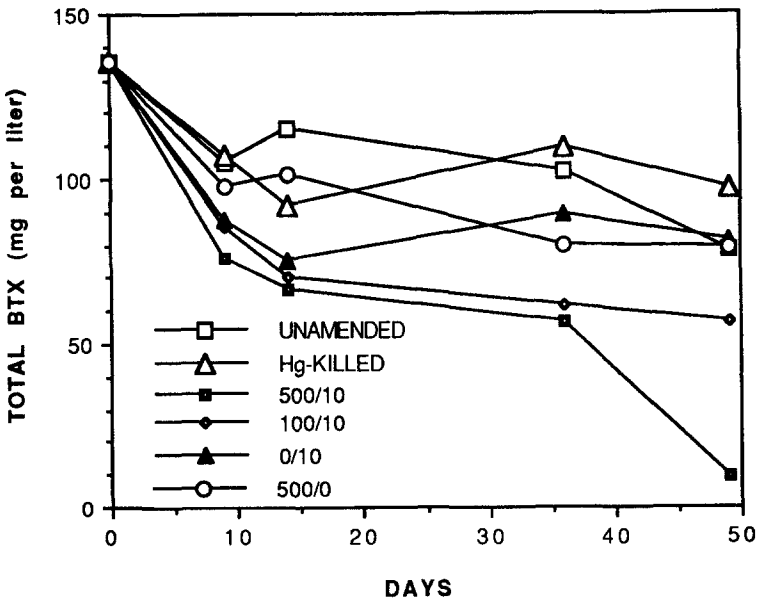

Figure 3. Loss of BTX in groundwater incubated anoxically with varying levels of nitrogen and phosphorus. Numbers given in figure (e.g. 500/10) represent the NO_3^--N and PO_4^{-3}-P levels (in mg L^{-1}), respectively. Hg-killed value is for samples treated with 100 mg L^{-1} Hg^{+2} to remove biological activity.

Table 2. Effect of iron and molybdenum addition on BTX loss under denitrifying conditions

Treatment	Percent BTX Remaining (as compared to unamended control)	
	31 days	53 days
N + P (500 mg L^{-1}N and 10 mg L^{-1}P)	13%	4%
N + P + Mo (0.1 mg L^{-1} Mo)	7%	5%
N + P + Fe (0.5 mg L^{-1} Fe)	7%	2%
N + P + Fe + Mo	7%	3%

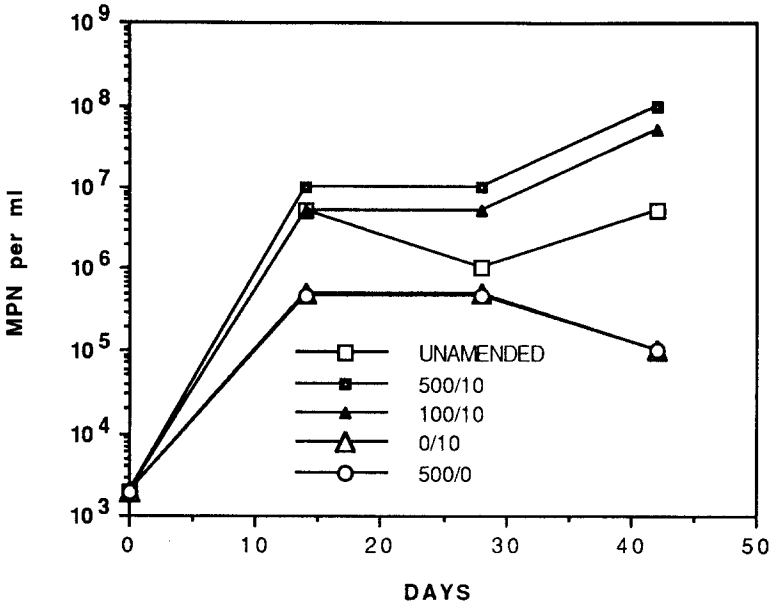

Figure 4. Levels of total denitrifying bacteria, expressed as most probable number (MPN) per mL, in groundwater incubated anoxically with varying levels of nitrogen and phosphorus. Numbers given in figure (e.g. 500/10) represent the NO_3^--N and PO_4^{-3}-P levels (in mg L^{-1}), respectively.

100 mg L^{-1} N and 10 mg L^{-1} P showed the next highest loss rate. BTX loss in the treatments with nitrate and no phosphorus, or phosphorous and no nitrate, were not significantly different than the unamended control. Moreover, the numbers of denitrifying bacteria were enhanced by more than an order of magnitude when both N and P were added together, but the counts in the treatments with N and no P, or P but no N, fell below the control level (Fig. 4).

Our results point to the capacity of the indigenous bacterial community to biodegrade BTX under denitrifying conditions. Since BTX compounds are among the 20 chemicals most often found at sites on the National Priority List, enhancement of biodegradation using nitrate could play an important future role in aquifer remediation.

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