



THE INFLUENCE OF THE GASOLINE OXYGENATE ETHANOL ON AEROBIC AND ANAEROBIC BTX BIODEGRADATION

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Abstract—Ethanol is frequently found along with benzene, toluene, and xylenes (BTX) in groundwater contaminated with gasoline. Yet, little is known about its effect on bioremediation of the toxic BTX contaminants. Aquifer microcosms were used to investigate the effect of ethanol on microbial degradation of representative BTX compounds under electron acceptor conditions commonly found in intrinsic bioremediation projects. Under aerobic conditions, ethanol retarded BTX biodegradation and exacerbated the biochemical oxygen demand (BOD). Anoxic conditions developed quickly when the BOD exerted by ethanol exceeded the available oxygen. This led to the persistence of benzene, which was also recalcitrant in denitrifying, sulfidogenic, and methanogenic microcosms during 99 days of incubation. Toluene was degraded under all anaerobic electron acceptor conditions tested, although the onset of relatively fast degradation always commenced after ethanol had been removed. Toluene degradation was not significantly affected by ethanol in denitrifying microcosms containing excess nitrate. Yet, no toluene degradation occurred when nitrate was limiting because nitrate was depleted while ethanol was being degraded. Ethanol also adversely affected toluene degradation in iron-reducing and methanogenic microcosms. Curiously, ethanol enhanced toluene degradation in sulfate reducing microcosms. This was hypothesized to be due to a low initial concentration of toluene degraders and their incidental growth during ethanol degradation. Albeit, the preferential utilization of ethanol and the accompanying depletion of electron acceptors suggest that ethanol would have a negative effect on passive BTX bioremediation. This is particularly important for the fate of benzene, which is the most toxic of the BTX and the most recalcitrant under anaerobic conditions. © 1998 Elsevier Science Ltd. All rights reserved

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INTRODUCTION

Groundwater contamination by petroleum product releases is a worldwide problem. In the United States, over 300 000 releases from underground storage tanks have been confirmed and more than 150 000 cleanups have been completed (USEPA, 1996). In Brazil, there are about 27 000 gas stations and concern about their impact on groundwater resources is increasing. The major concern is groundwater contamination by the toxic and water soluble components such as benzene, toluene, and xylenes (BTX), particularly in states such as São Paulo which derives 70% of its drinking water from aquifers. Comprehensive statistics on the magnitude of the BTX contamination problem in Brazil do not

exist. However, this problem is believed to be widespread because most tanks are older than 25 yr and are prone to leak (Corseuil and Alvarez, 1996).

Bioremediation, the use of microorganisms to degrade environmental pollutants *in situ*, holds great promise as an approach to manage BTX-contaminated aquifers. Indeed, this technique has been used successfully to clean up numerous BTX-contaminated sites in the United States (National Research Council, 1993). However, the extrapolation of this experience needs to consider differences in gasoline formulation that may affect the fate and transport of the target BTX contaminants. In this regard, the use of ethanol as a formulating ingredient is increasing worldwide, both as an indigenous substitute fuel for scarce hydrocarbons and as an oxygenate to minimize air pollution from combustion. For example, about 85% of the cars in Brazil run on gasoline containing 22% ethanol, and the other 15% runs on hydrated ethanol (Petrobrás,

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1995). Thus, ethanol is increasingly likely to be encountered in groundwater plumes containing BTX, and a better understanding of its effects on BTX bioremediation is warranted.

Ethanol is completely miscible in water and is often present in gasoline at much higher concentrations than BTX. Therefore, groundwater impacted by ethanol-amended gasoline is likely to have much higher ethanol than BTX concentrations. High ethanol concentrations in groundwater (>2%) can enhance the solubilization and migration of BTX (Corseuil and Alvarez, 1996). Such co-solvent effects have also been reported for methanol, a similar oxygenate used in M85 fuel and in other gasoline formulations in North America (Poulsen *et al.*, 1992).

Regarding biochemical effects, the need to understand substrate interactions between BTX and ethanol is very recent, and very few researchers have investigated the potential effects of ethanol on BTX degradation. Such research has been partially motivated by a trend in North America to substitute toxic and persistent gasoline oxygenates, such as methyl *t*-butyl ether (MTBE), with non-toxic and biodegradable ethanol. Using oxygen consumption as an indication of microbial activity, Hunt *et al.* (1997) reported that ethanol was not toxic to indigenous aquifer microorganisms at concentrations lower than 40 000 mg l⁻¹. However, ethanol at 20 mg l⁻¹ retarded aerobic benzene degradation, presumably due to a preferential utilization of ethanol. Barker *et al.* (1992) conducted related experiments involving controlled releases of BTX and methanol mixtures at the Borden site, Canada. They observed that BTX persisted longer in groundwater when methanol was present, and attributed this effect to microbial inhibition due to high methanol concentrations and to oxygen removal by methanol biodegradation.

With respect to site remediation, resource allocation problems have motivated a recent paradigm shift in the United States towards risk-based corrective action. This has stimulated an increase in the use of intrinsic bioremediation to control low-risk BTX contamination. In intrinsic bioremediation, the plume is monitored, but no enhancement of natural degradative processes is attempted as long as the pollutants do not present a serious risk (e.g. when biodegradation rates exceed migration rates and the plume is stable or receding). Due to the lack of oxygen addition in a passive system, anaerobic processes are believed to play an important role in containing and removing the BTX contamination (Rifai *et al.*, 1995). In such cases, indigenous microorganisms degrade BTX using electron acceptors preferentially in order of decreasing oxidation potential (Chapelle, 1993). Sequential depletion of electron acceptors could lead to successive transitions from aerobic to denitrifying, iron-reducing, sulfate reducing, and methanogenic conditions.

However, there are no reports in the literature regarding the effect of ethanol on BTX degradation under any of these electron acceptor conditions. A better understanding of such effects is warranted for the selection and design of appropriate remedial alternatives in places where ethanol is used as a gasoline additive. This study represents a first step towards achieving this goal.

METHODOLOGY

Microcosm preparation

Aquifer microcosms were prepared in triplicate to investigate the effects of ethanol on the biodegradation of representative BTX compounds under electron acceptor conditions commonly found in passive bioremediation projects. Emphasis was placed on monitoring BTX, ethanol, and electron acceptor concentrations to compare BTX degradation patterns with and without ethanol.

Aerobic microcosms were used to investigate the effect of ethanol on microbial degradation of benzene, toluene, and *o*-xylene under both oxygen limited and unlimited conditions. The microcosms were prepared with sandy aquifer material that had low organic content (0.2%) and no known previous exposure to BTX. The aquifer material was obtained from a scarcely populated area near Jurerê Beach, Florianópolis, Santa Catarina, Brazil. Samples were collected with sterilized tools from a 1.3 m depth at the phreatic surface, and stored at 4°C until use. 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⁻¹; total acidity = 26 mg l⁻¹ as CaCO₃; total alkalinity = 10 mg l⁻¹ as CaCO₃; hardness 433 mg l⁻¹ as CaCO₃; turbidity = 2 NTU; Cl⁻ = 10.7 mg l⁻¹; total phosphorus = 0.26 mg l⁻¹ as P; TKN = 16.3 mg l⁻¹; ammonia = 11.1 mg l⁻¹ as N. Microcosms were prepared in 120 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. Based on stoichiometric calculations, the theoretical oxygen demand of microcosms with initial ethanol concentrations of 0, 20, and 100 mg l⁻¹ was lower than the available oxygen in the headspace (i.e. 0.27 mg O₂ ml⁻¹ air at 25°C). Thus, aerobic conditions prevailed. However, microcosms fed 300 mg l⁻¹ of ethanol had a greater biochemical oxygen demand (BOD) than available oxygen and were thus oxygen-limited. BTX were fed separately or concurrently at about 20 mg l⁻¹ each using 10 μl gas-tight syringes. These are typical BTX concentrations near the source area following a recent spill. The microcosms were subsequently sealed with Teflon-lined septa and aluminum crimps. Controls were poisoned with 2,000 mg l⁻¹ sodium azide to discern biodegradation from volatilization losses. These experiments were also repeated with inorganic nutrient amendments, using the recipe of Corseuil and Weber (1994), to investigate the reproducibility of the observed trends under different nutrient conditions. All aerobic microcosms were incubated quiescently in the dark at 20°C.

Anaerobic microcosms were prepared by adding 20 g of soil and 200 ml mineral medium to 250 ml amber bottles. Denitrifying and iron-reducing microcosms were prepared with sandy soil obtained from a 1 m depth in the Pentacrest area of the University of Iowa, Iowa City. Sulfate reducing and methanogenic microcosms were prepared with sediments obtained from a pond near Iowa City. These sediments were anaerobic and had a distinct sulfide smell. The methanogenic reactors were also seeded

with a methanogenic mixed culture that had been enriched with acetate. For each electron acceptor condition, three sets of microcosms were prepared in triplicate and capped with Mininert valves. The first (treatment) sets were fed benzene and toluene at 0.4 to 1 mg l⁻¹ each, which are within the range of BTX concentrations commonly encountered in anaerobic plumes. Ethanol, which is completely soluble in water and is present at higher concentrations than BTX in Brazilian gasoline, was added at 100 mg l⁻¹. This ethanol concentration, which is much lower than what would be expected for groundwater equilibrated with Brazilian gasoline, was selected to facilitate experimentation under unlimited electron acceptor conditions. The second microcosm set (no-treatment control) was prepared equally, except that no ethanol was added to control for its effect on BTX biodegradation. The third set (sterile control) received BTX plus ethanol and was autoclaved and poisoned with 300 mg l⁻¹ HgCl₂. Additional non-sterile (sandy soil) microcosms were prepared with deoxygenated mineral medium lacking nitrate, ferric iron, or sulfate to control for the effect of adding these electron acceptors.

The mineral medium for the denitrifying microcosms was prepared as described by Alvarez *et al.* (1994). Nitrate was added at 200 or 400 mg l⁻¹ to, respectively, achieve limiting or unlimited electron acceptor conditions. The medium for iron-reducing bacteria was prepared as described by Lovley and Phillips (1988), except that MgCl₂ was substituted for MgSO₄ to preclude the potential for sulfate reducing conditions. This medium contained about 250 mM amorphous ferric oxyhydroxide, prepared as described by Lovley and Phillips (1986). Thus, ferric iron was provided in excess of the stoichiometric requirement to mineralize the added ethanol and toluene. The medium for sulfate reducing conditions was per the recipe of Parkin *et al.* (1990), and contained about 450 mg l⁻¹ of sulfate. While this concentration was sufficient to mineralize the added ethanol and BTX, background sulfate respiration in the sediment material required respiking sulfate after 27 d of incubation. The methanogenic medium was per the recipe of Hughes and Parkin (1996). All anaerobic microcosms were incubated under quiescent conditions at 25°C inside a (Coy) anaerobic chamber.

Analytical methods

BTX compounds were analyzed in 1 ml liquid samples using a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a Hewlett Packard 19395A headspace autosampler and flame-ionization and photoionization detectors in series. Separation was achieved using a J and W Scientific DB-WAX column isothermally at 35°C. Detection limits were approximately 0.1 mg l⁻¹ for each BTX compound. Ethanol was analyzed by direct injection of 5 µl liquid samples into a Varian D GC equipped with a Poropak Q 100/80 Mesh column and a flame-ionization detector. The detection limit for ethanol was about 3 mg l⁻¹. Nitrate, nitrite, and sulfate were analyzed with a Dionex 4500i ion chromatograph using an AS4A ion exchange column for separation followed by chemical suppression and conductivity detection. The detection limit for each of these anions was about 1 mg l⁻¹. The presence of methane in methanogenic microcosms was confirmed by headspace injection into a Hewlett Packard 5890 Series GC fitted with a molecular sieve and thermal conductivity detector. A YSI 5300 biological oxygen monitor, equipped with an Instech microchamber and an oxygen micro probe, was used to verify that oxygen was absent in anaerobic microcosms and that it had been depleted in aerobic microcosms designed to be oxygen limited. The limit of detection for dissolved oxygen was 0.1 mg l⁻¹.

RESULTS AND DISCUSSION

Aerobic degradation assays

Benzene, toluene, *o*-xylene, and ethanol removal in viable microcosms, but not in sterile controls (<5%), provided evidence of biodegradation. The fact that the aquifer material tested had an indigenous microbial population capable of rapidly degrading BTX without previous exposure reflects the ubiquitous nature of BTX degraders. In all cases, ethanol was preferentially utilized over all BTX compounds, and a lag period was generally observed during which ethanol was degraded before

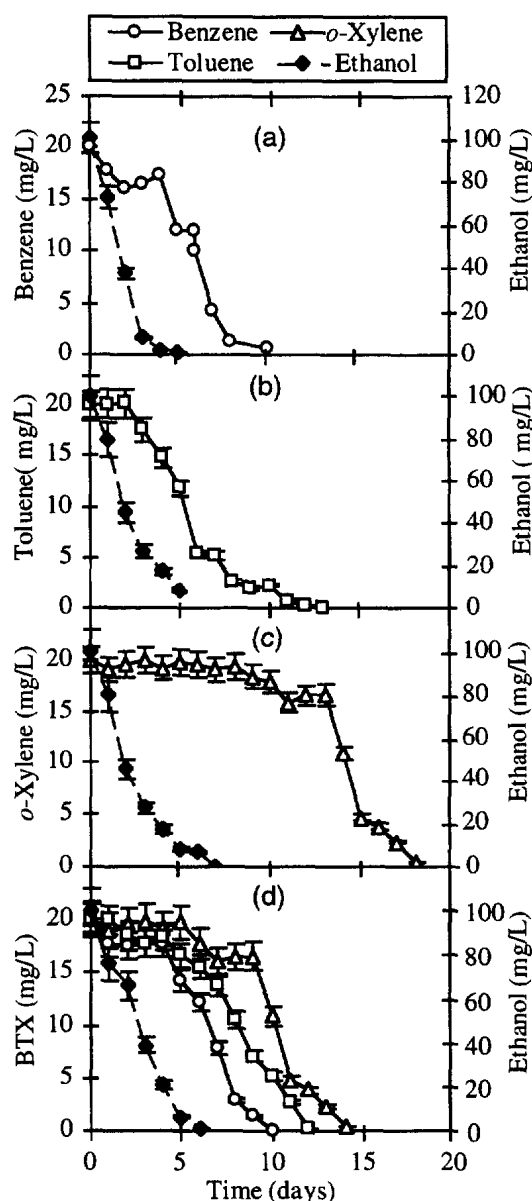


Fig. 1. Aerobic BTX degradation in microcosms fed 100 mg l⁻¹ ethanol. BTX losses in sterile controls were less than 5%. Error bars depict the standard deviation from the mean of triplicate microcosms. Error bars smaller than symbols are not depicted.

substantial BTX degradation occurred. Figure 1 shows a typical example, in which most of the added ethanol (100 mg l^{-1}) was degraded before the onset of BTX degradation, regardless of whether benzene (Fig. 1(a)), toluene (Fig. 1(b)), and *o*-xylene (Fig. 1(c)) were present individually or concurrently (Fig. 1(d)). The preferential degradation of ethanol over BTX may reflect the fact that ethanol is an easily degradable substrate than can be oxidized by constitutive enzymes through central metabolic pathways. BTX, on the other hand, are typically degraded by inducible oxygenase enzymes whose synthesis can be repressed by easily degradable substrates when present at high concentrations (Worsey and Williams, 1975; Duetz *et al.*, 1994). This suggests that, in bioremediation applications, the bacterial sub-population capable of degrading BTX would not fully express its catabolic potential while ethanol is present.

Lag periods for BTX degradation increased with the initial ethanol concentrations because ethanol removal was required prior to BTX degradation, and this took longer for higher ethanol concentrations. This effect was also observed in aerobic microcosms not amended with inorganic nutrients,

although the lags were longer. Figure 2 illustrates these effects using benzene degradation with and without nutrients amendment as an example. In the absence of ethanol, 20 mg l^{-1} of benzene was degraded within 3 days in microcosms amended with inorganic nutrients and trace minerals (Fig. 2(a)). The same amount of benzene lasted about 9 days without nutrient addition (Fig. 2(b)). In both cases, microcosms amended with 300 mg l^{-1} ethanol had a greater biochemical oxygen demand (BOD) than available oxygen. These microcosms became anoxic while degrading ethanol, and benzene persisted in the absence of molecular oxygen.

These latter 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. Because groundwater impacted by Brazilian gasoline is likely to have very high ethanol concentrations, there is a high probability that the available oxygen would be rapidly consumed before much aerobic BTX degradation can occur. This is particularly important for the fate of benzene, which is the most toxic of the BTX and degrades

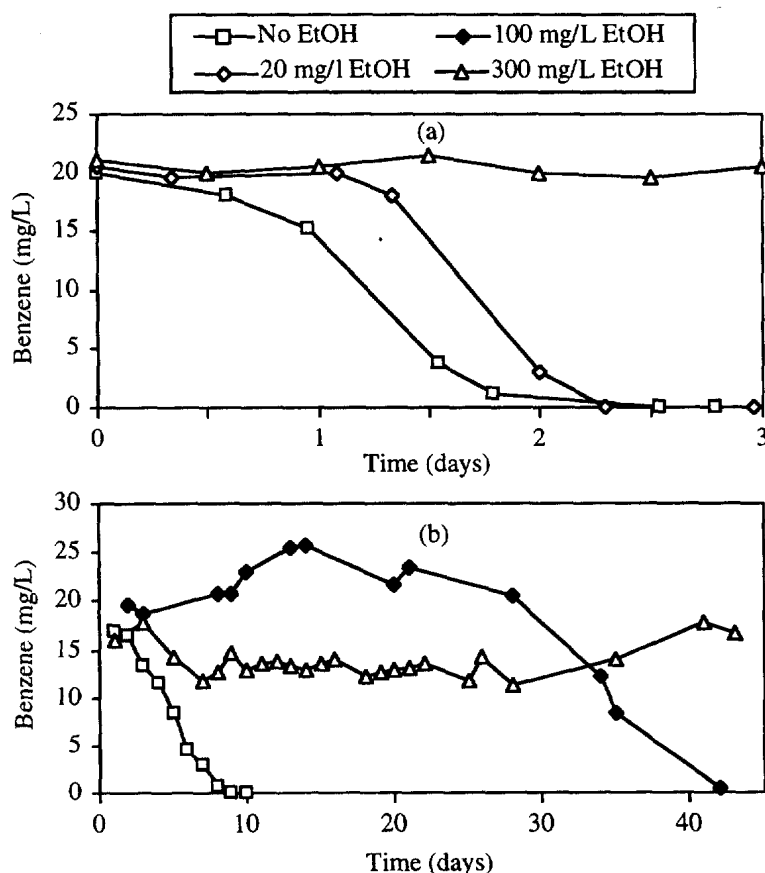


Fig. 2. Effect of ethanol and nutrients on aerobic benzene degradation. Degradation patterns are compared in microcosms amended with inorganic nutrients (a) vs microcosms prepared without nutrients (b). Microcosms fed 300 mg l^{-1} of ethanol became anoxic before benzene degradation occurred.

very slowly if at all under anaerobic conditions (Grbić-Galić and Vogel, 1987; Edwards *et al.*, 1992; Alvarez and Vogel, 1995; Reinhard *et al.*, 1997). This suggests that benzene could migrate without significant bioattenuation until most of the ethanol is biodegraded.

Because ethanol is likely to exacerbate the biochemical oxygen demand and deplete the available oxygen, the effect of ethanol on anaerobic BTX degradation was also investigated.

Anaerobic biodegradation assays

No benzene degradation was observed in denitrifying, sulfidogenic, and methanogenic microcosms during up to 99 d of incubation. Benzene mineralization has been reported in iron-reducing (Lovley *et al.*, 1996), sulfidogenic (Edwards and Grbić-Galić, 1992), and methanogenic microcosms (Grbić-Galić and Vogel, 1987), with lag periods sometimes exceeding one year (Kazumi *et al.*, 1997). However, the ubiquity of anaerobic benzene degraders has not been established. Thus, while the recalcitrance of benzene in these experiments could be due to insufficient incubation time to allow biodegradation

to proceed, it is also plausible that no anaerobic microorganisms capable of degrading benzene were present in the tested sediments.

Toluene, which is the most commonly reported BTX compound to degrade under anaerobic conditions, was degraded under all electron acceptor conditions tested. In all cases, toluene degradation coincided with the removal of the appropriate electron acceptor. However, no electron balances were conducted in this study because the electron acceptor demand from the added toluene was overshadowed by the higher and more variable background demand of the sediments.

Toluene was degraded faster in microcosms amended with stronger electron acceptors. In the absence of ethanol, denitrifying microcosms degraded toluene relatively fast, removing 0.9 mg l^{-1} of toluene within 3 d (Fig. 3(a)). Iron reducing microcosms were the second fastest, removing 0.7 mg l^{-1} of toluene in about 8 d (Fig. 4). Sulfate reducing microcosms were much slower, beginning to degrade toluene after 40 d of incubation (Fig. 5). Curiously, only about 45% of the added 0.7 mg l^{-1} of toluene was degraded, and toluene degradation

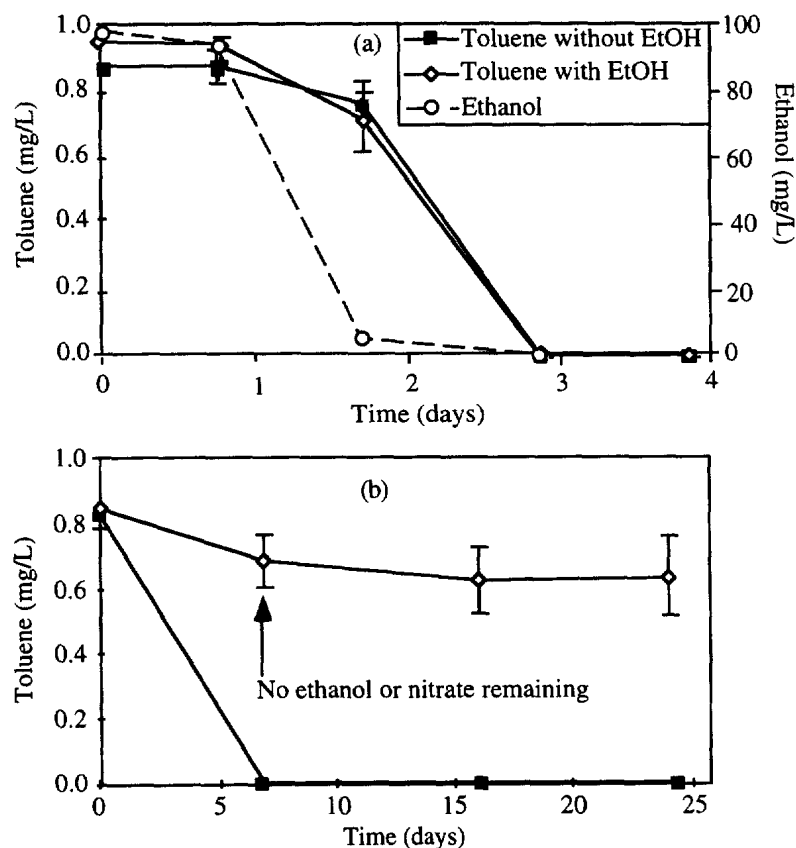


Fig. 3. Effect of ethanol on toluene degradation in denitrifying microcosms. Toluene degradation patterns are compared in denitrifying microcosms containing excess nitrate (a) and microcosms with insufficient nitrate to mineralize the added toluene plus ethanol (b). Data points are the average of three reactors, and the error bars represent one standard deviation. Toluene losses in controls not amended with nitrate were less than 5%.

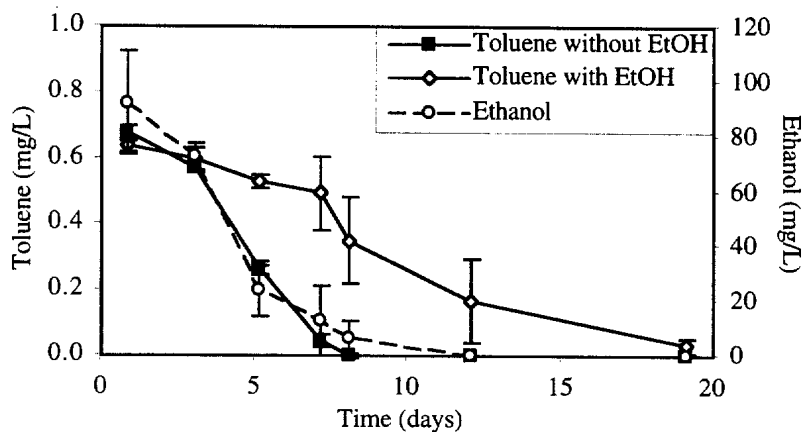


Fig. 4. Effect of ethanol on toluene degradation in iron-reducing microcosms. Data points are the average of three reactors, and the error bars represent one standard deviation. Toluene losses in controls not amended with ferric iron were less than 5%.

capabilities were lost in all three sulfidogenic microcosms not amended with ethanol after 50 d of incubation. Thus, 55% of the added toluene persisted after 99 d. The loss of toluene degradation ability cannot be attributed to a lack of electron acceptors. Although sulfate was consumed within three weeks by background respiration in the sediment material, more sulfate was added after 27 d to reach the original concentration of 450 mg l^{-1} , and $270 \pm 6 \text{ mg l}^{-1}$ remained in solution after toluene degradation stopped. Further studies would be required to determine why the degradation ability was lost, which in theory could be due to a build up of toxic byproducts and/or to microbial population shifts that eradicated toluene degraders. Methanogenic microcosms were the slowest in degrading toluene, exhibiting a 55 d lag and removing only 80% of the added 0.4 mg l^{-1} during the 99 d incubation period (Fig. 6). In all cases, toluene losses in sterile con-

trols and in anoxic microcosms prepared without electron acceptors were smaller than 10%.

Ethanol (100 mg l^{-1}) had a variable effect on anaerobic toluene degradation, even though it was always degraded preferentially and little toluene degradation occurred while ethanol was present (Figs 3–6). Toluene degradation patterns were not significantly affected by ethanol in denitrifying microcosms containing excess nitrate (400 mg l^{-1}) (Fig. 3(a)). Yet, little toluene degradation occurred when nitrate (200 mg l^{-1}) was limiting (Fig. 3(b)). In this case, nitrate was depleted within one week (while ethanol was being degraded) and no toluene degradation occurred thereafter. Ethanol retarded toluene degradation in iron reducing microcosms, which experienced an increase in the lag for toluene degradation from 3 to 7 days. The longer lag coincided with the time required to remove ethanol (Fig. 4). Methanogenic microcosms experienced a more pronounced adverse effect on toluene degra-

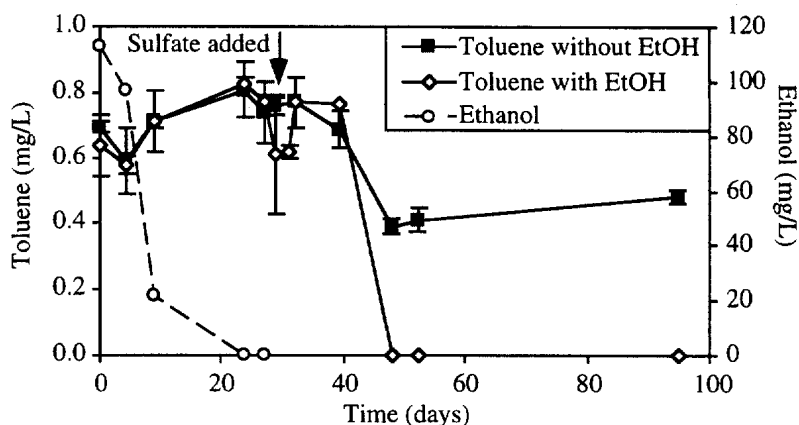


Fig. 5. Effect of ethanol on toluene degradation in sulfidogenic microcosms. Data points are the average of three reactors, and the error bars represent one standard deviation. Benzene losses were less than 5%.

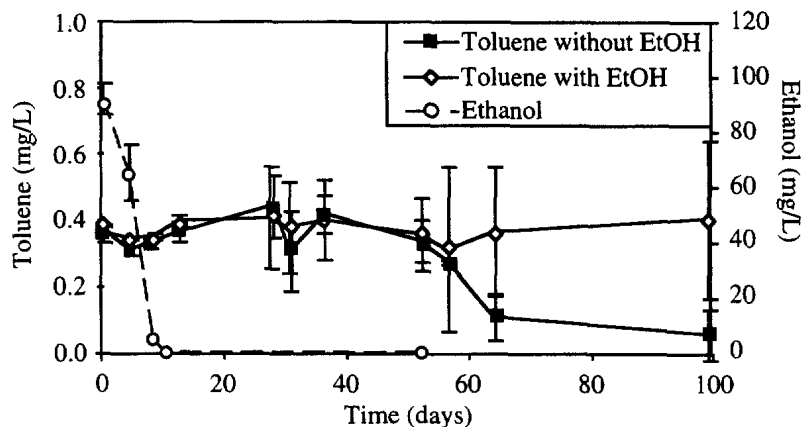


Fig. 6. Effect of ethanol on toluene degradation in methanogenic microcosms. Data points are the average of three reactors, and the error bars represent one standard deviation. Benzene losses were less than 5%.

dation. Although ethanol was degraded relatively fast under methanogenic conditions (within 10 days), no toluene degradation occurred in these microcosms within 99 days (Fig. 6). Further studies would be needed to determine if the persistence of toluene in these three microcosms was due to the utilization of critical growth factors or the build up of inhibitory byproducts during ethanol degradation, or whether a longer incubation time would have been required to observe toluene degradation.

Interestingly, the sulfidogenic microcosms show that there may be some exceptions to the detrimental effect of ethanol on BTX degradation (Fig. 5). Ethanol enhanced toluene degradation in all three microcosms, even though it was preferentially degraded. The reason for this enhancement is unclear, although it could be attributed to an incidental growth of toluene degraders during ethanol degradation. A higher concentration of toluene degraders would be conducive to faster toluene degradation rates once ethanol has been removed. This hypothesis does not imply that ethanol would select for BTX degraders, which is highly unlikely. Rather, the concentration of BTX degraders would increase after growth on ethanol, even though their fraction of the total heterotrophic consortium would likely decrease.

The above discussion represents a caveat against generalizations about the effect of fuel additives on BTX degradation patterns. Indeed, the diversity of microbial consortia may preclude generalizations about substrate interactions involving BTX degradation by mixed cultures (Alvarez and Vogel, 1991). Albeit, the preferential utilization of ethanol under all electron acceptor conditions tested, plus the associated depletion of the stronger electron acceptors, strongly suggest that the presence of ethanol in dissolved hydrocarbon plumes would generally hinder passive BTX bioremediation. It should be kept in mind, however, that other gasoline oxyge-

nates such as MTBE have become a serious environmental problem because of their toxicity and persistence in contaminated groundwater (Salanitro *et al.*, 1994). Indeed, the removal of MTBE from fuel-contaminated aquifers is a new and difficult challenge facing environmental remediation professionals in the United States. Thus, the potential negative effects caused by the presence of ethanol in the bioremediation of gasoline-ethanol mixtures may be well compensated by its biodegradability in aquifers and its beneficial effects on air pollution abatement.

SUMMARY AND CONCLUSIONS

This study investigated the effect of ethanol on the aerobic and anaerobic biodegradation of representative BTX compounds. On the basis of aquifer microcosm studies, the following conclusions were made:

(1) Ethanol will most likely be preferentially utilized over all of the BTX compounds under aerobic, denitrifying, iron-reducing, sulfate-reducing, and methanogenic conditions. This may prevent the bacterial sub-population capable of degrading BTX from fully expressing its catabolic potential and retard BTX degradation.

(2) Toluene was degraded under all electron acceptor conditions tested, and its degradation rate increased with the prevailing oxidation potential (i.e., aerobic > denitrifying > iron-reducing > sulfate reducing > methanogenic). Benzene, however, was only degraded in aerobic microcosms, and was recalcitrant under denitrifying, sulfidogenic, and methanogenic conditions during 99 d of incubation.

(3) Ethanol constitutes a significant additional electron acceptor demand to that exerted by other soluble components of gasoline, and is likely to cause the depletion of the preferred electron acceptors for BTX degradation. A decrease in the extent

of aerobic BTX degradation in oxygen limited aquifers 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.

(4) 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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REFERENCES

- Alvarez P. J. J. and Vogel T. M. (1991) Substrate interactions of benzene, toluene and *para*-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. *Appl. Environ. Microbiol.* **57**, 2981–2985.
- Alvarez P. J. J., Anid P. J. and Vogel T. M. (1994) Kinetics of toluene degradation by denitrifying aquifer microorganisms. *J. Environ. Eng.* **120**, 1327–1336.
- Alvarez P. J. J. and Vogel T. M. (1995) Degradation of BTEX and their aerobic metabolites by indigenous microorganisms under nitrate reducing conditions. *Water Sci. Tech.* **31**, 15–28.
- Barker, J. F., Hubbard, C. E., Lemon, L. A. and Voro, K. A. (1992) The Influence of methanol in gasoline fuels on the formation of dissolved plumes, and the fate and natural remediation of methanol and BTEX dissolved in groundwater. In *Hydrocarbon Contaminated Soils and Groundwater*, ed. E. J. Calabrese and P. T. Kosteki, pp. 103–113. Lewis Publishers, NY.
- Chapelle, F. H. (1993) *Groundwater Microbiology and Biochemistry*. Wiley, New York.
- Corseuil H. X. and Weber W. J. Jr. (1994) Potential biomass limitations on rates of degradation of monoaromatic hydrocarbons by indigenous microbes in subsurface soils. *Water Res.* **28**, 1415–1423.
- Corseuil H. X. and Alvarez P. J. J. (1996) Natural bioremediation perspective for BTX-contaminated groundwater in Brazil. *Wat. Sci. Tech.* **35**, 9–16.
- Duetz W. A., Marques S., de Jong C., Ramos J. L. and Van Andel J. G. (1994) Inducibility of the TOL catabolic pathway in *Pseudomonas putida* (pWWO) growing on succinate in continuous culture: evidence of carbon catabolite repression. *J. Bacteriol.* **176**, 2354–2361.
- Edwards E., Wills L. E., Grbić-Galić D. and Reinhard M. (1992) Anaerobic degradation of toluene and xylene under sulfate-reducing conditions. *Appl. Environ. Microbiol.* **58**, 794–800.
- Edwards E. and Grbić-Galić D. (1992) Complete mineralization of benzene under strictly anaerobic conditions. *Appl. Environ. Microbiol.* **58**, 2663–2666.
- Grbić-Galić D. and Vogel T. M. (1987) Transformation of toluene and benzene by mixed methanogenic cultures. *Appl. Environ. Microbiol.* **53**, 254–260.
- Hunt, C. S., Dos Santos Ferreira, R., Corseuil, H. X. and Alvarez, P. J. J. (1997) Effect of ethanol on aerobic BTX degradation. In *In Situ and Onsite Bioremediation*, ed. B. C. Alleman and A. L. Leeson, Vol. 4(1), pp. 49–54. Battelle Press.
- Kazumi J., Caldwell M. E., Sufita J. M., Lovley D. R. and Young L. Y. (1997) Anaerobic degradation of benzene in diverse anoxic environments *Environ. Sci. Technol.* **31**, 813–818.
- Lovley D. R. and Phillips E. J. P. (1986) Organic matter mineralization with the reduction of ferric iron in anaerobic sediments. *Appl. Environ. Microbiol.* **52**, 683–689.
- Lovley D. R. and Phillips E. J. P. (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl. Environ. Microbiol.* **54**, 1472–1480.
- Lovley D. R., Woodward J. C. and Chapelle F. H. (1996) Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. *Appl. Environ. Microbiol.* **62**, 288–291.
- National Research Council (1993) In *In Situ Bioremediation — When Does it Work?* National Academy Press, Washington, DC.
- Hughes J. B. and Parkin G. F. (1996) Concentration effects of chlorinated aliphatic transformation kinetics. *J. Environ. Eng.* **122**, 92–98.
- Parkin G. F., Lynch N. L., Kuo W. C., VanKueren E. L. and Bhattacharya S. K. (1990) Interaction between sulfate reducers and methanogens fed acetate and propionate. *Res. J. Water Pollut. Contr. Fed.* **62**, 780–788.
- Petrobrás (1995) *Relatório Anual de Atividades*, Rio de Janeiro, RJ.
- Poulsen M., Lemon L. and Barker J. F. (1992) Dissolution of monoaromatic hydrocarbons into groundwater from gasoline-oxygenate mixtures. *Environ. Sci. Technol.* **26**, 2483–2489.
- Rifai, H. S., Borden, R. C., Wilson, J. T. and Ward, C. H. (1995) Intrinsic bioattenuation for subsurface restoration. In *Intrinsic Bioremediation*, ed. R. E. Hinchee, F. J. Brockman and C. M. Vogel, Vol. 3(1), pp. 1–29. Battelle Press.
- Reinhard M., Shang S., Kitanidis P. K., Orwin E., Hopkins G. D. and Lebron C. A. (1997) *In situ* BTEX biodegradation under enhanced nitrate- and sulfate-reducing conditions. *Environ. Sci. Technol.* **31**, 28–36.
- Salanitro J. P., Diaz L. A., Williams M. P. and Wisniewski H. L. (1994) Isolation of a bacterial culture that degrades methyl *t*-butyl ether. *Appl. Environ. Microbiol.* **60**, 2593–2596.
- USEPA (1996) FY96 End of year activity report. U.S. Environmental Protection Agency, Office of Underground Storage Tanks, Washington, DC, November 6, 1996.
- Worsley M. J. and Williams P. A. (1975) Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence of a new function of the TOL plasmid. *J. Bacteriol.* **124**, 7–13.