Initiation of MTBE Biotreatment in Fluidized-Bed Bioreactors

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Abstract: Methyl *tert*-butyl ether (MTBE) is one of the most common ground water pollutants in the United States. Although MTBE has been characterized as a recalcitrant pollutant, it is now established that MTBE is biodegradable. A few bacteria that can grow on MTBE as a carbon and energy source have been identified and a host of bacteria that can cometabolize MTBE are known. There is very little information available concerning the biological treatment of MTBE contaminated ground water, despite the strong interest in applying biological treatment to the decontamination of MTBE laden water. In this paper we examine the treatment of contaminated ground water using a fluidized-bed bioreactor. Field studies demonstrated that the initiation of MTBE biotreatment was unpredictable, with one reactor starting to degrade MTBE immediately and a second reactor never degrading any MTBE. Laboratory studies were conducted to determine if a cosubstrate could be used to reliably enrich MTBE metabolizing microorganisms from a variety of environmental samples. It was determined that a number of compounds could enrich MTBE degrading populations, but that *iso*-pentane was the most reliable cometabolite of the compounds tested. *Iso*-pentane was used to initiate MTBE biotreatment in a laboratory fluidized-bed bioreactor. It was found that MTBE biotreatment continues even after *iso*-pentane addition was halted, suggesting that bacteria can gain maintenance energy from MTBE degradation. The reactor started with *iso*-pentane was as efficient as MTBE biotreatment as a reactor that started MTBE degradation without cosubstrate addition.

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Introduction

Methyl *tert*-butyl ether (MTBE) is now one of the most common groundwater pollutants in the United States (Squillace et al. 1996; 1999). Methyl-*tert*-butyl ether is added intentionally to gasoline to promote efficient combustion and may also be found incidentally in other petroleum products, such as jet fuel, heating oil, and diesel fuel, probably as a result of contamination during transport (Emelyanov et al. 1991; Robbins et al. 2000). MTBE plumes are commonly associated with gasoline contamination, but MTBE plumes have also been reported in groundwater contaminated with commercial jet fuel (Kang et al. 1999). Concern over the environmental recalcitrance of MTBE has escalated and the U. S. Environmental Protection Agency (USEPA) has issued a drinking water advisory for MTBE of 20–40 μg/L (Squillace et al. 1997; USEPA 1997, 1998). Many states are now instituting treatment requirements that may be as low as 5 μg/L (Lovett 1998; Martin 2001).

MTBE contamination has complicated the remediation of gasoline contaminated sites (Dernbach 2000). Water pumped from underground (as part of hydraulic containment or pump and treat actions) typically must be treated before discharge or reinjection. Biological treatment is a widely used technology for the removal of petroleum hydrocarbons from contaminated water (Voice et al. 1992) and there is a growing interest in applying biological treatment technology to MTBE remediation (Stocking et al. 2000). However, the efficiency and reliability of MTBE biological treatment is not well documented.

Several investigators have been able to maintain MTBE biodegrading treatment systems in the laboratory (Salanitro et al. 1994; Fortin and Deshusses 1999a,b; Acuna-Askar et al. 2000; Wilson et al. 2000; Kharoune et al. 2001; Pruden et al. 2001; Stringfellow 2002). In many cases, the reactors were difficult to start-up and were generally unstable, being easily subject to "upset" or loss of MTBE treatment efficiency. In one case, initiation of MTBE degradation was stimulated by the one time addition of a peat extract (Fortin and Deshusses 1999a). The applicability of laboratory results to field applications is not clear because most studies do not directly compare laboratory and field results (Stringfellow 2002).

Although there are several contaminated sites using biological treatment for the removal of MTBE from groundwater (R. Hines, personal communication 2000; K. O'Rielly, personal communication 2001), there is little published information on the subject. Tang and Sun (1997) conducted a study of the biological treatment of MTBE under field conditions using a complex waste stream generated as part of operations at a petroleum transfer terminal. The use of a suspended growth reactor was compared to a fixed-film, fluidized-bed reactor for the treatment of tank-water spiked with MTBE by the researchers. Suspended growth reactors could be used for MTBE removal, but it was concluded that fixed-film reactors were more practical for field application. The study demonstrated that fluidized-bed reactors, containing granular activated carbon (GAC) as a bed material, were able to treat

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MTBE contaminated waste streams to low μg/L effluent concentrations. However, MTBE degradation by the reactor was difficult to initiate, despite repeated inoculation with a laboratory mixed culture grown on MTBE as a sole carbon source. Additionally, the operational efficiency of the reactor was erratic (Tang and Sun 1997).

There are few other reports on field studies of MTBE biotreatment. At a fuel transfer station full-scale fluidized-bed reactors containing GAC were installed to remove benzene, toluene, ethylbenzene, and xylenes (BTEX) from gasoline contaminated ground water. The reactors began to remove MTBE only after 200 days of operation (Mosteller et al. 1997; Stocking et al. 2000). It was demonstrated that MTBE removal was the result of a combination of physical sorption and biodegeneration, but that biological degradation could account for the majority of MTBE treatment in the system (Stringfellow 1998). In fluidized-bed bioreactors, MTBE treatment can be inhibited by toluene and possibly other aromatic hydrocarbons found in gasoline (Stringfellow et al. 2000; 2001).

The exact mechanism of MTBE biodegradation is still not well defined. Organisms able to grow on MTBE as a sole carbon source have been described in several laboratories (Salanitro et al. 1994; Mo et al. 1997; Hanson et al. 1999; Pruden et al. 2001; Stringfellow 2002). Bacteria able to grow on MTBE as a sole carbon and energy source have been isolated from the field reactors included in this study (Stringfellow 2002). Bacterial growth on MTBE was poor and MTBE degrading cultures were difficult to enrich and maintain. It was concluded that MTBE biotreatment in these systems was dependent on a cometabolic process (Stringfellow 2002).

Many laboratories have identified organisms able to cometabolize MTBE (Hardison et al. 1997; Steffan et al.1997; Hyman et al. 1998; Hyman and O'Rielly 1999; Garnier et al. 1999, 2000; Corcho et al. 2000; Hyman et al. 2000; Piveteau et al. 2000; Solano-Serena et al. 2000; Stefan et al. 2000; Fayolle et al. 2001; Hernandez-Perez et al. 2001; Lui et al. 2001; Stringfellow 2002). It has been widely observed that microorganisms that are able to grow on alkanes found in gasoline (Potter and Simmons 1998) can frequently cometabolize MTBE (Hyman et al. 1998; Garnier et al. 1999; Hyman and O'Rielly 1999; Hyman et al. 2000; Solano-Serena et al. 2000; Lui et al. 2001; Stringfellow 2002). Propane, alcohols, ethers, and other compounds can also serve as co-metabolites for MTBE biodegradation (Steffan et al. 1997; Corcho et al. 2000; Piveteau et al. 2000; Steffan et al. 2000; Fayolle et al. 2001; Hernandez-Perez et al. 2001). Laboratory studies suggest that MTBE biodegradation in the pilot-scale, fluidized-bed bioreactors used in this study is a cometabolic process, linked to alkane degrading bacteria (Stringfellow 2002).

In this study, we examined the initiation of MTBE degradation in reactors treating gasoline contaminated round water at a fuel transfer station and document the unpredictable behavior associated with the onset of MTBE biotreatment. We then conducted laboratory studies to examine the cometabolism of MTBE by bacteria enriched on a variety of growth substrates. Bacteria grown on *iso*-pentane as a sole carbon and energy source were consistently able to cometabolize MTBE. Studies were conducted to evaluate strategies for using cometabolite addition to start MTBE biodegradation in reactors that did not treat MTBE spontaneously. The performance of the cometabolite-induced reactor was compared to the performance of a reactor that started degrading MTBE without inducement in side-by-side studies.

Materials and Methods

Reagents and Media

Ninety-eight% pure MTBE was purchased from EM Science (Gibbstown, NJ). All chemicals used in this study were reagent grade, purchased from either EM Science, J. T. Baker (Phillipsburg, N.J.), or Mallinckrodt (Paris, Ky). Sole Source Carbon Media (SSC media) was made by combining 2 g KH_2PO_4, 1.72 g Na_2HPO_4, 2.0 g NH_4Cl, 0.24 g $MgSO_4 \cdot 7H_2O$, 0.12 g $CaCl_2 \cdot 2H_2O$, and 2 mL of trace metal solution in 2 L of deionized water. Trace metal solution was made by adding 3.3 mg $MnSO_4 \cdot H_2O$, 6.2 mg $CuSO_4 \cdot 5H_2O$, 7.6 mg $ZnSO_4 \cdot 7H_2O$, 11.7 mg $Na_2MoO_4 \cdot 2H_2O$, 64.6 mg $FeSO_4 \cdot 7H_2O$, and 4.15 mL concentration HCl with 995.8 mL of deionized water. Nitrifier enrichment media was made by combining 4 g Na_2HPO_4, 4 g NH_4Cl, 0.24 g $MgSO_4 \cdot 7H_2O$, 0.12 g $CaCl_2 \cdot 2H_2O$, 4 g $NaHCO_3$, and 2 mL trace metals solution in 2,000 mL deionized water.

Analytical Procedures

MTBE was measured in the influent and effluent of the laboratory reactors three times per week by headspace analysis. On average, once per week split samples were provided to a U. S. EPA certified laboratory (Environmental Measurements Laboratory, Lawrence Berkeley National Laboratory, Berkeley, Calif.) to verify the results from the headspace analysis by Method 8260B (USEPA 1996). Influent and effluent were collected using procedures recommended for volatile organic compounds in I-CHEM EPA certified 40 mL sampling vials with Teflon/silicone septa screw caps (VOA vials, Nalgene, Rochester, N.Y.). For preservation of sampled water, 0.1 ml of water diluted HCl (1:1 v/v) was added to each vial. Influent and effluent samples were refrigerated at 4°C until analyzed. Sample-holding time was less than 2 weeks.

For headspace analysis, a gas-tight syringe was used to subsample the VOA vials and dispense 0.5 mL of sample into 2-mL autosampler vials in triplicate. A Varian 3400 gas chromatograph (GC) equipped with a flame ionization detector (FID) and an 8200 autosampler was used for static headspace analysis. The autosampler collected 50 μL headspace samples from the sample vial and injected the sample into the GC for separation on a DB-WAX capillary column (J & W Scientific, 30 m by 0.25 mm i.d., 0.25 μm film thickness). A blank water sample was run between samples to prevent any sample carry over. The temperature was maintained at 40°C and the injector and detector temperature were 150 and 225°C, respectively. Helium as a carrier gas flowed at 40 mL/min. MTBE had a retention time of 1.96 min and a detection limit of 0.1 ppm by this method.

For 8260B analysis in the laboratory reactor study, an Aglient 6890 GC with an Aglient 5973 network mass selective detector and a Tekmar 300 purge and trap unit was used to quantify MTBE and other volatile organics. Chromatographic separation was carried out with an RTX-WAX capillary column (Restek, 60 m by 0.25 mm i.e., 1.40 μm coating). The GC condition was set up according to the 8260B protocol and the MTBE reportable limit was calibrated to 5 μg/L.

Influent and effluent water samples from the field reactor were collected twice per week and sent to an EPA certified contract laboratory for analysis (Alpha Analytical, Reno, Nev.). Volatile organic compounds (including benzene, toluene, ethyl-benzene, xylenes, and MTBE) were measured by EPA Method 8260B. Extractable and purgable total petroleum hydrocarbons (TPH) were

measured by EPA Method 8015B. Total petroleum hydrocarbon was calibrated to a gasoline standard. Total and ferrous iron was analyzed by the same laboratory according to EPA Method 200.7 and Method SM 3500 FE D.

Development and Maintenance of Enrichment Cultures

Bacteria were enriched from soil and water samples using sterile technique and standard protocols. Soil samples were collected from gasoline contaminated sites in northern and southern California, a heavy oil contaminated site in Oklahoma, and a wooded hillside in Berkeley, Calif. Biomass and effluent water samples were collected from biological treatment plants treating MTBE contaminated ground water in southern California, Nevada, and laboratory reactors treating gasoline hydrocarbons.

For the enrichment of bacteria from soil and biomass samples, 1 g wet weight of sample was suspended in 60 mL of SSC media in a plastic centrifuge tube and placed on a shaker for approximately 24 h to extract bacteria from the soil. After shaking, the sample was centrifuged on a tabletop centrifuge to remove the larger particles of soil, and 10 mL of the supernatant was transferred to 200 mL of SSC media in a 500-mL flask. In the case of water samples, 10 mL of water was added directly to 200 mL of SSC media in a 500-mL flask.

For enrichment of bacteria able to grow on methanol, acetonitrile, octane, hexane, decane, dodecane, pentadecane, hexadecane, and tetradecane, 1.0 mL of compound was added directly to the flask containing 200 mL of SSC medium and the flask was capped with a beaker. For enrichment of cultures on toluene and *iso*-pentane, 0.1 mL of compound was added and the flasks were sealed with a screw cap to minimize volatile loss. The sealed flasks were opened and reaerated weekly and an additional 0.1-mL of compound added. For enrichment of nitrifying bacteria, water samples were inoculated into Nitrifier Enrichment medium and no additional carbon source was added. Methods used to test bacteria for growth on MTBE as a sole-carbon and energy source are described in Stringfelow (2002). Media without added substrate (SSC alone) did not grow significant amounts of heterotrophic or autotrophic bacteria.

When the enrichment became turbid, it was transferred to a new flask and allowed to grow again. After the first transfer, the enrichments were maintained as draw-fill cultures, by withdrawing 25 mL of culture each week and replacing the culture with fresh SSC media. Substrates were replaced weekly or as needed, as evidenced by the absence of a sheen (in the case of alkanes) or the absence of odor (in the case of volatiles). To prepare the enrichment cultures for the MTBE degradation assay, the cultures were placed in log-growth phase by drawing and filling 100 mL of SSC media and adding fresh substrate to the culture at an appropriate interval before the culture was harvested for the assay. In the case of the nitrifying enrichment, the draw-fill was made using Nitrifier Enrichment media.

Cometabolic Degradation of Methyl tert-Butyl Ether by Enrichment Cultures

Cometabolic biodegradation of MTBE was tested using a resting cell assay. Enrichment cultures were placed in a log growth phase, harvested, washed three times in SSC media, and diluted to a final concentration of approximately 250 mg/L dry-weight biomass. Triplicate biodegradation assays were assembled in a 20 mm×120-mm vial sealed with Mininert caps and incubated at 25°C on an orbital shaker. Each vial contained 5 mL of SSC media and 5 mL of sterile deionized water with a final MTBE and cell concentration of 7.6 and 25 mg/L, respectively. Degradation was determined by measuring MTBE in the headspace of the vial in relation to sample blanks (made with 10 mL deionized water only). Killed controls demonstrated that MTBE did not sorb to bacterial biomass. MTBE in the headspace was measured as described above on a Varian GC-FID, except that 100-μL headspace samples were collected and injected manually. MTBE headspace concentration was measured 1, 2, 5, and 7 days after the assays were started. Typically, it was evident by the second day of the assay if the enrichment culture was able to cometabolize MTBE. Optical density was measured at the end of the assay to determine if the culture was able to grow on MTBE. No enrichments exhibited detectable growth on MTBE.

Laboratory Reactor Operation

Two laboratory-scale, up-flow, fluidized-bed bioreactors (US Filter/Envirex, Waukesha, Wis.) were set up in parallel. The reactors were constructed of clear Flexi PVC glass with an internal diameter of 3.8 cm, a height of 1.7 m, and a volume of 1.56 L.

Granular activated carbon (GAC, Calgon Filtrasorb 400, Calgon Co., Pittsburgh, Pa) was used for bed material. GAC that had not been previously used in treatment (virgin GAC) was prewashed with running deionized water until fine particles were washed away. The two reactors were packed with the prewashed GAC to the bed height of 60 cm. Reactor operation was started without inoculum and the reactor temperature was maintained at ambient temperature (27–29°C).

The reactors were fluidized with MasterFlex L/S peristaltic pumps (Cole-Parmer Instrument Co., Barrington, Ill.) at a rate of 840 L/day. A metered flow of compressed oxygen was supplied through a mass flow controller (GFM-1700, Aalborg Instruments, Monsey, N.Y.) to maintain the oxygen concentration at a minimum of 4 mg/L in the reactors. Water was recirculated with a centrifugal pump (NPE-F, Goulds Pumps, Inc., Seneca Falls, N.Y.) in an oxygen contactor to maximize oxygen dissolution.

Influent to the reactor was delivered with a metering pump (RHV-O, Fluid Metering, Inc., Oyster Bay, N.Y.) at flow rates of between 5 and 20 L/day. The hydraulic residence time varied between 1.7 and 10.8 h. The influent feed to the reactor provided buffering capacity and nutrients to promote biofilm growth on the GAC in the reactor. The composition of the feed was 20 mg/L NH_4Cl, 40 mg/L KH_2PO_4, and 168 mg/L K_2HPO_4 in tap water. MTBE concentration in the feed was varied between 10 and 50 mg/L during the study (see results). A saturated solution of *iso*-pentane was made by adding 100 mL of *iso*-pentane to a bottle containing 700 mL of water. The water was allowed to reach equilibrium with the *iso*-pentane and then the saturated solution was pumped into the reactor using a MasterFlex C/L peristaltic pump.

Field Reactor Operation

The biological treatment system used in the field study was a Model 30 fluidized-bed bioreactor manufactured by USFilter/Envirex Products (Waukesha, Wis.). The reactor consisted of a 4.5 m tall by 51 cm diameter tower, an influent flow control pump, a pump to fluidize the bed material with recycled reactor water, a biomass control system, and an oxygen contactor designed to maintain high oxygen concentrations without stripping volatile organic compounds. The recycle flow was fixed at a rate of approximately 121 L/min to maintain fluidization of the bed mate-

Table 1. Groundwater Influent Characteristics during First and Second Start-ups of Pilot-Scale, Fluidized-Bed Bioreactor

	First Start-Up			Second Start-Up		
Parameter	Mean	Max	Min	Mean	Max	Min
MTBE (μg/L)	9,594	11,000	8,400	3,229	6,200	1,600
Purgable TPH (mg/L)	16.1	18.0	13.0	16.3	33.0	3.7
Extractable TPH (mg/L)	0.5	1.3	0.1	3.2	12.0	0.0
Total TPH (mg/l)	16.9	19.0	14.0	16.6	33.0	3.7
Benzene (μg/L)	5,738	7,200	490	2,335	4,500	340
Toluene (μg/L)	648	950	270	1,535	3,900	98
Ethyl-Benzene (μg/L)	140	280	20	195	940	0
m,p-xylene (μg/L)	567	760	370	1,455	3,200	110
o-xylene (μg/L)	293	400	210	677	1,500	130
COD (mg/L)	87.7	180.0	57.0	71.5	135.0	32.2
Total iron (mg/L)	4.7	5.5	3.4	3.9	12.0	1.5
Ferrous iron (mg/L)	2.0	4.6	0.6	2.8	5.0	1.5

rial as per the manufacturer's specifications. The oxygen delivery was on a feedback control to maintain an effluent concentration of 2.5 mg/L dissolved oxygen. The influent flow to the fluidized-bed reactor was set at 15 L/min and was limited to that rate by available groundwater flow. The temperature of the reactor was dependent on the ambient air temperature, influent water temperature and flow, and heating that occurs from compressed oxygen injection and recycle flow. In this study there was no attempt to control reactor temperature, except to avoid overheating during periods when influent flow was halted.

The tower was loaded with a solid bed material, in this case GAC, upon which bacteria are grown as a biofilm. For both of the field studies reported here, the reactors were inoculated with approximately 1.5 kilos of biologically active GAC from another fluidized-bed bioreactor treating contaminated ground water. The bioreactor that served as a source of inoculum had prior exposure to MTBE contaminated ground water (Stocking et al. 2000; Stringfellow et al. 2000).

Field Site Description

The field site was a fuel transfer station located in southern California. For the period included in this study, the maximum mean daily air temperature was 23.8°C and the minimum mean daily air temperature was 10.6°C (National Climatic Data Center 2000). The station is associated with a thoroughly characterized contaminant plume located in the shallow ground water table. The plume consists of gasoline hydrocarbons in mixture with MTBE (Table 1). Contaminated ground water is collected from a network of capture wells and pumped to a physical treatment plant on site. For this study, part or all of the captured ground water was diverted to a 20,000-gal equalization tank and then delivered to the biological treatment system. Influent samples were collected from the line between the equalization tank and the reactor.

Data Analysis and Calculations

Loading rate is determined by calculating total daily loads (mg/L influent concentration times daily flow in liters) and dividing by the reactor volume. Removal rate (rate) is calculated from the mg of each constituent removed per day (influent mg/L minus effluent mg/L, times flow) divided by the reactor volume. Volumetric loading and rate are expressed as mg of compound per liter reactor volume per day. The reactor volume was calculated by the volume to the bed control point rather than total reactor volume. Descriptive statistics and Student's *t*-tests were calculated using functions available in Excel (Microsoft Corp., Seattle, WA).

Results and Discussion

Initiation of Methyl tert-Butyl Ether Biotreatment in Field Reactors

In order to examine conditions influencing the treatment of MTBE contaminated ground water, a pilot scale fluidized-bed bioreactor was installed at a fuel transfer station in southern California (Stocking et al. 2000; Stringfellow et al. 2001). The biological treatment system received ground water that contained gasoline hydrocarbons and MTBE (Table 1). For the first start-up test, the reactor was loaded with 166 kg of Weststates coconut GAC, inoculated with GAC from another bioreactor (see methods), and forward flow to the reactor was 15 L/min. The reactor demonstrated an initial breakthrough of MTBE within the first 10 days of operation, as the absorption capacity of the carbon was depleted (Fig. 1). Between the 10th and 20th days of operation biological treatment of MTBE was evident and by the 45th day of operation the reactor was achieving steady treatment of MTBE (Fig. 1). Between days 40 and 80 the reactor demonstrated an average MTBE removal efficiency of 96%.

The rapid on-set of MTBE biodegradation was unexpected. Using an identical reactor, Tang and Sun (1997) were unable to initiate biotreatment of MTBE until after repeated inoculation with an MTBE degrading mixed culture developed at Shell Development Co. (Salanitro et al. 1994). In full-scale fluidized-bed bioreactors, significant MTBE treatment did not occur until hundreds of days after the reactor went on line for treatment of BTEX (Mosteller et al. 1997; Stocking et al. 2000). The rapid initiation of MTBE biodegradation was also unexpected in light of what is know about organisms that grow on MTBE. Bacteria that grow on MTBE typically have a slow growth rate, even under laboratory conditions, and would therefore not be expected to rapidly colonize the reactor (Salanitro et al. 1994; Mo et al. 1997). Tests on bed material (biofilms grown on GAC and flocculent from the top of the reactor) demonstrated that activity in the reactor could be attributed to cometabolic biodegradation (Stringfellow 2002).

For the second start-up, the reactor was emptied of GAC, rinsed with potable water, charged with 170 kg of Calgon coconut GAC, and inoculated in an identical manner as in the first start-up. The flow was again started at 15 L/min. In this case, MTBE

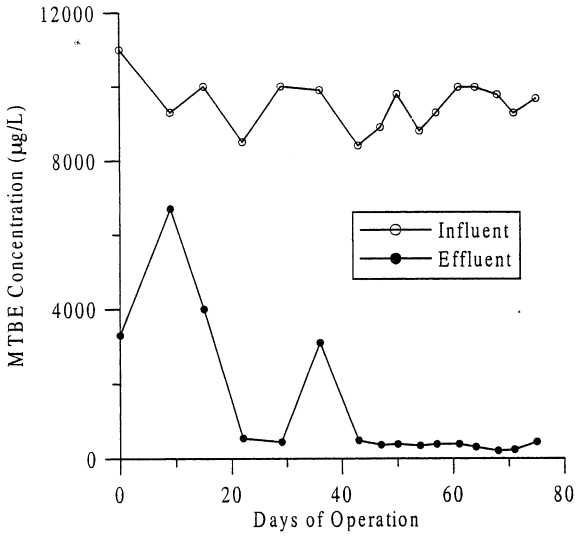

Fig. 1. Influent and effluent methyl *tert*-butyl ether concentrations during first start-up of pilot-scale, fluidized-bed bioreactor treating gasoline contaminated groundwater. Evidence of methyl *tert*-butyl ether breakthrough in first 10 days indicates depletion of sorptive capacity of the granular activated carbon bed material. By day 25 it is apparent that methyl *tert*-butyl ether biotreatment has started in bioreactor.

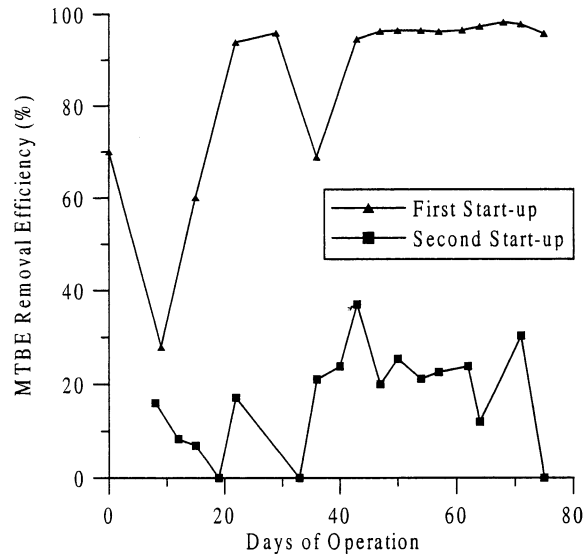

Fig. 3. Methyl *tert*-butyl ether removal efficiency as a function of time during first 75 days of operation during first and second pilot-bioreactor start-ups. The dramatic difference in the performance of same reactor during two sequential start-ups illustrates uncertain nature of methyl *tert*-butyl ether biotreatment. Uncertainty in the initiation of methyl *tert*-butyl ether biotreatment is unacceptable if methyl *tert*-butyl ether treatment is to be widely applied.

breakthrough was also apparent by the 10th day and complete breakthrough occurred by day 20 (Fig. 2). MTBE biotreatment did not start in this reactor, although it appeared that there was some improvement in MTBE removal over time (Fig. 2). This start-up was consistent with previous experience, which indicated that MTBE biodegradation potential would only develop in a reactor after an extended period of time (Tang and Sun 1997).

The differences in the start-up between the two reactors can be most clearly compared by plotting treatment efficiency as a function of time. In Fig. 3, the first 75 days of reactor operation are compared between the first and second start-ups. Although there was a difference in the amount of MTBE in the influent of the bioreactor between the two trials (Table 1), the difference was not significant enough to explain the dramatic difference observed between the two reactor start-ups (Stringfellow et al. 2001). The only other apparent difference between the two start-ups was the source of the carbon used to fill the reactors. Laboratory studies (described below) demonstrate that the difference in start-up patterns cannot be attributed to the difference in carbon suppliers.

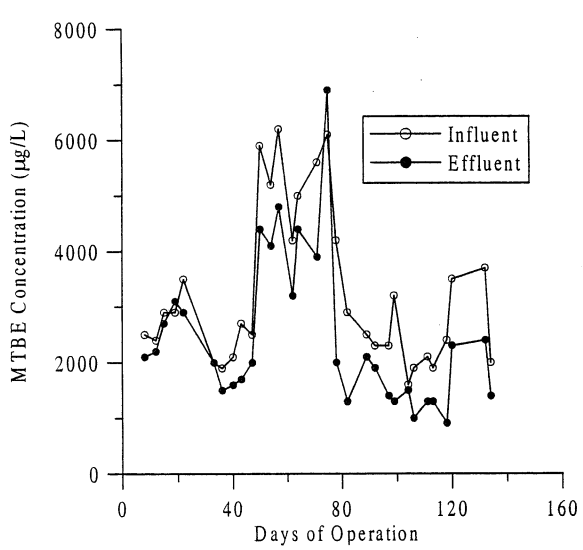

Fig. 2. Influent and effluent methyl *tert*-butyl ether concentrations during second start-up of pilot-scale, fluidized-bed bioreactor treating gasoline contaminated groundwater. Evidence of methyl *tert*-butyl ether breakthrough in first 10 days indicates depletion of sorptive capacity of the granular activated carbon bed material. Unlike the first start-up in this reactor (Fig. 1), methyl *tert*-butyl ether biotreatment is not apparent in this reactor.

It is apparent from this field study that the onset of biological treatment in fluid-bed reactors is unpredictable. The uncertainty associated with MTBE biological treatment needs to be resolved if the MTBE biotreatment is going to become an economical alternative for the treatment of MTBE contaminated ground water. We conducted laboratory studies to determine if the addition of cosubstrates could be used to induce MTBE biotreatment in fixed-film reactors that did not spontaneously initiate MTBE biodegradation.

Cometabolic Biodegradation of Methyl tert-Butyl Ether

In cometabolic biodegradation, bacteria grow on one compound and the enzymes induced to degrade the growth compound also are capable of degrading a second compound (MTBE) that does not serve as a growth substrate for the organism. Classically, the cosubstrate is not expected to provide energy to the bacteria and can even put the cometabolizing microorganism at a competitive loss (Chang and Criddle 1997). However, a broader definition of cometabolism includes the competitive metabolism of substrates that do not support growth of an organism, without reference to the potential of the substrate to provide energy under some conditions (Stringfellow and Aitken 1995; Aitken et al. 1998). Compounds that have been observed to serve as cosubstrates for MTBE degradation include linear-, branched-, and cyclic-alkanes,

Table 2. Enrichment Cultures Isolated from Diverse Environmental Sources were Tested for Their Ability to Cometabolize Methyl *tert*-Butyl Ether. Mixed Cultures Grown on *Iso*-pentane were Consistently Able to Cometabolize Methyl *tert*-Butyl Ether.

Growth substrate	Environmental sources of enrichment cultures[a]	MTBE cometabolism (positive/total)
Iso-pentane	Ground water treatment plants	7/7
	Gasoline contaminated soils	
	Oil contaminated soil	
	Uncontaminated soil	
Toluene	Ground water treatment plants	2/4
	Laboratory reactors	
Methanol	Ground water treatment plants	1/3
	Uncontaminated soil	
Acetonitrile	Uncontaminated soil	0/2
Ammonia	Ground water treatment plants	0/2
Octane	Ground water treatment plants	0/4
	Gasoline contaminated soil	
	Oil contaminated soil	
Hexane	Oil contaminated soil	0/1
Decane	Oil contaminated soil	0/1
Dodecane	Gasoline contaminated soil	0.2
	Oil contaminated soil	
Pentadecane	Gasoline contamined soil	0/2
	Oil contaminated soil	
Hexadecane	Gasoline contaminated soil	0/2
	Oil contamined soil	
Tetradecane	Gasoline contaminated soil	0/2
	Oil contaminated soil	

[a]See methods for further explanation.

aromatic hydrocarbons, alcohols, and other ethers (see the Introduction). Many compounds that serve as cosubstrates for MTBE degradation can be found in gasoline contaminated groundwater.

Biological treatment systems consist of complex bacterial communities (Dias and Bhat 1964; Taber 1976; Snaidr et al. 1997; Sakano and Kerkhof 1998; Stoffels et al. 1998). The addition of a biodegradable organic compound will result in the selection of bacteria able to most efficiently utilize the added substrate under the reactor conditions. Which particular bacteria will grow and dominate the reactor is not easily predicted (Stoffels et al. 1988). Therefore the selection of a cosubstrate to induce MTBE biodegradation in a biological treatment plant can be viewed as a probability problem. What compound will consistently produce a population of microorganisms that will also be able to degrade MTBE? For a cosubstrate to be effective at promoting MTBE treatment, it must promote the production of MTBE degrading enzymes in essentially all bacteria that are able to utilize the cosubstrate.

We conducted a series of enrichments to determine which substrates, when added to different initial sources of bacteria inoculum, would consistently produce MTBE cometabolizing populations of bacteria. Bacteria enrichments are a well-established technique in microbiology for examining individual populations of selected bacteria in environmental samples. Enrichment of specific populations of degrading bacteria also occur in reactors, so the use of batch enrichment techniques should give us an indication of what can happen in a reactor fed a selected cosubstrate. We selected a broad variety of potential cosubstrates to test. Enrichments were started from a number of sources, including uncontaminated soil, and the number of positive enrichments found for the total number of enrichments tested is presented in Table 2.

The results presented in Table 2 show that *iso*-pentane enriched bacteria have a very high probability of being able to cometabolize MTBE. Other researchers have shown that pure cultures of bacteria able to grow on *iso*-pentane cometabolize MTBE (Hyman et al. 1998; Hyman and O'Reilly 1999; Hyman et al. 2000; Solano-Serena et al. 2000; Fayolle et al. 2001). This study is the first to demonstrate that MTBE cometabolism is a consistent characteristic of mixed bacterial communities enriched on *iso*-pentane.

Some MTBE cometabolizing bacteria have been shown to grow on linear alkanes with a carbon number of C_6 or greater (Hyman and O'Reilly 1999; Solano-Serena et al. 2000). Garnier et al. (2000) demonstrated that MTBE cometabolism by a mixed culture enriched on gasoline was stimulated by the addition of hexane and heptane. The *iso*-pentane enrichments cultivated as part of this work were capable of oxidizing hexane and octane, and individual isolates from the enrichments were able to grow on C_6 to C_{16} linear alkanes (data not shown). However, it has not been determined if higher molecule weight linear alkanes can induce MTBE degrading enzymes.

We tested the hypothesis that bacteria enriched on higher molecular weight alkanes would be induced for MTBE cometabolizing enzymes. Bacteria enrichments grown on C_6–C_{16} linear alkanes did not degrade MTBE (Table 2). This result, in combination with the results from the *iso*-pentane enrichments, suggests that there are multiple enzyme systems available for alkane degradation and the enzymes used for the degradation of low-molecular weight alkanes are linked to activity against high molecular weight alkanes, but not vice versa. Recent publications suggest that multiple alkane-oxidizing enzymes occur in some alkane degrading bacteria (Hamamura et al. 2001). In any case, C_6 and above linear alkanes did not serve as cometabolites for MTBE degradation in mixed communities.

Toluene enrichments were developed that could degrade MTBE; however, toluene enrichment did not consistently produce MTBE degrading populations (Table 2). Toluene metabolism has been observed in MTBE degrading microorganisms, but the tolu-

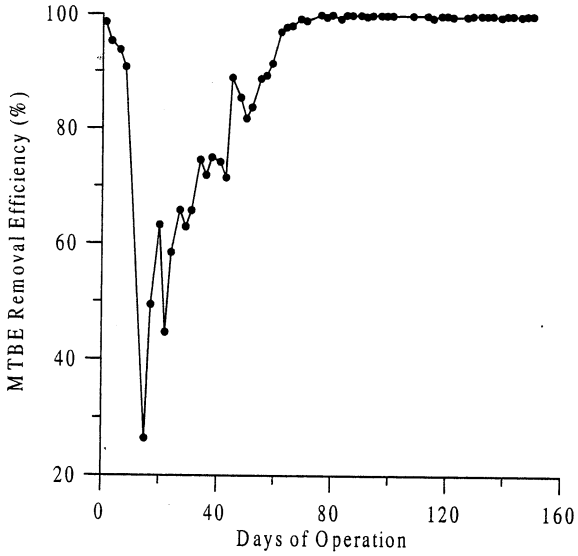

Fig. 4. Methyl *tert*-butyl ether removal efficiency as function of time during start-up of a laboratory Reactor 1. This reactor developed methyl *tert*-butyl ether degrading bacteria population without use of bacterial inoculum or cosubstrate stimulation.

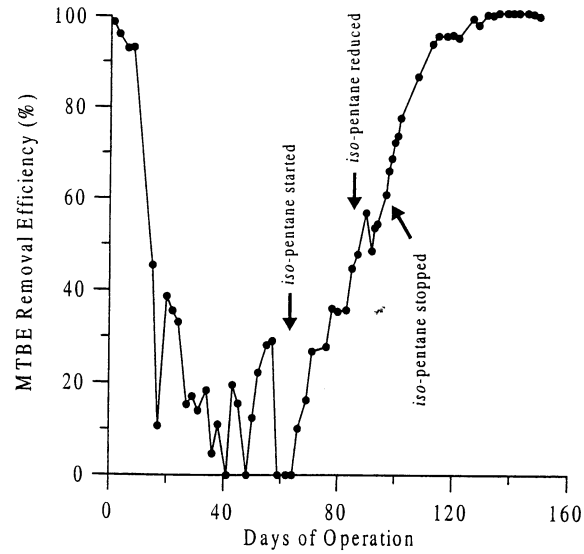

Fig. 5. Methyl *tert*-butyl ether removal efficiency as function of time during start-up of laboratory Reactor 1. This reactor did not spontaneously develop methyl *tert*-butyl ether biodegrading population. Addition of bacterial inoculum and *iso*-pentane as cosubstrate initiated biotreatment of methyl *tert*-butyl ether.

ene degrading enzymes are not believed to be responsible for MTBE degradation (Deeb et al. 2001). Toluene can inhibit MTBE metabolism in fluidized-bed bioreactors (Stringfellow et al. 2000). Toluene's potential as an inhibitor and its regulatory importance, combined with its lower probability of inducing MTBE cometabolism, make it a less promising substrate for the stimulation of MTBE metabolism in bioreactors.

Two common solvents, methanol and acetonitrile, were tested for their ability to enrich MTBE oxidizing populations (Table 2). Methanol is a gasoline additive and was suggested to be a possible candidate for MTBE cometabolism in gasoline contaminated environments. The acetonitrile culture was grown in our laboratory as part of another project and was tested as a matter of course, but was not found to cometabolize MTBE. The first methanol enrichment, from a treatment plant treating gasoline contaminated ground water, was able to oxidize MTBE, but subsequent methanol enrichments from other sources were not positive for MTBE cometabolism. It has been reported that bacteria able to grow on ethanol as a sole carbon and energy source were able to cometabolize MTBE (Piveteau et al. 2000); however, the role of alcohol degrading enzymes in MTBE metabolism has not been previously investigated.

The final growth substrate tested was ammonia. Ammonia-oxidizing bacteria (nitrifying bacteria) are common in biological treatment systems, including fixxed-film bioreactors (Snaidr et al. 1997; Sakano and Kerklof 1998). Early reports concerning MTBE biotreatment implicated nitrifying populations in MTBE metabolism (Salanitro et al. 1994). However, to our knowledge ammonia-oxidizing bacteria had not been tested directly for their ability to cometabolize MTBE. We enriched nitrifying bacteria from two different ground water treatment plants and were not able to find nitrifying populations that could cometabolize MTBE (Table 2).

Induction of Methyl-tert Butyl Ether Biotreatment in Laboratory Reactors

As a result of the enrichment experiments, we chose *iso*-pentane as the most promising substrate to use as a cometabolite for inducing MTBE biotreatment in fluidized-bed bioreactors.. A previous study has shown that *iso*-pentane addition could improve MTBE treatment efficiency in bioreactors that were already biodegrading MTBE, but were not meeting effluent target concentrations (Stringfellow 2002). Here, we tested the hypothesis that *iso*-pentane addition could be used to develop an MTBE-oxidizing population in fluidized-bed bioreactors that did not develop MTBE degrading population of bacteria spontaneously.

To test our hypothesis, we set up a pair of laboratory scale fluidized-bed bioreactors using virgin GAC from the same batch of GAC used in the second field experiment. The reactors were set up as described in the methods section and supplied a MTBE feed of approximately 10 mg/L in a mineral salts media (initial loading approximately 30 mg MTBE/L-reactor/day). In both reactors, there was an initial treatment of MTBE in the reactor due to sorption on carbon, but MTBE breakthrough was observed within approximately 20 days (Figs. 4 and 5), indicating that the sorptive capacity of the carbon was depleted rapidly.

In the first reactor (reactor 1) MTBE biotreatment started spontaneously. MTBE biotreatment was apparent by day 30 and treatment improved over the next 50 days, until by day 80 the reactor was treating MTBE with a greater than 98% efficiency (Fig. 4). In the second reactor (reactor 2), an MTBE degrading population did not develop and there was no indication of significant biotreatment of MTBE occurring even after 60 days (Fig. 5).

The on-set of MTBE biodegradation in reactor 1 was unusual for several reasons. Although the reactor had contained biologically active GAC in prior experiments (Stringfellow 2002), the reactors had been cleaned out thoroughly and disinfected with a concentration solution of hypochlorous acid between experiments. The reactors were loaded with virgin GAC, i.e., GAC direct from the manufacturer. There was no inoculum added to the reactor, except what small amount of bacteria entered the reactor in the feed, which was made with tap water and reagent grade chemicals. Notably, the identical reactor (reactor 2), assembled and started in the exact same way, at the exact same time, fed the exact same influent, and standing less than 4 ft away did not grow

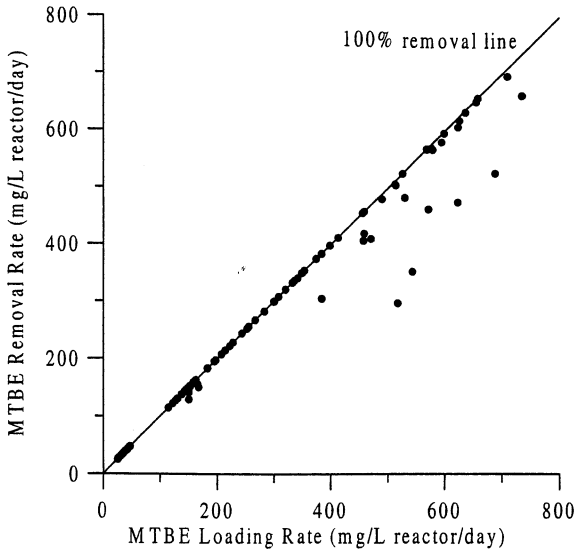

Fig. 6. Methyl *tert*-butyl ether removal as a function of methyl *tert*-butyl ether loading in Reactor 1. Reactor 1 exhibits efficient removal of methyl *tert*-butyl ether at both high and low loading rates. Loss of efficiency at higher loading is function of transitory loss of treatment as reactor responded to changes in hydraulic loading.

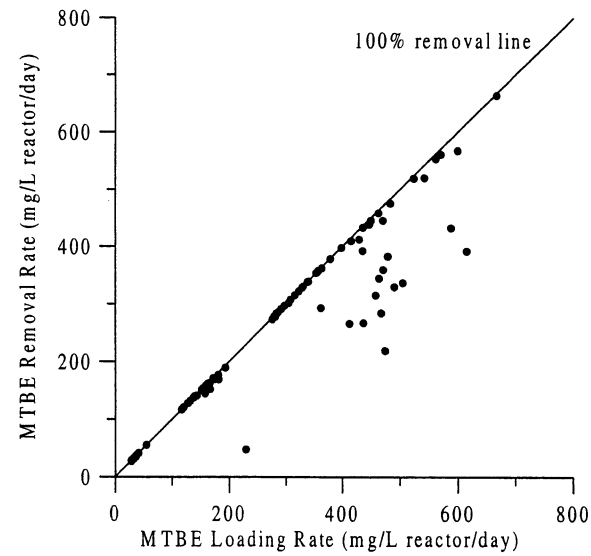

Fig. 7. Methyl *tert*-butyl ether removal as a function of methyl *tert*-butyl ether loading in Reactor 2, which was started with *iso*-pentane. Reactor 2 exhibits efficient removal of MTBE at both high and low loading rates, with some loss of efficiency as hydraulic loading changes. The performance of Reactor 1 and Reactor 2 appears identical, despite the difference in how they were started.

an MTBE biodegrading population. This result again demonstrates the unpredictability of the on-set of MTBE biotreatment, and can be directly compared to the results observed in the field studies.

By the 60th day of operation, Reactor 2 still had no discernable MTBE biotreatment activity. The reactor was biologically active, as demonstrated by the occurrence of nitrification (data not shown). At this time the reactor was inoculated with 75 mg (dry weight) of an *iso*-pentane degrading enrichment from a gasoline contaminated soil and an aqueous solution of *iso*-pentane was fed into the reactor at a rate equal to 390-mg *iso*-pentane/L-reactor/day. Within 10 days there was a noticeable improvement in MTBE treatment efficiency. Approximately 30 days after *iso*-pentane addition was started, the dose was reduced to approximately 39-mg *iso*-pentane/L-reactor/day. *Iso*-pentane feed was halted approximately 40 days after it was started. The addition of the inoculation alone was not tested because the iso-pentane degrading culture used was not able to grow on MTBE as a sole carbon and energy source in liquid culture.

When *iso*-pentane feed was halted, the treatment efficiency of the reactor was approximately 50%. MTBE treatment continued to improve over the next 30 days and the reactors stabilized at a treatment efficiency of 98% or better approximately 40 days after *iso*-pentane addition was halted. It is possible that the *iso*-pentane degrading bacteria could grow on MTBE under the conditions found in the reactor, but is also likely that the GAC in the reactor absorbed a significant amount of *iso*-pentane during the feed period and provided *iso*-pentane to the reactor for an extended period of time after the *iso*-pentane feed was halted. Even at the highest loading rates, *iso*-pentane was not observed in the effluent of the reactor. Both of these reactors have continued to treat MTBE without any further addition of cosubstrate.

The results of this test indicate that *iso*-pentane can be used to start MTBE biotreatment in reactors that do not initiate biotreatment spontaneously. *Iso*-pentane stimulated the growth of MTBE degrading microorganisms, but, once established, the bacteria are able to maintain themselves on MTBE, as indicated by the continued treatment of MTBE for over 300 days (see below). We have been unable to isolate bacteria from either laboratory reactor that can grow on MTBE as a sole-carbon and energy source. Both reactors contain significant populations able to grow on *iso*-pentane, even though Reactor 1 never received *iso*-pentane (data not shown). The characteristics of the bacterial communities in these reactors are currently under investigation.

Comparative Performance of Bioreactors

In order to investigate the performance characteristics of the two reactors, we varied the loading rates to the reactors by both increasing the hydraulic loading and by increasing the MTBE concentration in the influent. Between days 168 and 320 of operation, influent MTBE concentration was increased to 50 mg/L and the reactor flow was varied from 5 to 25 L/day. Both reactors responded to changes in hydraulic loading by a temporary loss of treatment efficiency followed by recovery to stable treatment. The performance efficiency of the reactors as a function of loading is shown in Figs. 6 and 7. The performance of the reactors, in terms of variation in treatment under changing loading conditions and removal efficiency, was not significantly different between the two reactors (Student's *t*-test, alpha=0.05). These results suggest that reactors started with *iso*-pentane can be expected to treat MTBE as efficiently as reactors containing a spontaneously established MTBE degrading population. There appears to be no disadvantage to using *iso*-pentane initiated bioreactors for treatment.

Summary and Conclusions

Biological treatment is a promising technology for the treatment of MTBE. However, the initiation of MTBE degradation in bioreactors needs to be predictable if there is going to be widespread application of biotreatment for the remediation of MTBE contaminated ground water. Fluidized-bed bioreactors containing

GAC can develop MTBE degrading populations spontaneously and demonstrate efficient and reliable treatment. However, in some cases, MTBE biotreatment does not start spontaneously and there is a need to apply novel techniques for starting MTBE biotreatment in these reactors.

An evaluation of mixed populations of bacteria enriched on a variety of carbon and energy sources demonstrated that bacteria able to grow on *iso*-pentane were consistently found to cometabolize MTBE. In a laboratory study, *iso*-pentane was used to start MTBE biodegradation in a fluidized-bed bioreactor. Once started, the MTBE degrading bacterial population in the bioreactor did not need to continued addition of *iso*-pentane to maintain activity, indicating that the bacteria developed using *iso*-pentane as a growth substrate were able to obtain maintenance energy from the degradation of MTBE, even though they could not grow on MTBE as a sole carbon and energy source. Comparison of the performance of an *iso*-pentane induced reactor with a reactor that developed an MTBE degrading population without inducement, indicated that there was no difference between the reactors in the ability to treat MTBE.

This study illustrates the unusual and complex nature of MTBE metabolism. In both field and laboratory studies, one reactor grew MTBE degrading bacteria spontaneously, while another reactor did not. The on-set of MTBE treatment is dependent on more than the introduction of a specific culture. The field reactors were both inoculated from the same source and the laboratory reactors were exposed to the same sources of bacterial inoculation (influent and air), yet in each case only one reactor spontaneously degraded MTBE. It is significant that bacteria from MTBE degrading reactors do not grow on MTBE in liquid culture, but are able to grow as a biofilm on GAC in the presence of a complex bacterial community which includes nitrifying bacteria. The GAC may be providing a supplemental source of organic carbon to the bacterial community in the reactor. The nitrifying bacteria fix carbon from the atmosphere and are well known to provide a source of carbon to the bacterial community as well. Therefore it is not necessary that MTBE metabolism in GAC filled fluidized-bed reactors must be dependent on bacteria able to grow on MTBE as a sole carbon and energy source.

MTBE metabolism by bacteria able to grown on *iso*-pentane is not well understood. It is apparent that *iso*-pentane stimulation of MTBE degradation follows some of the characteristics of a classic cometabolic system, i.e., in batch reactions MTBE alone does not maintain bacteria activity. However, the results of the laboratory reactor studies suggest that growth on MTBE may not be limited by energy. MTBE metabolism by *iso*-pentane grown bacteria does appear to meet the broader definition of cometabolism as the competitive metabolism of a growth and nongrowth substrate by a single enzyme (Stringfellow and Aitken 1995). Studies are currently underway to identify the enzymes involved in MTBE metabolism and to determine if *iso*-pentane and MTBE are truly competitive inhibitors.

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