Biodegradation **11:** 107–116, 2000.

Anaerobic benzene degradation

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Accepted 25 May 2000

Key words: bioremediation, Fe(III) reduction, methanogenesis, subsurfce microbiology, sulfate reduction

Abstract

Although many studies have indicated that benzene persists under anaerobic conditions in petroleum-contaminated environments, it has recently been documented that benzene can be anaerobically oxidized with most commonly considered electron acceptors for anaerobic respiration. These include: Fe(III), sulfate, nitrate, and possibly humic substances. Benzene can also be converted to methane and carbon dioxide under methanogenic conditions. There is evidence that benzene can be degraded under in situ conditions in petroleum-contaminated aquifers in which either Fe(III) reduction or methane production is the predominant terminal electron-accepting process. Furthermore, evidence from laboratory studies suggests that benzene may be anaerobically degraded in petroleum-contaminated marine sediments under sulfate-reducing conditions. Laboratory studies have suggested that within the Fe(III) reduction zone of petroleum-contaminated aquifers, benzene degradation can be stimulated with the addition of synthetic chelators which make Fe(III) more available for microbial reduction. The addition of humic substances and other compounds that contain quinone moieties can also stimulate anaerobic benzene degradation in laboratory incubations of Fe(III)-reducing aquifer sediments by providing an electron shuttle between Fe(III)-reducing microorganisms and insoluble Fe(III) oxides. Anaerobic benzene degradation in aquifer sediments can be stimulated with the addition of sulfate, but in some instances an inoculum of benzene-oxidizing, sulfate-reducing microorganisms must also be added. In a field trial, sulfate addition to the methanogenic zone of a petroleum-contaminated aquifer stimulated the growth and activity of sulfate-reducing microorganisms and enhanced benzene removal. Molecular phylogenetic studies have provided indications of what microorganisms might be involved in anaerobic benzene degradation in aquifers. The major factor limiting further understanding of anaerobic benzene degradation is the lack of a pure culture of an organism capable of anaerobic benzene degradation.

Introduction

The anaerobic degradation of benzene is of environmental significance primarily in petroleum-contaminated subsurface environments. Benzene is an important component of many petroleum products. When petroleum enters the subsurface as the result of surface petroleum spills or from leaks in underground storage tanks and pipelines, anaerobic conditions typically develop near the source of contamination (Anderson & Lovley 1997; Lovley 1997). The amount of oxygen that can be dissolved in groundwater even at saturation with air is low and rates of oxygen diffusion into the subsurface are slow. Therefore, the activity of aerobic hydrocarbon-degrading microorganisms can rapidly deplete oxygen. This is a concern because although benzene and other monoaromatic hydrocarbons are readily degraded under aerobic conditions in the subsurface (Salanitro 1993) the degradation of hydrocarbons under anaerobic conditions may be slow. This is of special concern with benzene, not only because of its high toxicity, but also because it is relatively water soluble and thus will move in the groundwater from the initial site of contamination.

Despite the importance of benzene as a contaminant in anaerobic aquifers, less is known about the anaerobic degradation of this hydrocarbon contaminant than many other hydrocarbons commonly found in petroleum products. As outlined in other articles in this volume, as well as in a recent review (Heider et al. 1998), there is considerable information available on the degradation of monoaromatic hydrocarbons other than benzene, as well as alkanes. Pure cultures of microorganisms capable of degrading these compounds under anaerobic conditions are available. In contrast, no pure culture of an anaerobic, benzene degrader has yet been reported and there is little information on the pathways by which benzene is metabolized under anaerobic conditions. Another confounding factor in the study of anaerobic benzene degradation is that the potential for benzene degradation is only found sporadically in subsurface environments. As detailed below, anaerobic benzene degradation is observed in some petroleum-contaminated aquifers, but not in others that appear to be geochemically similar. This contrasts with the apparent ubiquity of the potential for anaerobic degradation of toluene in petroleum-contaminated aquifers.

The purpose of this review is to summarize the information that is available on the anaerobic degradation of benzene and the factors controlling the rate and extent of this process. A review of the literature suggests that even though no anaerobic benzene-degrading organisms are available in pure culture, molecular analysis of anaerobic benzene-degrading communities is providing hints regarding what microorganisms are likely to be involved in anaerobic benzene degradation. Furthermore, studies of this metabolism in sediments has suggested several strategies for stimulating the activity of anaerobic benzene-degrading microorganisms in order to enhance the rate of benzene removal from contaminated subsurface environments.

Early evidence for anaerobic benzene degradation

The early studies of Grbic-Galic and Vogel (Vogel & Grbic-Galic 1986; Grbic-Galic & Vogel 1987) stimulated significant interest in the possibility for anaerobic benzene degradation. Methanogenic enrichment cultures that were initially established from sewage sludge with ferulate as the electron donor were found to partially mineralize benzene. When H_2O^{18} was incorporated in the culture medium, phenol labeled with O^{18} was recovered (Vogel & Grbic-Galic 1986). This demonstrated that the oxygen for the initial step in benzene oxidation could be derived from water rather than the molecular oxygen that is used in aerobic benzene degradation pathways. However, the proportion of O^{18}-labeled phenol that was produced was less than a third of the amount expected if all of the phenol had originated from anaerobic benzene oxidation with water supplying the oxygen (Vogel & Grbic-Galic 1986). The amount of methane produced in the enrichment cultures was much less than that expected if methanogenesis was the sole terminal electron-accepting process (TEAP) for benzene degradation (Grbic-Galic & Vogel 1987). This also raised questions about the actual pathway for benzene degradation in the cultures. Less than 6% of the [^{14}C]-benzene added to the cultures was converted to $^{14}CO_2$ as compared to the theoretical yield of 37.5% ($^{14}CH_4$ was not monitored) (Grbic-Galic & Vogel 1987). This suggested that there was in fact little benzene degradation taking place in the cultures and further tempered enthusiasm about the actual potential for anaerobic benzene degradation. Furthermore, many subsequent studies reported that benzene was not degraded under anaerobic conditions (Barker et al. 1987; Kuhn et al. 1988; Lee et al. 1988; Hutchins et al. 1991; Acton & Barker 1992; Barbaro et al. 1992; Anid et al. 1993; Flyvbjerg et al. 1993; Patterson et al. 1993; Thierrin et al. 1993). However, the suggestion by Grbic-Galic and Vogel that benzene could be degraded under anaerobic conditions provided the impetus for many of the later studies which did eventually provide evidence for significant anaerobic degradation of benzene.

Evidence for complete mineralization of benzene under anaerobic conditions: benzene oxidation coupled to the reduction of chelated Fe(III)

Evidence for complete oxidation of benzene to carbon dioxide coupled to the reduction of an electron acceptor other than oxygen came from studies with sediments from a petroleum-contaminated aquifer (Lovley et al. 1994). Benzene persisted in these sediments in which Fe(III) reduction was the predominant TEAP. However, when the Fe(III) chelator, nitrilotriacetic acid (NTA), was added to the sediments benzene was consumed after a lag period of ca. 100 days. When benzene was added back to the sediments that had been adapted for benzene degradation it disappeared more rapidly and without a lag period. The rate of benzene removal accelerated with continued re-additions of benzene. The rapid loss of benzene in all of the NTA-amended sediments but not in killed sediments or in live sediments which did not contain NTA indicated that the benzene removal could not be attributed to accidental oxygen contamination.

Studies with [^{14}C]-benzene demonstrated that the loss of benzene in the NTA-amended aquifer sediments was the result of benzene oxidation to carbon dioxide (Lovley et al. 1994). The stoichiometry of benzene oxidation and Fe(III) reduction in the benzene-adapted sediments demonstrated that benzene was oxidized with Fe(III) serving as the sole electron acceptor according to the reaction:

$$C_6H_6 + 30Fe(III) + 12H_2O$$

$$\rightarrow 6CO_2 + 30Fe(II) + 30H^+.$$

Current evidence suggests that NTA stimulated anaerobic benzene degradation in the aquifer sediments by making Fe(III) more available for microbial reduction. Fe(III) in aquifer sediments primarily exists in the form of insoluble Fe(III) oxides (Lovley 1991). If Fe(III)-reducing microorganisms need to establish direct contact with this insoluble Fe(III) oxide in order to reduce it, then this may kinetically constrain the activity of Fe(III)-reducing microorganisms. The addition of NTA to the sediments solubilizes some of the Fe(III), making it more accessible to the Fe(III)-reducing microorganisms (Lovley & Woodward 1996; Lovley 1997). When the Fe(III) is more accessible, the Fe(III)-reducing microorganisms in the aquifer sediments can reduce it faster (Lovley et al. 1994). Other Fe(III) chelators also stimulated the rates of benzene degradation (Lovley et al. 1996b). Further evidence that the stimulatory effect of NTA was the result of making Fe(II1) more available for reduction was the finding that benzene persisted when NTA was added to sediments which were depleted of microbially reducible Fe(III) and in which methanogenesis was the TEAP. However, benzene was degraded when soluble Fe(III) chelated with NTA was added to the Fe(III)depleted sediments (Lovley et al. 1994).

The rates of anaerobic benzene degradation in the NTA-amended aquifer sediments were comparable to those observed when the sediments were adapted for aerobic degradation of benzene. This demonstrated that anaerobic benzene degradation need not necessarily be a slow process.

Studies with freshwater aquatic sediments from the Potomac River first suggested that the addition of synthetic chelators is not always necessary in order to promote benzene oxidation coupled to Fe(III) reduction (Kazumi et al. 1997). The aquatic sediments were adapted for anaerobic benzene degradation within 50 days after the sediments were amended with poorly crystalline Fe(III) oxide. The benzene degradation in the presence of insoluble Fe(III) oxides contrasted with the studies with aquifer sediments in which an added synthetic chelator was also necessary for rapid benzene degradation. However, it is important to recognize that due to the addition of the Fe(III) oxides, the benzene degradation that was observed in the aquatic sediment incubations may not have been indicative of benzene degradation in situ.

Benzene oxidation coupled to the reduction of humic substances

Further studies on the role of chelators in stimulating anaerobic oxidation of benzene coupled to Fe(III) reduction revealed that the addition of humic substances (HS) stimulated benzene degradation in aquifer sediments better than any of the synthetic chelators evaluated (Lovley et al. 1996b). This was an unexpected result because the Fe(III) chelation capacity of the added HS was much less than that of the synthetic chelators that were evaluated.

Subsequent investigation demonstrated that HS can serve as an electron shuttle between Fe(III)-reducing microorganisms and insoluble Fe(III) oxides and that this was the likely explanation for the superior capacity of added HS to stimulate anaerobic benzene degradation (Lovley et al. 1996a). In the current model for electron shuttling via HS, Fe(III)-reducing microorganisms transfer electrons to quinone moieties in the HS (Lovley et al. 1996a; Lovley et al. 1998; Scott et al. 1998). The hydroquinone moieties that are produced can abiotically donate electrons to Fe(III) oxides. This not only reduces the Fe(III) in the sediments, but also regenerates the quinone moieties that can again serve as an electron acceptor for Fe(III)reducing microorganisms. In this manner, each HS molecule may undergo multiple cycles of reduction and oxidation. Thus, the concentration of HS or other extracellular compounds that contain quinone moieties that are necessary to stimulate Fe(III) reduction in aquifer sediments is low. Studies with the HS analog, anthraquinone-2,6-disulfonate (AQDS), have indicated that as little 10 μM AQDS (the lowest concentration tested;) can stimulate the reduction of Fe(III) oxides in aquifer sediments (Nevin and Lovley 2000). Electron shuttling via HS and related compounds can greatly accelerate Fe(III) reduction because the Fe(III)-reducing microorganisms need not contact the Fe(III) oxides in order to reduce them. The stimulation of Fe(III) reduction by HS is similar in this regard to the stimulation of Fe(III) reduction by Fe(III) chelators. However, overcoming the need for contact with the Fe(III) oxides via electron shuttling with HS is fundamentally different than the Fe(III) solubilization that takes place with Fe(III) chelators.

Although HS can chelate some Fe(III) (Benz et al. 1998; Lovley & Blunt-Harris 1999), the electron-accepting capacity of the Fe(III) in the HS is a minor fraction of the electron-accepting capacity that can be attributed to the quinone moieties (Lovley & Blunt-Harris 1999). Furthermore, unlike the quinone moieties, the iron bound in HS can not serve as an electron shuttle to Fe(III) oxides. Thus, Fe(III) chelated by HS is expected to be a minor component of the electron flow that proceeds through HS (Lovley & Blunt-Harris 1999).

The stoichiometry of benzene oxidation coupled to the reduction of HS has not been measured directly because the rapid oxidation of HS with Fe(III) prevents a ready measurement of electron flow to HS in benzene-degrading, HS-amended sediments. However, addition of the HS analog, AQDS, stimulated anaerobic benzene degradation in the same Fe(III)-reducing sediments in which benzene degradation was stimulated with the addition of HS (Anderson & Lovley 1999). This indicates that benzene oxidation can be coupled to the reduction of extracellular quinones, as AQDS does not have significant Fe(III) chelation capacity. Further evidence that Fe(III)-reducing microorganisms can couple the oxidation of aromatic hydrocarbons to the reduction of an extracellular quinone is the finding that the toluene-oxidizing, Fe(III) reducer, *Geobacter metallireducens,* can be grown with toluene as the electron donor and AQDS as the sole electron acceptor (R.T. Anderson, unpublished results).

In situ **benzene oxidation coupled to Fe(III) oxide reduction**

As noted above, benzene was only slowly degraded anaerobically, if at all, under the conditions naturally found in most of the petroleum-contaminated aquifers that were initially investigated. However, more recent studies have provided evidence for substantial anaerobic benzene oxidation under the in situ conditions in some aquifers and aquatic sediments. The petroleum-contaminated aquifer in which anaerobic benzene under in situ conditions has been most thoroughly investigated is the U.S. Geological Survey Toxics Study site in Bemidji, MN. Geochemical data collected by Baedecker and colleagues provided early evidence for the degradation of aromatic hydrocarbons at this site (Baedecker et al. 1989; Lovley et al. 1989; Cozzarelli et al. 1990; Baedecker et al. 1993). When [^{14}C]-benzene was added to sediments from this aquifer that were incubated under conditions that closely represented those in situ, the benzene was oxidized to $^{14}CO_2$ in some of the sediments from the Fe(III) reduction zone (Anderson et al. 1998; Anderson & Lovley 1999). The lack of a lag period prior to benzene oxidation in these sediment incubations suggested that the microorganisms were already adapted for benzene degradation and were probably oxidizing benzene in situ. Further evidence for in situ benzene degradation was depletion of benzene along the groundwater flow path within the Fe(III) reduction zone (Lovley & Anderson 2000). The potential for significant in situ benzene degradation was limited to the sediments from the Fe(III) reduction zone that were the furthest down-gradient from the source of contamination (Anderson et al. 1998). There was little or no anaerobic oxidation of [^{14}C]-benzene added to sediments in which Fe(III) reduction was the TEAP, but which were closer to the source of contamination. There was also no degradation of benzene in the sediments closest to the source of contamination in which methane production was the TEAP.

The localization of anaerobic benzene degradation to one portion of the Fe(III) reduction zone in the Bemidji aquifer could not be readily explained from an examination of geochemical parameters (Anderson & Lovley 1999). Higher concentrations of Fe(III) were not the primary explanation for the higher rates of benzene oxidation in the active sediments because there was no correlation between Fe(III) concentrations and rates of benzene degradation in this and other petroleum-contaminated aquifers (Anderson et al. 1998; Anderson & Lovley 1999). It was hypothesized that groundwater within the benzene degradation zone of the Bemidji aquifer might be enriched with Fe(III) chelators and/or electron shuttling compounds that would enhance anaerobic oxidation of benzene coupled to Fe(III) reduction, but there was no evidence for elevated concentrations of chelators or electron-shuttles in the groundwater (Anderson & Lovley 1999).

The one clear difference between the zone of anaerobic benzene degradation in the Bemidji aquifer and the rest of the aquifer was a significant enrichment of microorganisms in the genus Geobacter within the zone of benzene degradation (Anderson et al. 1998; Rooney-Varga et al. 1999). Analysis of the 16S rDNA sequences in the sediments demonstrated that *Geobacter* species were an important component of the microbial community in the benzene-degrading sediments (Rooney-Varga et al. 1999). The numbers of *Geobacteraceae* were more than two orders of magnitude higher in the sediments in which benzene was being actively degraded than in sediments from other portions of the Fe(III) reduction zone; and four orders of magnitude higher than in nearby aerobic sediments from an uncontaminated portion of the aquifer. The *Geobacteraceae* sequences recovered from the zone of benzene degradation were closely related to known species of *Geobacter* and were different than the *Geobacteraceae* sequences recovered from the uncontaminated sediments. These results suggested that the *Geobacter* species that predominated in the zone of anaerobic benzene oxidation might be responsible for the benzene degradation. Further evidence for this was the finding that a *Geobacter* sequence, similar to the one recovered in sediments, was the dominant sequence in a benzene-oxidizing, Fe(III)-reducing enrichment culture that was recovered from the benzene-degrading sediments (Rooney-Varga et al. 1999).

The finding that *Geobacter* species were associated with benzene degradation is in accordance with the fact that this is the only genera that is known to contain microorganisms that can oxidize aromatic compounds with the reduction of Fe(III) (Lovley 2000). Studies on the pathway for anaerobic benzene degradation in sediments in which Fe(III) reduction is the TEAP have demonstrated that there is no production of likely extracellular intermediates (D.R. Lovley, unpublished data). This suggests that just as *Geobacter metallireducens* oxidizes aromatic compounds completely to carbon dioxide (Lovley & Lonergan 1990), the benzene in Fe(III)-reducing sediments is directly oxidized to carbon dioxide within individual Fe(III)-reducing microorganisms, rather than by microbial consortia. However, none of the *Geobacter* species currently available in pure culture have been shown to oxidize benzene. Thus further studies are required to definitively determine whether any of the *Geobacter* species in the sediments are capable of anaerobic benzene oxidation.

Benzene oxidation coupled to sulfate reduction

Initial evidence suggesting that benzene could be anaerobically oxidized with the reduction of sulfate was the finding that benzene was oxidized to carbon dioxide in enrichment cultures established with aquifer sediments in which sulfate was provided as a potential electron acceptor (Edwards & Grbic-Galic 1992). However, sulfate depletion could not be documented and it could not be ruled out that other electron acceptors present in the sediment might have been responsible for the benzene oxidation (Edwards & Grbic-Galic 1992).

Benzene oxidation coupled to the reduction of sulfate was first documented in marine harbor sediments (Lovley et al. 1995). Sulfate reduction was the predominant TEAP in the sediments examined and benzene oxidation was coupled to sulfate reduction (Coates et al. 1996a; Coates et al. 1996b). In relatively pristine sediments there was a lag prior to benzene degradation, but in sediments with higher levels of petroleum contamination benzene oxidation proceeded without a lag (Lovley et al. 1995; Coates et al. 1996a). [^{14}C]-benzene was oxidized to $^{14}CO_2$ and the addition of molybdate, a specific inhibitor of sulfate reduction (Oremland & Capone 1988), completely inhibited the benzene oxidation (Lovley et al. 1995; Coates et al. 1996a; Coates et al. 1996b). Benzene oxidation was dependent upon the presence of sulfate. Approximately 80% of the benzene consumption in the sediments could be accounted for as benzene-dependent sulfate reduction. This suggested that the primary route for benzene oxidation was:

$$4C_6H_6 + 15SO_4^{=} + 12H_2O$$
$$\rightarrow 24HCO_3^- + 15HS^- + 9H^+.$$

Isotope trapping studies indicated that phenol, benzoate, *p*-hydroxybenzoate, cyclohexane, catechol, and acetate were not extracellular intermediates during benzene oxidation (Lovley et al. 1995). The lack of extracellular intermediates suggested that benzene was being completely oxidized to carbon dioxide within individual sulfate-reducing microorganisms.

Other marine sediments, as well as estuarine and freshwater aquatic sediments, were also found to contain microorganisms which could oxidize benzene to carbon dioxide with the reduction of sulfate (Phelps et al. 1996; Kazumi et al. 1997; Weiner & Lovley 1998a). The stoichiometry of benzene oxidation coupled to sulfate reduction was similar in all of the sediments. In the freshwater sediments, benzene appeared to be directly oxidized to carbon dioxide without the production of commonly considered potential extracellular intermediates as had been previously observed in marine sediments (Weiner & Lovley 1998a). An inoculum of the microorganisms from the freshwater, benzene-degrading sediments stimulated benzene degradation coupled to sulfate reduction in anaerobic aquifer sediments in which benzene otherwise persisted (Weiner & Lovley 1998a).

Although benzene often persists under sulfate-reducing conditions in petroleum-contaminated aquifers, as outlined below, the addition of sulfate to sediments from one petroleum contaminated aquifer greatly enhanced the rates of benzene oxidation coupled to sulfate reduction in both laboratory and field studies (Weiner et al. 1998; Anderson & Lovley 2000). As in all other studies on anaerobic benzene degradation, the microorganisms responsible for benzene oxidation were not recovered in pure culture. Analysis of dissimilatory sulfite reductase genes in the sediments suggested that sulfate-reducing microorganisms that were growing in response to the added sulfate were not closely related to any sulfate-reducing microorganisms currently available in pure culture (B. Methé and D. Lovley, unpublished data).

Benzene mineralization under methanogenic conditions

As summarized above, the earliest evidence for anaerobic benzene degradation under methanogenic conditions was provided by the studies of Grbic-Galic and Vogel (Vogel & Grbic-Galic 1986; Grbic-Galic & Vogel 1987). In a study with petroleum-contaminated aquifer sediments that were considered to be methanogenic, benzene disappeared during anaerobic incubations (Wilson et al. 1986), but the fate of the benzene was not determined and no data demonstrating that methanogenesis was the predominant TEAP were provided. Benzene was not degraded for the first 140 days of incubation, suggesting that the microorganisms in the sediment were not adapted for benzene degradation in situ.

Further evidence for the potential of benzene degradation under methanogenic conditions was the production of significant quantities of methane following the addition of benzene to slurries of aquifer sediments (Kazumi et al. 1997). The amount of methane produced in the presence of benzene was nearly the same as the methane produced from an equivalent quantity of benzoate. Benzoate was converted to methane without a lag period whereas methane production in the benzene-amended sediments lagged for several hundred days. [^{14}C]-benzene added to the sediments was converted to a combination of $^{14}CH_4$ and $^{14}CO_2$. The long lag period prior to significant benzene conversion to methane suggested that the microbial population had to be adapted for benzene degradation and was not degrading benzene in situ. Studies with other aquifer sediments found that benzene persisted under methanogenic conditions (Acton & Barker 1992; Edwards & Grbic-Galic 1994; Lovley et al. 1994; Kazumi et al. 1997; Anderson et al. 1998).

Evidence for in situ degradation of benzene under methanogenic conditions was presented in a study of methanogenic sediments from an aquifer that had been heavily contaminated with petroleum products for over 50 years (Weiner & Lovley 1998b). [^{14}C]-benzene was converted to $^{14}CH_4$ and $^{14}CO_2$ in proportions that were consistent with benzene degradation with methane production serving as the predominant TEAP (Weiner & Lovley 1998b) according to the reaction:

$$4C_6H_6 + 27H_2O \rightarrow 15CH_4 + 9HCO_3^- + 9H^+.$$

Benzene mineralization proceeded without a lag period, suggesting that the microbial community was already adapted for benzene degradation and was metabolizing benzene in situ.

In contrast to the apparent direct oxidation of benzene to carbon dioxide under Fe(III)-reducing and sulfate-reducing conditions, under methanogenic conditions, benzene is likely to be degraded by consortia of microorganisms, as are other monoaromatic compounds (Ferry & Wolfe 1976). The accumulation of phenol in a benzene-degrading methanogenic enrichment culture (Vogel & Grbic-Galic 1986; Grbic-Galic & Vogel 1987) was discussed above. Isotope-trapping studies demonstrated that [^{14}C]-phenol was produced from [^{14}C]-benzene in methanogenic aquifer sediments (Weiner & Lovley 1998b). These studies suggest that conversion of benzene to phenol may be the first step in the degradation of benzene in methanogenic systems. Extracellular [^{14}C]-acetate and [^{14}C]-propionate were also produced from [^{14}C]-benzene in the methanogenic, benzene-degrading aquifer sediments (Weiner & Lovley 1998b). There was no evidence for production of benzoate, *p*-hydroxybenzoate, or butyrate. It could not be definitively determined from the data available whether the acetate and propionate were produced from the degradation of the phenol generated from benzene or if they resulted from an alternative pathway in which the fatty acids were the first extracellular products of benzene metabolism. In either case, these results indicate that a consortium of microorganisms is probably involved in the conversion of benzene to methane. This consortium is likely to include: the benzene-degrading population(s); a phenol-degrading population to metabolize the phenol produced from benzene; acetogenic microorganisms to convert the propionate to acetate and hydrogen; and acetate- and H_2-utilizing methanogens.

It was speculated that the reason that benzene was readily converted to methane and carbon dioxide in the sediments from the aquifer that was heavily contaminated with petroleum, but that evidence for in situ benzene degradation under methanogenic conditions is lacking in many other aquifers, is that the sediments at this site have a long history of exposure to relatively high concentrations of benzene. An extended period of time may be required in order to develop the appropriate consortium of microorganisms that are hypothesized to degrade benzene under methanogenic conditions. Another factor might have been that the methanogenic sediments that readily degraded benzene were incubated with minimal disturbance of the sediments, whereas many previous studies significantly slurried and mixed the aquifer sediments. These procedures may have disrupted the potentially delicate consortia involved in benzene mineralization.

Benzene mineralization with the reduction of nitrate

Nitrate was the last of the commonly considered major electron acceptors in sedimentary environments found to serve as the terminal electron acceptor for anaerobic benzene oxidation. Early evidence consistent with a potential for anaerobic benzene degradation with the reduction of nitrate was the discovery of nitrate-dependent removal of benzene in anaerobic incubations of aquifer sediments or ground water (Major et al. 1988; Morgan et al. 1993). These studies did not determine whether the benzene was oxidized to carbon dioxide or whether benzene removal was associated with the removal of a quantity of nitrate consistent with benzene oxidation coupled to nitrate reduction. Furthermore, the initial observation of nitrate-dependent uptake at one of the sites (Major et al. 1988) could not be repeated in subsequent studies (Barbaro et al. 1992). This coupled with the finding that benzene was not degraded under nitrate-reducing conditions in many other studies (Kuhn et al. 1988; Hutchins 1991; Hutchins et al. 1991; Anid et al. 1993; Flyvbjerg et al. 1993) raised doubts about whether benzene oxidation coupled to nitrate reduction was possible.

However, a recent study with an enrichment culture established with a soil and groundwater inoculum has clearly demonstrated that benzene can be oxidized with nitrate serving as the sole electron acceptor (Burland & Edwards 1999). Benzene uptake in the culture was dependent upon the presence of nitrate. When [^{14}C]-benzene was added, over 90% of the radiolabel was recovered as $^{14}CO_2$. There was an increase in cell protein over time, indicating that the anaerobic benzene oxidation yielded energy to support cell growth. Nitrite accumulated in the cultures and the stoichiometry of benzene consumption and nitrate loss indicated that most of the benzene was oxidized according to the reaction:

$$C_6H^6 + 15NO_3^+ + 3H_2O$$

$$\rightarrow 6HCO_3^- + 15NO_2 + 6H^+.$$

There was somewhat less nitrate utilized than would be consistent with this reaction and N_2 was produced in the cultures. Therefore, it was also suggested (Burland & Edwards 1999) that some of the benzene oxidation may have been coupled to complete reduction of nitrate to N_2 as follows:

$$C_6H_6 + 6NO_3^- \rightarrow 6HCO_3^- + 3N_2.$$

These studies have shown the potential for anaerobic benzene oxidation in environments in which nitrate is present. It has yet to be determined whether there are environments in which this process is naturally important or in which a nitrate-based anaerobic bioremediation strategy for benzene contamination can be established.

Anaerobic strategies for remediation of subsurface benzene contamination

The finding that benzene can be degraded under anaerobic conditions has led to the suggestion of several potential strategies for anaerobic remediation of benzene in petroleum-contaminated aquifers and a successful field trial of one of these strategies. Anaerobic approaches for remediation of benzene contamination may be preferable over the more traditional aerobic bioremediation techniques, which can be technically difficult and expensive. For example, aerobic bioremediation strategies were unsuccessful in remediating an aquifer adjacent to a refinery that was heavily contaminated with petroleum products and in which methane production was the TEAP (Weiner et al. 1998; Anderson & Lovley 2000). Benzene was the primary contaminant of concern in the groundwater. As discussed above, there was evidence for in situ degradation of benzene under the methanogenic conditions found in the aquifer (Weiner & Lovley 1998b). However, laboratory studies demonstrated that benzene was more rapidly degraded under anaerobic conditions if Fe(III) oxide was added in conjunction with NTA or HS, or if sulfate was provided as an electron acceptor (Weiner et al. 1998). The potential of stimulating anaerobic benzene degradation with sulfate amendments was further evaluated because it was considered to be technically simpler for in situ remediation than the Fe(III)-based methods.

When aquifer sediments were incubated in columns under anaerobic conditions with a flow of benzene-containing groundwater moving through them, sulfate additions stimulated benzene degradation as it had in static incubations (Weiner et al. 1998). The removal of benzene and sulfate from the groundwater as it moved through the sediments was consistent with the stoichiometry expected for benzene oxidation coupled to sulfate reduction.

A field trial in the petroleum-contaminated aquifer demonstrated that addition of sulfate stimulated benzene degradation in situ (Anderson & Lovley 2000). The enhanced benzene removal was associated with the removal of the added sulfate from the groundwater. Comparison of benzene and sulfate losses suggested that benzene oxidation coupled to sulfate reduction could account for 42% of the sulfate depletion. Sulfate reduction was the predominant TEAP in the sulfate-amended sediments. Molybdate inhibited the oxidation of added [^{14}C]-benzene, which suggested that benzene oxidation was coupled to sulfate reduction. Sediments receiving sulfate had higher numbers of culturable benzene-degrading sulfate reducers than control sediments and there was an increase in the total sulfate-reducing population as indicated by an increase in the number of copies of dissimilatory sulfite reductase genes in the sediments. This indicated that the sulfate additions stimulated both the growth and the activity of benzene-oxidizing sulfate-reducing microorganisms. Thus, this anaerobic strategy provided a mechanism for remediating this site, which was not amenable to standard aerobic bioremediation approaches.

Anaerobic strategies for the remediation of benzene contamination are likely to be most applicable to aquifers that are heavily contaminated with petroleum. As previously outlined (Weiner et al. 1998), sulfate additions may be preferable to oxygen additions under such conditions because: 1) unlike oxygen, sulfate is not chemically consumed by reactions with reduced species such as Fe(II) and sulfide; 2) the addition of oxygen can cause plugging of the aquifer due to the formation of insoluble Fe(III) oxides, whereas sulfate will not react with Fe(II) to form Fe(III) oxides; 3) the solubility of sulfate is much higher than that of oxygen so it is easier to add higher concentrations to groundwater; 4) one mole of sulfate has twice the oxidizing (electron accepting) capacity of one mole of oxygen.

Laboratory studies of other aquifer sediments (Weiner & Lovley 1998a) have demonstrated that the addition of sulfate alone may not be sufficient to stimulate anaerobic benzene degradation in anaerobic aquifer sediments if the sediments do not contain a sufficient population of benzene-oxidizing sulfate reducers. In these instances, it may also be necessary to add an inoculum of the sulfate reducers, which laboratory studies have demonstrated can then promote benzene oxidation coupled to sulfate reduction (Weiner & Lovley 1998a). As previously noted in laboratory studies (Lovley et al. 1994; Lovley et al. 1996b; Weiner et al. 1998), stimulating Fe(III) reduction with the addition of chelated Fe(III), or a combination of Fe(III) and an electron-shuttling agent, may be alternative in situ remediation strategies for enhancing benzene degradation. However, these approaches have yet to evaluated in an in situ application.

Future directions in the study of anaerobic benzene degradation

A major factor limiting the further study of anaerobic benzene degradation is the lack of availability of a pure culture of an anaerobic, benzene-degrading microorganism. As noted above, studies with sediments have suggested that single organisms completely oxidize benzene in sediments in which sulfate reduction or Fe(III) reduction is the TEAP. Therefore, it should be possible to grow a benzene-oxidizing Fe(III)-reducing or sulfate-reducing microorganism in an appropriate medium with benzene as the electron donor and Fe(III) or sulfate as the electron acceptor. This seems even more likely given the recent isolation of a sulfate-reducing microorganism capable of anaerobically oxidizing naphthalene, another unsubstituted aromatic hydrocarbon (Galusko et al. 1999). The availability of anaerobic benzene-degrading microorganisms in pure culture would permit controlled physiological studies to determine what factors might control the rate and extent of benzene degradation in anaerobic environments. Such pure cultures would also provide molecular targets in the form of phylogenetic or metabolic genes which could aid in studying these organisms in situ. This would greatly expand the understanding of anaerobic benzene degradation beyond that available from the study of benzene-adapted sediments and enrichment cultures.

Acknowledgment

Fesearch from the author's laboratory summarized in this review was supported by grants from the National Science Foundation, the Office of Naval Research, and the American Petroleum Institute.

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