# Report for 2000MT9G: Enhancement of Fuel Oxygenate (MTBE) Biodegradation Potential in Groundwater

- Articles in Refereed Scientific Journals:
  - Veeh, Richard and Eric Kern, 2000, Enrichment, isolation, and identification of MTBE-degrading bacteria. Underground Tank Technology Update 14(5):6-8.
  - Kern, E. A., R.H. Veeh, H.W. Langner, R.E. Macur, and A.B. Cunningham. 2002. Characterization of methyl tert-butyl ether (MTBE)-degrading bacteria from a gasolinecontaminated aquifer. Bioremediation Journal 6(2):113-124.
- Dissertations:
  - Lang,Claus, 2000, Evaluating Transport and Biodegradation of MTBE in a Gasoline Contaminated Aquifer using Groundwater Flow and Transport Modeling, MS, Environmental Engineering, University of Stuttgart, Germany.

Report Follows:

**Abstract – Progress Report**

MTBE is degraded very slowly in the subsurface relative to other gasoline constituents. Previous research reports indicate that indigenous microbial populations may biodegrade MTBE and TBA at very low rates and after extended acclimation periods. Therefore, dissolved MTBE plumes in groundwater may impact larger areas compared to other gasoline constituents. The relative recalcitrance of MTBE combined with its high water solubility make it the single largest threat to ground water quality resulting from gasoline releases. Research team members collected field data and used chemical and environmental parameters to model degradation of MTBE and plume dynamics at a gasoline release site in Ronan, Montana. All pertinent historical, hydrogeological, and geochemical data were reviewed, and a limited characterization of the site microbial population was performed. This characterization focused on enrichment, isolation, and identification of bacterial strains and consortia that could biodegrade MTBE and its metabolites (e.g. TBA). This study included an educational component, in-depth scientific and engineering components, and a political/management component. Under the auspices of a graduate level class in Environmental Engineering (educational component) and with assistance and guidance from regulatory professionals within the Montana Department of Environmental Quality (political/management component), research advances to date support the following statements:

- A bacterial consortium capable of MTBE biodegradation is present in the subsurface at the Ronan, MT gasoline release site.
- MTBE-degrading isolates degraded MTBE at a higher rate in the presence of a co-substrate (2-propanol), indicating a possible relationship to MTBE-degrading, propane-oxidizing bacteria. MTBE metabolites, TBA and TBF, accumulated as MTBE was biodegraded.
- Genotypic characterization of the MTBE-degrading consortium indicated that at least five bacterial species initially contributed to MTBE degradation. However, over time under the selective pressure of MTBE as the sole carbon source, it appeared that *Rhodoferax fermentans* became the dominant species. Also, no metabolites were observed.
- Although an MTBE-degrading consortium was obtained from the site, the relatively low MTBE degradation rate observed in the laboratory indicates that microbial biodegradation is of minor importance to overall natural attenuation of MTBE in the field.
- Modeling efforts were somewhat successful in predicting MTBE plume dynamics.

Research advances made through this project have provided a mechanism to assess the biodegradation potential at another MTBE release site near Manhattan, MT. Enrichment cultures for MTBE-degradation from this site were initiated using protocols established with samples from the Ronan site. Experimental data suggest that there is also an MTBE-degrading consortium present at the Manhattan release site.

**Research Objectives**

The primary goal of this research project was to assess the biodegradation potential of MTBE and to determine what environmental factors may be limiting MTBE degradation at the gasoline release site near Ronan, MT. This work included a review of pertinent hydrogeological and geochemical data and characterization of any MTBE-degrading microbial species that could be identified and/or isolated through enrichment cultures. The objectives of this multi-faceted study included an educational component, in-depth scientific and engineering components, and a political/management component. Educational aspects of the project involved field site assessment, data collection, and transport modeling experience for interdisciplinary students enrolled in an MSU environmental engineering class. The political component of the project was designed to bring students into direct communication with consulting engineers and Montana Dept. of Environmental Quality (MDEQ) personnel both in the field and by presenting their findings in a workshop format of open discussion of logical management options. The MTBE release site was identified through the MDEQ-Underground Storage Tank Program as a threat to a pristine surface water receptor. In order to meet the overall goal, the research was planned to meet the following four general objectives:

<u>I. Site Data Review</u>

- Review of existing data from the Ronan, MT site to establish release history, groundwater hydrogeology, geochemistry, availability of potential electron acceptors, free product occurrence, and evidence of natural attenuation. This information was used to enhance interpretation of laboratory results and to allow site-specific recommendations for remedial action.

<u>II. Field Sampling and Data Collection</u>

- Collection of groundwater and/or soil samples from existing or subsequently installed monitoring well locations. Establishment/confirmation of existing plume dimensions. Samples were indexed and cross-referenced with geochemical conditions present at each sampling location.
- Analysis of site groundwater data for evidence of natural attenuation of MTBE, degradation products, and other petroleum compounds.

<u>III. Laboratory Experimentation and Modeling</u>

- Development and/or substantiation of gas chromatographic methods for analysis of MTBE and its known metabolites for monitoring MTBE degradation in enrichment cultures.
- Development of aerobic MTBE-degrading enrichment cultures using microcosms with minimal growth media and composite soil samples as microbial inocula.
- Identification of possible nutrient limitations (e.g. nitrogen, phosphorus, dissolved oxygen) and environmental limitations (e.g. temperature) to MTBE biodegradation at the field site.
- Identification of possible hydrocarbon co-substrates (e.g. propane) that may enhance MTBE biodegradation.
- Isolation and identification of specific MTBE/metabolite-degrading bacteria.
- Application of BIOSCREEN and MODFLOW models to determine predictive capabilities regarding existing plume dimensions, contaminant concentrations, etc.

IV. Education and Technology Transfer

- Organization of components of the laboratory and field research into a graduate level class in Environmental Engineering.
- Development and delivery of annual workshops/discussions on MTBE fate and transport in groundwater.

**Methodology**

*Site Characterization*

The release site is located at a fuel station east of U.S. Highway 93, one mile south of Ronan, MT. A 16,000-gallon underground storage tank removed in April, 1994 was estimated to have released approximately 10,000 gallons of gasoline over a one-year period. Historical data were retrieved to estimate initial MTBE concentration in the gasoline to aid in subsequent plume modeling efforts by students involved in the engineering class. The Montana Department of Environmental Quality established 20 monitoring wells at the site to identify plume boundaries, and eight additional wells were installed in 1997 for free product removal. A thorough review of reports containing several years of quarterly monitoring well data was performed. These reports originated from consultants (MSE; Butte, MT) for the Montana Dept. of Environmental Quality and contained complete gasoline contaminant concentrations as well as other pertinent chemical analyses, including dissolved oxygen.

The maximum depth to groundwater is approximately 15 ft. at the point of release. The dominant lithology identified from bore holes was silt and fine sand with scattered clay lenses, typical of lakebed deposits common to the area. Active remediation at the site included a combination of passive recovery skimmers, air sparging systems coupled with soil vapor extraction, and an interceptor trench.

*Enrichment Study Inocula*

Soil and aquifer materials used as inocula for MTBE-degrading bacterial enrichments were collected from two bore holes drilled to a depth of 1 m below the groundwater surface. One bore hole was near the down-gradient edge of the NAPL-phase gasoline plume at M-12 and the second was adjacent to M-19 near Spring Creek (the impacted surface water receptor). Samples were retrieved from above, at the interface with and below the groundwater surface, and were stored at 4ºC for one week prior to inoculation in enrichments. A pristine agricultural soil (Bozeman, MT) was also used as an inoculum in several "control" enrichments.

*Enrichment of MTBE-degrading bacteria*

Degradation experiments were conducted in closed 125-mL Erlenmeyer flasks under aerobic conditions. The flasks contained 25 mL of a minimal medium (SSE) designed to simulate a "typical" soil solution and contained $NH_4NO_3$ (1.25 mM), $CaSO_4$ (2 mM), $MgCl_2$ (2 mM), $KH_2PO_4$ (10 uM), KOH (1.25 mM), $FeCl_2$ (5 uM), supplemented with 100 uL $L^{-1}$ of micronutrient solution. Subsamples of the composite soil/aquifer material were used as inocula (1% w/v) for the MTBE-degrading microcosms. The slurries were spiked with 10 mg $L^{-1}$ MTBE and ~ 100,000 dpm $flask^{-1}$ [$^{14}$C]-MTBE. Radiolabeled $^{14}CO_2$ evolved from the slurries was captured and used to track MTBE degradation. Solutions of 0.5 M NaOH (0.3 mL) were utilized as $^{14}CO_2$-traps and were placed in cups suspended from the stoppers. The enrichment traps were analyzed weekly for $^{14}CO_2$ (scintillation analysis), and the aqueous phase was monitored for

MTBE disappearance using gas chromatography (GC). Treatments were tested in triplicate and compared to autoclaved controls that contained 250 mg $L^{-1}$ $HgCl_2$.

Due to headspace losses of MTBE by volatilization during sampling of the base traps, subsequent kinetic degradation experiments with the consortium RS24 were performed in 120-mL serum bottles with sealed teflon-coated septa. MTBE was introduced into 40 mL of SSE medium at concentrations ranging from ~10-70 mg $L^{-1}$ and 2-mL samples were aseptically taken for GC monitoring of MTBE degradation. At each sampling time, 3 mL of sterile air was also injected into the serum bottle headspace to maintain aerobic conditions.

*Gas chromatography*

Gas chromatographic analysis of 2-propanol, MTBE, TBA, and TBF was conducted using a Hewlett-Packard 5890 Series II gas chromatograph with a FID detector. A Porapak PS 80/100 mesh packed column (2 mm I.D. x 1.22 m glass; Supelco, Bellefonte, PA) was used with a He carrier gas flow rate of 18 mL $min^{-1}$ at 100ºC and a head pressure of 28 psi. The injector temperature was set at 190ºC and the detector temperature was 250ºC. The initial oven temperature was set at 100ºC for 4 min. and ramped at 10ºC $min^{-1}$ to a final temperature of 200ºC. 2-propanol, TBA, MTBE, and TBF peaks showed retention times of approximately 2.7, 4.5, 6.3, 8.3 min., respectively.

*Microbial cell counts*

0.5-mL samples of MTBE-degrading cultures were placed above a 0.2-µm, 25 mm-dia. black polycarbonate membrane (Poretics Products; Livermore, CA) clamped in a filter chimney assembly and vacuum manifold apparatus. A 100-µL aliquot of 10 mg $L^{-1}$ DAPI was added, allowed to stain the cells for 2 min. and rinsed 2-3 times to remove any unbound stain. The direct counts were conducted using a Nikon Plan Apo100x/1.40 oil lens (DIC H, WD 0.13, ∞/0.17) and a Nikon Eclipse E800 epifluorescence microscope with a mercury lamp UV source. The filter block used had an excitation bandwidth of 340-380 nm and an emission bandwidth of 435-495 nm. Total cell counts were based on a calibrated ocular grid, calculated total membrane area, and an average of 20 enumerated grid fields.

*Isolation of MTBE-degrading bacteria*

Soil-derived, MTBE-degrading enrichments were sampled and spread on both R2A and Noble agar (with basal salts) plates. Half of the Noble agar plates also received surface-applied treatments of 50 µL of 20 mg $L^{-1}$ 2-propanol prior to inoculation with bacteria. All of the Noble agar plates were incubated in closed metal containers and exposed to MTBE vapors by diffusion from MTBE-saturated filter paper. Single colonies from both types of plates were purified through repeated transfer under identical cultivation conditions. Colonies chosen for isolation were based primarily on differences in colony color and size. Isolated colonies were used to inoculate 120-mL serum bottles containing 40 mL of SSE amended with 10 mg $L^{-1}$ MTBE. 2-propanol (20 mg $L^{-1}$) was added as a cosubstrate in selected treatments. Gas chromatography was used to monitor MTBE disappearance and accumulation of degradation products.

*Nucleic acid extraction, polyerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and sequence analysis*

Cell lysis and DNA extraction protocols were based on established methods. Briefly, cells were lysed by mechanical disruption at 6.5 m $s^{-1}$ for 45 s using a beadbeater (Savant

Instruments, Qbiogene; Carlsbad, CA). The crude cell lysates were precipitated with 12M ammonium acetate, and the DNA in the resulting supernatant was precipitated with isopropanol. PCR was performed using primers shown to be highly conserved for numerous bacterial lineages, including the proteobacterial, Gram-positive, cyanobacterial, green nonsulfur, and *Cytophagales-Flavobacterium-Bacteriodes* lineages; and they amplify a hypervariable-containing rDNA region from nucleotide positions 1070 to 1392, based on the 16S rRNA sequence in *E. coli*. DGGE was performed using a 6-11% concentration gradient of acrylamide in addition to a urea/formamide gradient. DGGE bands were purified for sequence analysis, and band purity was confirmed by comparing the PCR products on a DGGE gel to the parent community. Sequencing of isolate DNA and DGGE bands was performed on an ABI Prism 310 Genetic Analyzer using a 47-cm capillary (PE Applied Biosystems, Foster City, CA) and Prism BigDye terminator cycle sequencing reaction kits (PE Applied Biosystems, Foster City, CA).

*MTBE plume modeling*

MTBE plume modeling efforts by engineering students involved the use of two groundwater models, BIOSCREEN and MODFLOW. Inputs to the models included parameters such as initial MTBE gasoline concentration, partitioning coefficients, groundwater flow rates, contaminant solubility, biodegradation rates, and amounts of free product associated with the site. Although some of these parameter values were based on laboratory experimental data, some of the parameter values were estimated.

**Research Results**

*Soil-derived enrichments*

A 12-treatment matrix of enrichment cultures amended with 10 mg $L^{-1}$ MTBE (cold) and $^{14}C$-MTBE as described was established to assay the presence of MTBE-degrading bacteria at the gasoline-contaminated site. The treatment matrix consisted of three inoculum types (M-12, M-19 and pristine) and two levels of nutrient amendment (1x and 10x SSE) (6 treatments). In addition, 2-propanol was added to an additional set of 10x SSE-treated inocula (3 treatments), and there was one set of killed controls (3 treatments). One flask (RS24) inoculated with soil from M-12 and amended with 2-propanol displayed significant MTBE degradation compared to sterile controls and other treatments. After an initial lag time of approximately 15 days, $^{14}CO_2$ analyses indicated a maximum of 33% MTBE degradation after 35 days. Other treatments displayed only minimal degradation over the 35-day trial relative to the sterile controls. Analysis of aqueous phase $^{14}C$-MTBE revealed significant losses from all enrichments, apparently due to headspace losses of volatilized MTBE while sampling the base traps. To minimize these losses, all subsequent experiments were performed in sealed serum bottles.

*MTBE degradation by consortium RS24*

During a subsequent 146-day period, the initial MTBE-degrading enrichment was enriched further (10% v/v inoculum transfer) with 14 mg $L^{-1}$ MTBE and approximately 20 mg $L^{-1}$ 2-propanol. Interestingly, the 2-propanol had been completely utilized after 7 days and small TBA and TBF peaks were evident. At the end of the second week, the TBA and TBF peaks were still evident; however, by the end of the third week no metabolite peaks were observed. Over this 3-week period, the MTBE concentration had decreased to 12 mg $L^{-1}$ and continued to decrease steadily at a rate of about 0.1 mg $L^{-1}$ $d^{-1}$. After this 146-day trial, another transfer (10%

v/v) was made and amended with 6 mg $L^{-1}$ MTBE without the addition of 2-propanol. An extended lag time (~18 days) was again observed before significant degradation occurred. However, with subsequent addition of MTBE on days 40, 47 and 53 the onset of degradation became increasingly rapid. As spiked amendments of MTBE increased from 10.3 mg $L^{-1}$ to 14.7 mg $L^{-1}$ to 66.4 mg $L^{-1}$, no significant decrease in degradation rate was observed. Beginning on day 47, MTBE disappearance was monitored daily and MTBE degradation rates of 0.15 mg $d^{-1}$ and 0.66 mg $d^{-1}$ were calculated from days 47-53 and days 53-57, respectively. Again, no metabolite peaks were observed in any of the chromatograms. In addition, the culture became noticeably more turbid over this time period suggesting microbial growth was coupled to MTBE degradation. To correlate MTBE disappearance with microbial growth, bacterial cell density was calculated for each sampling time from direct microscopic counts as described above. Increasing bacterial cell density during MTBE degradation in duplicate transfer cultures (10% v/v) of RS24 was observed. A gross estimate of biomass yield based on maximum MTBE utilization rates represented in the graphs was calculated. Assuming an approximate dry mass of $2 \times 10^{-10}$ mg $cell^{-1}$, a yield of 0.25 ± 0.02 mg dry biomass mg $MTBE^{-1}$ was observed.

A significant lag phase was repeatedly observed upon transfer of active MTBE-degrading cultures of RS24 to fresh medium. However, in re-spiking active cultures with MTBE no lag time was observed. To determine if a growth-related factor in the medium may be involved in the degradation of MTBE by RS24, degradation experiments were conducted with spent culture medium and fresh SSE mineral medium. Two actively growing duplicate MTBE-degrading cultures of RS24 were centrifuged at 8,000 rpm for 10 min at 4ºC. The supernatant (i.e. spent medium) was decanted and saved and the cell pellets were re-suspended in 3 mL of 1x PBS. Aliquots (1 mL) from each cell suspension were used as inocula for duplicate cultures with 40 mL spent growth medium, and 2-mL aliquots were used as inocula for duplicate cultures with 40 mL of fresh SSE medium. The four new cultures were amended with MTBE to a final concentration of 32 ± 6 mg $L^{-1}$. MTBE degradation with corresponding increases in cell density for the cultures with spent growth medium was observed. In contrast, the cultures with fresh SSE both show minimal MTBE degradation and much lower cell densities over the 60-day trial period. It should be noted that the average initial bacterial counts for duplicate cultures with fresh SSE ($2.85 \times 10^6$ cells $mL^{-1}$) were 2-fold greater than for duplicate cultures with spent media ($1.39 \times 10^6$ cells $mL^{-1}$).

*Isolation of MTBE-degrading bacteria*

Thirteen isolates were cultivated from the original MTBE-degrading enrichment. MTBE degradation kinetics were determined for each isolate in the presence and absence of 2-propanol. Degradation of MTBE was more rapid and complete in the presence of 2-propanol for all isolates tested. Since many isolates had similar colony morphologies and MTBE degradation kinetics, nucleotide sequence analysis of a 340-bp region of the 16S rRNA gene was performed to identify unique isolates. Of 9 isolates characterized, four isolates were 100% identical to *Pseudomonas* sp. Ant9 and five isolates were 100% similar to *Rhodococcus koreensis*. Figure 6 shows degradation kinetics of MTBE in the presence and absence of 2-propanol for two of the isolates identified as *Pseudomonas* sp. Ant9 and *Rhodococcus koreensis*. MTBE degradation by both isolates was minimal in the absence of 2-propanol as a cosubstrate. GC monitoring of isolates grown with 20 mg $L^{-1}$ 2-propanol revealed an initial decrease in 2-propanol concentration prior to the onset of MTBE disappearance, indicating a probable cometabolic

induction of MTBE degradation. Two metabolites observed during MTBE degradation with both isolates in the presence of 2-propanol were identified by GC/MS analysis as TBA and TBF.

*Molecular characterization of MTBE-degrading consortia*

DGGE profiles of RS24 and two successive subcultures taken from this consortium were generated. Sequence analysis of the DGGE bands revealed a phylogenetically diverse group of bacteria present in the consortium. A decrease in the total number of bands in the profiles was observed with successive subculturing. DGGE bands identified as *Pseudomonas* sp. Ant9 and *Rhodococcus koreensis* showed 100% sequence similarity to the two cultivated MTBE-degrading isolates identified above. Interestingly, the *Rhodococcus koreensis* band disappeared completely by the second subculture, while the band corresponding to *Pseudomonas* sp. Ant9 decreased in intensity with subculturing. However, due to bias inherent in the PCR, differences in DGGE band intensity may not reflect population abundance in the consortium. The band corresponding to *Rhodoferax fermentans* was present in the original consortium and appeared stable in both subcultures. After numerous attempts, bands A and B could not be sufficiently purified for sequence analysis. Bands H1 and H2 are believed to be heteroduplex molecules formed during the PCR reaction, as purification and re-amplification of both bands always yielded H1 and H2 in addition to band A and the band corresponding to *Rhodoferax fermentans*.

*MTBE plume modeling*

MTBE plume modeling using BIOSCREEN and MODFLOW were somewhat successful in describing the plume dynamics observed at the site. Value uncertainty for many of the input parameters to the models no doubt resulted in sub-optimal performance. Nevertheless, this study did show that transport modeling was a valuable tool for describing general plume behavior.

*Annual seminars and discussions*

In December of each year for the duration of the project, meetings were held either in Bozeman, MT or Helena, MT in which students presented the results of their work to site consultants and Montana DEQ personnel in a seminar format. These seminars ranged from topics including hydrogeology, biodegradation, modeling, toxicity, etc. Following the presentations, discussions were held among the students and other official representatives to clarify results and to receive input in guiding further research efforts.

**Discussion**

Enrichments designed to simulate MTBE and nutrient concentrations present in the aquifer revealed a diverse group of bacteria capable of degrading MTBE. Initial screening of gasoline/MTBE-contaminated soils from two monitoring well locations yielded a single enrichment capable of degrading MTBE in the presence of 2-propanol. Maintenance and transfer of this enrichment resulted in a bacterial consortium that could degrade MTBE at relatively high rates in the absence of 2-propanol as a cosubstrate. In this ultimate enrichment, cell growth coupled to MTBE degradation as the sole carbon source and no appearance of MTBE metabolites indicated the bacterial consortium derived energy from the complete catabolism of MTBE. Calculated gross biomass yields for this latter consortium of RS24 (~0.25 mg dry biomass mg $MTBE^{-1}$) were generally similar to the estimated biomass yield of 0.18 mg cells mg $MTBE^{-1}$ and 0.21-0.28 mg dry biomass mg $MTBE^{-1}$ reported by other researchers. A

previously reported bacterial strain capable of MTBE degradation was identified as a member of the Beta subgroup of Proteobacteria by 16S rRNA analysis. In our study DNA sequencing of a gel band of 16S rDNA amplified by PCR and separated by DGGE identified *Rhodoferax fermentans* as the organism probably most responsible for MTBE degradation in consortium RS24. Interestingly, the genus *Rhodoferax* also belongs to the Beta subgroup of Proteobacteria. Although in latter cultural transfers of RS24 without 2-propanol no TBA or TBF metabolites were observed during MTBE degradation, earlier cultures of RS24 displayed both TBA and TBF metabolites in the presence of 2-propanol. Also, accumulation of TBA was observed early in this study when one RS24-transferred culture was grown in the presence of 10 mg $L^{-1}$ benzene and 20 mg $L^{-1}$ 2-propanol. Because both TBA and TBF were always observed during MTBE degradation by the isolates, *Pseudomonas* sp. Ant9 and *Rhodococcus koreensis*, in the presence of 2-propanol and because these isolates seemed to be more prominent in DGGE gels of the earlier RS24 consortia, we believe that these two isolates are probably limited to cometabolic MTBE-degradation and eventually became subordinate to *Rhodoferax fermentans* under our experimental conditions. However, others have reported degradation of 28-29% of 200 mg $L^{-1}$ MTBE as the sole carbon source by three pure culture isolates over a two-week period (degradation rate ≈ 0.2 mg $d^{-1}$); and one of these isolates was identified as *Rhodococcus* sp. Also, they observed a reduction in MTBE degradation by this bacterium in the presence of butyl formate, *t*-butanol, and several other simple organic compounds. These results and results from our study showing an accumulation of TBA and TBF by *Rhodococcus koreensis* may indicate an inhibitory effect caused by accumulation of these metabolites.

The production of TBA and TBF support a previously proposed MTBE degradation pathway in which the enzyme, cytochrome P-450, oxidizes MTBE to *tert*-butoxymethanol, which then may be converted to TBF via an alcohol dehydrogenase. The TBF may then undergo hydrolysis to TBA. This latter hydrolysis step was also proposed by other researchers; however, they suggested that the initial formation of TBF may simply be a strict chemical oxidation by atmospheric oxygen. We believe our results do not support chemical oxidation as a major MTBE degradation step in that no TBA or TBF peaks were observed in our sterile controls in the presence or absence of 2-propanol.

The fact that no lag phase in MTBE degradation was observed for transferred cultures grown in spent supernatant suggests the presence of a factor in the medium that allowed uninterrupted MTBE degradation to occur. We propose that this mechanism may possibly involve either a repressor-regulated transcriptional system similar to that of the *lac* operon or activator-dependent regulation. In the first case, a constitutively expressed repressor protein would prevent transcription of genes involved in MTBE degradation. Presumably, production or activation of an inducer protein could be initiated by the presence of MTBE or an effector protein. Release of the inducer over time would increase its concentration and effect more binding to and release of the repressor protein and, thus, allow transcription of the MTBE degradation genes. This type of mechanism has already been proposed for other genes involved in contaminant biodegradation. For example, a model has been proposed for dichloromethane (DCM) degradation by *Methylobacterium* sp. in which production of the protein DcmA (responsible for DCM dehalogenase production) is normally repressed by binding of the regulatory protein DcmR to the *dcmA* promoter. De-repression (i.e. activation) of *dcmA* occurs by putative substrate-induced release of DcmR. Also, in another study research showed that the XylR protein has both repressor and activator roles in the control of toluene biodegradation by *Pseudomonas putida*. Undoubtedly, some of these regulatory mechanisms are strictly

intracellular in nature. However, we speculate that extracellular release of regulatory proteins acting as autoinducers such as those already identified in other types of quorum sensing and cell-cell communication would confer a selective advantage. For example, other researchers have suggested that quorum sensing may prevent unnecessary gene induction, allow a rapid sensing of changes in the environment, and serve to coordinate consortial metabolism among bacterial species. At the very least, we believe that this type of concentration-dependent phenomena could result in observations of MTBE degradation such those documented in this study.

Although several MTBE-degrading bacterial species were cultured from the Ronan aquifer, MTBE biodegradation in the aquifer is difficult to assess in relation to other processes of natural attenuation. However, because of the generally low quarterly dissolved oxygen (DO) measurements and low temperatures (4-12ºC), we concluded that biodegradation is relatively slow at the site. This conclusion is based on another study in which a low concentration of dissolved oxygen and low temperatures severely slowed MTBE degradation rate and growth rate of the mixed, MTBE-degrading bacterial culture. The low DO levels are likely due to seasonal fluctuations in the groundwater of the region as well as to oxygen utilization coupled to contaminant degradation. For example, DO measurements from the first to the fourth quarters of 1999 were consistently less than 0.5 mg $L^{-1}$ for MW-10. However, for the first quarter of 2000 a DO measurement of 3.3 mg $L^{-1}$ was recorded. Over this same time period for the same monitoring well, MTBE measurements first increased over an order of magnitude to 3,580 μg $L^{-1}$ and then gradually decreased again to < 300 μg $L^{-1}$. Likewise, TPH measurements increased almost two orders of magnitude to 10,900 μg $L^{-1}$ and then gradually decreased to < 900 μg $L^{-1}$. Because background DO levels obtained from MW-11 upgradient of the contaminant plume as well as from MW-6 and MW-16 (adjacent to MW-12 but outside the contaminant plume) were consistently between 4-6 mg $L^{-1}$, contaminant biodegradation inside the plume is indicated. However, specific evidence of MTBE biodegradation is confounded by the presence of other potential gasoline-related substrates; and no detection of MTBE metabolites has been recorded. Because measured DO levels indicate that oxygen may be limiting biodegradation at this site, we recommend that any applied treatment technology should investigate and/or include oxygen addition, although other important inorganic nutrients such as nitrogen and phosphorus may also be limiting the activity of MTBE-degrading bacteria. One report of enhanced MTBE degradation by injection of pure oxygen into an impacted aquifer supports the above recommendation.

This study has established the presence of bacteria capable of degrading MTBE in the gasoline-contaminated aquifer near Ronan, MT. Other studies targeting the identification of native bacterial populations with the capacity to degrade MTBE in natural environments will help to establish the ubiquity of these organisms and allow better prediction of the efficacy of biodegradation as a means to remediate MTBE-contaminated aquifers. In addition, further laboratory studies designed to elucidate factors that influence MTBE degradation, such as co-substrates or the concentration-dependent factor proposed in this study, may provide insight into more efficient methods of stimulating MTBE degradation by naturally occurring bacteria.

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- Elsa Meiser, MS Environmental Engineering, Montana State University (in progress)

- Approximately 20 students were involved in Dr. Al Cunningham's Environmental Engineering Investigations class (ENVE 534) at Montana State University over a 3-year period. These students received travel reimbursement for visits to the Ronan site for sampling and other data collection. As part of the course requirements, students generated reports on topics related to the site and its contaminants including site characterization, transport modeling, partitioning, biodegradation, natural attenuation, and health risk assessment. These students interacted with consulting engineers from MSE (Butte, MT) and with Montana Dept. of Environmental Quality personnel in the field and also presented their academic reports at annual meetings involving these and other state personnel.

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