ANAEROBIC BIODEGRADATION IN CONTAMINATED AQUIFERS: INFLUENCE OFPROTOZOAN PREDATION AND IRON BIOAVAILABILITY

By

Sreenivas Kota and Robert C. Borden
Department of Civil Engineering
North Carolina State University
Raleigh, North Carolina 27695

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Department of Civil Engineering
College of Engineering
North Carolina State University
Raleigh, North Carolina 27695
ABSTRACT

Recent research has shown that many petroleum hydrocarbon plumes biodegrade under anaerobic conditions. However, the major factors controlling the rate and extent of biodegradation are still poorly understood. In this study, we examine the effect of protozoan grazing and iron oxide bioavailability on the rate and extent of anaerobic biodegradation in contaminated aquifer sediment.

In a previous study, elevated numbers of aerobic and anaerobic protozoa were detected in a shallow aquifer contaminated with petroleum hydrocarbons, suggesting that protozoan predation might have a significant influence on bacterial populations and contaminant biodegradation rates. To evaluate the effect of protozoan predation on aerobic biodegradation rates, a sediment extract was fed a mixture of benzene, toluene, ethylbenzene, and xylene isomers (BTEX); and the extract then was incubated aerobically with and without protozoan inhibitors. After a 10-day incubation, over 89% of the added BTEX was degraded in incubations with the protozoan inhibitors compared to only 36% degraded in the incubations without protozoan inhibitors. The effect of protozoan predation on anaerobic biodegradation rates was evaluated in macrocosms (1 kg sediment and 600 mL liquid) that were repeatedly fed benzaldehyde over a 1000-hour period. In macrocosms containing the protozoan inhibitor cycloheximide, the number of total anaerobes and iron reducers was higher than in macrocosms that did not receive the inhibitor. However, benzaldehyde degradation rates and protozoan numbers were similar in the macrocosms with and without the protozoan inhibitor. These results suggest that protozoan grazing may limit biodegradation rates in aquifer sediment under aerobic conditions. However, protozoan predation was not significant in the experiments under anaerobic conditions.

To evaluate the effect of ferric iron—Fe(III)—bioavailability on contaminant biodegradation, anaerobic macrocosms were constructed with contaminated aquifer sediment. Repeated benzaldehyde additions initially resulted in an increase in ferrous iron—Fe(II)—and a decrease in Fe(III). However, after approximately 400 hours, total Fe(II) and Fe(III) stabilized, and both ethanol and benzylalcohol began to accumulate, indicating a shift in microbial processes from iron reduction to fermentation. The addition of Fe(III) as goethite, hematite, and amorphous oxyhydroxide to the Fe(III)-depleted sediment stimulated ethanol and benzylalcohol degradation, indicating that Fe(III) bioavailability was limiting contaminant biodegradation. Washing the sediment with CaCl₂ and NaAc to remove sorbed and/or precipitated Fe(II) also stimulated ethanol and benzylalcohol degradation, indicating that Fe(II) was fouling the sediment surface. These results suggest that fouling of the iron oxide surfaces by sorbed and/or precipitated Fe(II) may limit the amount of bioavailable iron, reducing the overall assimilative capacity of shallow aquifers.

key words: anaerobic, biodegradation, protozoa, iron oxides
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SUMMARY AND CONCLUSIONS

SUMMARY

Our current understanding of the processes controlling the natural attenuation of dissolved hydrocarbon plumes is limited. We know that many hydrocarbon plumes do biodegrade under ambient conditions without any active remediation. However, some dissolved gasoline plumes migrate thousands of feet with little attenuation of the most hazardous constituents. In this project, we examined factors potentially controlling the rate and extent of contaminant biodegradation in a gasoline-contaminated aquifer near Rocky Point, N.C. Groundwater at this site contains dissolved benzene, toluene, ethylbenzene, and xylene isomers (BTEX) associated with an underground storage tank release. Previous field and laboratory studies demonstrated that indigenous microorganisms were degrading the dissolved BTEX under ambient, anaerobic conditions. Toluene and o-xylene declined rapidly during transport through the first 100 m of transport. Benzene; m-, p-xylene; and ethylbenzene degraded more slowly in the plume and eventually migrated over 300 m downgradient. Near the source area, oxygen, nitrate, and sulfate are important electron acceptors. However, in most of the plume, ferric iron present in the aquifer sediment as Fe(II):Fe(III) clay mineral (glauconite) is the dominant electron acceptor. Microbial enumerations showed that large numbers of aerobic and anaerobic protozoa and iron-reducing bacteria are present in the contaminated portion of the aquifer. However, the number of iron reducers and protozoa varied greatly.

Two hypotheses were developed to explain the large variations in microbial populations and relatively slow contaminant biotransformation rates in this aquifer.

1. Grazing by aerobic and anaerobic protozoa is reducing the number of bacteria available for contaminant biodegradation and is reducing the overall biotransformation rate.

2. The mineral form of the iron present in the aquifer was limiting Fe(III) availability to the iron-reducing bacteria and was reducing the overall biotransformation rate.

In this project, a series of experiments was conducted to evaluate these two hypotheses and identify factors potentially limiting contaminant biotransformation in the contaminated aquifer at Rocky Point, N.C.

Aerobic Protozoan Grazing. In the first experiment, a sediment extract was incubated aerobically with BTEX and several different protozoan inhibitors to evaluate the significance of protozoan grazing under aerobic conditions. Laboratory incubations containing a sediment extract, and several different protozoan inhibitors were monitored for BTEX biodegradation. Biodegradation was most rapid in those incubations that contained a protozoan inhibitor. This indicates that when protozoan activity is suppressed, biodegradation is enhanced. BTEX biodegradation was most rapid in the cycloheximide treatment (> 90% BTEX biodegraded in 10 days), with somewhat slower degradation in the neutral red treatment. Amphotericin B did not enhance BTEX degradation.
A second set of aerobic incubations was conducted to confirm that bacteria were responsible for the observed BTEX biodegradation and to identify a cycloheximide concentration for use in follow-up anaerobic studies. As in the first experiment, BTEX biodegradation was more rapid in the cycloheximide treatments (95% degraded) than in the live-control incubations that did not receive a protozoan inhibitor (33% BTEX degraded). There was no significant difference in BTEX biodegradation between treatments that received 100 mg/L and 1000 mg/L cycloheximide, indicating that the higher concentration did not inhibit BTEX biodegradation. BTEX biodegradation was more limited in the incubations that received bacterial inhibitors (penicillin and gentamycin) than in the live controls, indicating that the observed BTEX losses were due to bacteria. MPN enumerations confirmed that the inhibitors were effective in controlling protozoan growth and predation.

Anaerobic Protozoan Grazing. A third predation experiment was conducted to evaluate the effect of protozoan grazing on substrate biodegradation under anaerobic, iron-reducing conditions. Two substrates were initially tested: benzaldehyde and toluene. However, there was no evidence of toluene biotransformation over a 1000-hour incubation, and all follow-up experiments were conducted with benzaldehyde. In the anaerobic macrocosms, benzaldehyde was rapidly degraded and microbial populations increased with and without the protozoan inhibitor (cycloheximide). Cumulative benzaldehyde degradation and microbial numbers were not statistically different in bottles with and without the inhibitor, indicating that protozoan grazing was not controlling bacterial populations or substrate degradation in these experiments. In bottles with and without the protozoan inhibitor, there was a two-order-magnitude increase in total anaerobic heterotrophs (10^9 cells/g increased to over 10^5 cells/g) and a three-order-magnitude increase in iron reducers (from 10^1 cells/g to over 10^4 cells/g). Populations in the presence and absence of cycloheximide were not statistically different after 360 hours (p = 0.05).

Iron Bioavailability. Anaerobic macrocosms were repeatedly fed benzaldehyde and monitored for depletion of ferric iron—Fe(III), production of solid- and aqueous-phase ferrous iron—Fe(II), benzaldehyde degradation, and accumulation of degradation intermediates. Ethanol and acetate were present in the sediment and groundwater used to construct the macrocosms and, consequently, were present in all live and control macrocosms. During the first 250 to 300 hours of incubation in the added-carbon macrocosms, a variety of parameters indicated generally more oxidizing conditions with Fe(III) as the dominant electron acceptor: Fe(II) increased, Fe(III) decreased, and ethanol was oxidized to acetate. Throughout the 700-hour incubation, benzaldehyde was transformed to both benzoate and benzylalcohol. However, during the first 250 to 300 hours, the benzylalcohol to benzoate ratio dropped steadily, indicating more oxidizing conditions. At about 250 to 300 hours, the dominant metabolic process shifted from iron reduction to fermentation. This shift was indicated by a gradual increase in the ethanol concentration, a gradual increase in the benzylalcohol to benzoate ratio, and a small decline in the benzaldehyde degradation rate. At approximately 400 hours, Fe(II) and Fe(III) stabilized, indicating that iron reduction had stopped. The increase in ethanol and benzylalcohol production after 300 hours was likely due to the accumulation of hydrogen. During the first 250 to 300 hours, H_2 was consumed via reduction of Fe(III) present on the aquifer solids. However, once Fe(III) reduction stopped, hydrogen began to accumulate causing a shift toward ethanol and benzylalcohol production.
The termination of iron reduction was surprising since a significant fraction of the original Fe(III) was still present in the sediment, suggesting that some other factor might be limiting Fe(III) availability. Three potential hypotheses were developed to explain the apparent shift from Fe(III) reduction to fermentation.

1. Fe(III) availability was not limiting Fe(III) reduction; some other unknown factor was causing this shift.

2. The mineral surfaces were being fouled by sorption and/or precipitation of Fe(II), reducing the bioavailability of the remaining Fe(III).

3. The mineral form of the remaining Fe(III) made it relatively unavailable for use by the sediment-associated microorganisms.

To evaluate hypothesis 1, several different Fe(III) forms (goethite, hematite, and amorphous oxyhydroxide) were added to separate incubations containing the sediment from the initial macrocosms, and the incubations were monitored for depletion of accumulated fermentation products and reduction of Fe(III). Ethanol and benzylalcohol degraded in all the iron-treated microcosms but not in the live-control microcosms without added iron. This indicated that the observed shift from iron reduction to fermentation was due to depletion of bioavailable iron. Ethanol and benzylalcohol concentrations dropped most rapidly in the iron oxyhydroxide- and goethite-treated microcosms. However, both compounds did eventually degrade in the hematite-treated microcosms. Fe(II) concentrations increased in all the iron-treated microcosms, with the largest Fe(II) increase in the iron oxyhydroxide- and goethite-treated microcosms.

To evaluate hypotheses 2 and 3, a portion of the sediment was washed with either CaCl₂ or NaAc to remove ion-exchanged and carbonate-precipitated Fe(II). This sediment was then blended 50:50 with unwashed sediment and used to construct microcosms. Parallel incubations contained nitrilotriacetic acid (NTA) to determine if the addition of this chelator could enhance Fe(III) availability. After approximately 1500 hours of incubation, there was no evidence of enhanced iron reduction in any incubation. At this time, amorphous iron oxyhydroxide was added to one replicate from each treatment. The iron oxyhydroxide addition stimulated iron reduction, resulting in complete degradation of ethanol and benzylalcohol within 600 hours. In microcosms containing CaCl₂- and NaAc-treated sediment that did not receive added iron, ethanol and benzylalcohol degradation started after approximately 2000 hours and iron reduction resumed. The enhanced biodegradation of ethanol and benzylalcohol and increased Fe(II) production in the CaCl₂- and NaAc-washed sediment indicates that iron reduction was limited by the fouling of the mineral surfaces, not because the Fe(III) was in a form that was not available to the iron reducers.

CONCLUSIONS

1. Protozoan grazing can control the maximum rate of contaminant biodegradation under certain conditions. In the aerobic grazing experiments, suppression of protozoan grazing with selective inhibitors substantially reduced the BTEX degradation rate. However, in the anaerobic experiments, protozoan grazing did not have a significant effect on the
benzaldehyde degradation rate. This may be because the benzaldehyde degradation rate was controlled by some other factor, possibly Fe(III) availability.

2. Iron-reducing bacteria from the petroleum-contaminated aquifer at Rocky Point were able to use the more difficult-to-extract crystalline Fe(III) mineral forms for oxidation of benzaldehyde. Use of selective extractants, including 0.5 N HCl and oxalate, will underestimate the amount of Fe(III) that is actually available to subsurface microorganisms. Extraction with 5.0 N HCl for 28 days will likely provide a more accurate estimate of total available iron.

3. Fe(III) may be limited by the fouling of the mineral surfaces with ion-exchanged and/or carbonate-precipitated Fe(II). In our experiments, evidence of Fe(III) limitation included accumulation of reduced fermentation products (ethanol and benzylalcohol) and termination of iron reduction. Addition of several different iron oxides, including hematite, stimulated ethanol and benzylalcohol oxidation via iron reduction.
RECOMMENDATIONS

1. The predation experiments demonstrated that, under certain conditions, protozoan grazing may control the maximum rate of contaminant biodegradation. However, it is not known under what environmental conditions or for what contaminants protozoan predation will be important. Additional research is needed to understand when predation is an important control on the rate and extent of contaminant biodegradation.

2. Our research shows that microorganisms can use the more difficult-to-extract crystalline iron mineral forms. As a consequence, selective chemical extractants such as 0.5N HCl or oxalate will underestimate bioavailable iron. Extraction with 5.0N HCl for 28 days should be used to provide a more accurate estimate of total available iron.

3. A large fraction of the Fe(II) produced during contaminant biodegradation in the subsurface will be present in a ion-exchanged or precipitated form. When attempting to identify the dominant electron acceptor in an environment, solid phase Fe(II) should be included in any electron balance calculations.

4. In our research, Fe(III) bioavailability was limited by the fouling of the mineral surfaces with Fe(II). Additional research is needed to identify conditions when Fe(II) fouling will be important.
1.0 INTRODUCTION

Natural attenuation has been widely adopted for the management of dissolved hydrocarbon plumes, in part, because of the tremendous costs associated with active remediation of thousands of underground storage tank (UST) releases. The U.S. Environmental Protection Agency (USEPA), Office of Research and Development and Office of Solid Waste and Emergency Response, defines natural attenuation as "the biodegradation, dispersion, dilution, sorption, volatilization, and/or chemical and biochemical stabilization of contaminants to effectively reduce contaminant toxicity, mobility, or volume to levels that are protective of human health and the ecosystem" (USEPA 1997).

Our current understanding of the processes controlling the natural attenuation of dissolved hydrocarbon plumes is limited. We know that many hydrocarbon plumes do biodegrade under ambient conditions without any active remediation. Reviews of UST site investigation reports in California and Texas indicate that most dissolved gasoline plumes are stable and do not migrate more than a few hundred feet (Rice et al. 1995; Mace et al. 1997). However, there are reports of some dissolved gasoline plumes migrating thousands of feet with little attenuation of the most hazardous constituents (Thierrin et al. 1995; Weaver et al. 1996).

The overall objective of this research is to improve our understanding of processes controlling the anaerobic bioattenuation of dissolved hydrocarbons in groundwater. In this project, we have focused on a dissolved gasoline plume in the Arvida Subdivision located near Rocky Point, N.C. Laboratory studies conducted with aquifer material from this site have shown that the indigenous microorganisms can degrade benzene, toluene, ethylbenzene, and xylene isomers (BTEX) under ambient, anaerobic conditions (Kota et al. 1997). Field monitoring has shown that the dissolved hydrocarbon plume is biodegrading using dissolved sulfate and iron oxides present in the aquifer sediment as the primary electron acceptors. However, natural attenuation processes at this site are not sufficient to control the migration of the plume, and high concentrations of benzene and other aromatic hydrocarbons have migrated over 1300 feet before they discharge to a small drainage channel.

In this study, we examined the effect of protozoan grazing and iron bioavailability on the rate of contaminant biodegradation in this aquifer. Specific objectives of this research include:

- Determining if protozoan grazing has a significant impact on the biodegradation rates in laboratory macrocosms under aerobic and iron reducing conditions.
- Evaluating factors potentially limiting the amount of Fe(III) available for microbial reduction.

The experimental design, methods, and results performed for each objective are presented in separate sections.
2.0 RESEARCH SITE BACKGROUND

2.1 SITE CHARACTERISTICS

The site examined in this study was a petroleum-contaminated Coastal Plain aquifer near Rocky Point, N.C. The groundwater throughout the study area is contaminated by BTEX, which originated from a UST release. The contaminant distribution immediately downgradient of the source was characterized in an 18-month groundwater sampling investigation, which indicated that dissolved hydrocarbons are being anaerobically degraded under mixed sulfate and iron-reducing conditions (Borden et al. 1995). No evidence for significant methanogenic activity was detected in the contaminant plume. Toluene and o-xylene decline rapidly during transport through the first 100 m of the 300-m-long contaminant plume after which these compounds remain relatively constant at low but detectable levels. Benzene and m-, p-xylene decline more slowly in the plume. The contaminant plume is characterized by the depletion of dissolved oxygen, nitrate, and sulfate; a negative redox potential; and elevated concentrations of dissolved carbon dioxide and iron.

The primary aquifer at the site consists of dark gray and green micaceous fine sand overlain by 1.5 to 4.5 m of silts, clays, and clayey sands that form a leaky surface-confining layer throughout the site. The sand is homogeneous with few exceptions and is composed of over 90% quartz sand with minor amounts of pyrite and muscovite flakes in a glauconitic-clay matrix. Some clay lenses are present in the sand aquifer. However, these lenses cannot be traced between closely spaced borings, indicating that they are of limited lateral extent. A more complete description of the site hydrogeology, geochemistry, and contaminant distribution is provided by Kota et al. (1997).

2.2 PREVIOUS EXPERIMENTS

Kota et al. (1997) conducted a series of laboratory and field experiments to (1) document that anaerobic BTEX biodegradation was occurring in the aquifer, (2) estimate the BTEX biodegradation rate under ambient conditions, and (3) study the microbial ecology of the contaminant plume. The biodegradation rates of BTEX were measured in anaerobic microcosms constructed with aquifer sediment recovered from three locations in the contaminant plume (source, mid-plume, and end-plume). Microcosms were designed to simulate ambient conditions to the maximum extent possible. In the source-area microcosms, none of the BTEX components degraded after 388 days of incubation. In the mid-plume microcosms, m-xylene biodegradation began with no lag, followed by toluene, o-xylene, and benzene. By day 140, both toluene and o-xylene had degraded to between 0.03 and 0.10 μM and then remained constant for the duration of the experiment (> 99% removal). Benzene degradation began after day 180, and by day 403 the benzene concentration was between 0.10 and 0.15 μM in each replicate (> 99% removal). First-order biodegradation rates ranged from 0.045 day\(^{-1}\) for toluene to 0.002 day\(^{-1}\) for ethylbenzene. Biodegradation was erratic in the end-plume microcosms. Some microcosms exhibited clear evidence of biodegradation while others did not.

In situ biodegradation rates were measured by using in situ test columns. Two experiments were conducted, each using two live columns and one abiotic column. In the first experiment, the columns were loaded with groundwater from one of the monitoring wells where the water
contained dissolved hydrocarbons at ambient concentrations. In the second experiment, columns were loaded with anaerobic groundwater spiked with benzene. In the first experiment, m-, p-xylene decreased by over 90%, benzene decreased by 50%, and pseudocumene decreased by 75 to 90%. There was no evidence of toluene, o-xylene, ethylbenzene, or mesitylene biodegradation in either live column. The absence of measurable toluene and o-xylene biodegradation was likely due to the low initial concentration of these compounds (< 50 µg/L). In the second experiment, benzene biodegradation began after a 41-day lag period, and by day 181 benzene had declined from over 1,000 µg/L to 180 µg/L in column 1 and 18 µg/L in column 2.

Two experiments were conducted to study the distribution of microorganisms in the contaminated aquifer. Enumerated populations included total microbes by acridine orange direct count, aerobic and anaerobic protozoa, iron reducers, and sulfate reducers. The objective of the first experiment was to evaluate spatial heterogeneity in closely spaced aquifer sediment samples from contaminated and pristine sections of the aquifer. In the second experiment, population distribution was measured in contaminated sediment samples spaced 0.3 m apart.

While there was little variability in microbial populations in closely spaced samples, there was significant variability in samples that were 0.3 m apart. Microbial populations were higher in the contaminated region than in the pristine region. In both experiments, the total bacterial population was on the order of $10^6$ per gram sediment, and no sulfate reducers were detected in any of the samples. The highest numbers of aerobic and anaerobic protozoa were 198 and 105 protozoa per gram of sediment, respectively. Iron reducers varied between $10^1$ and $10^5$ per gram sediment.

2.3 SUMMARY

Recent work at the Rocky Point site and the work of others have shown that dissolved hydrocarbons released from leaking USTs can and do biodegrade under ambient, anaerobic conditions. However, the major factors controlling the rate and extent of biodegradation are still poorly understood. We cannot estimate the rate of biodegradation from laboratory data or reliably predict the extent of biodegradation with even the most sophisticated mathematical models. For example, biodegradation rates at the Rocky Point site were measured using laboratory microcosms, in situ columns, and groundwater monitoring data; and the results are summarized in Table 1. As presented in this table, the match between the laboratory and field data is not good despite intensive efforts to simulate ambient field conditions in the laboratory microcosms. If the laboratory measured rates were representative of the field, then the plume would have degraded within a few hundred feet, yet the actual plume is over 1300 feet long. Given the type of data in Table 1, it is not possible to reliably predict the extent of contaminant migration, which is essential for the assessment of risk associated with a particular plume.
To reliably use intrinsic bioremediation for hydrocarbon plume management, techniques are required to measure a biodegradation rate that is applicable in the field. Development of such techniques requires a more thorough characterization of the factors that control biodegradation rates. In sections 3 and 4, we describe studies examining the effect of protozoan grazing and iron bioavailability on the rate of organic contaminant biodegradation.

Table 1. First-Order Biodegradation Rates (% per day)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Laboratory Microcosm</th>
<th>In situ Column</th>
<th>Field Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>2.4</td>
<td>0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Toluene</td>
<td>4.5</td>
<td>1.2</td>
<td>0.21</td>
</tr>
<tr>
<td>m-Xylene</td>
<td>2.0</td>
<td>1.4</td>
<td>0.13</td>
</tr>
<tr>
<td>o-Xylene</td>
<td>5.6</td>
<td>not measured</td>
<td>0.21</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>0.2</td>
<td>not measured</td>
<td>0.15</td>
</tr>
</tbody>
</table>
3.0 PROTOZOAN GRAZING UNDER AEROBIC AND ANAEROBIC CONDITIONS

Groundwater aquifers have been contaminated with a wide variety of hazardous organic chemicals as a result of both primitive waste disposal practices and accidental releases (Myers et al. 1994; Yang et al. 1995). Common contaminants include petroleum hydrocarbons, chlorinated solvents, ketones, and ethers. With the input of carbon into aquifers, the oxygen in the aquifer is typically depleted and anaerobic conditions often prevail. It has been demonstrated that the anaerobic degradation of organic contaminants occurs through microbially mediated reaction pathways including nitrate reduction (Kuhn et al. 1988; Hutchins et al. 1991), iron reduction (Lovley et al. 1989; Lovley and Lonergan 1990), sulfate reduction (Edwards and Grbic-Galic 1992; Haag et al. 1991), and methanogenesis (Wilson et al. 1986; Grbic-Galic and Vogel 1987). Biodegradation of organic contaminants has been reported, including benzene (Grbic-Galic and Vogel 1987; Wilson et al. 1986; Major et al. 1988; Cozzarelli et al. 1990; Vogel and Grbic-Galic 1986), chlorinated solvents (Major et al. 1991; McCarty 1994; McCarty and Wilson 1992, Wilson et al. 1994), and ketones under representative subsurface conditions. Thus, microbial processes play a significant role in the regulation of contaminant fate and transport in contaminated aquifers.

One strategy for the remediation of contaminated aquifers is natural attenuation, in which the activity of the indigenous microbial population is sufficient to control contaminant migration and ultimately to consume the contaminants. One factor limiting consideration of natural attenuation in remediation programs is uncertainty regarding the rate of contaminant biodegradation. Biodegradation rates calculated from field data vary widely, with values of 0.2 to 1% per day reported in a recent review (Rifai et al. 1995). For natural attenuation to be considered in the development of remediation programs for contaminated aquifers, a more fundamental understanding of factors controlling biodegradation is needed.

Protozoan grazing is one factor controlling the abundance of bacteria in freshwater and marine environments (Barcina et al. 1991; Sherr and Sherr 1987; Sherr et al. 1989; Sherr et al. 1988) and wastewater treatment plants (Curds et al. 1968; Wheale and Williamson 1980). Protozoan grazing on bacterial populations decreased p-nitrophenol biodegradation in lake water (Zaidi et al. 1989; Ramadan et al. 1990). Recent studies have shown that protozoa are common in both shallow (Beloin et al. 1988; Hirsch and Rades-Rohkohl 1983; Sinclair and Ghiorse 1987; Sinclair et al. 1990) and deep (over 200 m below surface) aquifers (Sinclair and Ghiorse 1989) and elevated protozoan populations have been detected in contaminated aquifers. This suggests that protozoan populations increased because they grazed on bacterial populations that were stimulated by an influx of carbon (Sinclair et al. 1993; Kinner et al. 1992; Thomas et al. 1997).

The objective of this study was to evaluate the influence of protozoan grazing on contaminant biodegradation rates in a gasoline-contaminated aquifer (Rocky Point, N.C.) undergoing intrinsic bioremediation.

3.1 EXPERIMENTAL DESIGN

Experiments were conducted to evaluate the influence of protozoan grazing under both aerobic and anaerobic conditions. The objective of the first experiment was to evaluate the significance of protozoan grazing under aerobic conditions and to evaluate a number of protozoan inhibitors that
could be used for experiments under anaerobic conditions. Laboratory microcosms (20-mL test tubes) were constructed with an extract of sediment known to contain protozoa as inoculum. Three protozoan inhibitors (cycloheximide, neutral red, and amphotericin B) were tested in duplicate over a range of concentrations. The inhibitor concentrations were selected based on the results of previous studies of protozoan grazing under aerobic conditions in aqueous systems. Inhibitor concentrations were 50, 100, 500, 1000, and 2500 mg/L for cycloheximide; 10, 50, and 100 mg/L for neutral red; and 10, 50, and 100 mg/L for amphotericin B. Cycloheximide inhibits 80S ribosomal protein synthesis, neutral red stimulates autocystosis and inhibits endocystosis, and amphotericin B is a polyene antifungal agent, which apparently binds to sterols and damages the osmotic barrier of the plasma membrane of eukaryotes (Tylor and Pace 1987; Sanders and Porter 1986).

In a subsequent experiment, tests were conducted with cycloheximide, which appeared to be the most effective inhibitor in the initial work. Treatments included triplicate live, abiotic (heat killed), live + protozoan inhibitor (cycloheximide at 100 and 1000 mg/L), and live + bacterial inhibitors (penicillin and gentamycin at 500 mg/L each) microcosms. In all experiments, effects were evaluated based on BTEX biodegradation, and the initial substrate concentration was 2 mg/L of benzene, toluene, ethylbenzene, o-xylene, and m-xylene. All sediment was collected from a contaminated region of the Rocky Point aquifer.

Tests under anaerobic conditions were conducted in 1-L bottles (macrocosms) that contained contaminated aquifer sediment. Treatments consisted of benzaldehyde or toluene as the carbon source, benzaldehyde + cycloheximide (1000 mg/L), a live control to which no carbon was added, and an abiotic control. Substrate biodegradation rates and both protozoan and bacterial populations were monitored over the course of the experiment. Benzaldehyde was selected as a rapidly degradable substrate that would amplify the rate and magnitude of population shifts relative to toluene. Its biodegradation in this aquifer sediment was verified in preliminary work. In addition, benzaldehyde is a suspected intermediate in toluene metabolism under iron-reducing conditions (Lovley and Lonergan 1990). In the toluene-treated macrocosm, protozoan and bacterial populations were compared with the behavior of the live control.

3.2 EXPERIMENTAL METHODS

3.2.1 Sediment and Groundwater Collection. Aquifer sediment from the contaminated region was obtained from a position approximately halfway between the source area and the end of the plume. Sediment samples for the aerobic and anaerobic experiments were obtained at depths from 2.13 to 2.74 m and 3.51 to 4.12 m, respectively. All sediment was obtained under anaerobic conditions by drilling below the water table with a hollow stem auger and then advancing a sterile coring tube. The tube was brought to the surface, immediately capped with sterile butyl rubber stoppers, and transported to the laboratory on ice where it was extruded in an anaerobic glove box (Ray Products, El Monte, Calif.) under N2 within 12 hours. The first and last 10 cm of the core were removed and the outer portions of soil pared away. The remaining sediment was anaerobically transferred into sterile mason jars and stored at 4°C. Sediment samples were homogenized in the anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) prior to use.
Groundwater was collected anaerobically from the same location where the sediment was collected. The well headspace was sparged with argon for 10 minutes before and during sample collection. Groundwater was pumped from the well through a closed system of polyethylene tubing equipped with a 0.45-µm filter (Gelman Sciences, Ann Arbor, Mich.) and was collected in a nitrogen-sparged 2.3-L (80-ounce) bottle. The water was transported to the laboratory on ice where it was stored at 4°C prior to use. All equipment and containers in contact with the water and sediment were presterilized.

3.2.2 Microcosm Construction. Microcosms were constructed in sterile 20-mL pressure tubes (Belco Biotechnology, Vineland, N.J.) that contained 15 mL of a sediment extract. The extract was formed by shaking 100 g of wet sediment in 1 L of sterile deionized water. The tubes were capped with sterile black butyl rubber stoppers and crimped with an aluminum seal. Abiotic treatments were constructed by autoclaving (121°C, 1 hour). All tubes were spiked with solutions containing the appropriate inhibitor and BTEX and were incubated at 25°C.

3.2.3 Macrocosm Construction. Macrocosms were constructed in 1-L bottles containing blended aquifer sediment (1000 g) and groundwater (600 mL) and capped with 3-cm-thick rubber stoppers. Benzaldehyde was repeatedly added to the two live treatments, while toluene was added once at 10 mg/L. The abiotic control was constructed by autoclaving for 1 hour on two consecutive days and adding HgCl₂ (250 mg/L). The abiotic (killed) control received toluene (10 mg/L), benzaldehyde (25 mg/L), and cycloheximide (1000 mg/L). All macrocosms were constructed in an anaerobic chamber using aseptic techniques. They were also incubated in the chamber at 25°C. Aqueous samples from the macrocosms were withdrawn at various time intervals and analyzed for benzaldehyde, toluene, cycloheximide, Fe(II), and CH₄ (aqueous). Duplicate sediment samples for enumeration of anaerobic protozoa, total heterotrophs, and iron reducers were removed from a macrocosm using a sterile, hollow glass rod.

3.2.4 Inhibitor Preparation. Stock solutions of eukaryote inhibitors were prepared in distilled water at concentrations of 10 g/L and refrigerated. Prior to use, cycloheximide was warmed to 60°C to increase its solubility. Required volumes of the stock solutions were filter-sterilized through 0.2-µm filters prior to use.

3.2.5 Microbial Population Enumeration. Microbial populations were enumerated for total heterotrophs, iron reducers, and aerobic and anaerobic protozoa by using most probable number (MPN) techniques. Although MPN techniques have inherent limitations in the fraction of the population measured (Foissner 1987), MPN values were used in this study as a relative measure to evaluate spatial and temporal differences in population. Inocula were prepared by shaking 10 g of sediment in 90 mL of sterile groundwater. Inocula were then diluted in sterile groundwater for MPN assays. MPNs were calculated by using a computer program (Russek and Colwell 1983).

The populations of aerobic and anaerobic protozoa were enumerated by using the five-well MPN procedure (Sinclair and Ghiorse 1987; Sinclair et al. 1993). The procedure is based on the addition of an inoculum to a glass ring immobilized with agar in a petri dish. Enterobacter aerogenes is grown separately on trypticase soy agar and added to each cylinder as a food source for protozoa. One mL of the inoculum was added to five replicate rings at each dilution. For anaerobic protozoa, petri dishes were incubated in the anaerobic chamber at 25°C. In most cases,
up to 10 days of incubation was necessary before protozoa could be observed, and each ring was checked by microscope for two months before they were counted as negative. For aerobic tests, samples were counted as negative if no protozoa were observed after 1 month. Encysted protozoa were enumerated by treating a separate subsample of the initial sediment extract with 10 mL of 0.55 N HCl for 15 minutes to kill vegetative protozoa. A greater MPN in the untreated sample relative to the treated sample indicated the presence of vegetative protozoa.

Total heterotrophs were enumerated by five-tube MPN tests. The medium was modified from Qian and Barlaz (1996) and consisted of the following (grams per liter): glucose (10), yeast extract (1), tryptase peptones (2), KH$_2$PO$_4$ (1.61), Na$_2$HPO$_4$ . 7H$_2$O (3.18), NH$_4$Cl (1), NaCl (0.9), MgCl$_2$ . 6H$_2$O (0.2), CaCl$_2$ . 2H$_2$O (0.1), NaHCO$_3$ (3.5), cysteine hydrochloride (0.5). In addition the medium contained trace minerals (Kenealy and Zeikus 1981) with the addition of Na$_2$WO$_4$ (0.033) and vitamins (Wolin et al. 1963). Resazurin (2-mL, 0.1 %) was added to the medium and the pH was adjusted to 6.6. The medium was boiled under N$_2$ that had been passed through a heated copper column to remove traces of oxygen. Nine mL of medium was dispensed in anaerobic pressure tubes, which were then sealed with black butyl rubber stoppers and aluminum crimps and autoclaved (121°C, 20 minutes). Tubes were scored positive based on elevated optical density after 1 month.

Iron reducers were enumerated using a ten-tube MPN assay with medium selective for Fe(III) reducers (Lovley and Phillips 1986b). The medium was sparged with N$_2$ to remove dissolved oxygen and the pH was adjusted to 7. Finally, 9 mL were dispensed into anaerobic pressure tubes, sealed with black butyl rubber stoppers and aluminum crimps, and autoclaved (121°C, 20 minutes). Tubes were incubated for 2 months, after which Fe(II)$_{aq}$ was measured in each tube. Tubes were considered positive if Fe(II)$_{aq}$ exceeded that in uninoculated controls at the 99% confidence interval. A separate set of three controls was analyzed at each dilution. For Fe(II) analysis, a 0.5-mL sample was digested in 5 mL of 0.75 N HCl (anaerobically). After 3 hours when the liquid was clear, tubes were removed from the anaerobic hood and 5.5 mL of phosphate buffer (1 12.7 g KH$_2$PO$_4$/L and 144.9 K$_2$HPO$_4$/L) containing phenanthroline (1 powder pillow/25 mL) was added. A$_{510}$ was then read after 15 minutes and compared to a standard curve.

3.2.6 Inhibitor Effects on Protozoa. To verify the effectiveness of cycloheximide as a protozoan inhibitor, replicate dilutions of the sediment extract were incubated in glass rings immobilized in petri dishes as for the MPN procedure. The survival of protozoa was compared in the presence and absence of 100 and 1000 mg cycloheximide/L. Survival was assessed by observation of swimming using a microscope. Since it cannot be assumed that swimming implies feeding, growth, or reproduction; protozoan suspensions were monitored for several days for survivors, which implies feeding. Liquid samples from the petri dish were observed for protozoa after 1, 7, and 10 days. MPNs were calculated for the active protozoa after 10 days.

3.2.7 Monitoring and Analytical Methods. Microcosms were analyzed for BTEX by first removing approximately 0.2 mL of free liquid in a gas-tight syringe. The sample was diluted to 5 mL and analyzed for BTEX using a Tekmar Purge-and-Trap Model LSC 2000 and a Perkin Elmer autosystem gas chromatograph equipped with a 75-m DB-624 megabore capillary column (J & W Scientific, Folsom, Calif.) and flame ionization detector. The injector temperature was maintained at 250°C. The oven was initially set at 40°C for 2 minutes, increased at 20°C per
minute to 90°C and then increased at 20°C per minute to a final temperature of 140°C and held for 5 minutes. The detection limit for BTEX compounds was 1 μg/L.

To analyze macrocosms for toluene, benzaldehyde, and cycloheximide, 2 mL of free liquid was removed using a gas-tight syringe while puncturing the stopper with a needle so a vacuum did not develop. Sampling was conducted in the anaerobic chamber and sample volume was replaced with sterile groundwater. One μL of liquid from the target vial was analyzed by direct injection to the gas chromatograph described above. The injector temperature was maintained at 250°C. The oven was initially at 40°C for 2.5 minutes, increased at 10°C per minute to 75°C, and then increased at 20°C per minute to a final temperature of 200°C and held for 5 minutes. The detection limits for toluene, benzaldehyde, and cycloheximide were 0.7, 1, and 10 mg/L, respectively.

3.3 RESULTS

3.3.1 Predator-Prey Relationships. In this section, microbial population data (Kota et al. 1997) are used to evaluate whether protozoan-bacteria ratios are consistent with the potential for protozoan gazing to be important. Theoretical models of steady-state phagotrophic food chains suggest that there is a characteristic ratio between the biomass of predators and their prey (Kerr 1974; Platt and Denman 1977). The value of this ratio is derived from the ratios of individual predator and prey sizes and the size-dependent rates of metabolism and growth, making this ratio a function of growth efficiencies. Specifically, the ratio between predator and prey biomass is proportional to the gross growth efficiency of the predator (i.e., yield = assimilated C/consumed C).

Predator-prey biomass ratios were calculated for contaminated and pristine samples. The average total bacterial population was about 2 x 10^6 cells per gram of sediment (Kota et al. 1997). Assuming the biovolume of a bacterium to be 0.58 μm^3 (Massana and Pedros-Alio 1994) and a biovolume-to-biomass conversion factor of 0.35 pg/μm^3 at 50% carbon/dry wt. (Bjornsen 1986), the total bacterial biomass is 4.06 x 10^4 mg/g sediment. Foissner (1987) measured the density and biomass of wheat fields/meadows that were conventionally farmed and found the density and biomass of testacea (protozoa) to be 751 protozoa/g-dry wt. soil and 0.0279 mg cells/g dry wt. soil, respectively. Assuming aerobic and anaerobic protozoa have the same biomass as testacea, predator-prey biomass ratios for all samples were calculated (Figure 1).

To assess the potential significance of protozoan grazing, the ratios shown in Figure 1 are compared to ratios reported in the literature. In (aerobic) plankton food chains with a fairly simple community size structure, theory predicts a predator-prey biomass ratio of slightly less than unity (Kerr 1974; Platt and Denman 1977). Fenchel and Finlay (1990) calculated the biomass ratios between protozoa and their food organisms in water bodies with anoxic bottom waters and calculated the ratio to be about 0.24 (standard deviation—S.D. = 0.07 and range = 0.14 to 0.36). The lower predator-prey ratio in the anaerobic system is likely due to the lower growth efficiency of anaerobic metabolism relative to aerobic metabolism.
For samples (A to F) only aerobic protozoan biomass was taken into account because aerobic protozoa were dominant. Calculations indicate that samples A, B, and C have a predator-prey biomass ratio well above 1.0, the critical ratio presented above. Similarly, for Samples (I to VI) only anaerobic protozoan biomass was taken into account because anaerobes were dominant. Samples II and V have a predator-prey biomass typical of other anaerobic environments (Fenchel and Finlay 1990). However, Samples III and IV have a predator-prey ratio that is 20 to 40 times higher than what has been typically measured which indicates the potential significance of protozoan grazing.

3.3.2 Aerobic Experiment. The effects of amphotericin, neutral red, and cycloheximide on aerobic BTEX biodegradation are presented in Figure 2. The reported BTEX losses can be attributed to biological effects as the data have been corrected for abiotic losses. While BTEX biodegradation was measured in all live treatments, biodegradation was more complete in treatments that received protozoan inhibitors relative to the live treatment. These data indicate that when protozoan activity is suppressed, biodegradation is enhanced, presumably due to removal of one regulator of bacterial growth. Similar observations were reported for p-nitrophenol biodegradation in lake water (Zaidi et al. 1989; Ramadan et al. 1990).

Cycloheximide was the most effective treatment because greater than 90% of added BTEX biodegraded compared to the live treatment in which only 50% degraded. The slight decrease in BTEX biodegradation (86%) at the highest cycloheximide concentration may indicate some inhibition of bacteria. t-Tests showed that BTEX biodegradation at 2500 mg/L was different from 100, 500, and 1000 mg/L of cycloheximide (\( \alpha = 0.05 \)). Neutral red was also effective; and based on the trend in BTEX biodegradation, concentrations greater than 100 mg/L may have further enhanced biodegradation. Amphotericin B did not appear to enhance BTEX degradation (Figure 2).
A second experiment was conducted to reconfirm the effects of cycloheximide and to compare the effect of cycloheximide with that of bacterial inhibitors (penicillin and gentamycin). BTEX depletion after correction for abiotic losses is presented in Figure 3. As above, treatments that received cycloheximide degraded greater than 95% of the added BTEX, while live microcosms degraded only 33%. Assuming that BTEX biodegradation occurred as a result of bacterial metabolism, the addition of bacterial inhibitors should reduce BTEX biodegradation. As presented in Figure 3, both penicillin and gentamycin appear to have inhibited BTEX biodegradation. t-Tests \( (p = 0.05) \) showed that penicillin and gentamycin were different from live controls (one outlier omitted in live controls). Thus, the measured BTEX losses can be attributed to bacteria. The BTEX degradation that did occur in the bacterial inhibitor treatments (penicillin and gentamycin) can be attributed to either the slow response of the inhibitor, antibiotic-resistant bacteria, or an insufficient dose of the bacterial inhibitor.

The sediment extract used for the microcosms was enumerated for protozoa in the presence and absence of 100 and 1000 mg cycloheximide/L. After 1 day, most of the amoebae and flagellates did not appear in tests at both 100 and 1000 mg/L. In contrast, amoebae and flagellates were observed in the cycloheximide-free tests. After 7 days, no protozoa were observed to be swimming and the numbers were significantly lower than the cycloheximide-free control. MPNs calculated after 10 days for treatments that received cycloheximide were 1 and 0.44 cells/g of sediment at 100 and 1000 mg/L, respectively, while the population was 109 cells/g of sediment in the control. Similar observations were reported by Acea and Alexander (1988) when cycloheximide was added to glucose-amended soil. Cycloheximide caused a decline in the number of protozoa and a rise in the number of protozoan cysts (Acea and Alexander 1988).
Figure 3. Effect of cycloheximide and bacterial inhibitors (penicillin and gentamycin) on aerobic BTEX biodegradation rates.

All treatments have been corrected for abiotic losses. The error bars indicate ± 1 standard deviation.

The use of a eukaryote inhibitor for grazing experiments is predicated on three assumptions (Tremaine and Mills 1987). First, the target heterotrophic eukaryotes are inhibited; second, the nontarget microorganisms (autotrophic bacteria and heterotrophic bacteria) are not inhibited; and third, bacterial growth rates are not stimulated either directly by use of the inhibitor as a substrate or indirectly by the inhibitor lysing cells and thereby increasing bacterial substrate concentrations. The first assumption is accurate, as the MPNs in the presence and absence of cycloheximide show that it was an effective protozoan inhibitor. The second assumption is also accurate, as cycloheximide did not inhibit bacterial activity at concentrations up to 1000 mg/L given the increased BTEX biodegradation in its presence relative to cycloheximide-free controls. While cycloheximide could have stimulated overall bacterial growth by serving as a carbon source or by lysing cells (third assumption), it seems unlikely that this would cause a significant increase in the BTEX degradation rate. If BTEX degraders grow on cycloheximide, then cycloheximide concentration would be expected to have a greater effect on BTEX degradation. In fact, there was little effect of cycloheximide concentration on the extent of BTEX biodegradation (Figures 2 and 3).

In summary, the enhancement of BTEX biodegradation in the presence of a protozoan inhibitor and the inhibition of degradation in the presence of bacterial inhibitors both suggest the potential for protozoan grazing to reduce BTEX biodegradation.

3.3.3 Anaerobic Experiment. Benzaldehyde was rapidly consumed under anaerobic conditions in both live macrocosms (benzaldehyde only and benzaldehyde + cycloheximide). Benzaldehyde
was repeatedly fed to the macrocosms for over 1000 hours (Figure 4). Depletion of benzaldehyde in the live macrocosms relative to the abiotic (data not shown), suggested that bacterial populations were utilizing benzaldehyde for growth.

Figure 4. Benzaldehyde concentrations and cumulative benzaldehyde degradation in the anaerobic macrocosms.

Microbial populations (total heterotrophs, iron reducers, and anaerobic protozoa) were enumerated at various time points during the first 360 hours. Figure 5 shows the variations in microbial populations over the course of the incubation. Error bars are 95% confidence intervals from the MPN enumeration. In both treatments, total heterotrophs increased from $10^3$ cells per gram sediment to greater than $10^5$ cells per gram sediment. Similarly, iron reducers increased from $10^1$ to greater than $10^4$ cells per gram of sediment. Populations in the presence and absence of cycloheximide were not statistically different after 360 hours ($p = 0.05$). The increase in iron reducers corresponded with increases in Fe(II)$_{aq}$ concentrations in both benzaldehyde and benzaldehyde + cycloheximide treatments (Figure 6). Protozoan populations in the benzaldehyde-only treatment fluctuated around 20 cells per gram of sediment (Figure 5), while no protozoa were measured in the benzaldehyde + cycloheximide treatment.
Figure 5. Microbial populations in benzaldehyde-only and benzaldehyde + protozoan inhibitor treatment.

Figure 6. Aqueous Fe(II) measurements in the macrocosms, anaerobic experiment.
It was hypothesized that the presence of a protozoan inhibitor would stimulate bacterial populations (total heterotrophs and Fe-reducers); however, this did not occur. Thus, in the anaerobic macrocosms, protozoa did not control bacterial populations. The absence of a predation effect is also evident in the benzaldehyde uptake results (Figure 4). The amount of benzaldehyde consumed at the end of 360 hours in benzaldehyde-only and benzaldehyde + cycloheximide macrocosms was 57 and 62 mg, respectively. Continuous respiking of benzaldehyde in these two treatments over a period of 1000 hours did not show a significant difference in benzaldehyde uptake.

In the macrocosm containing toluene, no evidence of toluene degradation was observed after 1000 hours. Therefore, no microbial measurements were made. The absence of toluene degradation may be due to the presence of other easily degradable carbon sources present in the system because sediment from the same location has been shown to degrade toluene in previous microcosm (Hunt et al. 1998) and in situ column (Hunt et al. 1997) studies. The presence of other degradable carbon is apparent from the production of a similar amount of Fe(II) in the macrocosms with added toluene and no added carbon (Figure 6).

Cycloheximide concentrations in benzaldehyde + cycloheximide and abiotic control treatments decreased during the course of the experiment (data not shown), presumably due to sorption to the sediment. However, no protozoa were detected in the benzaldehyde + cycloheximide treatment, and the cycloheximide concentration was still greater than 500 mg/L after 360 hours, indicating that the cycloheximide treatment was effective in controlling protozoa. Finally, no inhibition of bacterial populations was observed as evidenced by the similarity between treatments with and without benzaldehyde, suggesting that cycloheximide was an effective protozoan inhibitor for this system.

3.4 DISCUSSION

Aerobic microcosm experiments indicated that the presence of protozoa resulted in decreased biodegradation rates. Given previous reports on the presence of protozoa in contaminated aquifers, it seems plausible that protozoan grazing may be a regulator of in situ biodegradation rates. Sinclair et al. (1993) reported elevated protozoan populations in a section of an aquifer amended with H₂O₂ relative to an untreated area. Similarly, elevated numbers of protozoa were observed in a sewage-contaminated aquifer (Kinner et al. 1992).

Anaerobic protozoa were present in the aquifer sediment tested (Kota et al. 1997). This is consistent with previous reports by Thomas et al. (1997) that both aerobic and anaerobic protozoa were detected in a fuel-contaminated aquifer and indicates that protozoa may be active under a wide variety of electron acceptor conditions (Thomas et al. 1997). However, in the anaerobic macrocosm incubations, protozoan numbers did not increase. The absence of protozoan growth is surprising given the two-order magnitude increase in total heterotrophs and three-order magnitude increase in iron reducers. The absence of protozoan growth could be due to a build up of inhibitory degradation products or some other limiting factor.

Protozoan grazing did not adversely affect the rate of anaerobic benzaldehyde biodegradation in the anaerobic macrocosms. The absence of a grazing effect may be related to the rapid
degradability of benzaldehyde. Because it degraded rapidly, microbial growth may have been sufficiently rapid to negate any adverse effects of grazing. In a study to determine the role of anaerobic ciliates on bacteria in an anaerobic lake waters, less than 0.1% of bacterial biomass was consumed per day (Massana and Pedros-Alio 1994). Low growth efficiency of anaerobic protozoa was suggested to be the most probable reason. Review of available field studies by Massana and Pedros-Alio (1994) further suggested that this conclusion could be extrapolated to most other anoxic systems. The study also concluded that bacterial assemblages seem to be controlled by bottom-up mechanisms; they can apparently grow to their carrying capacity, and the large biomass attained is maintained by low growth and low predation losses.

The absence of an effect due to benzaldehyde may not necessarily be extended to toluene. The absence of toluene biodegradation in the macrocosms for over 900 hours illustrates the relatively slow growth of toluene-degrading microorganisms. The slower growth rate would make them more susceptible to protozoan grazing. This, plus reports of both aerobic and anaerobic protozoa in a fuel-contaminated aquifer (Thomas et al. 1997), indicate that protozoa may be active under a variety of electron-accepting conditions. Further studies of the importance of protozoan-grazing in fuel-contaminated aquifers undergoing anaerobic BTEX biodegradation are required to determine whether protozoa are a significant regulator of in situ biodegradation rates.
4.0 BIOAVAILABILITY OF IRON IN CONTAMINATED AQUIFER SEDIMENT

Fe(III) reduction is an important electron sink in the oxidation of organic contaminants in a wide variety of sedimentary environments (Lovley 1995a; Lovley 1995b; Cook 1984; Crosby et al. 1983; Jones et al. 1983; Jones et al. 1984; Lovley and Phillips 1986a). Fe(III) is an abundant, if not the most abundant, potential electron acceptor in many soils, aquatic sediments, and aquifers (Lovley 1991). However, most of the Fe(III) present in these environments is thought to be unavailable for microbial reduction (Lovley 1987).

There are a multitude of Fe(III)-oxide minerals that vary widely in their degree of crystallinity, particle size, available surface area, reactivity, and oxidation state (Schwertmann 1991; Bromfield and David 1978; Burns and Burns 1981; Eirlich et al. 1973; Jones and Browser 1978; Ponnampereuma et al. 1967; Langmuir and Whittemore 1971). Rates of Fe(III) reductive dissolution appear to be inversely proportional to the degree of crystallinity (hematite < goethite < ferric hydroxide) (Munch and Ottow 1980; Jones et al. 1983; Lovley and Phillips 1986a; Lovley and Phillips 1986b). The greater bioavailability of the less crystalline forms may be due to their greater surface area. Arnold et al. (1988) and Roden and Zachara (1996) have shown that differences in the reduction rates of different Fe(III) oxides can be related to differences in the mineral surface area.

Most previous attempts to estimate the amount of bioavailable iron in aquifer sediments were based on the use of selective extractions to determine the most chemically reactive fraction. These included 0.5 N HCl extraction for 1 hour and 24 hours (Lovley and Phillips 1987b; Heron et al. 1994a); oxalate extraction for 4 hours (Lovley and Phillips 1986b; Phillips and Lovley 1987); and Ti(III)-EDTA extraction (Heron et al. 1994b). Heron et al. (1994a) found that 0.5 N HCl extraction for 24 hours dissolves only ferrihydrite and partly akageneite and concluded that this technique may be used as a nonquantitative indicator for the presence of poorly crystalline Fe(III) in sediment. Ammonium oxalate has been used to estimate the amount of amorphous or poorly crystalline iron oxide in soils and sediments (Schwertmann 1964; McKeague and Day 1966) and has been suggested as a method for estimating the fraction of bioavailable Fe(III) (Phillips and Lovley 1987). However, more recent work has shown that Fe(II) can catalyze the dissolution of crystalline oxides by oxalate, resulting in an overestimation of the amount of bioavailable Fe(III) (Phillips et al. 1993). Heron et al. (1994a) and Phillips et al. (1993) have shown that oxalate in the presence of Fe(II) can extract highly crystalline Fe(III) oxides such as akageneite, magnetite, goethite and hematite. Since aquifer sediments often contain Fe(II) compounds, ammonium oxalate cannot be used for determining poorly crystalline Fe(III) in such systems (Heron et al. 1994a). Other workers have estimated bioavailable Fe(III) based on accumulation of Fe(II) in the aqueous phase. However, this approach will likely underestimate Fe(III) availability, since much of the Fe(II) produced remains in solid forms (Brannon et al. 1984; Ponnampereuma et al. 1967).

Iron bioavailability could also be limited by fouling of the mineral surfaces by sorbed or precipitated Fe(II). Typically, most of the Fe(II) produced in sediments is not present in the aqueous phase (Lovley and Phillips 1988; Nealson 1983; Ponnampereuma 1972; Van Breeman 1988) but is present in the solid phase as Fe(II) adsorbed onto the Fe(III)-oxide surfaces or in a
variety of reduced minerals, including magnetite (Fe₃O₄), siderite (FeCO₃), and vivianite (Fe₃PO₄ • 8 H₂O) (Pfanneberg and Fischer 1984; Lovley et al. 1987; 1989; Fischer 1988; Lovley and Phillips 1988). However, the effect of sorption and/or precipitation of Fe(II) on Fe(III) bioavailability is not well understood. In one study, washing a Fe(II)-fouled iron oxide with NaAc to remove adsorbed and/or precipitated Fe(II) stimulated Fe(III) reduction (Roden and Zachara 1996).

The physical form of the iron oxide may also affect bioavailability. Iron oxide minerals are usually concentrated in the clay size fraction (< 2 μm) of most soils (Lindsay 1988). The small particle size and large surface area of clays should increase the reducibility of these iron oxides (Lovley 1991). However, direct contact between the Fe(III)-phase and the reducing microorganism may be required and may limit the rate of iron reduction in some aquifers. Studies with Fe(III)-reducing bacteria have shown considerable iron reduction when direct contact was allowed, but none when the Fe(III)-oxide phase was enclosed in dialysis tubing (Munch and Ottow 1983). However, work by Caccavo et al. (1997) demonstrated that irreversible cell adhesion is not requisite for microbial reduction of amorphous Fe(III) oxide. The percentage of cells that adhered to amorphous Fe(III) oxide were < 50 and 100% for S. alga RAD 20 and S. alga BrY, respectively, but the rates of Fe(III) reduction were similar. Also, pore spaces in finer grained sediments may be too small for easy access by microorganisms, potentially limiting iron reduction.

In this research, the various factors controlling Fe(III) bioavailability were evaluated in aquifer material from a petroleum-contaminated aquifer near Rocky Point, N.C. Specific objectives of this work were to: (a) determine the amount of Fe(III) in the sediment available for microbial reduction, (b) relate Fe(III) depletion to shifts in microbial processes, (c) evaluate the effect of Fe(II) sorption and/or precipitation on Fe(III) availability, and (d) identify extraction methods that correlate with the amount of Fe(III) that is actually reduced.

4.1 EXPERIMENTAL DESIGN

Sediment, from a portion of a petroleum-contaminated aquifer in which Fe(III) was the predominant electron acceptor, was initially characterized using several different extraction methods (0.5 N HCl extraction for 1 and 24 hours; oxalate extraction for 4 hours, 24 hours, and 7 days; and 5.0 N HCl extraction for 28 days) to determine the amount of potentially bioavailable Fe(III). Macrocosms containing this sediment were then repeatedly fed benzaldehyde and monitored for depletion of Fe(III), production of solid- and aqueous-phase Fe(II), benzaldehyde degradation, and accumulation of degradation intermediates. Benzaldehyde was chosen as the carbon source since it is readily biodegradable and is presumed to be an intermediate in toluene biodegradation via iron reduction (Lovley and Lonergan 1990). Treatments consisted of three live added-carbon, one live-control (no-added-carbon), and one abiotic (benzaldehyde-added) macrocosms.

After an approximate 400-hour incubation, iron reduction slowed and fermentation products began to accumulate. However, a significant amount of Fe(III) still remained in the sediment. Three potential hypotheses were developed to explain the apparent shift from Fe(III) reduction to fermentation.
1. Fe(III) availability was not limiting Fe(III) reduction, and some other unknown factor was causing this shift.

2. The mineral surfaces were being fouled by sorption and/or precipitation of Fe(II), reducing the bioavailability of the remaining Fe(III).

3. The mineral form of the remaining Fe(III) made it relatively unavailable for use by the sediment-associated microorganisms.

To evaluate hypotheses 1 and 3, several different Fe(III) forms (amorphous-oxyhydroxide, goethite, and hematite) were added to separate incubations containing the sediment from the initial macrocosms and monitored for depletion of accumulated fermentation products and reduction of Fe(III). To evaluate hypothesis 2, a portion of the sediment was washed with either CaCl2 or NaAc. CaCl2 should remove ion-exchanged Fe(II), and NaAc should remove both carbonate-precipitated and ion-exchanged Fe(II) (Heron et al. 1994a; Roden and Zachara 1996). As these treatments were expected to kill or remove most of the indigenous microbes, the washed sediment was then mixed with unwashed sediment, incubated, and monitored for depletion of fermentation products and reduction of Fe(III). For both the Fe(III) mineral addition and sediment washing experiments, untreated live controls and heat-killed abiotic controls were incubated in parallel. Live treatments were constructed in duplicate and single abiotics were constructed for each live treatment.

4.2 MATERIALS AND METHODS

4.2.1 Groundwater and Sediment Collection. All sediment was obtained under anaerobic conditions by drilling below the water table with a hollow stem auger and then advancing a sterile coring tube. The tube was brought to the surface, immediately capped with sterile butyl rubber stoppers, and transported to the laboratory on ice where it was extruded in an anaerobic glove box (Ray Products, El Monte, Calif.) under N2 within 12 hours. The first and last 10 cm of the core were removed and the outer portions of soil pared away. The remaining sediment was anaerobically transferred into sterile mason jars and stored at 4°C. Prior to use, soil from each core was mixed in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.).

Groundwater was collected anaerobically from the same location where the sediment was collected. The well headspace was continually purged with oxygen-free argon for 10 minutes before and during sample collection. Groundwater was pumped from the well, through a closed system of polyethylene tubing equipped with a 0.45-μm filter (Gelman Sciences, Ann Arbor, Mich.), and collected in a nitrogen-sparged 2.3-L (80-ounce) bottle. The water was transported to the laboratory on ice where it was stored at 4°C prior to use. All equipment and containers in contact with the water and sediment were presterilized.

4.2.2 Macrocosm and Microcosm Construction. The initial macrocosm set was constructed in 1-L bottles containing blended aquifer sediment (1000 g) and groundwater (600 mL). Benzaldehyde was repeatedly added to three live and one abiotic macrocosm. One additional live macrocosm was operated with no added carbon to measure the effect of background organic carbon. The abiotic macrocosm was constructed by autoclaving sediment on two consecutive
days for 1 hour at 121°C and 20 psi and adding HgCl₂ (0.9 mM). All macrocosms were constructed in an anaerobic glove chamber using aseptic techniques and incubated in the same chamber at 25°C. CO₂ was trapped in separate serum bottles containing 2M NaOH by connecting the serum bottles to the macrocosms with rubber tubing. Aqueous samples were analyzed for dissolved organics, Fe(II), and CH₄. Sediment for solid-phase iron analysis was obtained from the macrocosms by blending triplicate 3-g samples retrieved using a hollow glass rod. Whenever the rubber stopper was opened, the 15-mL headspace in the macrocosms was flushed with nitrogen to eliminate the hydrogen that is present in the headspace of the anaerobic chamber.

The iron mineral addition microcosms were constructed in 160-mL serum bottles consisting of 100 g of sediment and 100 mL of water from selected macrocosms. Microcosms prepared with sediment from the added-carbon macrocosm (benzaldehyde treated) were amended with approximately 540 μmole Fe(III)/g. Microcosms prepared with sediment from the no-added-carbon (benzaldehyde-treated) macrocosm received approximately 54 μmole Fe(III)/g. Treatments consisted of iron oxyhydroxide, goethite, hematite, live control (no iron added), and an abiotic. As above, all microcosms were assayed for dissolved organics, iron (solid and aqueous), CH₄ (aqueous and headspace), and CO₂.

The washed-sediment microcosms were constructed in 60-mL serum bottles containing 20 g of treated sediment, 20 g of untreated sediment and 40 mL of liquid. The sediment used to construct these microcosms was obtained from one of the added-carbon macrocosms that was no longer producing Fe(II). Fe(II) present on the sediment surfaces was removed by mixing 75 g of sediment with 0.6 L of anaerobic treatment solution for 24 hours and then washing the treated sediment with anaerobic deionized water to remove the remaining solution. Ion-exchanged Fe(II) was removed with 1 M CaCl₂ (pH 7.0) (Heron et al. 1994a) and carbonate precipitated Fe(II) was removed with 1 M NaAc (pH 7.0) (Roden and Zachara 1996). A third treatment contained 40 g of untreated sediment and 4 mM nitrilotriacetic acid/kg of sediment to evaluate whether nitrilotriacetic acid (NTA) would make Fe(III) more available. A fourth treatment contained only unwashed sediment with no amendments as a live control. Each treatment consisted of two live and one abiotic microcosm.

4.2.3 Iron Mineral Forms. Amorphous Fe(III) oxide was prepared by neutralizing a FeCl₃ solution with 1 M NaOH and washing the suspension with deionized water to remove NaCl (Lovley and Phillips 1986a). Goethite was prepared according to Schwertmann (1991). FeCl₂·4H₂O (9.9 g) was dissolved in 1 L of distilled water after first purging with N₂ gas for 30 minutes to remove dissolved oxygen. The solution was then neutralized with 110 mL of 1 M NaHCO₃, and N₂ purge gas was replaced with air at a flow rate of 30 to 40 mL/minute. The solution was stirred continuously and oxidation was complete within 48 hours during which the color of the suspension changed from green-blue to ochre. X-ray diffraction analysis of a freeze-dried sample confirmed the mineral identity. Hematite was purchased from Fisher Scientific Co. and siderite was purchased from Ward’s Natural Science Establishment, Inc. (Rochester, N.Y.). Goethite and hematite were sieved to a particle size < 100 μm, and siderite was sieved to < 250 μm.

4.2.4 Microbial Enumerations. The number of total anaerobic heterotrophs, iron reducers, and methanogens (H₂ utilizers and acetate utilizers) were determined by MPN enumeration. Inocula
were formed by shaking 10 g of sediment in 90 mL of groundwater; dilution series were formed in groundwater. One mL portions of each dilution were inoculated in 9 mL of medium. MPNs were calculated using a computer program (Russek and Colwell 1983). Total heterotrophs and iron reducers were enumerated as described in section 3.2.5. Methanogens were enumerated using a five-tube MPN procedure (Barlaz et al. 1989). For acetate-utilizing methanogens, sodium acetate (80 mM) was added and for hydrogen-utilizing methanogens the tubes were pressurized with 2 kPa of H₂:CO₂ (80:20). Tubes were scored positive if the headspace methane concentration exceeded 0.5% after 2 months.

4.2.5 Analytical Methods. Fe(II) and Fe(III) concentrations on the sediment were determined by using a 28-day extraction with 5.0 N HCl. Heron et al. (1994a) reported that a 21-day extraction with 5.0 N HCl completely dissolves crystalline and noncrystalline iron oxides, siderite, and magnetite and partially dissolves Fe(II) and Fe(III) bound to clays and silicates. We selected a 28-day extraction period based on preliminary work that showed Fe(II) and Fe(III) stabilized after 28 days.

To quantify Fe(II) and Fe(III), 2 g of wet sediment was digested in 50 mL of HCl (5 N, anaerobic) for 28 days. The acid was first deaerated by boiling under a stream of nitrogen. After 28 days, 0.5 mL of the extract was filtered to remove particles greater than 0.2 μm and added to acetate buffer at pH 5 (Stookey 1970) containing phenanthroline. As above, the Fe(II) concentration was determined by reading absorbance at 510 nm and comparing to a standard curve. Total iron was determined by adding 1 mL of the filtered extract to 10% hydroxylamine-hydrochloride solution that served as a reductant. After 20 minutes, this sample was analyzed by the phenanthroline assay. The same procedure was also used for the 1-hour and 24-hour 0.5 N HCl extractions.

Oxalate-extractable iron was determined according to Phillips and Lovley (1987). Two g of sediment were added to 50 mL of a solution of ammonium oxalate (28 g/L) and oxalic acid (15 g/L), which was deaerated by bubbling with N₂. After 4 hours, 1 day, and 7 days, liquid samples from three replicates were analyzed for Fe(II) and total iron as above.

Aqueous concentrations of Fe(II) were determined by adding 0.1 mL of filtered sample to the acetate buffer containing phenanthroline solution as described above. Aqueous samples were analyzed for benzaldehyde, benzoate, benzylalcohol, hexanoic acid, caproic acid, butyrate, propionate, acetate, and ethanol. Samples were first derivatized with meth-prep I (Altech, Deerfield, Ill.) to convert the organic acids to the corresponding methyl esters. For a 1-mL sample, approximately 40 μL of meth-prep I was added together with 3 μL of internal standard (heptanoic acid). One μL of the treated sample was then analyzed by direct aqueous injection with a Shimadzu 9A gas chromatograph equipped with a flame ionization detector and a DB-624 Megabore capillary column (J & W Scientific, Folsom, Calif.). The injector temperature was maintained at 250°C. The oven was initially at 40°C for 5 minutes, increased at 15°C per minute to 165°C and then increased at 40°C per minute to 220°C and held for 5 minutes. Compounds were determined by comparison to external standards. The detection limit for these compounds ranged from 0.01 to 0.02 mM. Out of the organics compounds analyzed in the macrocosms, hexanoic acid, caproic acid, butyrate, and propionate were not detected. The identity of organic
acids and biodegradation intermediates were periodically confirmed using a gas chromatograph equipped with an ion selective detector (Hewlett Packard G1800A). Methane (aqueous and headspace) concentrations were analyzed as described by Hunt et al., (1997).

Dissolved inorganic carbon in the CO₂ traps was measured by using a Rosemount Dohrmann (DC-190) carbon analyzer (Santa Clara, Calif.). Total and organic carbon were analyzed using the boat sampler attachment to the carbon analyzer. Inorganic carbon was determined as the difference between total carbon and total organic carbon. For total organic carbon, 1-g samples were air dried (anaerobically), acidified with HCl (1 N) to remove inorganic carbon, and dried at 60°C overnight before analysis. Each sample was run multiple times to yield a standard deviation of no greater than 20% of the mean.

4.3 RESULTS

4.3.1 Sediment Characterization. The sediment used as a microbial inoculum and Fe(III) source in this study was obtained from a petroleum-contaminated aquifer near Rocky Point, N.C. Groundwater at the sediment sampling location contains no measurable dissolved oxygen, low sulfate (< 0.01 μM), low methane (< 0.1 μM), high dissolved Fe(II) (1.4 to 1.9 μM), and dissolved BTEX (0.07 μM). The aquifer material is of marine origin and consists of fine quartz sand (over 94% greater than 0.1 mm) in a clay matrix with minor amounts of pyrite and muscovite flakes. X-ray diffraction has shown that most of the iron in the sediment is present as a mixed Fe(II):Fe(III) clay mineral—glaucnite, with smaller amounts of the Fe(II)-rich clay-berthierine and possibly iron-rich illite (Becker 1992). In incubations with sediment from this location, Hunt et al. (1997) measured anaerobic degradation of toluene, o-xylene, m-xylene, and benzene with Fe(III) as the predominant electron acceptor. The number of total heterotrophs in the sediment inoculum was low (2.3 x 10² cells/g) with few Fe(III) reducers and methanogens (Table 2). The amount of easily extractable (0.5 N HCl or oxalate) Fe(III) in the sediment varied from 2.8 to 6.0 μmoles/g depending on the extraction technique used (Table 3). These values are lower than literature reports of bioavailable Fe(III) at other iron-reducing sites (18 to 49 μmoles/g: Heron et al. 1994a; 2 to 18 μmoles/g: Albrechtsen et al. 1995; and 0 to 110 μmoles/g: Lovley and Phillips 1987b). However, the total amount of Fe(III) present in the sediment from Rocky Point was comparable to the literature reports, suggesting that the Fe(III) present in the Rocky Point sediment was less available. Since the aquifer sediment had been exposed to anaerobic, organic rich water for over 10 years, some of the original Fe(III) may have been reduced. Pristine sediment collected 50 m outside the contaminant plume contained a little higher percentage of Fe(III), indicating that previous exposure to the contaminant plume may have reduced the amount of bioavailable iron.

4.3.2 Macrocosp Experiment. Macrocossms were constructed using contaminated aquifer sediment and contaminated groundwater and were incubated to determine the amount of Fe(III) available for microbial reduction. Ethanol and acetate were present in the sediment and groundwater used to construct the macrocosms and, consequently, were present in all macrocosms. In the killed-control macrocosm (abiotic), ethanol, acetate, and benzaldehyde did not degrade (Figure 7), and both Fe(II) and Fe(III) remained constant (Figure 8) over 700 hours of incubation.
Table 2. Microbial Populations from Phase I Incubations (cells/g dry wt. of sediment)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Heterotrophs</th>
<th>Iron Reducers</th>
<th>H₂/CO₂-Utilizing Methanogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>2.3 x 10²</td>
<td>1.3 x 10¹</td>
<td>4.4</td>
</tr>
<tr>
<td>Macrocosms with no added carbon after 400 hours</td>
<td>3.3 x 10⁴</td>
<td>1.0 x 10³</td>
<td>89.0</td>
</tr>
<tr>
<td>Macrocosms with added benzaldehyde after 400 hours</td>
<td>1.2 x 10⁶</td>
<td>7.5 x 10⁴</td>
<td>&lt; 2.1</td>
</tr>
</tbody>
</table>

Table 3. Extractable Iron (Fe) Present in Contaminated and Pristine sediment (µmoles/g dry sediment)

<table>
<thead>
<tr>
<th>Extraction Procedure</th>
<th>Pristine Sediment</th>
<th>Contaminated Sediment</th>
<th>Sediment from Added-Carbon Macrocosms after 400-Hour Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe(II)</td>
<td>Fe(III)</td>
<td>ΣFe</td>
</tr>
<tr>
<td>0.5 N HCl for 1 hour</td>
<td>1.90</td>
<td>4.00</td>
<td>4.90</td>
</tr>
<tr>
<td>0.5 N HCl for 1 day</td>
<td>2.98</td>
<td>7.72</td>
<td>10.70</td>
</tr>
<tr>
<td>Oxalate for 4 hour</td>
<td>1.36</td>
<td>0.00</td>
<td>1.36</td>
</tr>
<tr>
<td>Oxalate for 1 day</td>
<td>2.10</td>
<td>3.63</td>
<td>5.70</td>
</tr>
<tr>
<td>Oxalate for 7 days</td>
<td>2.94</td>
<td>4.69</td>
<td>7.63</td>
</tr>
<tr>
<td>5.0 N HCl for 28 days</td>
<td>7.70</td>
<td>47.81</td>
<td>55.51</td>
</tr>
</tbody>
</table>

*Contaminated sediment was used in all incubations.

*NA: Not Applicable. Results of oxalate extractions at 400 hours are not representative since Fe(II) produced during incubation catalyzed the extraction of Fe(III).
In the live-control (no-added-carbon) macrocosm, the ethanol concentration dropped from 2.5 mM to less than 0.2 mM, and acetate increased from 2 mM to 5 mM by 400 hours (Figure 9). Over this same period, Fe(II) increased from 10.4 μmoles/g to 17.4 μmoles/g sediment (Figure 8). During the first 100 hours, aqueous Fe(II) increased from 1.1 mM to 2 mM while solid Fe(II) remained fairly constant (data not shown). However, from 100 to 400 hours, solid-phase Fe(II) increased along with aqueous Fe(II).

We hypothesize that ethanol was fermented to acetate in these macrocosms by the following reaction.

\[
\text{CH}_3\text{CH}_2\text{OH (ethanol)} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- \text{ (acetate)} + \text{H}^+ + 2 \text{H}_2
\]

(1)

Acetate and ethanol concentrations were routinely monitored by direct aqueous injection after derivatization. The identity of both compounds was periodically confirmed by analysis on a gas chromatograph equipped with a mass selective detector. Unfortunately, instrumentation was not available to measure H₂.

Figure 7. Variation in aqueous concentration of benzaldehyde, ethanol, and acetate in abiotic macrocosms.
Figure 8. Variation in Fe(II), Fe(III), and total iron (Fe) in added-carbon (live), no-added-carbon (live-control), and abiotic macrocosms over time.

**Fe(II) - (Solid + Aqueous)**

**Fe(III) - (Solid)**

**Fe(Total) - (Solid)**

Total Fe and Fe(II) are the sum of aqueous and solid-phase measurements. Fe(III) is from solid phase only. In abiotic macrocosms there was no significant change in Fe(II) or Fe(III) over time ($p = 0.05$). However, in the no-added-carbon macrocosms there was a measurable increase in Fe(II) and decrease in Fe(III). In the added-carbon macrocosms, iron reduction slowed after 400 hours.
Both ethanol and acetate were present in the groundwater used to construct the macrocosms.

The cause of the acetate accumulation (Figure 7) is not known but may be related to the low pH of the sediment (~5.9) and the low number of methanogens (Table 2). Acetate is an important substrate for methanogenic- and iron-reducing bacteria in anoxic environments and is known to be a major precursor of methane in sediments (Winfrey and Zeikus 1979; Jones et al. 1989). However, other workers have noted acetate accumulation in aquifer sediments. In incubations of unamended sediment slurries, Jones et al. (1989) observed acetate accumulation over time. Elevated concentrations of acetate were reported in contaminated aquifers (Cozzarelli et al. 1994; McMohan et al. 1995), and acetoclastic methanogenesis was suggested to be the rate limiting step at these site. In a separate study, Lovley et al. (1989) observed the inability of A. putrefaciens to completely oxidize multicarbon substrates to carbon dioxide under iron-reducing conditions. Lactate and pyruvate were oxidized to acetate, which was not metabolized further.

Over the first 400 hours, the ethanol concentration declined by 2.3 mM, while acetate increased by approximately 3.0 mM. The somewhat greater than stochiometric acetate production could be due to degradation of background organic carbon, release of sorbed acetate from the solid-phase and/or minor analytical errors. Fe(III) reduction is believed to be the primary terminal electron acceptor in the no-added-carbon microcosms since: (1) oxygen, nitrate, and sulfate were below detection throughout the incubation and (2) Fe(II) produced during the first 400 hours was equivalent to 62% of the theoretical H₂ production from ethanol in equation 1. In the no-added-carbon macrocosms, total heterotrophs and iron reducers increased by two orders of magnitude with iron reducers continuing to be the dominant population (Table 2).

The added-carbon macrocosms were fed approximately 7 mM benzaldehyde over a 700-hour period by repeatedly respiking whenever benzaldehyde was depleted (Figure 10). The benzaldehyde uptake rate increased up until ~400 hours and then decreased. Total Fe(II) increased and Fe(III) decreased until about 400 hours after which Fe(II) production was nearly
zero and there was no detectable Fe(III) depletion. Substantially more Fe(II) was produced in the added-carbon macrocosms than in the no-added-carbon controls, indicating that benzaldehyde addition stimulated iron reduction (Figure 8). Total heterotrophs increased by 4 orders of magnitude and iron reducers increased by 3 orders of magnitude in the added-carbon macrocosms (Table 2). The large increase in iron reducers correlates with the large amount of Fe(II) produced in the added-carbon macrocosms. No methane was produced in these macrocosms, and the number of methanogens remained below detection.

Figure 10. Variation in benzaldehyde in added-carbon macrocosms.

![Graph showing variation in benzaldehyde concentrations over time](image)

The rate of benzaldehyde degradation appears to slow after 400 hours.

Acetate increased from about 2 mM to a maximum of ~4 mM between 250 and 300 hours and then remained constant or declined slightly (Figure 11). Ethanol was also present in the added-carbon microcosms and decreased from approximately 2.7 mM to 0.2 mM by 100 hours and then remained steady until 250 to 300 hours. After 300 hours, the ethanol concentration began to increase (Figure 11). Ethanol production was stimulated by benzaldehyde as a similar increase was not measured in the no-carbon-addition macrocosms (Figure 9).
Benzaldehyde was transformed to benzoate and benzylalcohol (Figure 12) which were not biodegraded further in the macrocosms. Benzoate is a known intermediate in the anaerobic biodegradation of both naturally occurring and xenobiotic aromatic compounds (Young and Frazer 1987) and has been shown to degrade under Fe(III)-reducing conditions (Lovley et al. 1989). However, *A. putrefaciens* was unable to couple the oxidation of benzoate to Fe(III) reduction even though it was able to oxidize multitude of other electron donors (Lovley et al. 1989). In a study examining the fate of hydrocarbon metabolites, Cozzarelli et al. (1995) found that benzoate did not degrade when Fe(III) and sulfate were added as electron acceptors even though iron reduction and sulfate reduction were important processes in situ and occurred in the microcosms. However, benzoate did degrade when nitrate was supplied as an electron acceptor. Cozzarelli et al. (1995) concluded that the biogeochemical fate of specific organic acids observed in groundwater varied with the structure of the acid and the availability of electron acceptors. Using sediments from the deep terrestrial subsurface sediments, Jones et al. (1989) found that benzoate degraded in only those sediments that harbored methanogens and persisted in all nonmethanogenic samples. Benzoate and acetate disappearance always occurred concomitantly. Therefore, the persistence of benzoate can be attributed to the unavailability of a specific electron acceptor, nonmethanogenic conditions, or the absence of the required microorganisms.
Figure 12. Variation in aqueous benzoate and benzylalcohol concentrations over time in added-carbon macrocosms.

Both benzoate and benzylalcohol were produced throughout the 700-hour incubation (Figure 12). However, the ratio of benzylalcohol to benzoate varied considerably over time (Figure 13). During the first 150 hours when ethanol was being depleted, the benzylalcohol-to-benzoate ratio dropped steadily, indicating more oxidizing conditions. During this same period, Fe(II) increased and Fe(III) decreased, indicating active iron reduction. However, at about 250 to 300 hours, the ethanol concentration began to increase and the benzylalcohol-to-benzoate ratio began to increase, indicating more reducing conditions. Shortly after this (at ~ 400 hours), Fe(II) and Fe(III) stabilized indicating that iron reduction had stopped.

We hypothesize that the increase in benzylalcohol production after 300 hours is due to the accumulation of hydrogen as illustrated in the biochemical reactions presented in Table 4. Hydrogen will be produced during the initial transformation of benzaldehyde to benzoate (reaction 1). During the first 250 to 300 hours, this H₂ was consumed via reduction of Fe(III) present on the aquifer solids (reaction 2). However, once Fe(III) reduction stopped, we hypothesize that hydrogen began to accumulate causing a shift toward ethanol and benzylalcohol production (equations 3 and 4). Numerous studies have shown that when microbial processes shift from oxidative to fermentative, there is a change in product formation (Reddy et al. 1972; Iannotti et al. 1973; Weimer and Zeikus 1977; Ishimoto et al. 1973). Jones et al. (1984) showed that addition of Fe(III) to growth media caused a shift from ethanol to volatile fatty acid production.
After 400 hours incubation, sediment from the added-carbon macrocosms was analyzed to determine changes in Fe(II) and Fe(III) (Table 3). Total Fe(II) (aqueous plus 28-day extraction with 5.0 N HCl) increased by 6.4 μmoles/g of sediment while sediment Fe(III) decreased by 4.7 μmoles/g. Assuming ethanol and benzaldehyde were oxidized to acetate and benzoate, 5.2 μmoles/g of iron should have been reduced. The close match between predicted and observed iron reduction indicates that Fe(III) was the primary electron acceptor in this system. However, it is not clear why Fe(III) reduction stopped after 400 hours.

Over the 400-hour period of active iron reduction, between 4.7 and 6.4 μmoles/g of iron were reduced. This compares reasonably well with the amount of readily extractable Fe(III) present in the original sediment (2.8 to 6.0 μmoles/g) and suggests that all the bioavailable Fe(III) may have been reduced. However, a significant fraction of the 0.5 N HCl extractable Fe(III) and over 85% of the total Fe(III) was still present in the sediment after 400 hours. This suggests that some other factor might be limiting Fe(III) availability.

Of the total Fe(II) produced in the incubations, 34% was in the aqueous phase and 66% was sorbed or precipitated on the solid phase (data not shown). The form of the solid-phase Fe(II) was not known. Equilibrium calculations using MINTEQA2 (Allison et al. 1991) predicted the precipitation of siderite (FeCO₃).
Table 4. Hypothesized Biochemical Reactions in the Added-Carbon Macrocosms

<table>
<thead>
<tr>
<th>Reaction</th>
<th>( \Delta G'_0 ) (kJ per ( H_2 ))^*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H(_2) Production:</strong></td>
<td></td>
</tr>
<tr>
<td>1. ( C_6H_5CHO + H_2O \rightarrow C_6H_5COO^- + H^+ + H_2 )</td>
<td>-61^a,b</td>
</tr>
<tr>
<td><strong>H(_2) Consumption:</strong></td>
<td></td>
</tr>
<tr>
<td>2. ( 2 \text{Fe(III)} + H_2 \rightarrow 2 \text{Fe(II)} + 2 \text{H}^+ )</td>
<td>-202^c</td>
</tr>
<tr>
<td>3. ( CH_3COO^- + H^+ + 2 H_2 \rightarrow CH_3CH_2OH + H_2O )</td>
<td>-24^a</td>
</tr>
<tr>
<td>4. ( C_6H_5CHO + H_2 \rightarrow C_6H_5CH_2OH )</td>
<td>-41^b</td>
</tr>
<tr>
<td>5. ( \text{HCO}_3^- + 4H_2 + H^+ \rightarrow \text{CH}_4 + 3H_2O )</td>
<td>-34^a</td>
</tr>
<tr>
<td><strong>Fermentation:</strong></td>
<td></td>
</tr>
<tr>
<td>6. ( C_6H_5CHO + H_2O \rightarrow C_6H_5COO^- + H^+ + C_6H_5CH_2OH )</td>
<td>-102^a,b</td>
</tr>
</tbody>
</table>

*Standard Gibbs free energy at pH 7 calculated from standard free energies of formation given in: ^aThauer et al. (1977); ^bSandler (1989); and ^cStumm and Morgan (1981).*

To provide some indication of the chemical composition of the solid-phase Fe(II), sediment samples were extracted for 24 hours by using anaerobic solutions of CaCl\(_2\) (1 M, pH 7.0) and sodium acetate (1 M, pH 5.0). The CaCl\(_2\) solution was intended to extract material that ion exchanged onto clays and recovered 2.9 \( \mu \)moles Fe(II)/g sediment. The NaAc solution was intended to extract both ion-exchanged and amorphous carbonate minerals and recovered 4.8 \( \mu \)moles Fe(II)/g. In separate analyses, the sediment inorganic carbon was found to increase by 2.8 \( \mu \)moles/g over the course of the incubation. (Note: An increase in inorganic carbon was detected only on the solid phase but not in aqueous phase.) These results indicate that approximately 60% (2.9 \( \mu \)moles/g) of the Fe(II) is ion exchanged onto the sediment with the remaining fraction present as a carbonate mineral with the same stochiometry as siderite (FeCO\(_3\)). However, Heron et al. (1994a) reported that NaAc at pH 5 does not extract siderite. Heron’s observation was confirmed in our own laboratory using ground siderite (<250 \( \mu \)m) obtained from Ward’s Natural Science Establishment, Inc. (Rochester, N.Y.) We hypothesized that the FeCO\(_3\) present in the sediment was in an amorphous form that could be extracted with NaAc. To evaluate this hypothesis, fresh FeCO\(_3\) was prepared by titrating 1 M FeCl\(_2\) with 1 M Na\(_2\)CO\(_3\) to pH 7.0. The resulting precipitate was washed with deionized water until the chloride concentration in the
associated water dropped below 1 mM. Fe(II) analysis on the precipitate confirmed the
stoichiometry of the precipitate to be FeCO$_3$. Fe(II) extracted with anaerobic CaCl$_2$ (1 M, pH 7.0)
and NaAc (1 M, pH 5.0) for 24 hours was 7 and 82% of the total Fe(II), respectively. This
indicates that a substantial portion of the solid-phase Fe(II) generated in the added-carbon
incubations was present in an in a NaAc extractable form of FeCO$_3$.

4.3.3 Addition of Iron Mineral Forms. At the completion of the initial macrocosm incubations,
it was known that reduced fermentation products began to accumulate about the same time that
iron reduction slowed. However, it was not clear whether the accumulation of fermentation
products was due to a reduction in bioavailable iron or some other unknown factor.

Separate microcosms were constructed using sediment and water from the previous no-added
carbon- and added-carbon macrocosms. The microcosms were amended with different iron
mineral forms to determine if an added electron acceptor could shift the microbial activity away
from fermentation and back to iron reduction. Ethanol and benzylalcohol degraded in all the iron-
treated microcosms but not in the live-control microcosms without added iron (Figures 14a and
14b). This indicates that the observed shift from iron reduction to fermentation was due to

Figure 14. Effect of iron mineral addition on biotransformation of fermentation products in
sediment from added-carbon macrocosms: (a) ethanol and (b) benzylalcohol.
depletion of bioavailable iron. Ethanol and benzylalcohol concentrations dropped most rapidly in the iron oxyhydroxide-and goethite-treated microcosms. However, both compounds did eventually degrade in the hematite-treated microcosms. Fe(II) concentrations increased in all the iron-treated microcosms (Table 5). The largest increase in Fe(II) was in the iron oxyhydroxide- and goethite-treated microcosms followed by hematite-treated microcosm. In the live-control microcosm (no iron added) and abiotic microcosm, Fe(II) concentrations increased slightly. These results are consistent with previous reports that Fe(III) present in the more highly crystalline mineral forms is less bioavailable (Lovley and Phillips 1986a; Lovley and Phillips 1987a; Munch and Ottow 1980).

Table 5. Fe(II) Increase in Iron Mineral Addition Incubations (μmoles/g dry wt. sediment)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Solid Phase</th>
<th>Aqueous Phase</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxyhydroxide</td>
<td>10.4</td>
<td>7.1</td>
<td>17.5</td>
</tr>
<tr>
<td>Goethite</td>
<td>9</td>
<td>3.4</td>
<td>12.4</td>
</tr>
<tr>
<td>Hematite</td>
<td>5</td>
<td>1.4</td>
<td>6.4</td>
</tr>
<tr>
<td>Live Control</td>
<td>1.9</td>
<td>0.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Abiotic</td>
<td>1.4</td>
<td>0.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Acetate and benzoate did not degrade in any of the added-carbon macrocosms, including those with added iron minerals (Figure 15), indicating that acetate and benzoate degradation is not limited by iron bioavailability. However, there was an initial drop in the aqueous concentration of Fe(II), acetate and benzoate, and Fe(II) (data not shown) because of sorption to the added iron minerals. This is consistent with results by Cornell and Schwertmann (1996) who showed that iron oxides adsorb organic acids and inorganic ions.

Acetate was degraded under methanogenic conditions in microcosms constructed with sediment that had been previously incubated for 700 hours without any added benzaldehyde (Figures 16 and 17). However, benzoate remained constant in all incubations (data not shown). Acetate degraded and methane accumulated in all microcosms constructed with this sediment regardless of whether or not iron minerals were added. Low recovery of methane for the amount of acetate metabolized may be due to losses associated with rubber tubing connecting the microcosms to the NaOH trap. There was no increase in total Fe(II), indicating the low methane recovery was not due to acetate utilization by Fe reducers. In the previous macrocosm incubations with no-added-carbon, there was roughly an order of magnitude increase in the number of methanogens grown on a mixed substrate of acetate and H₂/CO₂ (Table 2). However, there was no increase in the methanogen population in the added-carbon macrocosms and no detectable methane production in both the added-carbon and no-added-carbon macrocosms.
Figure 15. Effect of iron mineral addition on biotransformation of fermentation products in sediment from added-carbon macrocosms: (a) acetate and (b) benzoate.
Figure 16. Effect of iron minerals on biotransformation of fermentation products in microcosms prepared with sediment and water from no-added-carbon macrocosms.

Figure 17. Methane production in microcosms prepared with sediment and water from no-added-carbon macrocosms.

Concentrations represent aqueous + headspace measurement calculated with respect to aqueous volume.
Methanogens utilizing either acetate or H₂/CO₂ were enumerated separately at the end of the iron-added incubations because of the observed depletion of acetate. In the microcosms without added carbon, H₂/CO₂-utilizing methanogens increased to 4.7 x 10² cells/g, and acetate-utilizing methanogens increased to 2.1 x 10³ cells/g, which corresponds with the observed methane production. However, in the microcosms with added carbon, there was only a slight increase in H₂/CO₂-utilizing methanogens to 32 cells/g, and there were no detectable acetate-utilizing methanogens. Again, this corresponds with the observed persistence of acetate in the added-carbon microcosms.

The long lag period before the onset of methanogenesis in all incubations was likely due to the low pH. During the initial 700 hour incubation, the pH of both the added-carbon and no-added-carbon macrocosms increased from 5.9 to 6.4, and it remained constant during the added-iron-microcosm incubations. Methanogens are known to be active in the pH range of 6.8 and 7.4 (Zehnder 1976). In a study evaluating factors influencing methanogenesis in an anoxic aquifer, Beeman and Suflita (1990) observed that methanogenesis was severely inhibited at pH values < 7. Moreover, acetoclastic methanogens are known to have long generation times, and low cell yields from growth on acetate (Wolfe and Higgins 1979). In the added-carbon incubations, the high benzoate levels may also have inhibited methanogenic activity. Fang et al. (1997) found that at pH 7, total benzoate concentrations greater than 40 mM (equivalent to 0.04 mM benzoic acid at this pH) reduced methane production by over 50% in 100 hours in anaerobic granules adapted to methanogenic conditions. In the added-carbon incubations, the sediment was exposed to greater than 0.1 mM benzoic acid for over two months.

4.3.4 Sediment Fouling by Fe(II). Results of the added-iron experiments demonstrated that Fe(III) availability was limiting iron reduction, causing an accumulation of reduced fermentation products. However, it was not known whether the Fe(III) still present in the sediment was in a mineral form that was not available to the iron reducers or if fouling of the sediment surfaces by Fe(II) had reduced the Fe(III) availability.

To evaluate the effect of Fe(II) fouling on iron bioavailability, sediment from the added-carbon macrocosms was washed with either CaCl₂ or NaAc to remove Fe(II) present on the mineral surfaces. This sediment was then blended 50:50 with unwashed sediment and used to construct microcosms. Parallel incubations contained 4 mM NTA/kg of sediment to determine if addition of this chelator could enhance Fe(III) availability. Unwashed sediment and killed-control microcosms were also incubated. There was no evidence of ethanol or benzyalcohol biodegradation in any of the abiotic incubations (Figure 18).

After approximately 1500 hours of incubation, there was no evidence of enhanced iron reduction in any live incubation. At that time, amorphous iron oxyhydroxide (545 μmoles/g sediment) was added to one replicate from each treatment. The iron addition stimulated iron reduction, resulting in complete degradation of ethanol and benzylalcohol within 600 hours (Figure 19).

In microcosms containing CaCl₂- and NaAc-treated sediment that did not receive added iron, ethanol and benzylalcohol degradation started after approximately 2000 hours (Figure 20) and iron reduction resumed (Table 6). The long lag time prior to the resumption of iron reduction is
No indication of depletion of organic compounds in any treatments.
Figure 19. Variation in organic compounds over time in washed-sediment microcosms spiked with iron oxyhydroxide at 1500 hours.

Ethanol and benzyl alcohol degradation resumed, indicating iron availability was limiting.
Figure 20. Variation in organic compounds over time in washed-sediment microcosms.

Ethanol and benzylalcohol were depleted after 2100 hours in CaCl₂- and NaAc-treated sediment but persisted in NTA- and untreated sediment.
Table 6. Fe(II) Produced in Washed-Sediment Incubations (μmoles/g dry wt.)

<table>
<thead>
<tr>
<th>Washing Treatment*</th>
<th>Solid phase</th>
<th>Aqueous phase</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>3.82</td>
<td>2.51</td>
<td>5.33</td>
</tr>
<tr>
<td>NaAc</td>
<td>4.18</td>
<td>2.94</td>
<td>7.12</td>
</tr>
<tr>
<td>NTA</td>
<td>1.00</td>
<td>2.18</td>
<td>3.12</td>
</tr>
<tr>
<td>Unwashed Control</td>
<td>0.63</td>
<td>0.87</td>
<td>1.50</td>
</tr>
<tr>
<td>CaCl₂-Abiotic</td>
<td>0.73</td>
<td>0.33</td>
<td>1.06</td>
</tr>
<tr>
<td>NaAc-Abiotic</td>
<td>0.55</td>
<td>0.76</td>
<td>1.31</td>
</tr>
<tr>
<td>NTA-Abiotic</td>
<td>-0.54</td>
<td>3.36</td>
<td>2.82</td>
</tr>
<tr>
<td>Unwashed Control-Abiotic</td>
<td>-0.09</td>
<td>0.33</td>
<td>0.24</td>
</tr>
</tbody>
</table>

*These microcosms correspond to Figure 20 and did not receive added Fe(III) at 1500 hours.

potentially due to two factors. First, the number of active iron reducers may have been reduced by holding the sediment without bioavailable Fe(III) for > 2000 hours prior to the start of these incubations. Second, the remaining Fe(III) was probably in a mineral form that was less available to the microorganisms. In the Fe(III) addition experiments, the lag period prior to ethanol and benzylalcohol biodegradation was significantly longer for hematite treatment than for the oxyhydroxide and goethite treatments (Figure 14), indicating that the iron mineral form may influence the lag period prior to resumption of iron reduction.

In the NTA-treated and untreated sediment microcosms, there was no evidence of ethanol and benzylalcohol biodegradation or iron reduction. Other researchers have found that NTA and ethylenediamine tetra acetic acid can enhance the extent of Fe(III) reduction by complexing with Fe(II). In our work, the concentration of NTA added (4 mM or ~ 4 mmoles/kg sediment) was probably not sufficient to mobilize a substantial portion of the sediment Fe(II) given the high aqueous-phase concentration of Fe(II) (5 mM). A higher NTA concentration could not be used in these incubations because of potential problems with NTA toxicity (D. Lovley, personal communications). In the abiotic treatments, all monitored parameters remained constant throughout the course of the experiment (data not shown). No methane was produced in any of the sediment washing incubations.

The enhanced biodegradation of ethanol and benzylalcohol and increased Fe(II) production in the CaCl₂- and NaAc-washed sediment indicates that iron reduction was limited by fouling of the
mineral surfaces, not because the Fe(III) was in a form that was not available to the iron reducers. These results should not be surprising given that the addition of the highly crystalline iron oxide hematite stimulated iron reduction. Although re-initiation of Fe(III) reduction was expected in NaAc microcosm with NaAC-washed sediment, the mechanism for enhancing Fe(III) reduction in CaCl₂-washed microcosm is less clear. Theoretically, washing the sediment with CaCl₂ should only substitute Fe(II) with Ca(II) on the mineral surfaces.

4.4 DISCUSSION

In the sediment macrocosms, ethanol and benzylalcohol began to accumulate when iron reduction slowed because of a decline in Fe(III) bioavailability. Calculations of the free energy yield indicate that ethanol should begin to accumulate at a H₂ concentration of 8 μM. Shifts in microbial processes from iron reduction to methanogenesis have been reported to occur in the range of 0.2 to 2 nM (Lovley et al. 1994; Lovley and Goodwin 1988; Vroblesky et al. 1997. Therefore, accumulation of ethanol suggests absence of methanogenic conditions and serves as a geochemical signature indicating depletion of bioavailable iron in this system.

One surprising observation was the high concentrations of ethanol present in groundwater at the start of our incubations. To our knowledge, ethanol accumulations in contaminated groundwater have not been previously reported. In ongoing work in another petroleum-contaminated aquifer, high ethanol concentrations (~130 mg/L) were observed in the microcosms (unpublished data). The high ethanol concentrations present in the groundwater suggest that iron reduction in the aquifer is limited by Fe(III) bioavailability. However, when the macrocosms were constructed with groundwater and aquifer sediment, ethanol was rapidly degraded via iron reduction. We hypothesize that the rapid ethanol degradation observed in the macrocosms was due to the mixing of the aquifer sediment that exposed fresh mineral surfaces, which enhanced iron bioavailability. Additional studies are underway in our laboratory to evaluate the effect of spatial variations in aquifer characteristics on biotransformation rates.

Other workers have reported the presence of acetate in contaminated aquifers (Cozzarelli et al. 1994; McMohan et al. 1995). Cozzarelli et al. (1994) studied aquifers contaminated with crude oil, creosote, and gasoline. In the aquifers contaminated with crude oil and gasoline, acetate concentrations were in the range of 2 μM and < 2 μM, respectively. However, at the creosote contaminated aquifer, aliphatic acids had accumulated (> 600 μM), and acetate (506 μM) was the major intermediate of creosote degradation under methanogenic conditions. At a petroleum-hydrocarbon contaminated aquifer near Hanahan, S.C., acetate concentrations were between 3 and 7 mM at six out of nine locations sampled (McMohan et al. 1995). In our incubations, acetate concentrations were in the range of 2 to 3 mM. High acetate concentrations may persist because of the depletion of other electron acceptors and because of low pH, which inhibits development of a significant methanogenic population. Lack of acetate oxidation using Fe(III) as the electron acceptor may be due to microbial consortia present in this system. Acetate is known to be readily utilized under Fe(III)-reducing conditions. However, in a study conducted to investigate the ability of A. putrefaciens to couple the oxidation of potential donors to the reduction of Fe(III), Lovley et al. (1989) observed that A. putrefaciens could couple the oxidation of various electron donors to Fe(III) reduction, yet acetate produced from lactate and pyruvate oxidation was not metabolized further.
In many aquifers, Fe(III) is potentially one of the most important electron acceptors for contaminant biodegradation. However, there are no reliable methods for assessing the amount of bioavailable Fe(III) and the extent of iron reduction that is actually occurring. Over the course of the initial macrocosms incubations and follow-up washed-sediment incubations, approximately 13 μmoles/g of Fe(II) were produced or approximately 37% of the total Fe(III) present. Prior to the start of these incubations, selective chemical extraction of the sediment with 0.5 N HCl and oxalate for different time periods had generated estimates of the total bioavailable Fe(III) that varied between 2.8 and 6.0 μmoles/g. At the end of the washed-sediment incubations, the amount of Fe(III) that was reduced was 9.4 μmoles/g. Clearly these selective extraction techniques underestimated the amount of Fe(III) that was actually bioavailable. Even in the initial macrocosm experiments, Fe(II) production exceeded the Fe(III) extracted with 0.5 N HCl or oxalate.

The most common approach used to estimate the extent of iron reduction in contaminated aquifers is to compare aqueous Fe(II) concentrations in contaminated and uncontaminated portions of the aquifer. Using this approach, Wiedemeier et al. (1995) evaluated the significance of iron reduction at 25 natural attenuation sites and found that Fe(III) reduction was dominant at only 2 sites while sulfate reduction and methanogenesis were dominant at the 23 remaining sites. However, in a landfill-leachate-polluted aquifer, Heron and Christensen (1995) found that over 98% of the Fe(II) produced was not dissolved. In our system over 97% of Fe(II) produced accumulated on solid phase. This Fe(II) was present as an ion exchangeable phase and as a NaAc extractable material consistent with ferric carbonate. Fe(II) precipitation has been reported at several different organic-contaminated aquifers. Baedecker et al. (1992) reported the presence of authigenic Fe(II)-bearing minerals in creosote-and crude oil-contaminated aquifers. Pyrite and siderite were precipitated in the creosote-contaminated aquifer because of the high concentrations of SO₄²⁻, aqueous Fe(II) and HCO₃⁻ in the groundwater. In the crude oil-contaminated aquifer, magnetite was found in one sample and ferroan calcite was found throughout the contaminated zone. Heron et al. (1994a) reported the presence of high concentrations of pyrite in a leachate-contaminated aquifer. Geochemical data indicated that the groundwater was supersaturated with siderite; however, this authigenic siderite was not observed in any sediment samples. These results indicate that the use of only aqueous measurements of Fe(II) will often significantly underestimate the extent of iron reduction.

At present, the significance of Fe(II) fouling of iron oxide surfaces is poorly understood. In our work, fouling of the mineral surfaces with ion-exchanged and precipitated Fe(II) reduced iron availability, resulting in an accumulation of reduced fermentation products. In a related study, Roden and Zachara (1996) found that over 90% of the Fe(II) produced by a dissimilatory Fe(III) oxide-reducing bacterium was either incorporated as an adsorption complex or as a precipitate on the Fe(III) oxide surface. Iron reduction slowed after approximately 2.7% of the available Fe(III) was reduced due to fouling of the iron oxide. However, washing the iron oxide with NaAc re-initiated the Fe(III) reduction process.

However, it is not clear whether Fe(II) fouling is an important limitation on iron bioavailability in contaminated aquifers since other factors, including advective flushing, may limit Fe(II) buildup in the aqueous phase. Analysis of pristine and contaminated aquifer sediment at the Rocky Point site
showed some depletion of Fe(III) in the contaminated zone but no detectable increase in Fe(II) (Borden et al. 1994). At the Vejen landfill in Denmark, decades of exposure to leachate resulted in essentially complete depletion of the iron oxides originally present in the methanogenic zone immediately downgradient of the landfill (Heron and Christensen 1995). This occurred even though the groundwater was saturated with siderite, and pyrite was precipitating near the landfill (Heron et al. 1994a).
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