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Implications of the Presence of Ethanol on Intrinsic Bioremediation of BTX Plumes in Brazil

HENRY X. CORSEUIL,¹ JOSÉ R. AIRES,² and PEDRO J.J. ALVAREZ³

¹Universidade Federal de Santa Catarina Departamento de Engenharia Sanitária e Ambiental CEP 88049, Florianópolis Santa Catarina, Brasil ²Petrobrás/CENPES/DIGER Cidade Universitária, QD.7 Ilha do Fundão 21949-900 Rio de Janeiro, RJ, Brasil ³The University of Iowa Department of Civil and Environmental Engineering Iowa City, IA 52242-1527

ABSTRACT

Intrinsic bioremediation, the use of indigenous microorganisms to degrade hazardous substances within aquifers without engineered stimulation, shows great promise as a cost-effective approach to hydrocarbon plume management. This technique requires thorough site characterization and monitoring to verify that the natural attenuation processes continue to provide adequate risk protection. Significant progress has been made towards understanding the hydrogeochemical and microbiological factors that influence the feasibility of intrinsic bioremediation of fuel-contaminated aquifers in North America and Europe. Nevertheless, this experience should be extrapolated with care to contaminated sites in Brazil, where gasoline contains about 22% of ethanol. Preliminary laboratory studies show that ethanol can enhance the solubilization of BTX in water, and it might exert diauxic effects during BTX biodegradation. A better understanding of the biochemical, physical, and ecological effects of ethanol is needed to develop a rational basis for the selection, mathematical modeling, and monitoring of appropriate intrinsic bioremediation systems in Brazil.

INTRODUCTION

Groundwater contamination by petroleum hydrocarbons is a widespread occurrence. To put the magnitude of this problem in perspective, it is estimated that 600,000 out of 2 million underground tanks storing gasoline in the USA are or will soon be leaking [1]. In Brazil, it is not known how many of about 135,000 underground tanks are leaking [2]. Up to now, the main concerns are concentrated in the State of São Paulo, where groundwater is used by about 70% of the population. In the 1970's economic boom, during the so called "Brazilian miracle", there was a large increase in the number of gas stations in the country. Considering that the mean life time of underground storage tanks is about 20 years, Brazil will probably be facing soon similar soil and groundwater contamination problems as

those currently faced by the USA and Europe. A major concern with leaking gasoline tanks is groundwater contamination by the toxic and water soluble components such as benzene, toluene, and xylenes (BTX). These hydrocarbons have relatively high pollution potential because of their significant concentration in gasoline, relatively high water solubility, and chronic toxicity. All BTX compounds are powerful depressants to the central nervous system, and benzene can cause leukemia in humans [3]. Consequently, BTX are priority pollutants, and their common occurrence in aquifer drinking water supplies has lead to the development of several physical, chemical, and biological methods for their removal. Many of these technologies, however, are difficult to implement in developing countries such as Brazil because of economical and technical limitations. A need exists for an easy, cost-effective method for mitigating the risk to public health associated with BTX contaminated groundwater.

Developing countries usually base their selection of environmental restoration technologies on the extensive experience accumulated in North America and Europe. The extrapolation of this experience to Brazil, however, will need to consider differences in the formulation of Brazilian gasoline, which contains 22% of ethanol. Furthermore, ethanol is widely used as fuel for cars in Brazil, and is usually stored in neighboring tanks which may also be leaking. Therefore, ethanol is likely to be present in hydrocarbon plumes. This paper presents a perspective on intrinsic bioremediation as an approach to BTX pollution control in Brazil with an emphasis on the implications of the presence of ethanol in BTX plumes.

WHY INTRINSIC BIOREMEDIATION?

Traditional processes for removing BTX from aquifers involve pumping the contaminated groundwater for above ground treatment with activated carbon or air strippers. Nevertheless, BTX compounds are moderately hydrophobic and tend to sorb to the aquifer material. This makes them difficult to withdraw by pumping, and serves as a slow-release mechanism for sustained groundwater contamination. Pump-and-treat technologies alone can result in prohibitively long time periods for the removal of the residual contamination, and are often economically unfeasible [4]. In addition, treatment with activated carbon or air strippers merely transfer the contaminants from one phase to another, rather than transforming it into less harmful compounds. In situ bioremediation, which involves the use of indigenous microorganisms to degrade the target compounds within the aquifer, is receiving increasing attention due to its potential cost-effectiveness. Other advantages of bioremediation include minimum land disturbance, treatment moves with the groundwater, it does not dewater the aquifer due to excessive pumping, and it is environmentally sound because it ultimately transforms the target BTX into harmless products such as carbon dioxide and water [5].

The common approach to in situ bioremediation is to engineer the environment to overcome limitations to natural degradative processes. For example, fertilizers and oxygen can be injected into contaminated aquifers to overcome the insufficient supply of nutrients and electron acceptors. This approach is In some cases, however, natural conditions at called active or engineered bioremediation. contaminated sites meet all the essential environmental factors so that bioremediation can occur without This process is called natural or intrinsic human intervention to stimulate microbial activity. bioremediation, and differs from no-action alternatives in that it requires thorough documentation of the role of microorganisms in eliminating the target contaminants. This is accomplished via tests and monitoring at field sites or on-site derived samples of soil, sediment, or water to ensure that the natural attenuation process continues to provide adequate risk protection. Intrinsic bioremediation is becoming increasingly popular with liable parties because it is the least expensive approach to control BTX plumes. It should be emphasized, however, that this technique is not a panacea that is applicable to all situations. Indeed, its successful application is site-specific and requires fulfillment of the following conditions.

REQUIREMENTS FOR THE SUCCESS OF INTRINSIC BIOREMEDIATION

1. Presence of microorganisms with potential to biodegrade the target compounds. Hydrocarbons have a natural pyrolytic origin and have been in contact with microorganisms throughout evolutionary periods of time. Thus, it is not surprising that many bacteria have acquired the ability to utilize hydrocarbons as food. The ability of microorganisms to degrade BTX has been known since 1908, when Stormer isolated the bacterium *Bacillus hexabovorum* by virtue of its ability to grow on toluene and xylene aerobically [6]. In an early review, Zobell [7] identified over 100 microbial species from 30 genera that could degrade hydrocarbons. The existence of BTX degraders is a widely accepted fact. Furthermore, they are widely distributed. The ubiquity of soil bacteria capable of degrading BTX was first demonstrated in 1928 by Gray and Thornton, who reported that 146 out of 245 uncontaminated soil samples contained bacteria capable of metabolizing hydrocarbons [6. Therefore, this requirement is easily met.

2. Accessibility of target pollutants to the microorganisms. A common limitation of natural degradative process is the lack of adequate contact between pollutants and microorganisms. The target pollutants must be accessible in various aspects, including physicochemically (e.g., desorption from aquifer solids to enhance bioavailability), structurally (e.g., bonds requiring cleavage must be exposed and not be sterically blocked by large atoms such as chlorine), and biochemically (e.g., the target pollutant must be able to pass through the cellular membrane). In regards to BTX, these requirements are generally met. It is not known, however, whether the presence of ethanol in a hydrocarbon plume can exert a sufficient cosolvent effect to enhance BTX bioavailability.

3. Induction of appropriate degradative enzymes. This process involves activation of specific regions of the bacterial genome. When some target substrates are present, they initiate a cascade of biochemical reactions that result in the transcription of genes that code for the synthesis of the necessary degradative enzymes. In regards to BTX degradation, many enzymes require induction, and the inducer (e.g., toluene) must be present at a higher concentration than the minimum threshold for induction [8]. In general, this threshold is very low and enzyme induction is rarely a limiting factor in BTX bioremediation. Furthermore, BTX contamination is often discovered several years after the fact, and significant microbial acclimation and enzyme induction can occur during this time [9]. Nevertheless, the presence of easily degradable substrates could exert diauxic effects (i.e., preferential substrate utilization) which would hinder the induction of BTX degrading enzymes. This may be an important factor in Brazil where gasoline contains 22% of ethanol, an easily degradable substrate. In this case, a lag period may be observed during which ethanol is degraded before any significant BTX degradation occurs (Figure 1).

4. Availability of electron acceptors. Hydrocarbons are in a reduced state, and their oxidation is thermodynamically very feasible. Microorganisms mediate their oxidation using electron acceptors during natural respiratory processes. The following preferential utilization has been observed, and reflects a decreasing oxidation potential of the potential electron acceptor:

oxygen > nitrate > ferric iron > sulfate > carbon dioxide.

In general, the kinetics of hydrocarbon oxidation is faster for electron acceptors with higher oxidation potential.

i) <u>Biodegradation using molecular oxygen</u>. Aerobic BTX degradation is usually fastest. Using toluene as an example, half lives associated with its aerobic degradation typically vary between 1 and 20 days [11, 12, 13, 14]. Yet, it is not uncommon that half lives fall out of this range, depending on the active microbial concentration and mass transfer limitations. Aerobic toluene mineralization follows the following stoichiometry:

$$C_7H_8 + 9 O_2 \rightarrow 7 CO_2 + 4 H_2O$$



Figure 1. Effect of ethanol concentration on aerobic benzene degradation in batch aquifer microcosms. Microcosms were prepared as described by Alvarez and Vogel [10]. The lag time for benzene degradation increased with the initial ethanol concentration, possibly due to diauxy. Longer lags were not due to ethanol toxicity because rapid ethanol degradation, indicated by oxygen consumption, was observed (data not shown). The microcosm fed 300 mg/L of ethanol had a greater oxygen demand than available oxygen, and became anoxic before the onset of benzene degradation.

Therefore, the complete mineralization of toluene requires about 3.1 mg/L for each mg/L of toluene (or the other BTX compounds). The extent of aerobic BTX degradation is controlled by the amount of contamination released, the rate of oxygen transfer into the subsurface, the background oxygen concentration of the groundwater (usually 6 to 12 mg/L), and the occurrence of alternate substrates [10, 15]. The presence of ethanol in Brazilian gasoline represents a significant additional oxygen demand by the soluble components, and is likely to decrease the extent of aerobic BTX degradation in oxygen limited aquifers (Figure 1).

ii) Biodegradation using nitrate. Once oxygen is depleted, some facultative denitrifiers can replace oxygen with nitrate as the terminal electron acceptor during BTX degradation. Half lives for toluene degradation under denitrifying conditions are a little longer, typically ranging from 20 to 50 days [16, 17], but degradation rates could be as fast as under aerobic conditions if the denitrifier concentration is sufficiently high (Figure 2).

The mineralization of toluene coupled to the complete nitrate reduction to nitrogen gas is represented by the following reaction:

$$C_7H_8 + 7.2 \text{ NO}_3^- + 7.2 \text{ H}^+ \rightarrow 7 \text{ CO}_2 + 7.6 \text{ H}_2\text{O} + 3.6 \text{ N}_2$$

Numerous laboratory and field studies have shown that toluene, m-, p-, and o-xylene, ethylbenzene, and naphthalene can be degraded under strictly anaerobic denitrifying conditions. However, benzene, which is the most toxic of the BTX, is recalcitrant and requires microaerophilic conditions for its degradation with nitrate as electron acceptor [18, 19, 20].

<u>iii) Biodegradation using ferric iron</u>. When oxygen and nitrate have been used up, some indigenous microorganisms can use ferric iron [Fe(III)] as the electron acceptor during BTX degradation [21, 22]. Large amounts of ferric iron are present in mineral forms in most aquifers, which constitute a large potential electron acceptor pool for hydrocarbon oxidation. For example, the mineralization of toluene coupled to the reduction of ferric hydroxide can be represented as follows:

$$C_7H_8 + 36 \text{ Fe}(\text{OH})_3 + 72 \text{ H}^+ \rightarrow 7 \text{ CO}_2 + 36 \text{ Fe}^{+2} + 94 \text{ H}_2\text{O}$$



Figure 2. Effect of initial denitrifiers concentration (Xo) on toluene degradation. Denitrifying microcosms were prepared as described by Alvarez et al. (1994). Sterile controls had a 12% loss of toluene over 150 days. A shorter lag time was observed with a higher initial concentration of denitrifiers, even though the initial toluene concentration was higher. Numerical simulations with appropriate biokinetic coefficients [16] indicate that the longer lag for Xo = 0.00005 mg/L reflects the time required to grow sufficient microorganisms capable of exerting measurable degradation rates.

Although the mechanisms of hydrocarbon degradation under iron-reducing conditions are not fully understood, field evidence suggests that this is an important mechanism in the subsurface biodegradation of dissolved BTX [21]. Similar to other anaerobic processes, however, degradation by iron reducers is greatly underestimated by current mathematical bioremediation models [15].

iv) Biodegradation using sulfate. Laboratory studies have shown that BTX can be degraded under sulfate reducing conditions [23, 24]. Anaerobic toluene mineralization under sulfidogenic conditions is described by the following stoichiometric equation:

$$C_7H_8 + 4.5 \text{ SO}_4^{-2} + 3 \text{ H}_2\text{O} \rightarrow 7 \text{ HCO}_3^{-1} + 2.25 \text{ H}_2\text{S} + 2.25 \text{ HS}^{-1} + 0.25 \text{ H}^+$$

This process is relatively slow, and its extent and significance in aquifers has not yet been quantified. When it occurs, it is usually near the center of the plume, farthest from the surrounding uncontaminated (aerobic) groundwater.

<u>v) Biodegradation using carbon dioxide</u>. Laboratory studies have also shown that BTX can be degraded under methanogenic conditions [25, 26]. The reaction for toluene can be represented as:

$$C_7H_8 + 5 H_2O \rightarrow 2.5 CO_2 + 4.5 CH_4$$

Similar to BTX degradation under sulfate reducing conditions, this process is relatively slow and its significance as an attenuation mechanism in aquifers has not been proven. This may be due, in part, to the high sensitivity of methanogens and sulfate reducers to a wide variety of environmental conditions, including pH, temperature, and toxicants (including oxygen in this case). An imbalance in any of these factors could easily inhibit these anaerobic BTX degradation processes.

5. Availability of inorganic nutrients. Microorganisms need macronutrients to synthesize cellular components, such as nitrogen for aminoacids and enzymes, phosphorus for ATP and DNA, sulfur for some coenzymes, calcium for stabilizing the cell wall, and magnesium for stabilizing ribosomes. A C:N:P ratio of 30:5:1 is generally sufficient to ensure unrestricted growth in aquifers

[27]. Microbes also need micronutrients to perform certain metabolic functions. For example, trace metals such as Fe, Ni, Co, Mo, and Zn are needed for some enzymatic activities. In general, aquifer minerals contain sufficient nutrients to support microbial activity. Nevertheless, geochemical analyses and laboratory biodegradation assays should be performed to verify that the presence of inorganic nutrients is sufficient for the success of intrinsic bioremediation.

6. Adequate pH. Enzymes are polymers of aminoacids, and their activity requires the proper degree of aminoacid protonation. This is controlled by the pH. Optimum groundwater pH is usually near the neutral value of 7.0, but most aquifer microorganisms can perform well between pH values of 5 and 9. Groundwater is typically well buffered within this range, so that the microbial physiological requirement for adequate pH is generally met in aquifers [9]. Nevertheless, aquifers contaminated by municipal landfill leachates may contain elevated concentrations of organic acids and pH's as low as 3.0. In these cases, pH may represent a significant environmental problem to the indigenous bacteria.

7. Adequate temperature. Temperature is one of the most important environmental factors influencing the activity and survival of microorganisms. Low temperatures reduce the fluidity and permeability of the cellular membrane, which hinders nutrient (and contaminant) uptake. Higher temperatures are associated with higher enzymatic activity and faster biodegradation rates, up to an optimum value which is species specific. In this range, BTX degradation rates can triple as a result of a temperature increase of 10°C [28]. If the temperature rises much beyond the optimum value, proteins, enzymes, and nucleic acids become denatured and inactive. The temperature of the upper 10 m of the subsurface may vary seasonally; however, that between 10 and 100 m approximates the mean annual air temperature of a particular region [5]. Groundwater temperatures in Brazil typically vary between 20 to 25°C, which is a very favorable range for intrinsic bioremediation.

8. Absence of toxic substances. Some contaminants can be present in aquifers at sufficiently high concentrations that inhibit microbial activity. For example, it is not uncommon for aquifer microorganisms to encounter potentially toxic heavy metals such as Pb, Hg, Cd, and Cr. While heavy metals are required in trace quantities for nutritional purposes, they can be bactericidal if present in soluble form at concentrations greater than about 1 mg/L. Although Brazilian gasoline has no lead, geochemical analyses and laboratory degradation assays with representative aquifer samples should be performed to verify the absence of inhibitory substances.

9. Faster biodegradation than migration rate. This condition is necessary to ensure that the hydrocarbon plume will recess rather than expand and reach potential groundwater users. These relative rates depend on the type and concentration of the contaminants, the indigenous microbial community, and the subsurface hydrogeochemical conditions. BTX compounds enter groundwater primarily by solubilization from the gasoline that reaches the water table. In Brazil, the solubility of BTX in groundwater can be enhanced by the cosolvent effect of ethanol, as illustrated for *o*-xylene in Figure 3. Therefore, higher BTX concentrations should result in groundwater that is in equilibrium with Brazilian gasoline compared to North American or European fuel. Once BTX are dissolved, they are transported by the moving groundwater. Their transport can be retarded by sorption onto aquifer materials, especially in aquifers with a high organic carbon content. It is not known, however, whether the presence of ethanol in the plume can decrease BTX retardation and facilitate their transport.

The primary mechanisms that limit BTX migration are biodegradation and, to a lesser extent, volatilization [13]. Abiotic reactions such as hydrolysis are not significant because BTX are stable under environmental conditions. In intrinsic BTX bioremediation, the rate-limiting attenuation mechanism is frequently the influx of oxygen, which in turn limits aerobic BTX degradation kinetics [29]. Nevertheless, the presence of a free phase is a critical factor influencing whether a plume will expand or recess. For example, a fluctuating water table can continue to flush BTX into the plume, which would hinder its recession. Consequently, the removal of the free-phase and the sorbed



Figure 3. Effect of ethanol concentration on the water solubility of *o*-xylene. Fifty mL of distilled water plus 5 mL of *o*-xylene were added to 100-mL serum bottles. Following ethanol addition at various concentrations, the bottles were shaken periodically and stored inverted at 25°C for 5 days. Emulsification of *o*-xylene was visually apparent in the bottles amended with 10% ethanol or more, but not with 1% ethanol or less.

hydrocarbons from the source area source is an important prerequisite for the successful implementation of intrinsic bioremediation.

CONCLUSIONS

At present, there are only a few well documented, full-scale demonstrations of unaided intrinsic bioremediation. Indeed, this emerging technique has not yet achieved pedagogical maturity. Nevertheless, intrinsic bioremediation shows great promise as a practical and cost-effective alternative for managing low-risk, BTX contaminated aquifers in Brazil and elsewhere; provided that it is implemented with adequate site characterization, analysis, and monitoring.

While some progress has been made towards understanding the hydrogeochemical and microbiological factors that influence the feasibility of intrinsic BTX bioremediation in North America and Europe, this experience should be extrapolated with care to contaminated sites in Brazil, where gasoline contains about 22% of ethanol. To take full advantage of intrinsic bioremediation in Brazil will require further basic and applied research. In particular, research is needed to gain a better understanding of (1) How does the presence of ethanol affect BTX solubilization, transport, microbial community structure, enzyme induction, and biodegradation kinetics under different electron acceptor conditions; (2) When and where is intrinsic bioremediation the appropriate choice?, (3) How well can we predict whether a given plume will expand or recess?, and (4) What is the critical hydrogeological, geochemical, and microbiological information needed to address these questions? A better understanding of these issues should lead to the development of a rational basis for the selection, mathematical modeling, and monitoring of appropriate intrinsic bioremediation systems.

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Address reprint requests to:

Professor Pedro J.J. Alvarez Department of Civil & Environmental Engineering The University of Iowa Iowa City, IA 52242-1527