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Chapter 17

Applying Co-Metabolic Biological Reactions for the Ex-Situ Treatment of Methyl *tert*-Butyl Ether Contaminated Groundwater

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Abstract

Methyl *tert*-butyl ether (MTBE) has become a widespread ground water contaminant in the United States. The rapidly increasing impact on public drinking water supplies has created an urgent need to develop an economical treatment technology that can treat large volumes of water to low MTBE concentrations. Prior research has shown that MTBE can be degraded by bacteria either as a carbon and energy source or co-metabolically. In this paper, fluidized-bed bioreactors treating MTBE contaminated ground water were examined to determine the mechanism (growth based or co-metabolic) by which MTBE was biodegraded. It was determined that the predominant mechanism was a co-metabolic degradation process. Further investigation revealed that fatty acids (lactate and acetate) and alkanes (*iso*-pentane) could serve as co-metabolites for stimulating MTBE biodegradation. Field and laboratory testing indicates that *iso*-pentane is the superior co-metabolite for stimulating MTBE removal, because of it's selectivity for MTBE degraders.

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Introduction

Approximately 70% of all gasoline in the United States contains methyl *tert*-butyl ether (MTBE) and, as a consequence, MTBE has become a widespread ground water contaminant (*1, 2, 3, 4*). Only selected individual compounds such as *iso*-pentane (*ca.* 12%) toluene (*ca.* 8%), 2-methyl pentane (ca. 5%), m-xylene (*ca.* 4 %) and *n*-pentane (*ca.* 3%) approach the concentrations of MTBE (15 %) found in reformulated gasoline. Unlike the natural components of gasoline, MTBE can migrate long distances in ground water (*1, 4*).

Ground water contamination problems are especially of concern in California and other Western states where oxygenated fuels are used extensively and the population relies heavily on ground water for their drinking water. California is responsible for 25% of the global MTBE consumption, which is largely utilized in gasoline (*5*). A survey published in 1998 demonstrated that MTBE contamination is now found at the majority of leaking underground fuel tank sites in California (*1*). MTBE has been detected in municipal drinking water supplies throughout California and in some cases supply wells have been closed due to high levels of MTBE contamination (*1, 4*). MTBE contamination of ground and surface water has created a political up-roar in California (*6*). The level of contamination is considered of great enough concern that the Governor of California has issued an executive order restricting the use of MTBE in gasoline. MTBE use is to be completely banned in California by the end of 2002 (*7*). However, even if MTBE was eliminated from gasoline immediately, there will remain many sites in California alone that will potentially require *in-situ* or *ex-situ* remediation (*1*).

Biological treatment of MTBE contaminated ground water has only recently been considered as a potentially applicable technology. Several investigators have been able to maintain MTBE biodegrading treatment systems in the laboratory (*8, 9, 10, 11*). These laboratory reactors are typically fed MTBE as the primary or sole carbon source. In all cases the reactors exhibited slow biomass accumulation, were difficult to start, and are generally unstable, being easily subject to a loss of MTBE treatment efficiency. Similar problems have been found to plague reactors used in the field.

The most complete study available on the biological treatment of MTBE in a complex waste stream is a study in which use of a suspended growth reactor was compared to a fixed-film reactor for the treatment of tank-water spiked with MTBE (*12*). Suspended growth reactors could be used for MTBE removal, but it was concluded that fixed-film reactors were more efficient at retaining the slow growing MTBE degrading population (*12*). Due to the slow growth and low yield of MTBE degraders, it is generally agreed that fixed-film systems, such as fluidized-bed reactors, should be more practical for MTBE treatment in the field.

Full-scale biological treatment of MTBE contaminated ground water has been reported (*13, 14, 15*). Two parallel fluidized-bed reactors with a combined design capacity of 540 gpm were installed at a fuel transfer terminal in Nevada to remove benzene, toluene, ethyl-benzene, and xylenes (BTEX compounds) from gasoline contaminated ground water. The reactors began to remove MTBE after approximately 200 days of operation (*15, 14*). It was demonstrated that MTBE removal was the result of a combination of physical sorption and biodegradation, but that biological removal rates could account for the majority of MTBE treatment in the system (*16*). The system in Nevada has been treating MTBE contaminated groundwater for almost four years (*13, 14*).

Bacterial cultures capable of degrading MTBE have been described only in the last few years. Salanitro *et al.* (*11*) reported that they were able to maintain an MTBE degrading culture in a suspended growth reactor. These bacteria were shown to grow on MTBE as a sole carbon source, exhibited a slow growth rate (<0.01 day^{-1}), and were able to oxidize MTBE to CO_2 with the transitory production of *tert*-butyl alcohol (TBA) as a measurable intermediate. Bacteria capable of growth on MTBE as a sole carbon source have now been grown in a number of laboratories (*9, 8, 17*).

All cultures that grow on MTBE are characterized by slow growth rates and low cell yields (*8, 11, 17*). These characteristics are not optimal for the development of full-scale biological systems treating low influent concentrations and high flows, as is needed in most ground water treatment systems. There is also very little information available concerning how bacteria capable of growing on MTBE as a sole carbon source will respond in systems receiving other compounds in addition to MTBE. MTBE contaminated ground water may also be contaminated with gasoline, alcohols, and other more easily degraded carbon sources, as well as potentially toxic substances such as chlorinated solvents. Even in fixed film systems, slow growing organisms must compete with faster growing organisms, such as toluene degraders, for nutrients, oxygen, and space. Slow growing organisms will also be more susceptible to wash-out, as influences such as shear forces and iron deposition limit biofilm growth, attachment, and cell retention time. This competition may partially explain why MTBE degrading bioreactors are difficult to maintain in the field.

It is now well established that enzymes found in many microorganisms can act upon a broad range of substrates, in a non-specific manner not typically found in higher organisms. If the substrate transformed by the enzyme does not become a useful product that can serve as an resource for the organism and promote growth, the transformation is termed "co-metabolic." Classic examples of co-metabolic transformations that are useful in *in-situ* and *ex-situ* biological treatment include trichloroethylene transformations by toluene degraders and the transformation of benzo[a]pyrene and other high molecular weight polynuclear aromatic hydrocarbons by phenanthrene degraders (*18, 19, 20*).

It has been determined that MTBE can be degraded by microorganisms co-metabolically. Steffan *et al.* (*21*) reported that MTBE could be degraded by camphor degraders and propane oxidizing bacteria that could not use MTBE as a growth substrate. Propane oxidizers were considered the most efficient MTBE degraders and were found to metabolize MTBE to TBA and other intermediates. Hardison *et al.* (*22*) demonstrated that diethyl ether degrading fungi could also co-metabolize MTBE.

It has been shown that MTBE co-metabolism is a common characteristic of alkane degrading bacteria (*23, 24, 25*). Bacteria able to grow on *iso*-butane and *iso*-pentane, as well as propane and *n*-pentane, are active for MTBE degradation. There is evidence that MTBE is oxidized by the same oxygenase enzymes that are expressed by microorganisms to initiate growth-related oxidation of alkanes (*21, 23, 24, 25*). During the oxidation of MTBE, there is the production of an initial stable oxidation product, TBA. As is found with organisms that grow on MTBE, TBA is a transitory intermediate that is further degraded with time.

The objective of the research presented here was to determine the mechanism by which MTBE was being degraded in full-scale fluidized bed bioreactors treating MTBE contaminated ground water. Once the mechanism was determined, field and laboratory tests were conducted to evaluate improved techniques for MTBE biological treatment.

Methods

Biological reactors included in this study are up-flow, fluidized bed reactors which use granular activated carbon (GAC) for their bed material. The reactor at the California site (approximately 2,000 L) and the laboratory reactors (approximately 4 L) are essentially identical to the reactors used by Tang and Sun (*12*). At the Nevada site, there are two full-scale (10,000 L) reactors operating in parallel. Both the laboratory and field reactors are manufactured by Envirex/U. S. Filter (Waukesha, WI).

Bioreactors at both field sites receive MTBE contaminated ground water without pretreatment (*14, 13*). No additional MTBE is added to the reactors, but the ground water is supplemented with nitrogen and phosphorous to stimulate biological treatment. Laboratory bioreactors receive a synthetic feed containing gasoline and MTBE, supplemented with inorganic nutrients.

Samples of biologically active materials (GAC and floc) were collected from the fluidized-bed bioreactors located at the two field sites and shipped overnight on ice to the laboratory. These materials were used in MTBE up-take experiments and as inoculum for enrichment cultures.

Enrichment cultures were selected by inoculating field samples into Erlenmeyer flasks containing mineral salts media and the compound of interest.

Mineral salts medium was made by combining 1 g KH_2PO_4, 0.86 g Na_2HPO_4, 1 g NH_4Cl, 0.06 g $MgSO_4$, 0.06 g $CaCl_2$-$2H_2O$ and 1 mL trace metal solution in 1 liter of distilled-deionized water. Trace metals solution was made by combining 3.3 mg $MnSO_4$-H_2O, 6.2 mg $CuSO_4$-$5H_2O$, 7.6 mg $ZnSO_4$-$7H_2O$, 11.7 mg Na_2MoO_4-$2H_2O$, and 64.6 mg $FeSO_4$-$7H_2O$ in 1 liter of 0.05 N HCl.

MTBE enrichments were made at high MTBE concentrations, where MTBE was added as a vapor in constant excess, and at low concentrations where MTBE was added at less than 10 mg/L. MTBE degrading organisms were isolated on mineral media agar in an MTBE atmosphere and purified on R2A agar in a MTBE atmosphere. Culture purity was also confirmed on R2A agar without MTBE. Cultures were identified using FAME analysis (MIDI Inc., Newark, DE).

MTBE up-take experiments were conducted by combining 1.5 grams of bed material (either floc or GAC), mineral salts media, and MTBE in a 40 mL vial fitted with a Mininert™ valve. MTBE removal over time was measured by headspace analysis using GC/FID. Activity measurements on MTBE degrading cultures were conducted in a similar manner.

Results and Discussion

The original hypothesis of this project was that organisms able to grow on MTBE as a sole carbon and energy source were responsible for MTBE treatment in fluidized-bed bioreactors. The major piece of evidence supporting this argument was the extended period of time (over 200 days) it took for MTBE removal to begin in the reactor at the Nevada site (*15, 14*).

Enrichment cultures were started using MTBE as a sole carbon and energy source. After an extended period, some of the enrichments at high MTBE concentrations became slightly cloudy and others developed a waxy culture of bacteria at the air-water interface (dubbed "White-top"). These enrichments were plated on mineral salts agar in an MTBE atmosphere for isolation. It was found that many organisms from these enrichments were able to grow in trace organic compounds in the agar, and it was necessary to further screen the isolated organisms for MTBE removal in an MTBE up-take experiment. In the up-take experiments, it was found that several organisms exhibited growth (an increase in turbidity) without demonstrating significant MTBE degradation. Three strains of bacteria and a fungus were found to be able to degrade MTBE in liquid culture (Table 1). Two of the isolates (strains 18a and 35) were identified as *Nocardioides luteus* and *Rhodococcus erythropolis*. The "White-top" strain and the fungus could not be identified by FAME or Biolog™ analysis, however "White-top" was clearly another nocardioform.

Table 1: Screening of isolated cultures for the ability to degrade MTBE in liquid media. Percent removals in bold were significantly greater than controls.

Culture	*Turbidity*	*% Removal*
White-top	+++	**51.2**
18a	+++	**17.9**
Fungus	++	**12.5**
35	++	**11.3**
41	+	5.2
23	++	5.0
27	++	4.9
40	++	4.8
37	++	4.3
11	+	4.1
1	++	3.7
22	+	2.7
7	+++	2.1
28	+	1.3
30	+++	0.4
3a	++	-0.1
31	++	-1.2
29	+	-3.7
45	+	-4.4

The growth of bacteria on MTBE was poor. In a typical incubation, the organisms exhibited no growth or MTBE degradation for over a week and then would begin to degrade MTBE over an extended period that could last an additional 20 days or more (Figure 1). In many cases, MTBE enrichments would not maintain MTBE degrading activity after multiple transfers. Growth on MTBE gave extremely low yields of bacterial biomass. These results were consistent with results obtained by Salanitro (*11, 26*).

The poor growth, low activity, and instability of the MTBE degrading cultures raised several issues concerning the relative importance of growth based transformation processes in MTBE degrading fluidized-bed bioreactors. It was also apparent that trying to develop novel process control strategies for MTBE treatment would be difficult if the treatment were solely dependent on growth based processes.

It was concluded that organisms able to grow on MTBE as a sole carbon source did exist in the Nevada bioreactors, but it seemed unlikely that a growth based mechanism could account for all of the activity observed in the field. An alternative hypothesis, that co-metabolic MTBE degradation was the predominate mechanism for MTBE treatment in the Nevada bioreactors, was formulated and tested.

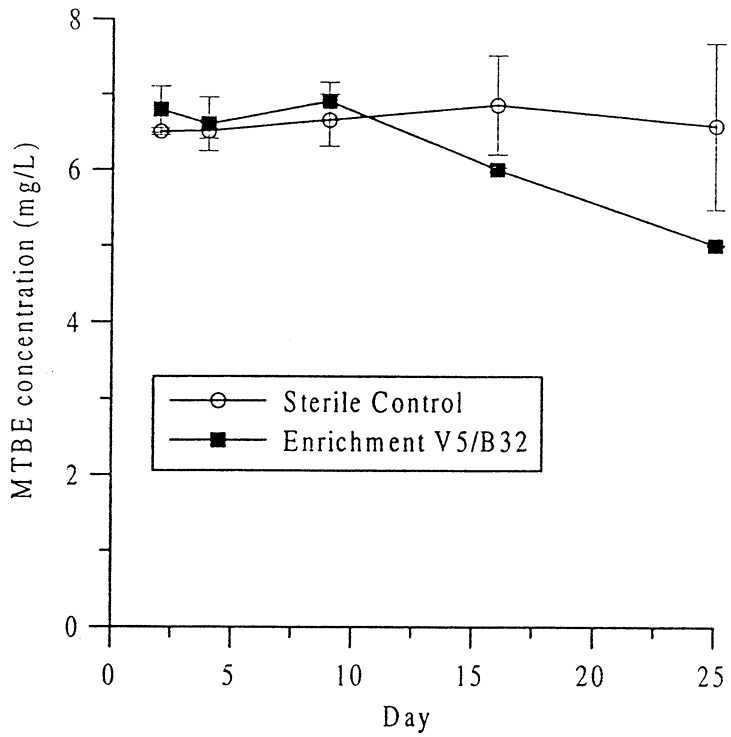

Figure 1: Degradation of MTBE by an MTBE enrichment culture. Mean plotted with error bars of one standard deviation

Ground water being treated by bioreactors at the field sites in this study is characterized by a diverse organic carbon content. In addition to MTBE, the ground water can contain benzene, toluene, ethyl-benzene, xylenes, and other petroleum hydrocarbons such as alkanes (*14, 13*). At the sites included in these studies, there are sufficient alternative carbon and energy sources to support the co-metabolism of MTBE.

A kinetic evaluation was made of samples taken from fluidized-bed reactors exhibiting good MTBE treatment efficiency (Figure 2). Fitting the Michaelis-Menten kinetic model to the data reveals that the K_m for MTBE degradation was approximately 60 mg/L. This number is surprisingly high and is much more consistent with a co-metabolic mechanism than a growth base mechanism.

Further evidence that a co-metabolic mechanism is the dominant mechanism for MTBE removal in the field reactors was obtained by directly testing samples of bed material for their ability to maintain MTBE degrading activity when repeatedly supplemented with MTBE. In one experiment, MTBE was added to bed material in a standard batch degradation assay and MTBE degradation was monitored over time. When MTBE concentration went below 0.1 mg/L, the vial was re-aerated, more MTBE was added to the culture, and MTBE removal was again followed. Results from this experiment are presented in Figure 3. As one can see, the MTBE biodegradation potential of this culture declined over time as the culture was re-supplied with MTBE. The data indicate that MTBE is not

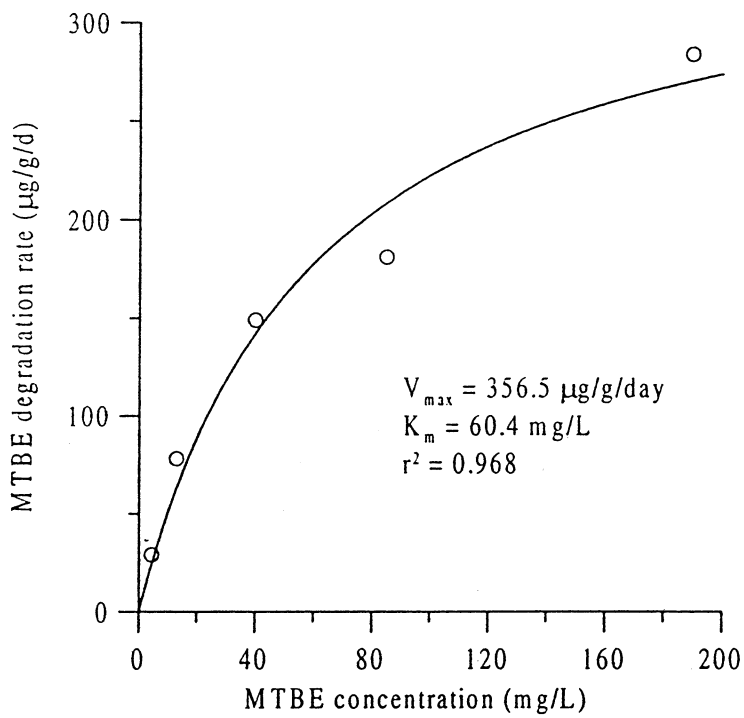

Figure 2: MTBE degradation kinetics exhibited by bed material taken from a fluidized bed bioreactor treating gasoline contaminated ground water

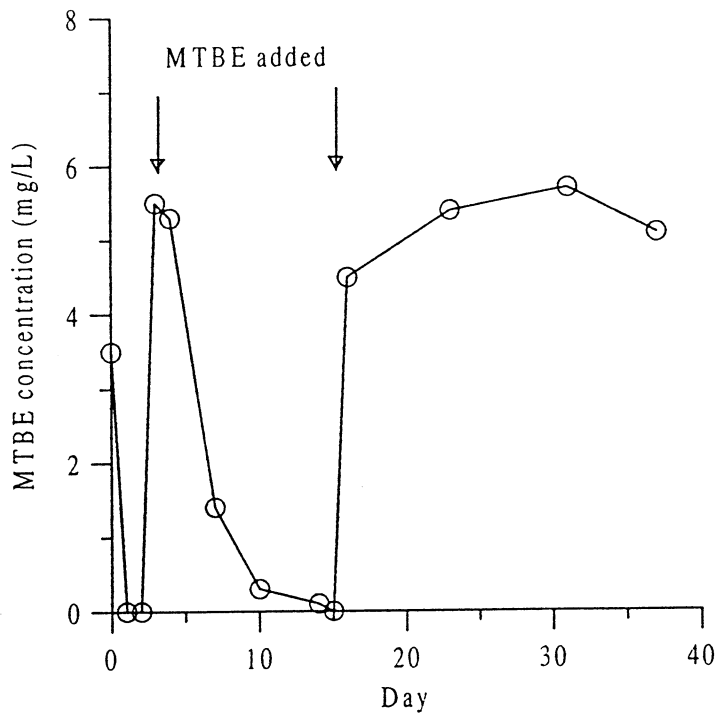

Figure 3: MTBE biodegradation activity declines over time when MTBE is supplied as a lone supplement

serving as an efficient energy source for this culture, as would be expected if MTBE degradation were a co-metabolic process. These results can be contrasted to those of Hanson *et al.* (*17*), where bacteria that grow on MTBE were able to maintain activity for an indefinite period.

It appeared from the evidence that co-metabolic biodegradation was the dominant mechanism for MTBE treatment in the field. With this realization, the focus of this research shifted to determining what supplemental carbon sources could serve as co-metabolites for MTBE degradation. Two approaches were taken. In one approach, bacteria were enriched from the field reactors on different carbon sources and then tested for their ability to degrade MTBE. In the second approach, bed material that had lost MTBE degradation activity was tested for MTBE degradation with and without an additional carbon source, to determine if the addition of the carbon source stimulated MTBE removal. A number of carbon substrates were tried as both supplements and as enrichment media, including toluene, p-xylene, methanol, *tert*-butyl alcohol, hexane, *iso*-pentane, lactate, acetate, glycerol, and glucose.

Enrichment cultures grown on *iso*-pentane consistently demonstrated MTBE degradation activity (Figure 4). MTBE degradation appears to be a constant characteristic of *iso*-pentane degraders. This result is consistent with the results of experiments conducted by others (*23, 25*).

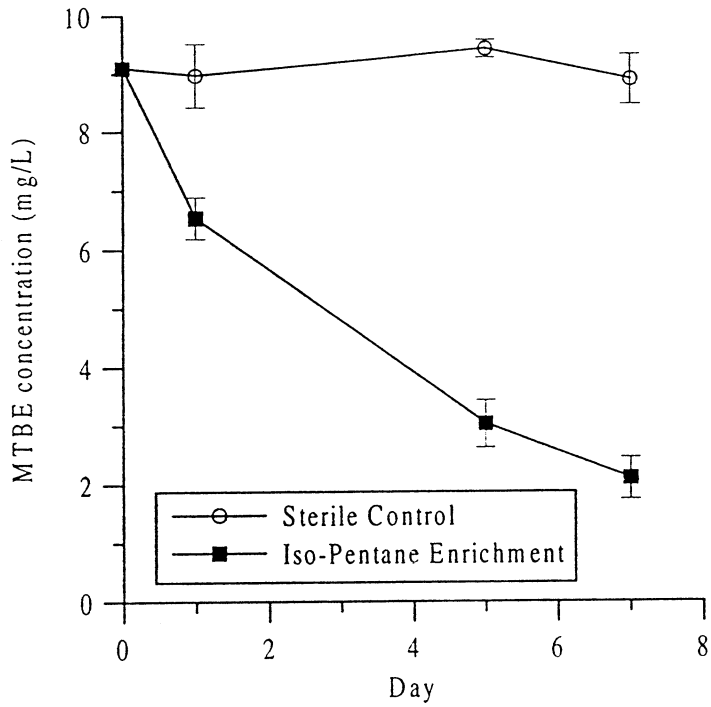

Figure 4: Degradation of MTBE by an iso-pentane enrichment. Mean plotted with error bars of one standard deviation

In the stimulation experiments, the fatty acids lactate and acetate were found to restore or enhance MTBE removal by bed material. The stimulation of MTBE removal by fatty acids was a surprising result, given that other general growth substrates, such as glycerol and glucose, did not stimulate MTBE degradation. Laboratory experiments were conducted with lactate under a variety of conditions. The use of lactate in combination with ferrous iron addition was particularly effective for stimulating MTBE removal. The combination of lactate and reduced iron is typically used for the cultivation of iron-bacteria, which are part of the microbial community in the fluidized bed bioreactors.

A field test was conducted in Nevada to determine if lactate could be used to stimulate MTBE treatment efficiency in a full-scale reactor. Lactate was added continuously at an average concentration of 20 mg/L to one reactor for a 21 day period. The MTBE treatment efficiency of the treated reactor was compared to the (control) reactor that did not receive lactate. The reactor that received lactate demonstrated a stimulation of MTBE removal efficiency in comparison to the reactor that did not receive lactate (Figure 5). However, the stimulation of MTBE removal by lactate addition was only transitory under these conditions. It is believed that lactate is not sufficiently selective for MTBE degraders and that, as lactate addition continues, MTBE degrading bacteria are over-grown by other microorganisms that can also use lactate, but do not

degrade MTBE. Further research on the use of lactate as a stimulant of MTBE degradation is focusing on the use of lactate in combination with *iso*-pentane and other alkanes.

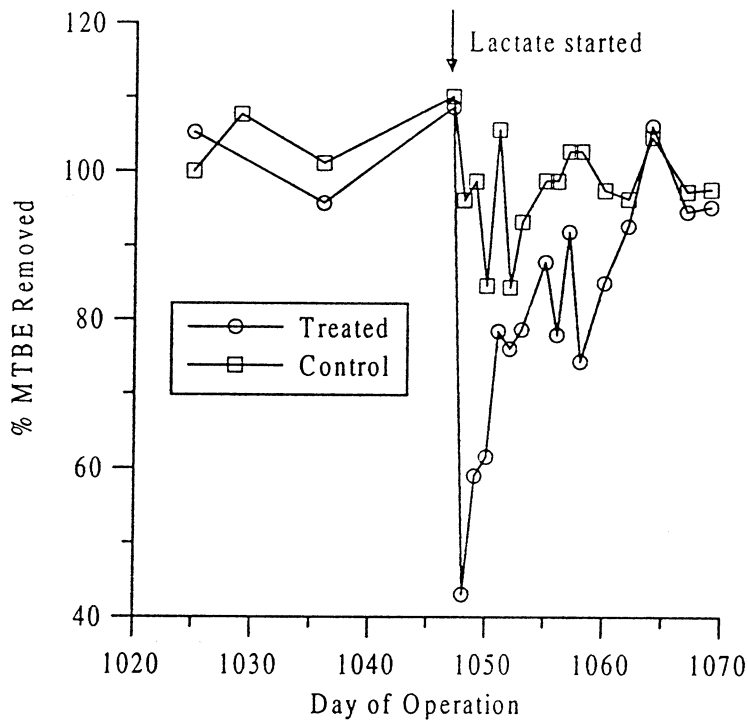

Figure 5. Lactate stimulation of MTBE removal efficiency in a full-scale reactor. Lactate addition caused a transitory improvement in MTBE treatment.

Experiments and field tests are being conducted to examine the use of *iso*-pentane as a co-substrate for MTBE degradation. Tests in laboratory reactors have shown that *iso*-pentane addition can stimulate MTBE removal (Figure 6). The impact of *iso*-pentane stimulation is greatest in poor performing reactors and is dose dependent (data not shown). Field tests of *iso*-pentane as a stimulant for MTBE biodegradation are being conducted.

Summary and Conclusions

Laboratory and field data support the argument that the primary mechanism for MTBE removal in fluidized-bed reactors treating contaminated groundwater containing gasoline hydrocarbons will be co-metabolic biodegradation. Gasoline range alkanes, particularly *iso*-pentane, can serve as reliable co-substrates for the stimulation of MTBE biodegradation. Other substrates, specifically lactate and acetate, can also stimulate MTBE degradation. However, application of lactate

as a co-substrate under field conditions is problematic, because lactate does not specifically enrich for MTBE degraders. Future research will focus on the use on *iso*-pentane as a co-substrate under field conditions and the use of lactate in combination with alkanes.

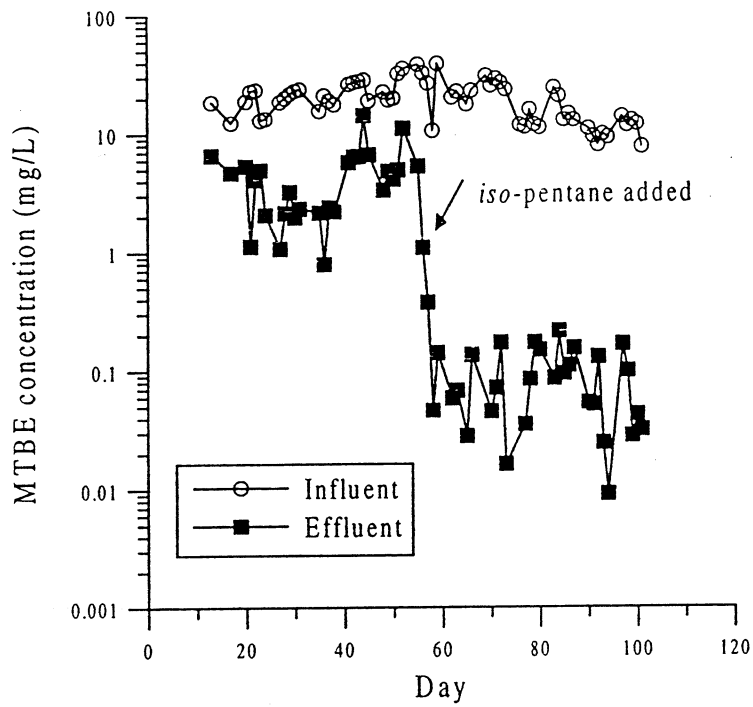

Figure 6: Iso-pentane stimulation test in laboratory reactor. Iso-pentane was added as a slug dose over two days.

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