BIODEGRADATION AND INHIBITORY EFFECTS OF METHYL-TERTIARY-BUTYL ETHER (MTBE) ADDED TO MICROBIAL CONSORTIA

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ABSTRACT

The environmental fate and biological effects of the fuel oxygenate methyl-tertiary-butyl ether (MTBE) is of concern because, among other factors, it is highly soluble in water. Thus ground and surface water contamination may occur through accidental exposures and spills. Little is known about the biodegradability and ecotoxicity of MTBE. We found that MTBE added to carbon-limited microbial consortia increased oxygen consumption but that concentrations approaching 740 mg/L inhibited the mineralization potential of another fuel constituent (hexadecane) by up to 50%. These results indicate that MTBE can be metabolized in the environment but that toxicity may adversely affect the overall biodegradation of hydrocarbon constituents of liquid fuels.

KEY WORDS

MTBE, hexadecane, radiorespirometry

INTRODUCTION

Methyl-tertiary-butyl ether (MTBE) satisfies the United States Clean Air Act Amendment of 1990 that requires fuel oxygenation in gasoline mixtures to reduce carbon monoxide air pollution. MTBE has been used throughout the United States and Canada as an octane enhancer but it is now primarily used to increase oxygen content in gasoline. The Oil and Gas Journal [1] estimates that oxygenated gasoline will eventually comprise about one third of the gasoline consumed in the United States. Controversy surrounds the type of oxygenated gasoline which best satisfies consumer needs and air quality requirements. Besides MTBE, ethanol also satisfies the Clean Air Act requirements. Both ethanol and MTBE are attractive additives for gasoline because they have high octane characteristics, burn clean and can be manufactured from abundant resources [2]. Ethanol, a renewable resource, currently produced through the microbial fermentation of corn, is biodegradable.

As a group, alkyl ethers including MTBE are chemically stable and little information on their biodegradation is available. Salanitro et al. [3] described a mixed microbial culture apparently growing on MTBE as its sole carbon source in continuous culture, but little else is available in the literature.

MTBE has a high vapor pressure (3.35×10^4 Pa at 25ºC; [4]), a high water solubility (4.8×10^4 mg/l at 20ºC; [5]) and a low log octanol/water partition coefficient (1.3; [6]). MTBE is miscible in gasoline and soluble in water, alcohol and ether.

Accidental spills of gasoline containing MTBE or leaks from storage tanks can pose a hazard to groundwater supplies [7]. Studies of the biodegradation of MTBE in

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subsurface ground vary. Yeh and Novack [8] evaluated biodegradation in static soil/water microcosms and found that the biodegradation potential of MTBE varied with site and depth and was the most difficult to degrade of the oxygenates studied. Bacteria and fungi able to degrade naturally occurring organics are widespread. Indigenous microbial populations represent a primary mechanism in which petroleum hydrocarbon pollutants are eradicated from the environment. A better understanding of the microbial communities with respect to MTBE can be helpful in dealing with the future problem of MTBE site contamination.

Hexadecane is a representative alkane found in many petroleum products including crude oil, fuel oil and gasoline. Many studies have shown that hexadecane is easily biodegraded under aerobic conditions [9]. The microbial degradation of a linear hydrocarbon such as hexadecane (C_{16} H_{34}) involves the oxidation of the hydrocarbon in a series of steps that yield the corresponding alcohol, aldehyde and fatty acid. The fatty acid then is decomposed by a reaction sequence called beta oxidation.

In this study I investigated the interaction between MTBE and the degradation of a representative alkane hydrocarbon, hexadecane, using radiorespirometry protocols and attempted to select for a microbial consortia capable of metabolizing MTBE using continuous culture enrichment techniques. This radiorespirometric assay was designed to minimize many of the complex factors regulating microbial metabolism such as carbon availability, temperature, etc., but not the in situ microbial biomass and its potential to degrade the organics in the sample [10]. If the use of MTBE reduces carbon monoxide emissions but causes human health problems [7] or ecotoxicity, its further use will prove futile.

THEORY

The rate of $^{14}CO_2$ (r*; dpm/da) production from a specific radiolabeled organic chemical varies with the total rate of CO_2 production (R; ug/da) and the specific activity of the added radiotracer (A*/(Sn+A); dpm/ug), where A* is the total radioactivity added, Sn is the in situ organic chemical concentration (ppm soil or ppm in water) and A is the concentration of organic chemical added with the radiolabeled chemical.

$$r^* = \frac{A^*}{(Sn + A)} \times R \quad (1)$$

The actual rate of CO_2 production from all carbon sources in the sample (R) also depends on organic chemical "availability" represented by (Sn+A), initial biomass, and the respiratory activity of that biomass. The respiratory activity is controlled by incubation conditions that dictate the growth rate limiting factor.

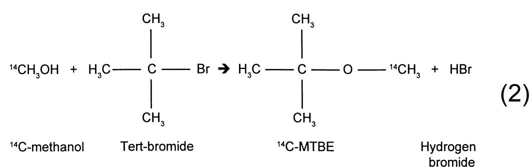
By adding a large amount of a labeled organic chemical to each sample to be assayed, A in Equation 1 will be large enough to make r* primarily dependent on A, rather than Sn, for all but the most grossly contaminated samples [10]. By incubating the microorganisms or contaminated soils in nutrient-rich medium under identical conditions for relatively short periods, the effects of most other external factors, including microbial growth rate, are reduced. Therefore, r* is the potential for the microbial community derived from a soil sample to metabolize the particular chemical added in the assay (A). The mineralization potential of soils, water and marine sediments for hexadecane, phenanthrene, naphthalene, benzene and benzo[a]pyrene has been assayed based on the above rationale [10].

PROCEDURE

Bushnell-Haas (BH) basal medium (Difco, Detroit, MI) was used for batch cultures. It contains: $MgSO_4$-$7H_20$ (0.2 g), $CaCl_2$ (0.02

g), KH_2PO_4 (1 g), K_2HPO_4 (1 g), NH_2NO_3 (1 g) and $FeCl_3$-$6H_20$ (0.05 g) for every 1000 ml of water. Carbon sources were added to BH according to the protocol. The pH was adjusted with NaOH to 7.0 and checked with litmus paper. Water lost in autoclaving was replaced by bringing the flask up to volume with sterile water. All culture media were prepared with distilled and deionized water (American Society for Testing Standards type 1 quality). The MTBE (99.8%) was obtained from Sigma-Aldrich Chemicals in liquid form.

Carbon-14 labeled MTBE was prepared by the Department of Chemistry, University of Northern Iowa, from $^{14}CH_3OH$ (Sigma Chemical Company, St. Louis, MO) by the following reaction:



(2)

Note that the MTBE was labeled on the carbon of the methyl group. Neat ^{14}C-MTBE was added to an acetone solution containing 2 g/L of MTBE. This stock solution had a specific activity of approximately 300,000 dpm/mg MTBE

{1-14C}-hexadecane was obtained from Sigma Chemical Company (St. Louis, MO). The source material had a specific activity of 5 mci/mmol. The neat hexadecane was added to an acetone solution containing 2 g/L of hexadecane. This stock solution had a specific activity of approximately 600,000 dpm/mg hexadecane.

An inoculum for the enrichment culture was obtained from an oiled sediment sample (KN 213B, B lower) collected by Dr. Edward Brown on Knight Island in Prince William Sound, Alaska. A consortium was enriched with 1.25 grams of either L-valine or L-(+)glutamic acid (Fisher Scientific, Chicago, IL) added to BH basal medium. These carbon sources were chosen so that catabolite repression, which is a mechanism where the presence of a readily degradable, non-toxic carbon source inhibits induction of the catabolic enzyme system necessary for degradation of a less readily degradable carbon source (potentially MTBE), is minimized [11].

Soil samples used directly for radiorespirometry were collected by Emy Monroe from a crude oil-contaminated terrestrial site in interior Alaska. Some of the samples were processed immediately while others were stored at 4ºC until needed.

The culture apparatus used in the enrichment experiments was a Braun Biostat 2 benchtop "fermentor." (B. Braun Biotech Inc., Allentown, PA) The medium, stored in a 20 L plastic carboy, was pumped via a variable speed peristaltic pump through silicone tubing into a 2 liter working volume reactor vessel. The culture vessel is made of borosilicate glass with a height/diameter of about 2/1. Waste cells and medium were removed from the system as fresh medium was added from the peristaltic pump. All experiments were conducted at a constant temperature of 25ºC. The medium carboy and reactor flask were autoclaved for each experiment and connected to each other using aseptic techniques.

The flow rate (F, in ml/hr) delivered by the pump was determined by allowing the effluent to drain into a graduated cylinder for a specific amount of time. The flow rate was measured several times to assure a constant delivery by each pump at a given flow rate. The dilution rate (D) was calculated as the flow rate divided by the volume of the medium in the culture vessel. This value, D, is equivalent to the specific growth rate of the microorganisms when growing at steady-state (u). After at least three residence times (R; where R = 1/D) with no change in pH, O_2, and cell mass measured by absorbance at 540 nm, a steady-state was assumed, and samples of

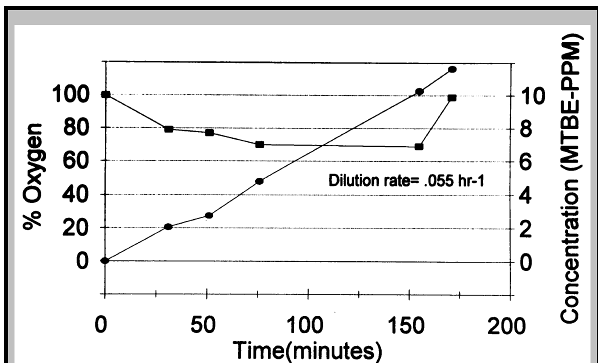


Figure 1. Carbon-limited consortium from enrichment culture. At t = 0 MTBE was added to the feed as the sole carbon source (1.25 g/L) to the feed. ■ shows % oxygen and ● shows the MTBE concentration in the culture vessel with respect to time after MTBE addition started.

the reactor vessel contents were removed for microbiological and chemical analyses. At steady state, MTBE replaced the valine or glutamic acid as the carbon source in the feed, and oxygen was monitored continuously.

For some experiments 40 ml of enrichment consortia from the fermentor culture vessel were added to I-Chem vials, injected with 50 µl ^{14}C-MTBE stock solution (100 ppm) and incubated for prescribed incubation times (0, 24, 48, 72 and 96 hrs). After the desired incubation period, ten milliliters of each sample were transferred into another I-Chem vial fitted with a center well and a NaOH wetted filter paper. The sample was acidified to stop metabolism and evolve CO_2. Each of these vials was left unopened for twenty-four hours before the filter paper was transferred to a scintillation vial and assayed for $^{14}CO_2$ using the same protocol as described for the soil samples (see text).

Using a protocol modified from Brown et al. [10] ^{14}C-MTBE was also added to Alaskan soil slurries. A fraction of each soil sample was mixed 1:10 (w/w) in a sterile 500 ml bottle containing BH broth. After vigorous shaking by hand for one minute, 10 ml aliquots of the slurry were pipetted into sterile 40 ml precleaned glass incubation vials fitted with Teflon-lined septa and center wells containing a small filter paper strip wetted with 5 N NaOH (Kontes, Vineland, NJ, and I-Chem Research, Hayward, CA). Triplicate vials were prepared for 0, 24, 48, 72 and 96 hour incubations. Each vial was then injected with 50 µl of the ^{14}C-MTBE stock solution to result in 100 ppm MTBE/vial (100 ppm MTBE wet soil and 10 ppm slurry).

After injecting the vials with radiolabeled MTBE, one ml of 3 N HCl was injected into the first three vials of each concentration series for time zero. The remaining vials in each series were incubated at room temperature without shaking for the predetermined time and then acidified with HCl and left unopened for 24 hours before removing the filter paper out of the center well and transferring to a scintillation vial. Ten milliliters of ScintiSafe (Fisher Scientific, Chicago, IL) was added to the vials. The vials were then counted in the liquid scintillation counter (Beckman LS 5000 TD).

Using the general protocol procedure, Alaskan soil slurries were also injected with 50 µl of the radiolabeled hexadecane stock solution. The resulting initial concentration of added hexadecane was 100 ppm/vial (100 ppm wet sediment, 10 ppm slurry). With hexadecane, each of four groups of fifteen vials were injected with one of four concentrations of MTBE. The four different concentrations are: 1) 10 µl MTBE (737 ppm soil slurry) 2) 25 µl MTBE (1840 ppm soil slurry) 3) 35 µl MTBE (2600 ppm soil slurry) 4) 50 µl of MTBE (3690 ppm soil slurry).

RESULTS

Oxygen concentrations in the continuous culture vessel were measured after MTBE was added to carbon-limited enrichments. Figure 1 shows the results of a typical experiment run at D = 0.055 hr^{-1}. The left y-

axis shows percent oxygen in the culture vessel, and the right y-axis shows the concentration of MTBE in the culture vessel. The x-axis shows the time (min) after the MTBE addition started. Figure 1 shows that oxygen in the culture vessel decreases as the concentration of MTBE in the culture vessel increases to about 10 parts per million (ppm). The MTBE concentration in the culture vessel (S) is a function of the feed concentration, S_0, dilution rate, and microbial metabolism. Assuming no microbial metabolism, the highest MTBE concentration possible at time (t) is :

$$S = S_o - S_o e^{-D(t)} \quad (3)$$

The results in Figure 1 provide indirect evidence that microbial consortia are able to, at least, partially degrade MTBE until levels are reached which inhibit oxygen consumption (approximately 10 ppm for these low biomass, A_{540} = 0.01, carbon-limited cultures).

In order to determine if the MTBE is being mineralized (metabolized to CO_2) by the consortium, 40 ml subsamples from stable populations in the fermentor culture vessel were assayed for production of $^{14}CO_2$ from radiolabeled MTBE. The results from these experiments are shown in Table 1. Six subsample vials were used for each incubation period of 0, 24, 48, 72 and 96 hours.

Using the radiorespirometry protocol, soil samples from Alaska were also analyzed for their potential to mineralize MTBE. The total aerobic heterotrophic microbial population in these samples measured by Most Probable Number ranged from 10^7-10^8 heterotrophs/g soil, which is two to three orders of magnitude higher than the population contained in the enrichment cultures when MTBE was added. ^{14}C-MTBE was added to 1:10 slurries (six for each incubation period), incubated and then counted. The results from this experiment are shown in Table 2.

Figure 2 shows the mineralization potential of hexadecane by the same soil from Alaska. Hexadecane mineralization increases with time to almost eighteen percent of the labeled hexadecane being recovered as $^{14}CO_2$ after 96 hours.

In Figure 2b-e the same concentration of hexadecane was added to the samples from the soils along with various concentrations of MTBE. The amount of hexadecane mineralized decreases as a function of the added MTBE.

Enrichment Time (hrs)	% Recovery of CO_2*								
	#1	#2	#3	#4	#5	#6	mean $\bar{x}$		sd $\bar{s}$
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00
24	0.03	0.14	0.02	0.01	0.05	0.00	0.04	±	0.05
48	0.22	0.26	0.32	0.36	0.18	0.23	0.26	±	0.07
72	1.50	1.40	0.78	1.01	0.46	1.32	1.08	±	0.40
96	2.20	2.40	1.20	1.08	1.94	2.39	1.87	±	0.59

$$*\%CO_2 = \frac{^{14}CO_{2\,at\,time\,t} - {}^{14}CO_{2\,at\,time\,o}}{Total\ Added\ ^{14}C - MTBE} \times 100$$

Table 1. Enrichment culture mineralization of MTBE.

DISCUSSION AND CONCLUSIONS

In an attempt to reduce the atmospheric environmental consequences of fuel burning in internal combustion engines, oxygenate chemicals are being introduced into gasoline. Oxygenates are organic compounds that are designed to increases the oxygen content of gasoline. The Clean Air Act Amendments mandate that oxygenates be seasonally added to gasoline in certain parts of the country to help reduce overall CO emissions [12]. One of the most commonly used oxygenates is MTBE. It is by far the most popular ether oxygenate due to its low cost, ease of production, transfer and blending characteristics [13].

The atmosphere is not the only area where additives and combusted fuels are released [13]. Large amounts of gasoline are used each day in the United States (approximately 300 million gallons) and the transportation system required to transport the gasolines is likely to have spills both on the surface and in the subsurface [14]. Since MTBE is soluble in water, once it is spilled, it is likely to reach groundwater and surface waters. If the use of MTBE reduces carbon monoxide emissions but causes human health problems [7] or ecotoxicity from ground and surface water contamination, alternative oxygenates may need to be investigated for use.

Recent literature [3, 13] provided evidence that while single species cultures could not biodegrade MTBE, multiple species growing in a consortium would degrade MTBE to produce CO_2. I sought to confirm this finding using continuous culture enrichments in this study. In addition, because it is well known that both single species and consortia living in hydrocarbon-contaminated sites will easily degrade hexadecane to CO_2, I measured whether or not MTBE affected the potential for microbial consortia to degrade hexadecane.

I found that oxygen was utilized by a low biomass carbon-limited consortium when small amounts of MTBE were added as the sole carbon source. This provides indirect evidence that MTBE is capable of being metabolized by microbial populations since oxygen is the terminal electron acceptor in aerobic metabolism. However, I was unable to stabilize any consortia with MTBE as the sole carbon source and, in fact, found that when MTBE in the culture vessel reached approximately 10 ppm, the enrichment stopped using O_2 and began to "wash out." I further investigated aerobic metabolism of MTBE by adding radiolabeled ^{14}C-MTBE to samples from the cul-

Enrichment	% Recovery of CO_2*								
Time (hrs)	#1	#2	#3	#4	#5	#6	mean $\bar{x}$		sd $\bar{s}$
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00
24	0.04	0.01	0.01	0.01	0.00	0.03	0.02	±	0.02
48	0.32	0.19	0.30	0.21	0.32	0.25	0.27	±	0.06
72	0.54	0.72	0.72	0.72	0.63	1.06	0.73	±	0.18
96	1.17	1.97	1.70	1.55	1.91	1.44	1.62	±	0.30

$$*\%CO_2 = \frac{^{14}CO_{2\,at\,time\,t} - {}^{14}CO_{2\,at\,time\,o}}{Total\ Added\ {}^{14}C - MTBE} \times 100$$

Table 2. Natural soil consortium mineralization of MTBE.

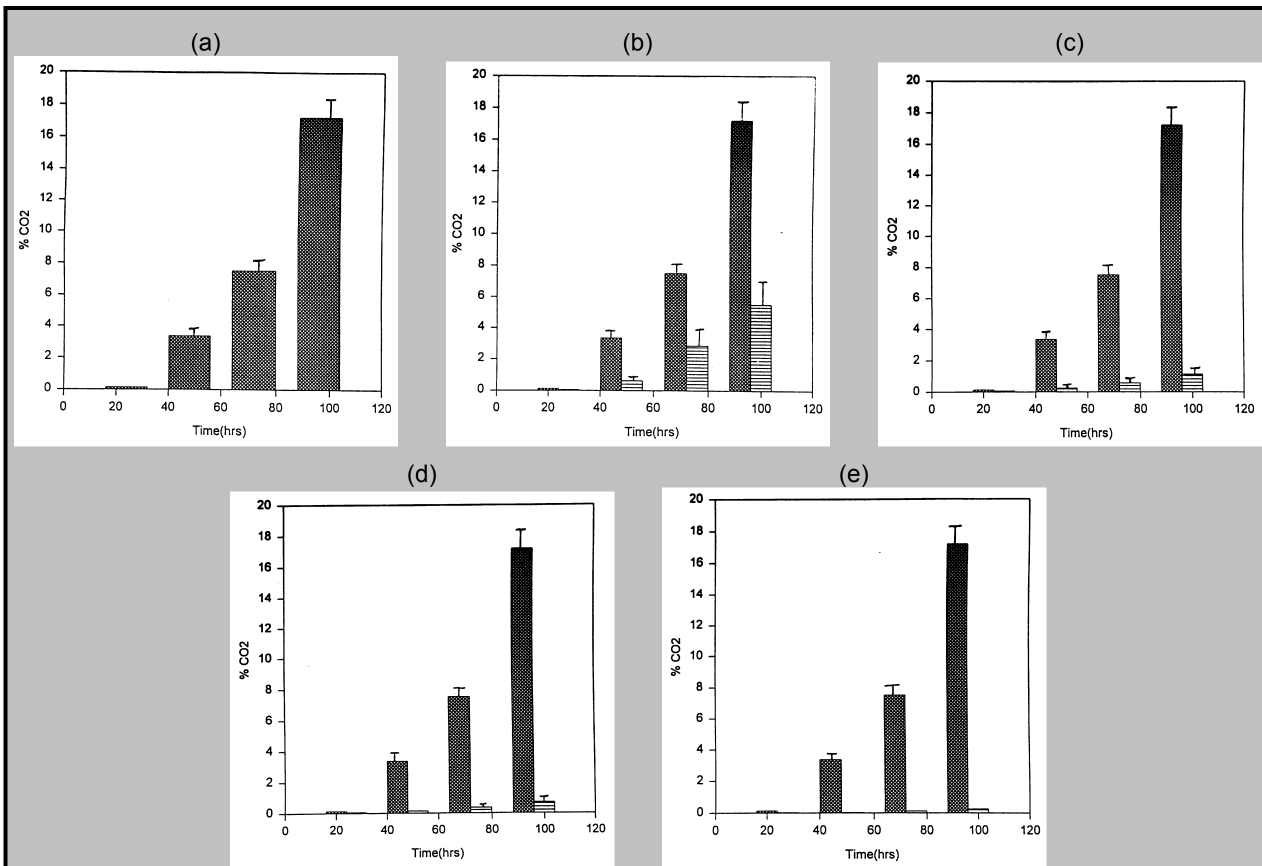


Figure 2. Hexadecane mineralization potential as a function of time and MTBE addition. Fifty microliters of radio-labeled hexadecane were added to 10 ml of soil slurries yielding a concentration of 10 ppm hexadecane-slurry, 100 ppm hexadecane-wet soil. MTBE additions were (a) 0 ppm; (b) 737 ppm; (c) 1840 ppm; (d) 2600 ppm; or (e) 3690 ppm.

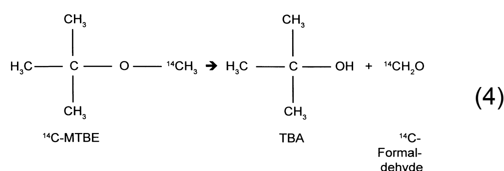
ture vessel to see whether $^{14}CO_2$ could be detected. The basic premise behind the experiment was that if the oxygen depletion was indeed aerobic metabolism, the end product CO_2 might be recovered.

I found (Table 1) that only about 2% of the radiolabeled ^{14}C-MTBE was converted to $^{14}CO_2$. It could be argued from these results that mineralization did indeed occur, but its environmental significance would be minimal. The combined results from the enrichment culture and mineralization experiments indicate that while aerobic metabolism of MTBE may occur to some extent by consortia, it occurs at a very slow rate. Further, MTBE or its metabolites appear to inhibit metabolism of MTBE at concentrations exceeding 10 ppm when no other carbon sources are present. Thus it is unlikely that MTBE can serve as a sole carbon source for microorganisms.

The samples collected from oiled soils in Alaska were also investigated to see if natural populations with a much higher biomass than we were able to maintain in enrichment cultures would produce $^{14}CO_2$ from ^{14}C-MTBE. Table 2 shows little to no mineralization of the ^{14}C-MTBE compared to hexadecane.

According to Anderson [12], MTBE absorbed into the mammalian body is rapidly converted to tertiary butyl alcohol (TBA), which is eliminated from the body in urine. That microorganisms would follow this conversion pathway is not unreasonable [3, 13] and such a pathway may not be detected

by our protocol since the radiolabeled formaldehyde produced may or may not be converted to radiolabeled CO_2.



(4)

Suflita and Mormile [14] found that in anaerobic degradation, oxygenates containing a tertiary or quaternary carbon atom proved more recalcitrant than unbranched or moderately branched analogs but would degrade eventually. Other studies have shown the MTBE is completely recalcitrant to degradation [15]. My results indicate that MTBE may slowly be metabolized to CO_2 aerobically in the environment, but only when conditions preclude it or its metabolites from exerting toxicity to the population of microorganisms capable of degrading it. The rate and extent of degradation of organic components in gasoline will influence the type of clean-up efforts which will be appropriate when it is spilled. MTBE does effect the microbial degradation of hexadecane, but only when present in relatively high concentrations.

My results show (Figure 2) that when hexadecane is added to hydrocarbon-contaminated soil, approximately eighteen percent is converted to CO_2 in ninety-six hours using the protocol described. Eighteen percent conversion to CO_2 is actually substantial considering that besides CO_2, hexadecane will be metabolized for other uses such as synthesizing new cell material [10].

Jensen and Arvin [15] showed that MTBE had a weak inhibitory effect on the degradation of aromatic hydrocarbons at a concentration of 200 mg/L. My results are consistent with the results of Jensen and Arvin [15]; however, the mechanism of inhibition is not clear.

In summary, when MTBE was added to a carbon-limited enrichment consortium, oxygen levels in the culture vessel decreased, providing indirect evidence of MTBE metabolism. When radiolabeled MTBE was added to subsamples from the enrichments or soil slurries, only small amounts of $^{14}CO_2$ were produced. When MTBE was added to soil slurries containing radiolabeled hexadecane, the production of $^{14}CO_2$ from hexadecane decreased. These characteristics are important to know when considering the expanded use of MTBE in the gasoline-blending processes.

ACKNOWLEDGMENTS

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