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Anaerobic Biodegradation of Methyl *tert*-Butyl Ether Under Iron-Reducing Conditions in Batch and Continuous-Flow Cultures

Amy Pruden, Marie A. Sedran, Makram T. Suidan, Albert D. Venosa

ABSTRACT: The feasibility of biodegradation of the fuel oxygenate methyl *tert*-butyl ether (MTBE) under iron-reducing conditions was explored in batch and continuous-flow systems. A porous pot completely-mixed reactor was seeded with diverse cultures and operated under iron-reducing conditions. For batch studies, culture from the reactor was transferred anaerobically to serum bottles containing either MTBE alone or MTBE with ethanol (EtOH) and excess electron acceptor. In the continuous-flow reactor, MTBE conversion to *tert*-butyl alcohol (TBA) was observed after 181 days of operation, and stable removal was achieved throughout the remainder of the study. Simultaneously, both the MTBE only and the MTBE and EtOH iron-reducing batch serum bottles also began to degrade MTBE. Bottles were respiked and the degradation rate was determined to be $2.36 \pm 0.10 \times 10^{-4}$ mmol MTBE/min·kg VSS. The EtOH present with MTBE degraded faster ($7.76 \pm 0.08 \times 10^{-3}$ mmol EtOH/min·kg VSS) but did not have a noticeable effect on the rate of MTBE degradation. No evidence of TBA degradation was observed by the iron-reducing cultures. Stoichiometry of iron utilization was determined from the iron balance of the continuous-flow reactor, and it was found that the bulk of the electron acceptor was required for energy and maintenance with little remaining for cell synthesis. This is consistent with a yield coefficient of less than 0.1. Molecular analysis of the iron-reducing culture by denaturing gradient gel electrophoresis indicated that uncultured strains of δ -Proteobacteria were dominant in the reactor. *Water Environ. Res.*, 77, 297 (2005).

KEYWORDS: Fuel-oxygenates, methyl *tert*-butyl ether, ethanol, anaerobic biodegradation; iron reduction

Introduction

The fuel oxygenate methyl *tert*-butyl ether (MTBE) has become a prevalent groundwater contaminant because of its widespread application in reformulated fuels and because of the tendency of underground fuel storage tanks to leak. Groundwater is typically low in dissolved oxygen under natural conditions, and the oxygen that is available is often even further reduced in the presence of hydrocarbon spills. As demonstrated in previous studies (Deeb et al., 2001; Sedran et al., 2002), benzene, toluene, ethylbenzene, and xylene (BTEX) compounds are preferred substrates over MTBE by microorganisms. These compounds are also present in gasoline and are frequently observed co-contaminants with MTBE. Microbial preference for BTEX over MTBE is also generally observed in the field (Schirmer et al., 1999). The presence of more readily biodegradable substrates, such as BTEX, further depletes the oxygen available for MTBE biodegradation. Because the majority of MTBE contamination is present under oxygen-limiting conditions, anaerobic

biodegradation is of special interest. Successful natural attenuation of MTBE is often considered to be dependent on the ability of microorganisms to degrade MTBE anaerobically.

Several studies have addressed anaerobic degradation of MTBE, but the results are not generally conclusive. Anaerobic microcosm studies have been done with varying results (Mormile et al., 1994; Ruiz-Aguilar et al., 2002; Suffita and Mormile, 1993; Yeh and Novak, 1994). One of the first studies indicating anaerobic biodegradation of MTBE was done in a methanogenic microcosm with Ohio River sediment, but degradation was only observed in one of the triplicates, and this after a 152-day acclimation period (Mormile et al., 1994). The MTBE was observed to be converted to *tert*-butyl alcohol (TBA), which was not further degraded. Several oxygenates were investigated in this study, and the results suggested that compounds containing *tert*-butyl groups were especially resistant to anaerobic degradation. Conversely, a study done by Yeh and Novak (1994) indicated that TBA degraded readily under anaerobic conditions, whereas MTBE was recalcitrant. However, the results of this study were not confirmed with radiolabeled studies nor by correlating substrate use with electron receptor depletion.

Anaerobic mineralization of MTBE has only recently been reported. Bradley et al. (2001b) reported mineralization of MTBE in microcosms containing surface-water sediment with amended nitrate for denitrifying conditions. Approximately 20 to 30% of the theoretical radiolabeled MTBE was recovered as carbon dioxide in these microcosms with no indication of TBA buildup. Later, a more elaborate sediment microcosm study was conducted under methanogenic, sulfate-, manganese-, and iron-reducing conditions, in addition to denitrifying conditions (Bradley et al., 2001a). Mineralization was observed under all redox conditions except methanogenic conditions, where only conversion to TBA was observed. Based on the results of this study, the following electron acceptor hierarchy was suggested: oxygen > nitrate > manganese (IV) > iron III > sulfate [$O_2 > NO_3 > Mn(IV) > Fe III > SO_4$]. A similar study was also recently carried out by Finneran and Lovely (2001) using anaerobic petroleum-contaminated aquifer sediments. Mineralization of both ^{14}C -MTBE and ^{14}C -TBA were reported and were recovered as both radiolabeled carbon dioxide ($^{14}CO_2$) and radiolabeled methane ($^{14}CH_4$). Methanogenesis and sulfate and iron reduction were reported to be taking place simultaneously. In this study, humic substances were suggested to be an enhancing agent for MTBE degradation under iron-reducing conditions by acting as an electron shuttle between MTBE and insoluble iron oxides.

As reports of anaerobic mineralization of MTBE are only recently emerging, research is still needed to confirm the phenomenon and to verify its consistency of occurrence and applicability to bioremediation. This study explores the feasibility of MTBE biodegradation under iron-reducing conditions in batch and continuous-flow systems seeded with diverse cultures.

Methodology

Enrichment of Iron-Reducing Culture in Porous-Pot Reactor. The continuous-flow reactor was seeded with diverse cultures to optimize the likelihood of encountering microorganisms capable of anaerobic MTBE biodegradation. The reactor was seeded with 100 mL of the active biomass from five aerobic MTBE-degrading cultures enriched under various substrate conditions (which had been seeded from Port Hueneme sediment washwater, bioreactor sludge provided by J. Salanitro, Shell Equilon Corporation (Houston, Texas), and Cincinnati Municipal Sewer District (MSD) activated sludge; see Pruden et al., 2001), along with a mixture of denitrifying, methanogenic, and sulfate-reducing reactor cultures, which had been exposed to MTBE for over one year in our laboratory, but which did not demonstrate stable removal of MTBE. These reactors had been seeded with a similar mixture from the aerobic reactors along with activated sludge for the denitrifying reactor and anaerobic digester sludge for the methanogenic and the sulfate-reducing reactor. The iron-reducing reactor was operated for 56 days before transferring the majority of the biomass to serum bottles for the batch studies. At this time, the reactor was restarted with the remaining culture.

The culture was enriched in a porous-pot completely-mixed reactor, which allowed for efficient separation of hydraulic retention time (HRT) and solids residence time (SRT) (described in Pruden et al., 2001 and Wilson et al., 2000). The total liquid volume in the reactor was maintained at 10 L with a culture volume of 6 L enclosed within a 0.2- μ m polyethylene porous pot. The HRT of the reactor was 4.2 days, and the SRT was 200 days (minimal biomass wasting was incurred only for total suspended solids [TSS] and volatile suspended solids [VSS] analysis—approximately 30 mL/week—throughout operation). The headspace of the reactor was flushed thoroughly with nitrogen gas (pre-purified grade), and sealed from the atmosphere using silicone sealant. The headspace outlet was connected with ultra-low permeability tubing (Cole Palmer, Vernon Hills, Illinois) to a gas-tip meter (Rebel Point Wet Tip Gas Meter Company, Nashville, Tennessee) to monitor any gas production by the system. In addition, all feed solutions were purged with nitrogen gas (industrial purity) until a dissolved oxygen reading of zero was obtained. The headspaces of both the buffer and nutrient reservoirs to the system were continuously flushed with nitrogen gas (industrial purity), and sodium sulfide was added to the buffer solution to further scavenge any remaining dissolved oxygen (0.16 g/L reactor concentration). The total flowrate was 2.37 L/d, with a nutrient composition identical to that described in Sedran et al. (2004), except that they were provided as chloride salts rather than sulfate salts. This included the addition of vitamins mentioned in this reference. The nitrogen source was ammonium chloride [NH₄Cl]. The pH was maintained in the range 6.8 to 7.3 through the addition of sodium hydrogen carbonate [NaHCO₃] and sodium hydroxide [NaOH] into the buffer feed solution. The substrate conditions were as follows: the reactor was initially operated at 1.1 mM EtOH (50 mg/L) with 0.11 mM MTBE (10 mg/L) for one month, followed by MTBE as the sole carbon substrate at 0.11 mM for the remainder of operation. All substrates were provided to the reactor via syringe pump into the buffer line to eliminate volatile losses.

Iron-reducing conditions were established through amendment of ferric chloride (Fisher, Fair Lawn, New Jersey) to the nutrient solution. Ferric iron was provided at a target concentration of 8.0 mM, well in excess of the theoretical amount necessary for the mineralization of the influent substrates. The theoretical amount was determined based on the stoichiometry of the electron donor and acceptor half reactions. In addition, 0.15 g/L humic acid sodium salt (Aldrich, Milwaukee, Wisconsin) was added to the buffer of the iron-reducing reactor to stimulate possible electron shuttling activity, as described in Finneran and Lovely (2001). Later, the humic substances were removed from the buffer to determine the effect.

Analysis. For aqueous and gas-phase MTBE and intermediate analysis above 1.1 μ M (0.1 mg/L), a Hewlett Packard 5890 Series II gas chromatograph (GC) (Hewlett Packard, Palo Alto, California) flame ionization detector (FID) was used. This instrument was equipped with a custom made 60/80 Carbowax B/5% Carbowax 20 M glass column (column number 2-1641 packed with 11766) (Supelco, Bellefonte, Pennsylvania). Two of the major intermediates of MTBE were also detectable by this method (TBA and *tert*-butyl formate). All samples were filtered using 0.45-mm MAGNA nylon membrane filters (Micron Separations, Inc., Westboro, Massachusetts) before injection to the GC. Substrate was measured in the influent line and in the effluent of the reactor to be sure that any losses of MTBE observed in the effluent were a result of biodegradation and not due to losses in the influent line.

The pH (Model 720A pH meter, Orion Research Co., Boston, Massachusetts), dissolved oxygen (Corning Checkmate II, Corning, New York), and the suspended solids concentrations (Methleth 2540D; APHA et al., 1998) were determined weekly. Volatile fatty acids (acetic, propionic, and butyric acid) were also monitored weekly in the effluent by analysis on an Agilent 6890 GC/FID with a megabore HP-FFAP column for fatty acid detection (Agilent, Palo Alto, California).

Total iron and iron (II) analyses were performed using the phenanthroline method according to standard methods (Skoog et al., 2000) on a diode array UV-VIS spectrophotometer (Hewlett Packard, model 4852). Total iron and iron (II) was measured in both the influent and effluent of the reactor. Iron (III) was determined as the difference between the total iron and iron (II). Headspace of the reactors was also monitored weekly for carbon dioxide, nitrogen, oxygen, and methane using a HP GC 5890 with a thermal conductivity detector (TCD) equipped with two custom-built molecular sieve packed columns (Supelco, Bellefontane, Pennsylvania) placed in series and connected by a relay valve. This allowed for close monitoring of the gases both to ensure that the reactor remained anaerobic and to provide information about the carbon balance of the system.

Batch Studies. As mentioned above, the majority of the biomass from the porous-pot reactor was sacrificed on day 56 of operation for setting up the batch studies. Anaerobic batch serum bottles were prepared under iron-reducing conditions in 60-mL amber serum bottles with 40 mL anaerobic culture and 20 mL of anaerobic headspace. A total of 72 active bottles in each treatment group were prepared with an equal number of killed bottles to provide enough bottles for triplicate sampling on a monthly basis for one year. All of the bottles received 0.057 mM (5 mg/L) of MTBE, and half of the active bottles and half of the killed bottles also received 0.87 mM (40 mg/L) of ethanol (EtOH). Ferric iron was provided in the form of ferric chloride (FeCl₃) at 9.0 mM to all bottles in excess of the amount necessary for substrate utilization. Killed controls received 12.5 g/L mercuric chloride (HgCl₂).

Just before the batch test, the reactor was reseeded with an additional 100 mL of activated sludge from the MSD. The reactor was drained anoxically by applying pressure to the reactor with nitrogen (pre-purified grade) and forcing the biomass into a nitrogen purged buret and transferring it to an anaerobic glovebox. The glovebox was purged with 20 cycles with pre-purified nitrogen gas followed by 5 to 10 additional cycles of a nitrogen and carbon dioxide mix, with the final target headspace carbon dioxide concentration being the same as that of the reactor (approximately 5%). The glovebox was also equipped with a palladium catalyst (activated with 2 cycles of 1% hydrogen (H_2) before the final nitrogen carbon dioxide mix cycles), which served to scrub any residual oxygen present in the system. Bottles were sealed with 0.25-mm- (1-in.-) thick butyl rubber septa (Bellco Glass, Vineland, New Jersey) and aluminum crimp seals, and were stored upside-down to keep the septa moist and prevent gas leakage from the atmosphere.

Bottles were sacrificed one day after setup, followed by one month, and subsequently every other month. The total length of the batch studies was 380 days, with a total of eight sampling events. At the time of sacrifice, the headspace of the bottles was analyzed on the TCD for carbon dioxide, nitrogen, oxygen, and methane by piercing through the septa with a gas sampling syringe (Valco, Inc., Houston, Texas). This allowed us to confirm the absence of oxygen and methane in the bottles. Headspace was also monitored for gas phase MTBE and TBA on the GC/FID packed column. In the aqueous phase, the pH, dissolved oxygen, total organic carbon (TOC), VSS, and the total and ferric iron concentrations were analyzed at time of sacrifice. However, the iron measurements from the batch tests were found to be unreliable because of the small sample volume and the unavoidable presence of biomass in the samples (organic matter is known to interfere with the phenanthroline iron test).

Because the batch tests were set up on the 56th day of reactor operation, there is a 56-day differential for direct culture comparison between the activity of the porous-pot reactor and the batch study (day 1 for the batch study = day 56 for the reactor, etc.).

Spike Test. A spike test was performed on the iron-reducing batch bottles to confirm degradation of MTBE and to determine the rate. The spike test took place from day 197 to day 238 of the iron-reducing batch study. After MTBE was confirmed to be absent on the previous sampling event (day 189), the remaining active and killed-batch study bottles were all spiked anaerobically with 0.07 mM MTBE or 0.07 mM MTBE and 0.66 mM EtOH. All bottles also received an additional 9.0 mM (500 mg/L) of Fe(III). A total of 21 bottles were spiked in each treatment group. Bottles were sampled randomly in triplicate with time to obtain the degradation curves. Concentrations were monitored by removing sample through the septa (without bottle sacrifice) using a gastight syringe.

Denaturing Gradient Gel Electrophoresis. Denaturing gradient gel electrophoresis (DGGE) microbial community analysis was carried out the porous-pot reactor culture as described in Pruden et al. (2001) using universal polymerase chain reaction (PCR) primers first described in Muyzer et al. (1993). Sequences were aligned to closest matches and reference organisms using ClustalX (Thompson et al., 1997). Phylogenetic trees were constructed by maximum likelihood analysis with 100 bootstrap replicates using PAUP Star version b10 (Sinaur Associates, Sunderland, Massachusetts).

Results and Discussion

Performance of Iron-Reducing Porous-Pot Reactor. A plot of the performance of the iron-reducing reactor is shown in Figure 1. The MTBE removal in the iron-reducing reactor was first observed

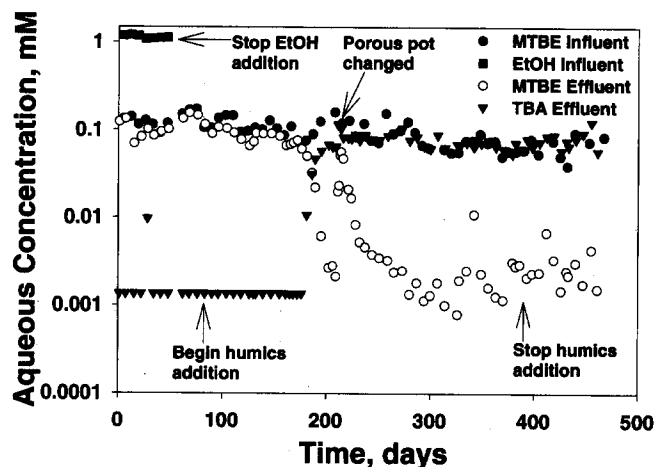


Figure 1—Performance of iron-reducing porous-pot reactor with time. Significant changes in operation or performance are noted.

on day 181 of operation and was maintained throughout the remainder of the study. The MTBE was converted to TBA, however, which was not observed to degrade further.

Shortly after MTBE removal was first observed, permeability of the porous pot became hindered such that biomass began to overflow from the pot and was detected in the effluent. Immediately upon this discovery, the reactor was drained anaerobically and a new pot was installed. The system recovered thereafter, and effluent MTBE returned to previous levels after one month. This incident provided a good indication of the resilience of the system to disturbance. The VSS and TSS were observed to increase steadily both before and after the porous pot changing, but declined slightly after the humics were removed from the feed, suggesting that humics may have increased the estimate of the VSS and TSS and also may have contributed to clogging of the system.

While MTBE degradation began during a period when humic acids were present in the feed, removal of humic acids had no apparent effect on MTBE degradation. However, considering the VSS and TSS data, it is likely that the porous pot was retaining humic acids. In this case, more than 200 days would be required for one full sludge age to pass and for the humic acids to wash out of the system. Therefore, because the reactor was maintained only 80 days after removing humic acids from the feed, it cannot be concluded from the reactor data whether or not the humic acids truly had an effect.

Iron(III) Utilization Associated With Methyl *tert*-Butyl Ether Degradation. A plot of the iron balance of the porous-pot reactor is shown in Figure 2 and confirms that iron-reducing conditions were established in the system. Effluent iron (II) was observed to be significantly higher than the influent iron (II), indicating that iron (II) was being produced as expected. While iron (II) production was initially unstable during startup and after EtOH was removed from the feed, it did stabilize after consistent MTBE removal was observed (Figure 2). A decrease in iron (II) production concurrent with the reactor upset on day 203 (porous pot change) was also observed and recovered as the MTBE removal recovered.

The average iron used by the system, in terms of the concentration of iron (II) observed in the effluent minus the iron (II) fed to the reactor during stable periods of MTBE removal, was

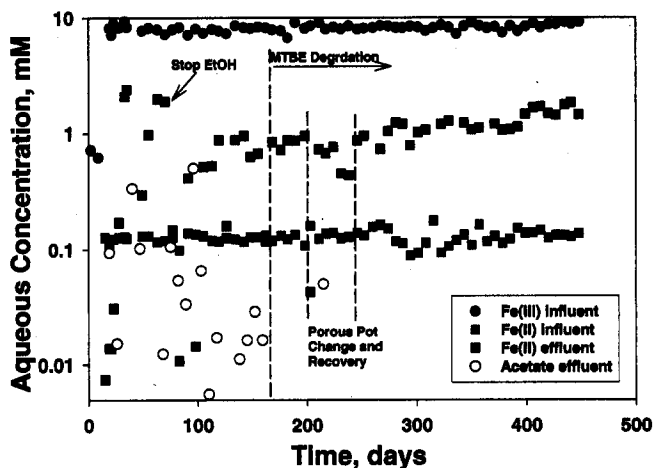
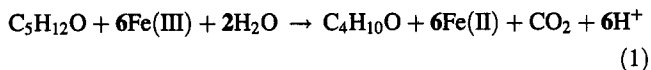
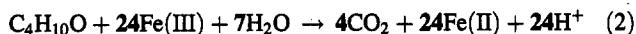


Figure 2—Plot of influent total iron and iron (II) and the effluent iron (II) of the iron-reducing porous-pot reactor. Acetate is also plotted as an indicator of instability during initial operation of the system. Changes in system operation and performance are indicated.

0.741 ± 0.178 mM. The following presents the proposed energy reaction, or the acceptor half reaction minus the donor half reaction ($R_e = R_a - R_d$), for conversion of MTBE to TBA.



If 0.11 mM of MTBE is initially present (the influent concentration of the iron-reducing reactor), then 0.66 mM of iron (III) are required, which corresponds to a production of 0.66 mM iron (II). The average stable iron (II) observed in the effluent is very close to this estimate, and is well within one standard deviation. If the conversion of the remaining TBA is assumed to take place in the same way, then the following represents the energy reaction for this transformation.



In this case, by the same reasoning, 2.64 mM iron (III) is still required for the complete mineralization of TBA. As the influent iron (III) was provided at an average of 7.9 ± 1.4 mM, ample iron (III) remained available for TBA degradation throughout the study. Both of these calculations are based solely on combining the donor (R_d) and acceptor (R_a) redox half reactions, and thus does not consider partitioning of electrons from the donor (MTBE) between the acceptor and the formation of new cells. As described in Rittmann and McCarty (2001), f_e is the fraction of electrons from the donor which are transferred to the electron acceptor, and f_s is the fraction incorporated to new biomass. Overall stoichiometry of substrate utilization is thus described as follows.

$$R = f_s R_c + f_e R_e - R_d \quad (3)$$

Where R_c , R_e , and R_d are the reduction half reactions for the cells, electron acceptor, and the electron donor, respectively. Considering that, in this case, the observed iron (III) utilization in the conversion of MTBE to TBA is stoichiometrically supported solely by $R_e - R_d$, this suggests that f_s can be approximated by 0 and f_e as 1. This is logical because f_s is analogous to the observed yield, which is known to be quite low for aerobic utilization of

MTBE (~0.1 to 0.2; Fortin et al., 2001), and thus is likely to be even lower for anaerobic MTBE degradation. Unfortunately, we were not able to estimate observed yield based on culture growth because the iron and humics interfered with accurate VSS measurements.

Though it is observed that, on average, more iron (II) was produced during initial operation with an influent of 1.1 mM EtOH (as would be expected), there is not sufficient stable data to determine the stoichiometry of EtOH degradation by iron reduction. It is also likely that iron reducers were not the sole-dominating microorganisms during startup because the reactor was not seeded specifically with an iron-reducing culture. Acetate was noted to be present at unstable levels in the effluent throughout the first 189 days of operation (Figure 2), and propionate during the first 30 days (data not shown), indicating the presence of fermenters in the system. High concentrations of acetate were observed to correlate with reactor upset and low iron (II) effluent concentrations, indicating a possible competition between iron reducers and fermenters (Figure 2). Such upsets were observed during reactor startup and also when the porous pot was changed. Other than this upset, the last day that acetate was detectable in the effluent was on day 189, at the same time MTBE was first noted to start degrading in the system. Thereafter, the acetate and propionate in the effluent remained below detection during stable MTBE removal. Utilization of fermentation byproducts, such as acetate, may thus account for the unstable iron utilization by the system before MTBE degradation began. Therefore, iron-utilization calculations only included data during times of stable removal of MTBE and no detectable production of acetate.

Iron-Reducing Batch Study. In the batch study, strict anaerobic conditions could be ensured, and the risk of reactor maintenance (such as cleaning of the porous pot) exposing the culture to oxygen was eliminated. Also, conducting the study under batch conditions allowed for close monitoring of culture activity and carbon balance.

Simultaneous to the commencement of MTBE to TBA conversion in the iron-reducing porous-pot reactor, conversion of MTBE to TBA was also observed in both the MTBE and MTBE/EtOH batch study. This is shown in Figure 3 on sampling day 133, which corresponds to day 188 of the porous-pot reactor. Therefore, approximately the same acclimation period was observed for the cultures having the same origin in both the batch and continuous-flow systems. As was the case in the reactor, no evidence of TBA degradation was observed in the batch study. The average TBA remaining at the end of the study was approximately equimolar with the MTBE that had been added. This was confirmed based on the total moles of MTBE present in both the aqueous phase (Figure 3) and the gas phase (data not shown) of the kills, compared with the moles of TBA in the active bottles in both phases. Mormile et al. (1994) also observed a resistance of TBA to anaerobic degradation, while more recently Bradley et al. (2002) specifically described TBA to be recalcitrant under iron-reducing conditions. As demonstrated in the present study, other alcohols, such as ethanol, are readily degradable anaerobically. This suggests that the *tert*-butyl group may be the responsible factor for inhibiting degradation.

The presence of EtOH did not appear to have any effect on the degradation of MTBE, though EtOH had degraded to completion in the iron-reducing porous-pot reactor by the time of the second sampling event (day 22). Humic substances were never present in the iron-reducing batch studies, which may provide a better indicator than the reactor data that they were not necessary to stimulate iron reduction.

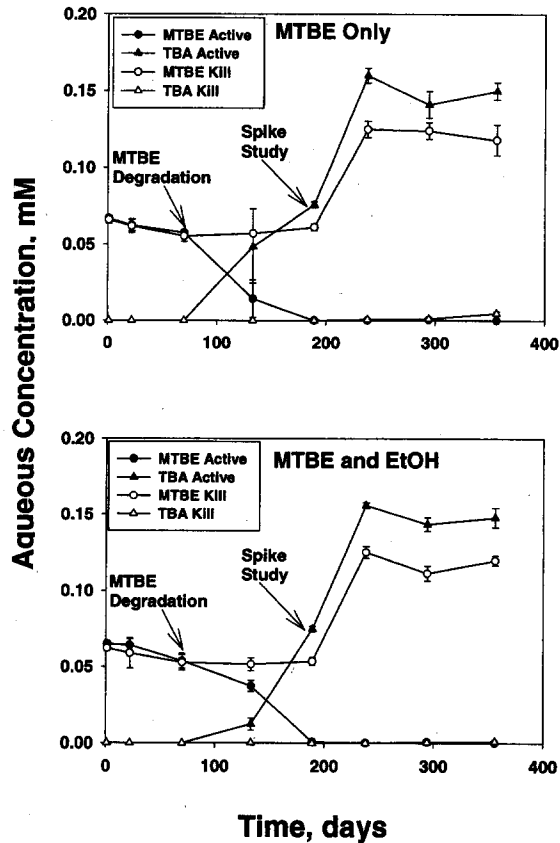


Figure 3—Anaerobic batch MTBE and TBA plots for the MTBE and MTBE-and-EtOH experiments under iron-reducing conditions. The EtOH is not shown.

Iron-Reducing Spike Test. The plots obtained from the spike study are presented in Figure 4, with the days corresponding to those for the batch study (Figure 3). Ethanol was observed to degrade spontaneously, without a lag period. The MTBE exhibited a degradation lag in both the bottles with and without EtOH, but eventually converted completely to TBA. The final MTBE concentrations were in the range of 11 to 23 μM (10 to 20 $\mu\text{g/L}$). The degradation rates obtained from this study and the observed degradation lags are reported in Table 1. Rates were determined as the slope of the linear portion of the degradation curve, as estimated using the linear regression tool in Sigma Plot 8.0 (SPSS, Inc., Chicago, Illinois). The lag was determined as the x -axis coordinate (time) of the intersection of the linear regression line with the corresponding y -value of the initial concentration point. The rate was normalized to the initial VSS concentration, which was measured at the beginning of the study with four replicates ($977 \pm 10 \text{ mg/L}$). The ethanol rates determined from this study were approximately 33 times that of the MTBE rates. The rate of MTBE degradation was the same for both the studies with and without ethanol, but there is some indication that ethanol may have had the effect of decreasing the lag period before MTBE degradation. The lag time was almost twice as long without ethanol ($142 \pm 5.7 \text{ h}$) than with ethanol ($80 \pm 1.2 \text{ h}$). The VSS normalized rates are all considerably lower than aerobic rates reported in the literature (Fortin et al., 2001; Hanson et al., 1999; Sedran et al., 2002; Wilson et al., 2002).

Characterization of the Iron-Reducing Culture. Microbial community analysis of the iron-reducing reactor culture was done

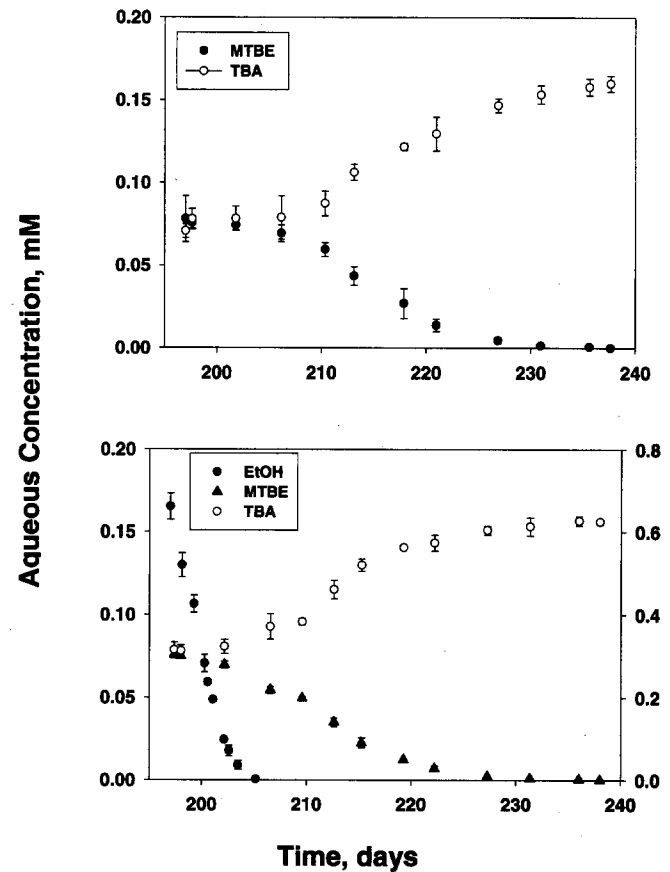


Figure 4—Degradation plots obtained from respiking iron-reducing serum bottles. Top: MTBE only spiked at 0.07 mM; Bottom: MTBE spiked at 0.07 mM and EtOH spiked at 0.66 mM. Days correspond to days in the batch study (Figure 3).

using DGGE. DNA was extracted from the iron-reducing reactor on a monthly basis after the first observation of MTBE degradation. Unfortunately, obtaining PCR products was especially challenging, given that humic substances are known PCR inhibitors, in addition to other inhibitors commonly present in anaerobic systems. Five weak PCR products were obtained from the reactor culture and were resolved on a DGGE gel. Because of the weak PCR products, the banding patterns were too faint to be able to identify definitive changes in the culture with time according to changes in the banding

Table 1—Rates of degradation, iron-reducing batch spike study.

	mmol/day ⁻¹	VSS norm (mmol/min-kgVSS)	Lag Time (hours)
MTBE (no EtOH)	$1.32 \pm 0.05 \times 10^{-4}$	$2.36 \pm 0.10 \times 10^{-4}$	142 ± 5.7
MTBE (with EtOH)	$1.30 \pm 0.02 \times 10^{-4}$	$2.32 \pm 0.03 \times 10^{-4}$	80 ± 1.2
EtOH	$4.37 \pm 0.04 \times 10^{-3}$	$7.76 \pm 0.08 \times 10^{-3}$	0 ± 0.02

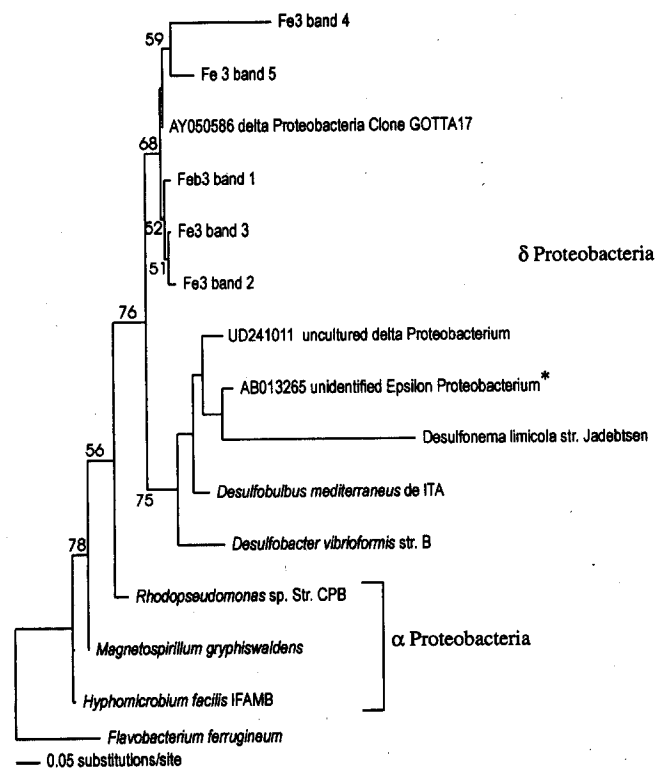


Figure 5—Phylogenetic tree of DGGE band sequences obtained from the iron-reducing reactor. Sequences obtained in this study are labeled Fe3 band 1 to Fe 3 band 5. Tree was constructed by maximum likelihood analysis, with bootstrap values determined with 100 replicates and are reported for major nodes where values exceeded 50, using Paup Star v. b10. *Flavobacterium ferrugineum* is the outgroup. Major Bacterial divisions are indicated with brackets. An asterisk (*) is currently classified as an ϵ Proteobacterium.

pattern. The net effect is that the detection limit of DGGE in this study would be above the 1% of the total community that has been demonstrated previously (Brüggenmann et al., 2000). Often, these detectable microbes present in the community at 1% or greater are referred to as "dominant" microbes. Therefore, the microbes detected in this study would certainly be dominant in the community, though it cannot be claimed that all members of the reactor culture above 1% were detected.

Five total unique bands could be visualized in the SybrGold (Molecular Probes, Inc., Eugene, Oregon) stained DGGE gel when exposed to UV light. These were excised from the gel, reamplified, and sequenced. All bands yielded unambiguous DNA sequence data but did not closely match any known cultured microorganisms. All five sequences were submitted to GenBank under the following accession numbers: AY140533–AY140536. A 98% match was found with several uncultured δ -Proteobacteria associated with chlorobenzene (GenBank No.: AY050586; Alfreider et al., 2002), trichloroethylene (GenBank No.: AY133065 and AF529133; Carroll and Zinder, 2002) and dihaloethane (Genbank No.: AY122597; Reiss and Guerra, 2002) degradation. All five of the isolated DGGE bands showed above 90% similarity to these microorganisms, which are most closely associated with the sulfate-reducing bacteria of this subclass (*Desulfocapsa*, *Desulfobulbus*). The δ -Proteobacteria are

known to contain several well-studied iron-reducing bacteria of environmental significance within the family *Geobacteraceae*, which includes several genera capable of iron-reduction: *Desulfuromonas*, *Desulfuromusa*, *Pelobacter*, and *Geobacter* (Lovely, 2002).

Confirmation that the microorganisms identified by DGGE in this study are indeed iron-reducers capable of MTBE degradation would require isolation in pure culture, but this initial identification will allow for comparison with similar strains found by other researchers. A phylogenetic tree including the DGGE bands identified in this study and their closest matches is presented in Figure 5. This data represents the first attempt to characterize an iron-reducing reactor culture actively degrading MTBE.

Conclusions

Conversion of MTBE to TBA under iron-reducing conditions was observed in batch and continuous-flow systems. This is the first known report of MTBE degradation under iron-reducing conditions in a continuous-flow culture. Rates of MTBE biodegradation via iron reduction were determined and found to be significantly lower than rates obtained in aerobic studies. Also, according to stoichiometry, the yield is estimated to be even lower than what has typically been observed in aerobic cultures ($\ll 0.1$ versus 0.1 to 0.2). Dominant, identifiable microorganisms present in the system were found belong to the δ -Proteobacteria.

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