



Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers

DR Lovley

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003, USA

Laboratory incubations of aquifer material or enrichments derived from aquifer material as well as geochemical data have suggested that, under the appropriate conditions, BTEX components of petroleum (benzene, toluene, ethylbenzene and xylene) can be degraded in the absence of molecular oxygen with either Fe(III), sulfate, or nitrate serving as the electron acceptor. BTEX degradation under methanogenic conditions has also been observed. However, especially for benzene, the BTEX contaminant of greatest concern, anaerobic degradation is often difficult to establish and maintain in laboratory incubations. Although studies to date have suggested that naturally occurring anaerobic BTEX degradation has the potential to remove significant quantities of BTEX from petroleum-contaminated aquifers, and mechanisms for stimulating anaerobic BTEX degradation in laboratory incubations have been developed, further study of the organisms involved in this metabolism and the factors controlling their distribution and activity are required before it will be possible to design rational strategies for accelerating anaerobic BTEX degradation in contaminated aquifers.

Keywords: benzene; toluene; groundwater; Fe(III) reduction; sulfate reduction

Introduction

Contamination of shallow aquifers as the result of terrestrial oil spills and leaking underground storage tanks is a serious environmental problem. The benzene, toluene, ethylbenzene, and xylene (BTEX) components of petroleum products are of particular concern because of their toxicity and because these compounds are relatively soluble and thus migrate with the groundwater. Therefore, there is substantial interest in mechanisms which can remove BTEX from contaminated groundwater, either naturally or under engineered conditions. All BTEX components can be degraded by aerobic microorganisms and aerobic degradation can play an important role in removing BTEX from groundwater naturally [54]. Furthermore, addition of oxygen to contaminated aquifers in order to stimulate aerobic degradation of BTEX is a common bioremediation practice [32].

Significant portions of petroleum-contaminated aquifers are anaerobic as the result of microbial respiration consuming the low concentrations of oxygen that can dissolve in ground water and because rates of reoxygenation as a result of oxygen diffusion from the atmosphere are relatively slow. As detailed below, within the last decade it has become apparent that BTEX can be degraded in these anaerobic zones and that such anaerobic BTEX degradation may be responsible for significant removal of at least some BTEX components from contaminated groundwater. However, until recently anaerobic BTEX degradation was considered to have only limited usefulness as a bioremediation strategy because numerous studies [1,4,8,9,22,29,31,32,50,56] indicated that benzene, the most toxic of the BTEX

components, was not degraded under anaerobic conditions. The recent finding that benzene can be rapidly degraded under Fe(III)- and sulfate-reducing conditions [3,17,39,43,44,45] has enhanced the potential benefit of anaerobic metabolism to BTEX remediation as it demonstrates that if the appropriate conditions are supplied, benzene might be removed from anoxic aquifers without the requirement of adding oxygen, which can be technically difficult and expensive [32,54]. The purpose of this review is to summarize recent advances in the understanding of anaerobic BTEX degradation in petroleum-contaminated aquifers.

Potential electron acceptors for anaerobic BTEX oxidation

Potential electron acceptors for anaerobic oxidation of organic matter in subsurface environments include: nitrate, Mn(IV), Fe(III), and sulfate [36]. With the exception of Mn(IV), one or more BTEX components can be oxidized with the reduction of each of these electron acceptors, as detailed below. Although it does not appear to have been investigated, it is likely that BTEX oxidation coupled to Mn(IV) reduction is also possible as at least one Fe(III) reducer that can oxidize aromatic compounds is known to use Mn(IV) as an electron acceptor for the oxidation of other electron donors [40]. Of these electron acceptors, Fe(III) typically provides the greatest potential electron-accepting capacity in shallow aquifers [14,33,34]. However, as discussed below, it is possible that nitrate and sulfate reduction can be important in some instances, especially if they are added to groundwater in order to stimulate BTEX oxidation coupled to nitrate or sulfate reduction. Furthermore, some BTEX components may also be degraded under methanogenic conditions. The reduction of nitrate, Mn(IV), Fe(III), or sulfate as well as methano-

genesis are referred to as terminal electron-accepting processes (TEAPs) [36,37].

In aquifers contaminated with petroleum or other organic pollutants such as landfill leachate, the anaerobic TEAPs are generally segregated into distinct zones (Figure 1) [5,6,11,37,47]. Such zonation is most likely the result of the availability of electron acceptors and the competition of microorganisms for electron acceptors [36]. In aquifers that have been contaminated for some time, methanogenesis typically predominates upgradient, closest to the source of contamination. This is because near the source of contamination, potential alternative electron acceptors (Fe(III), sulfate, etc) have already been depleted by BTEX degradation. If Fe(III) or sulfate are available, methanogenesis is generally excluded because Fe(III) reducers and sulfate reducers are able to outcompete the methanogenic community for electron donors [36]. This competition was evident from geochemical data on a petroleum-contaminated aquifer in Bemidji, Minnesota [35]. It appeared that shortly after contamination and the development of anaerobic conditions immediately downgradient from the contaminant source, Fe(III) reduction was the predominant TEAP in the anaerobic zone. However, as Fe(III) was depleted from the upgradient sediments closest to the source of the contamination, Fe(III) reduction moved further downgradient and methanogenesis became the predominant TEAP upgradient. It seems likely that this progression (Figure 2) is a common phenomenon in petroleum-contaminated aquifers.

As discussed below, sulfate concentrations in some groundwaters are sufficiently high that sulfate reduction can be an important TEAP. When Fe(III) is available, Fe(III) reducers can often outcompete sulfate reducers for electron donors or sulfate reducers may preferentially reduce Fe(III) [15,36]. Thus, when there is significant sulfate in the groundwater, a sulfate reduction zone can be expected

downgradient from the sulfate-depleted methanogenic zone but upgradient from the zone in which Fe(III) is still available for Fe(III) reduction (Figure 1). When present, Mn(IV) and/or nitrate-reducing zones are expected to be downgradient from the Fe(III) reduction zone. This is because Fe(III) reduction is generally excluded in the presence of Mn(IV) and nitrate due to: 1) preferential utilization of nitrate and Mn(IV) by Fe(III) reducers; 2) the ability of nitrate reducers to outcompete Fe(III) reducers for electron donors; and 3) rapid oxidation of Fe(II) by Mn(IV) preventing net Fe(III) reduction until Mn(IV) is depleted [36].

The TEAP zones in anaerobic aquifers can sometimes be roughly discerned through standard geochemical measurements of loss of electron acceptors (sulfate, nitrate) from groundwater or accumulation of dissolved endproducts (Fe(II), Mn(II), sulfide, methane). However, reduced endproducts produced in upgradient zones tend to persist in anaerobic groundwater as it moves further downgradient and this can obscure the true TEAP distributions [14,37]. Measurements or calculations of redox potential are also not effective in delineating the TEAP distribution because there is no consistent relationship between redox potentials and the predominating TEAPs in sedimentary environments [41].

Thus, in the past it has generally been necessary to obtain cores from aquifers to accurately determine the TEAP. One way in which this can be done is to perform incubations of sediments in which rates of all of the various anaerobic processes are measured. However, this is labor-intensive and time-consuming. A simpler method is to add [^{14}C] acetate to the sediments and monitor the production of $^{14}\text{CO}_2$ and $^{14}\text{CH}_4$ [37]. From the production of radiolabelled end products and simple geochemical measurements the predominant TEAP in the sediments can be rapidly and simply determined (Figure 3).

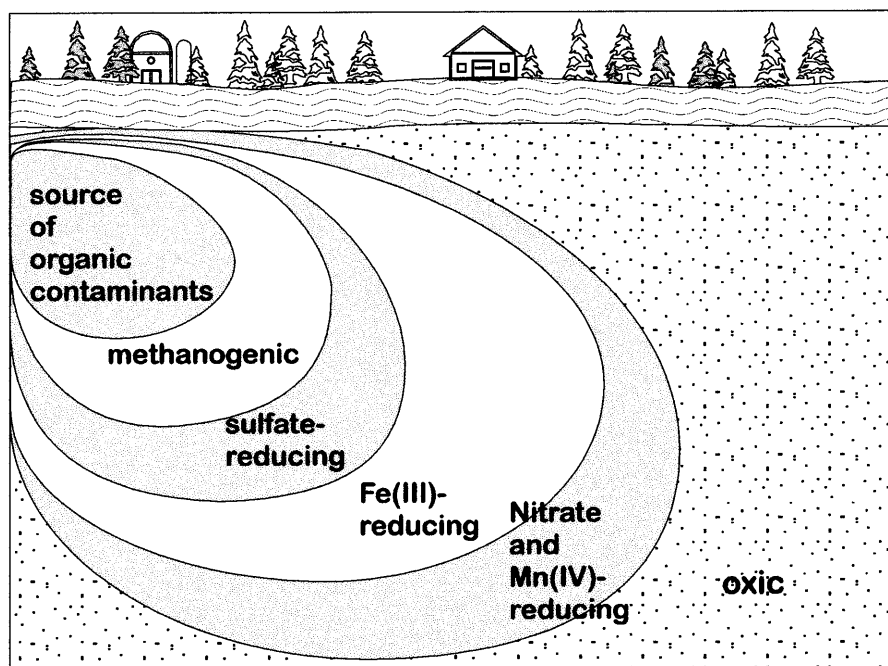


Figure 1 Typical distribution of anaerobic terminal electron-accepting processes (TEAPs) in a petroleum-contaminated aquifer.

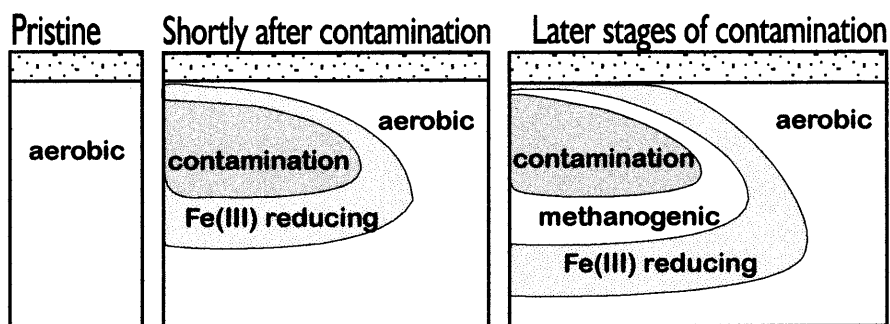


Figure 2 Evolution of TEAP distribution at a petroleum-contaminated aquifer following the introduction of crude oil.

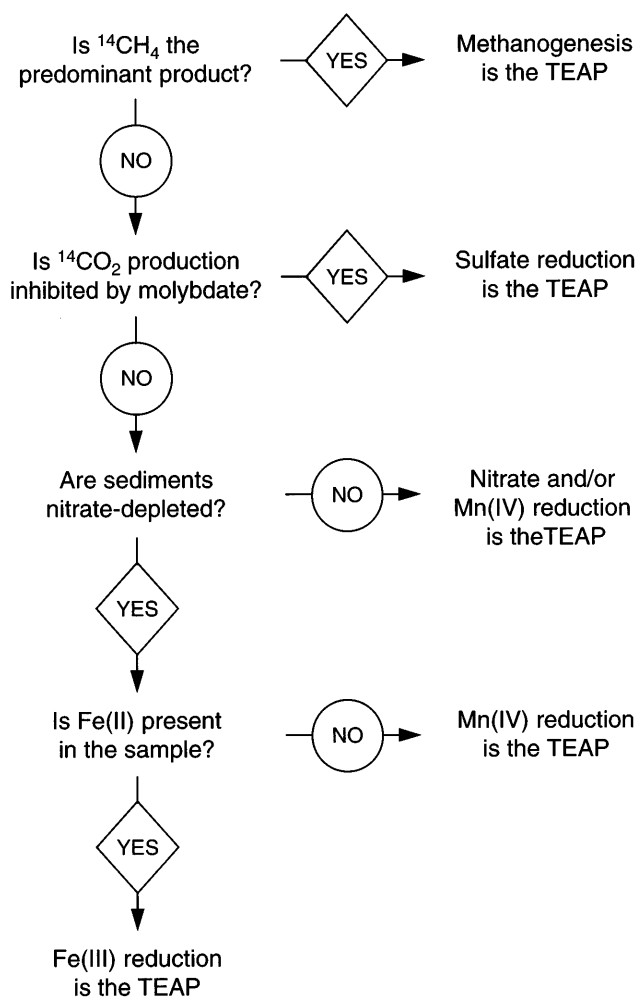


Figure 3 Decision tree for determining the TEAP in sediments with [2-¹⁴C]-acetate.

A simpler technique for determining the TEAP taking place in a particular anaerobic zone of an aquifer that does not require sampling of the sediments has recently been developed [37]. This approach relies on the fact that, under quasi steady-state conditions in anaerobic sedimentary environments, there are specific concentrations of dissolved H₂ that are characteristic of methanogenic, sulfate-reducing, Fe(III)-reducing, and Mn(IV)- and/or nitrate-reducing conditions [37,41]. Comparisons of predictions of TEAPs derived from measurements of dissolved H₂ and direct

measurements of TEAPs in laboratory incubations of aquifer sediments demonstrated that dissolved H₂ measurements could accurately predict TEAPs in petroleum-contaminated aquifers in which it was impossible to determine the TEAP by measuring concentrations of microbial end products or by estimates of redox potential [37].

BTEX oxidation coupled to Fe(III) reduction

As discussed above, with the development of anaerobic conditions in petroleum-contaminated aquifers, Fe(III) is generally the most abundant potential electron acceptor for organic matter oxidation. A pure culture of a toluene-oxidizing, Fe(III)-reducing microorganism known as *Geobacter metallireducens* has been described [35,40,42]. *G. metallireducens* was the first organism found to degrade aromatic hydrocarbons under strict anaerobic conditions. It oxidizes toluene directly to carbon dioxide with Fe(III) as the sole electron acceptor without the production of detectable extracellular intermediates. Pure cultures of Fe(III) reducers capable of oxidizing other BTEX components have not been described. However, in sediment enrichments of benzene-oxidizing Fe(III) reducers, the stoichiometry of benzene oxidation and Fe(III) reduction was consistent with complete oxidation of benzene to carbon dioxide with Fe(III) serving as the sole electron acceptor [44].

One of the first suggestions that Fe(III) reduction could be important for BTEX degradation in petroleum-contaminated aquifers came from geochemical data collected by Baedeker and colleagues at the US Geological Survey's Groundwater Toxics Site in Bemidji, Minnesota [6,7,35]. These studies demonstrated loss of BTEX components in the anaerobic portion of the aquifer that could not be attributed to sorption or volatilization. The initial accumulation of isotopically light carbon dioxide in the anaerobic ground water suggested that the early anaerobic loss of BTEX components was the result of their oxidation to carbon dioxide. Preliminary evidence that at least some of the BTEX degradation was coupled to Fe(III) reduction was the finding that Fe(II) accumulated in the water over time and that the sediments in the contaminated portion of the aquifer contained much less Fe(III) than sediments from a nearby uncontaminated portion of the aquifer. Subsequent geochemical modeling suggested that even in the most contaminated portion of the aquifer, some of which was already depleted of Fe(III), approximately half of the BTEX oxidation could be attributed to Fe(III) reduction [6].



Recently, direct evidence for BTEX degradation in the Fe(III) reduction zone of the Bemidji aquifer was obtained by monitoring the production of $^{14}\text{CO}_2$ from [ring- ^{14}C]-toluene and [^{14}C]-benzene injected into sediments incubated under anaerobic conditions. Both aromatic hydrocarbons were rapidly oxidized to $^{14}\text{CO}_2$ without a lag [3]. This is the first documented instance of rapid and extensive anaerobic benzene oxidation in laboratory incubations without long lag periods or extensive manipulation of the sediment. These data strongly suggest that benzene can be rapidly oxidized to carbon dioxide in the anaerobic portions of some aquifers.

Further geochemical evidence for involvement of Fe(III) reducers in BTEX degradation was the disappearance of BTEX components and the accumulation of Fe(II) in ground water at a North Carolina site [12]. In two aquifers contaminated with a variety of organics, including BTEX, there was significant loss of BTEX components, including benzene, as the ground water moved through the zone in which Fe(III) reduction was considered to be the TEAP [46,53]. There was little or no loss of BTEX in the upgradient methanogenic/sulfate-reducing zones.

At a petroleum-contaminated aquifer located in South Carolina, toluene was degraded in laboratory incubations of sediments from the Fe(III)-reducing zone, but there was no degradation of benzene, even after extended incubations [44]. However, benzene was rapidly oxidized to carbon dioxide with the reduction of Fe(III) if synthetic Fe(III) chelators were added to the sediments [44,45]. The chelators stimulate benzene and toluene oxidation coupled to Fe(III) reduction by solubilizing Fe(III) [43]. The soluble Fe(III) is much more accessible to the Fe(III) reducers than the insoluble Fe(III) oxides that are the natural source of Fe(III) in aquifer material. Addition of chelated Fe(III) as an electron donor in order to stimulate anaerobic degradation of BTEX has been proposed as an alternative to oxygen addition for remediation of petroleum-contaminated aquifers [44].

Addition of humic acids also stimulates benzene degradation under anaerobic conditions [45]. Although humic acids can chelate Fe(III), the primary mechanism by which they stimulate Fe(III) reduction and, thus presumably benzene oxidation coupled to Fe(III) reduction, is not by solubilizing Fe(III). Rather, the humics act as an electron shuttle between the Fe(III)-reducing microorganisms and the Fe(III) oxides [38]. Fe(III) reducers are able to donate electrons to humics which then can nonenzymatically reduce Fe(III), regenerating an oxidized form of the humic. Fe(III) reducers can conserve energy to support growth from the electron transport to the humics. Thus, this represents a novel form of microbial respiration. It may also have practical significance as the addition of humics to petroleum-contaminated aquifers in order to stimulate BTEX degradation coupled to Fe(III) reduction may be environmentally more acceptable than addition of synthetic Fe(III) chelators.

BTEX oxidation coupled to sulfate reduction

Although groundwaters in freshwater aquifers are often low in sulfate, sulfate concentrations may be increased due to high sulfate concentrations in the rainwater providing

recharge, fertilizer amendments to soils, contamination with septage or landfill leachate, or proximity to saltwater sources [10,19,56,58]. Furthermore, because of the good solubility of sulfate, high concentrations of sulfate could readily be added to an aquifer, if it was found that provision of this electron acceptor could stimulate BTEX degradation.

A pure culture of a toluene-oxidizing sulfate reducer has been isolated from marine sediments [51]. This organism which has requirements for high concentrations of sodium and magnesium is unlikely to be important in toluene oxidation in freshwater aquifers.

However, degradation of toluene and xylenes has been documented in laboratory incubations of aquifer material under sulfate-reducing conditions [10,13,19,22,26,50]. In some instances [10,19] oxidation of aromatics was confirmed with radiolabelled tracers, and stoichiometries of loss of the aromatic and reduction of sulfate were consistent with sulfate serving as the sole electron acceptor for oxidation. Rates of toluene loss along a groundwater flow path in the sulfate reduction zone of a petroleum-contaminated aquifer were consistent with measured rates of toluene oxidation estimated by the injection of [U- ^{14}C]-toluene into laboratory incubations of aquifer sediments [13].

Benzene degradation under sulfate-reducing conditions has also been observed in some instances. An enrichment established with aquifer sediments oxidized benzene to carbon dioxide after lag periods of 30–60 days [17]. Keys to establishment of the benzene-oxidizing enrichment were omitting the addition of toluene and xylenes to the sediments along with the benzene and maintaining benzene concentrations below 200 μM . Although it was not demonstrated that benzene oxidation was coupled to sulfate reduction, subsequent studies with marine sediments demonstrated that benzene can be rapidly oxidized to carbon dioxide with sulfate serving as the sole electron acceptor [39]. A pure culture which can serve as a model for this metabolism has yet to be isolated.

Very slow rates of ^{14}C -benzene oxidation to $^{14}\text{CO}_2$ were observed in sediments from the sulfate-reduction zone of a petroleum-contaminated aquifer. These slow rates were in accordance with the finding that there was little removal of benzene from the groundwater as it moved through the sulfate reduction zone [13].

BTEX degradation under methanogenic conditions

Loss of each BTEX component has been observed under methanogenic conditions in incubations of contaminated aquifer material [1,18,59,60]. Mineralization of toluene and *o*-xylene have been confirmed with radiolabel [18,59] and stoichiometric production of methane from these substrates has also been reported [18]. Although benzene disappearance from aquifer material under methanogenic conditions has been reported [59,60], the fate of the benzene that disappeared was not determined and other studies have found that benzene persists under methanogenic conditions [1,18,44]. Further evidence for the potential for benzene degradation under methanogenic conditions includes the finding of partial mineralization of benzene to carbon diox-

ide and methane in enrichment cultures [25] and river sediments [57].

BTEX oxidation coupled to nitrate reduction

Although nitrate is typically not available in high concentrations in petroleum-contaminated aquifers, it could readily be added as an electron acceptor. Thus, there has been substantial investigation into BTEX degradation under nitrate-reducing conditions in aquifers. Pure cultures of denitrifying microorganisms capable of oxidizing toluene, ethylbenzene, and *m*-xylene have been reported [2,16,20,23,52,55] and metabolism of *o*- and *p*-xylenes have been documented in enrichment cultures [21,27]. No well-substantiated benzene-degrading denitrifying enrichment or pure cultures have been identified.

The potential for degradation of toluene and *m*-xylene under denitrifying conditions in aquifers was first demonstrated in laboratory columns of aquifer material [30,31,61]. Degradation of other xylenes and ethylbenzene, as well as further confirmation of toluene and *m*-xylene degradation, have subsequently been observed in laboratory incubations of aquifer sediments or ground water [4,8,22,28,29,31,48–50].

The potential for benzene oxidation under denitrifying conditions is less clear. The vast majority of studies failed to detect benzene oxidation under denitrifying conditions [4,8,22,28,29,31]. Although another study [24] has been cited [49] as evidence for benzene oxidation coupled to nitrate reduction, no data specifically demonstrating benzene degradation were presented.

To date, the best evidence for benzene oxidation with the reduction of nitrate are two studies which demonstrated nitrate-dependent loss of benzene in anaerobic incubations of aquifer sediments or ground water [48,49]. However, neither of these studies demonstrated oxidation of benzene to carbon dioxide or established stoichiometries of benzene loss with the expected reduction of nitrate. Thus, anaerobic benzene oxidation coupled to nitrate reduction is less well-documented than benzene degradation with other TEAPs. As an indication of the often fickle nature of benzene degradation, at one of the sites where nitrate-dependent benzene loss had been observed [48], a subsequent study could not repeat this observation [8].

Conclusions

Perusal of the peer-reviewed literature demonstrates that there is the potential for all of the BTEX components to be degraded in petroleum-contaminated aquifers with either Fe(III), nitrate, or sulfate as the electron acceptor and degradation has even been observed under methanogenic conditions. In some instances, especially for benzene metabolism, the evaluation of this metabolism has been at a relatively superficial level. This is due, in part, to the fact that benzene oxidation is not consistently seen in samples from anaerobic aquifers. Furthermore, in many cases where benzene uptake has been observed there have been no follow-up studies to further investigate this metabolism. Isolation of an anaerobic benzene-oxidizing organism would greatly benefit research in this area.

More field-oriented research is necessary, in order to truly understand BTEX degradation under anaerobic conditions in petroleum-contaminated aquifers. This is especially true for benzene degradation. For example, it is questionable whether benzene degradation in laboratory incubations after long lag periods is a reliable indication that benzene is being degraded *in situ*. Future studies should focus on well-designed field experiments which can provide a more controlled and detailed accounting of BTEX removal than what has been provided to date by geochemical modeling of existing petroleum-contaminated sites.

A major microbiological goal should be to determine the factors which control the distribution and activity of BTEX-degrading organisms. Why benzene can be rapidly degraded under anaerobic conditions in some sediments as soon as they are collected, whereas others develop the capacity after extended laboratory incubations, and some never develop this capacity will probably be answered, at least in part, by differences in the populations of benzene-degrading microorganisms in the sediments. However, before such studies on population dynamics can be conducted, BTEX-degrading microorganisms need to be isolated or at least enriched from aquifer sediments so that appropriate markers for these populations (such as 16S rRNA sequences) can be identified.

Further study of anaerobic BTEX degradation is warranted not only to aid in a better understanding of the naturally occurring anaerobic degradation of BTEX in petroleum-contaminated aquifers, but also to develop solutions for more rapid remediation of petroleum-contaminated aquifers. Although there is an increasing reliance on naturally occurring degradation (generally referred to as intrinsic bioremediation or natural attenuation) to prevent the spread of BTEX in contaminated aquifers, this approach is not always feasible. A limitation of natural attenuation which is not always appreciated is that it is slow. Thus, if regulators require continuous monitoring of a contaminant plume, the long-term costs of natural attenuation may be very high. Furthermore, economic or regulatory pressure may require faster remediation of a site than natural attenuation can accomplish. As outlined above, the very limited investigation into anaerobic BTEX degradation that has taken place to date has suggested that when the appropriate conditions are achieved, all BTEX components can be rapidly degraded under anaerobic conditions. Further research in this area could suggest simple ways in which fast, consistent anaerobic BTEX degradation could be established for quick remediation of petroleum-contaminated aquifers.

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