Ann. Microbiol., **52**, 207-221 (2002)

Methyl *tert*-butyl ether (MTBE) bioremediation studies

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Abstract – The massive production of methyl *tert*-butyl ether (MTBE), a primary constituent of reformulated gasoline, combined with its mobility, persistence and toxicity, makes it an important pollutant. It was considered recalcitrant until a few years ago, but recently MTBE biodegradation in aerobic conditions has been demonstrated with both mixed and pure cultures. Mixed cultures are generally the more effective for MTBE removal, and the addition of a suitable co-substrate to the cultures can enhance the removal. The slow degradation of MTBE has been observed under anaerobic conditions, but only in the presence of humic substances. In recent work, degradation possibilities have been demonstrated when MTBE is the sole carbon and energy source, although the biodegradation was slow. In several cases the addition of a co-substrate such as *n*-alkane, particularly pentane, improved MTBE degradation. The biological treatment of MTBE contaminated soil, water and groundwater could provide a simpler, less expensive alternative to chemical and physical processes for MTBE removal, and the possibility of such an approach has led to investigations into the microbial consortia able to degrade this compound, and the best environmental conditions for its removal. Over the last few years the state of the art of MTBE bioremediation studies has expanded rapidly and numerous articles have been published. Field experiments have revealed the possibility of removing MTBE completely, though the process is slow. Particularly important are treatments involving biostimulation, diffusing oxygen in contaminated groundwaters, and bioaugmentation, that has been demonstrated to improve MTBE removal. The aim of this paper has been to provide a summary of the literature available on MTBE biodegradation in batch systems, reactors and in field.

Key words: methyl *tert*-butyl ether (MTBE), microorganisms, cometabolism, bioremediation, bioaugmentation, biostimulation.

INTRODUCTION

Methyl *tert*-butyl ether (MTBE) is a fuel oxygenate commonly added to gasoline at 11-15% concentration to improve fuel combustion. It is preferred by the petro-

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leum refinery industry to other oxygenates as it is less expensive, easier to use and creates a gasoline of lower vapour pressure than the others; furthermore its use results in fewer volatile organic compound (VOCs) and nitrogen oxide emissions that can cause health and air quality problems (Ainsworth, 1992).

The production of MTBE has increased enormously over the past few years, and since 1993 it has been the second most produced organic chemical manufactured in the United States, 17 billion pounds (approx. 7,700,000 tonnes) being generated there in 1995 (Anon, 1996). In 1997 the annual production volume of MTBE in the European Union was 3,000,000 tonnes, while the consumption was over 2,300,000 tonnes (EFOA, 2000).

The release of MTBE into the environment can occur at industrial sites involved in manufacturing the chemical, or blending it with gasoline and, downstream, nearer the consumer, during the storage, distribution and transfer of MTBE-blended gasoline, or when, at automotive service stations, there are spills, leaks or fugitive emissions (Garret *et al.*, 1986).

MTBE is a clear flammable liquid of low viscosity with a terpene odour. Chemically, it is an ether based molecule ($C_5H_{12}O$) containing 18% by weight oxygen; its boiling point is 55.2 °C, water solubility 51.26 g/L, Koc 12.3 and Log Kow 1.24. Some of its physical and chemical characteristics are akin to those of other common gasoline constituents like benzene, toluene, ethyl-benzene and the xylenes (BTEXs), with which it is associated in environmental pollution, while other properties are different. Table 1 allows a comparison to be made.

TABLE 1 – MTBE and BTEX properties

Physical and chemical properties	Benzene	Toluene	Ethyl-benzene	*o*-Xylene	MTBE
Molecular weight	78.11	92.14	106.17	106.17	88.15
Water solubility (mg/L)	1730	534	161	175	43,000-51,260
Henry law constant	0.23	0.272	0.336	0.212	0,057
Log Koc	1.18-2.16	1.56-2.25	1.98-3.04	1.68-1.83	1.035-1.091
Log Kow	2.36	2.73	3.24	3.10	1.24
VP(mmHg at 25 °C)	76.95	28.4	9.53	6.6	245

In general, when MTBE escapes into the environment through gasoline release it is subject to volatilisation, however some of its characteristics, such as mobility, persistence and toxicity, make it a potential groundwater pollutant. It is more resistant to biodegradation than most other petroleum constituents and much more soluble in water; in fact in soil it tends to exhibit very high mobility and it leaches groundwater. Once in the groundwater, MTBE travels at about the same rate as the water itself, whereas benzene and other petroleum constituents tend to adsorb to soil particles. In surface waters MTBE is subject to rapid volatilisation.

TOXICOLOGY AND ECOTOXICOLOGY

The effect of the compound on human and environmental health depends on the quantity of MTBE present, and the length and frequency of exposure.

With regard to environmental effect, the addition of MTBE to gasoline improves air quality in that MTBE enhances combustion, reducing the release of carbon monoxide and benzene, however the emissions of other pollutants like formaldehyde can increase (U.S. EPA, 1993). Moreover, being a volatile organic compound, MTBE can itself contribute to the formation of photochemical smog in the presence of other VOC (U.S. EPA, 1994a).

MTBE has been reported to have low toxicity for aquatic organisms, the lethal concentration generally having to be greater than 100 mg/L. Little information is available on the toxicity of MTBE for terrestrial organisms (U.S. EPA, 1994b).

The MTBE effect on human health requires further studies: its effect associated with breathing is not known. However laboratory studies on small mammalian animals have shown that respiratory exposure has an adverse effect on the nervous system and on foetus development, and can lead to kidney damage. In addition, there is evidence supporting the role of MTBE as a possible human carcinogen (U.S. EPA, 1999). In December 1997, as a result of these findings, the EPA (Environmental Protection Agency) issued a Drinking Water Advisory on MTBE, advising water suppliers to ensure that MTBE levels do not exceed 20-40 μg/L (U.S. EPA, 1997). More recently, the California Office of Environmental Health Hazard Assessment set a public health goal of 5 μg/L (U.S. EPA, 1999).

As far as concerns MTBE in waters, the MTBE Water Quality Criteria Work Group, constituted by representatives from private companies, trade associations and EPA, defined preliminary criteria for fresh and marine waters. The calculated preliminary freshwater criteria for acute (Criterion Maximum Concentration) and chronic (Criterion Continuous Concentration) exposure effect protection are 151 and 51 mg/L, respectively. Calculated preliminary marine criteria for acute and chronic exposure effect protection are 53 and 18 mg/L, respectively (Mancini *et al.*, 2002).

Given these risks, interest in the biodegradability of this compound and, consequently, in its removal from the different environmental compartments, has recently increased. Several studies in recent years (Prince, 2000; Fayolle *et al*., 2001) have been directed towards investigating the biodegradability of MTBE that, until the early nineties, was considered completely recalcitrant.

PHYSICAL REMOVAL OF MTBE FROM WATER AND SOIL

Although the procedures are often difficult and time consuming, MTBE can be removed from soil and water using existing technologies (Montgomery-Watson, 1996; Prince, 2000; Fayolle *et al*., 2001), e.g., soil vapour extraction (SVE), air stripping, granular activated carbon (GAC) and selected zeolites.

In SVE technology air is injected through the soil to volatilise the contaminant. The contaminant vapours are then extracted or vacuumed from the soil, collected, treated in the appropriate manner, and disposed of to prevent further con-

tamination. Unfortunately, compared to the other components of gasoline, the relative low volatility of MTBE makes it difficult to remove. Air stripping is a process in which contaminated water is passed through a column filled with packing material and an upward-flow of air removes the chemicals from the water. Also in this case the vapours are not released directly into the air before the appropriate treatment.

The GAC treatment techniques consist of pumping contaminated water through a bed of activated carbon to remove organic contaminant compounds. However as MTBE does not adsorb well to organic fractions, its effective removal requires the repeated passing of enormous water volumes through a GAC system.

On the contrary, selected zeolites with high SiO_2/Al_2O_3 ratios were recently shown to be effective for MTBE removal from water (Anderson, 2000). Laboratory studies have demonstrated that zeolites with high SiO_2/Al_2O_3 ratios are capable of adsorbing MTBE and other organic contaminants from water at levels up to over an order of magnitude more efficiently than activated carbon. Furthermore, zeolites are stable over a wide range of conditions, including elevated temperatures and acidic conditions (Newsam, 1986), and can be regenerated more easily than activated carbon.

After each of these physical removal treatments the extracted MTBE and/or its produced intermediates can be degraded via microbial degradation, a cheap and effective process.

MTBE MICROBIAL DEGRADATION

Until the early nineties MTBE was believed to be recalcitrant to biodegradation processes (Jensen and Arvin, 1990). However when investigations revealed the health risks associated with the use of this compound, researchers started working on its biodegradability and on verifying the effectiveness of MTBE contaminated site remediation (Bewley *et al.*, 1989).

Recent studies (Salanitro *et al.*, 1994; Mo *et al.*, 1997; Steffan *et al.*, 1997; Hardison *et al.*, 1997; Garnier *et al.*, 1999) have proved that, under aerobic conditions, specialised *ad hoc* microbial culture mixtures are able, like some pure cultures, to degrade MTBE. It has been suggested that the difficulty in removing MTBE lies in the poor affinity between oxygen and MTBE degrading cultures (Fortin *et al.*, 2001). Some work is also in progress on anaerobic conditions, but MTBE removal appears much more difficult in such conditions. The bulk of present-day knowledge indicates oxygen dependent processes as being the more worthy of consideration for MTBE removal.

Biodegradation of MTBE under anaerobic conditions

Various attempts at biodegrading MTBE under anaerobic conditions have been made, but the results have not been very satisfactory (Suflita and Mormile, 1993; Mormile *et al.*, 1994; Yeh and Novak, 1994). A few rare cases of appreciable anaerobic removal involving extremely long incubation times have been reported, but by very few researchers (Franklin *et al.*, 2000; Prince, 2000).

Mormile *et al.* (1994) investigated *Acetobacterium woodi* and *Eubacterium*

limosum, bacteria known to degrade phenyl methyl ethers under anaerobic conditions, to see whether they could also metabolise MTBE. In fact methyl butyl ether was completely depleted in both sulfate-reducing and methanogenic incubations, and a small amount of methyl *tert*-butyl ether did undergo partial transformation to *tert*-butanol after a long period of acclimatisation, 152 days, and the formed product resisted further anaerobic decay. The authors themselves considered these results a "rare occurrence".

In these same years Yeh and Novak (1994) showed MTBE being attacked under methanogenic conditions, in soils of low organic content and low pH (5.5). More recently MTBE was seen to be attacked by microorganisms in iron-reducing conditions (Prince, 2000), but this does not appear to be a widespread phenomenon.

However recent laboratory scale investigations (Finneman and Lovley, 2001) have confirmed the biodegradability of MTBE and *tert*-butyl alcohol (TBA) in iron-reducing conditions. The experiments were carried out using aquatic sediments to which were added: MTBE, at a concentration of 50 mg/L, Fe(III) and humic substances (HS). Over a period of 275 days the MTBE was depleted to below 1 mg/L. These sediments, amended with HS and additional MTBE (17 mg/L), were able to degrade the MTBE slowly but not completely (60%); the same sediments, but not amended, removed TBA (50 mg/L) without any lag period. Humic substances play an important role in the anaerobic degradation of organic matter. In fact, the Fe(III)-reducing microorganisms oxidise MTBE by transferring electrons to HS and, once reduced, the HS then abiotically transfers electrons to the Fe(III); HS is thus regenerated in an oxidised form.

Biodegradation under aerobic conditions using pure and mixed cultures

Pure cultures

Using pure cultures, MTBE, as the only carbon and energy source, is biodegraded *in vitro*. Three pure MTBE degrading bacterial cultures were isolated by Mo *et al.* (1997) from activated sludge and the fruit of Ginko trees, the bacteria belonging to the genera *Methylobacterium, Rhodococcus* and *Arthrobacter*. Although these cultures were found to attack and transform 29% MTBE added to media at a concentration of 200 mg/L in two weeks, the mineralisation of the chemical was very low (8% after a week). Moreover, in the presence of more easily biodegradable carbon sources, the rate of MTBE degradation decreased significantly.

A bacterial strain, (PM1) isolated by Hanson *et al.* (1999) was capable of utilising MTBE, supplied at different concentrations, as the sole carbon and energy source. In presence of 20 mg/L the PM1 strain converted 46% of MTBE to CO_2 in 5 days. By 16S rDNA analyses the strain resulted a member of the β subgroup of *Proteobacteria* (Bruns *et al*., 2001).

Garnier *et al*. (1999) isolated a MTBE degrading strain, identified as *Pseudomonas aeruginosa*, from a consortium able to mineralise pentane. The strain metabolised MTBE only in the presence of pentane and there was no degradation of the alkyl ether without the alkane. Thus, the controlled addition of a metabolite to foster MTBE degradation appears a most interesting possibility.

Steffan *et al.* (1997) tested the ability of several pure propane oxidising bacterial cultures to degrade gasoline oxygenates, including MTBE. After growth on propane, all the tested strains degraded MTBE, at the concentration of 20 mg/L, and the degradation percentages ranging from 20 to 60% (after 24 hour of incu-

bation); TBA was the first intermediate isolated. Neither MTBE nor TBA acted as an effective growth substrate for the propane oxidisers, that preferred propane as the carbon and energy source. This is in agreement with the recent data of Hyman *et al*. (1998), who indicated that MTBE is principally co-metabolically oxidised by mono-oxygenase enzyme activity induced by other substrates, such as the *n*-alkanes, under aerobic conditions. Experiments performed by Yang *et al.* (1998) also confirmed that as oxygen availability increases, the MTBE degradation rate increases, following Michaelis-Menten kinetic.

Mixed cultures

MTBE biodegradation appears far more interesting using mixed cultures, rather than pure cultures. In fact with mixed cultures one microbial population is able to transform a compound into a metabolite that can then be taken over, and further degraded, by another population.

For instance, Salanitro *et al.* (1994) reported the selection of an aerobic mixed bacterial culture, named BC-1, from a plant bio-treating sludge. This culture metabolised MTBE to CO_2 (20%) and cell mass (40%). The BC-1 culture was able to sustain a population of autotrophic ammonia-oxidising bacteria that nitrify NH_4^+ and use, as carbon source, the CO_2 produced from the metabolism of MTBE. The BC-1 culture contained four or five microorganisms, including coryneiforms, pseudomonads and achromobacters; note that none of these isolates are able to grow when MTBE is the only carbon and energy source. However, the isolates grew in the presence of acetate and MTBE, degrading the latter (120 mg/L) at rate of 34 mg/g of cell per hour within 4 hours. The primary metabolite isolated from the MTBE breakdown was TBA. Other researchers have also reported selected mixed cultures able to degrade MTBE (Cowan and Park, 1996, Eweis *et al*., 1997).

Other mixed cultures degrade in batch MTBE at the concentration of 100 mg/L, the residual concentration at 80 hours ranging from below the detection limit (1 μg/L) to 50 μg/L. A very small quantity of TBA was detected (65 μg/L). The specific activity of the consortium ranged from 7 to 52 mg MTBE/g dry wt per hour (corresponding to 19-141 mg COD/g dry wt per hour); 79% of MTBE was converted in to carbon-carbon dioxide (Fortin *et al*., 2001). In any case the growth of both mixed and pure cultures in the presence of MTBE as the only carbon and energy source is always poor, as evidenced by several authors (Fortin *et al*., 2001; Hanson *et al*.,1999; Fortin and Desshues, 1999a, b).

For example Fortin *et al*. (2001) showed that mixed cultures grown in presence of MTBE supplied as sole carbon and energy source showed a 0.1/day growth rate and 0.11 g dry wt/L yield biomass, corresponding to 0.040 g dry wt/g COD/L. The poor growth is, according to Fortin *et al*. (2001), due to an inefficient carbon assimilation pathway.

MTBE BIODEGRADATION PATHWAY

Investigations carried out with propane-oxidising pure cultures (Steffan *et al*., 1997) have demonstrated that these bacteria are able to degrade MTBE in aerobic conditions. The first metabolite identified is *tert*-butyl alcohol (TBA). The con-

version of MTBE into TBA seems to be mediated by a P-450 enzyme, most likely a mono-oxygenase, an enzyme that appears to have a broad range of hosts (Wackett *et al.*, 1989; Fayolle *et al*., 2001). Experiments in the presence of P-450 cytochrome inhibitors revealed a significant decrease in MTBE conversion (64-85%). Further experiments performed in the absence of oxygen led to the same results. Besides propane-oxidising bacteria also *Pseudomonas putida*, that encodes the P-450cam mono-oxygenase, oxidises MTBE; the first metabolite, TBA, is further oxidised to 2-methyl-2-hydroxy-1-propanol and then to 2-hydroxyisobutyrate (HIBA), neither of these being degraded further by propanotrophs. The accumulation of the two intermediates is a significant rate limiting step in the mineralisation of MTBE and TBA. Their further degradation could require one of three proposed reactions: decarboxylation resulting in the formation of 2-propanol; dehydration resulting in the formation of methacrylic acid; or hydroxylation resulting in the formation of 2,3-dihydroxy-2-methyl propionic acid. The proposed MTBE pathway is shown in Fig. 1 as reported by Hardison *et al*. (1997).

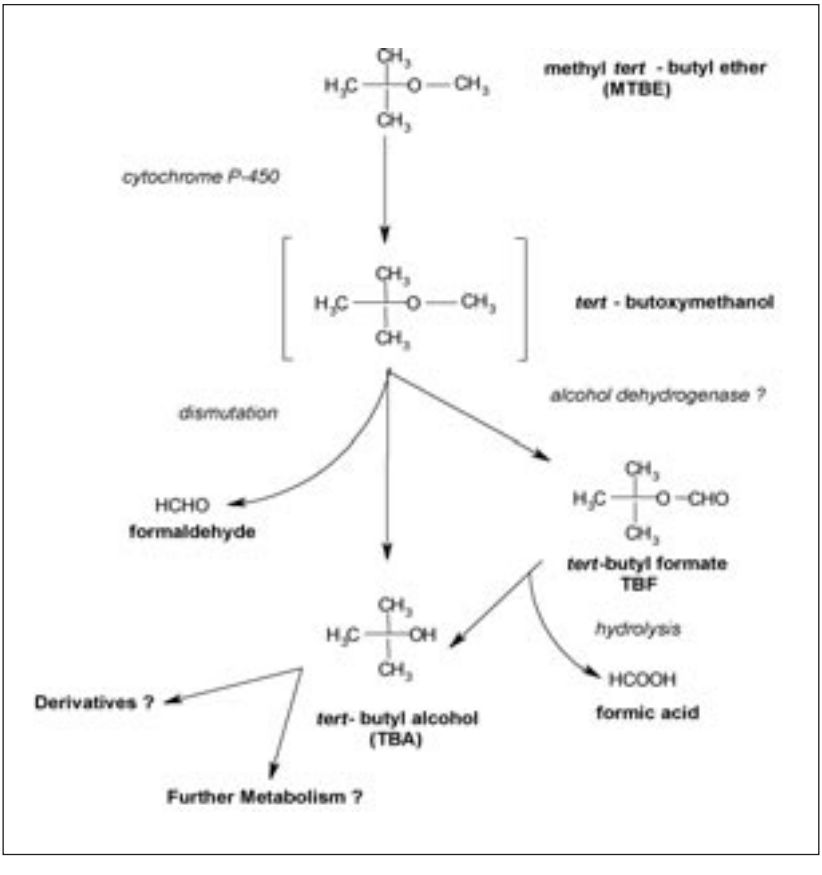

FIG. 1 – MTBE degradation pathway.

The likely involvement of the cytochrome P-450 in MTBE degradation is supported by other studies showing that mammalian P-450 can hydroxylate gaseous *n*-alkane and can *o*-dealkylate both diethylether (Chenglis and Neal, 1980) and MTBE (Bradley *et al.*, 1999).

Hardison *et al*. (1997) studied the biodegradation of MTBE by a filamentous fungus, a *Graphium* sp. strain, under cometabolic conditions in the presence of pentane. They suggest that MTBE oxidation is initiated by cytochrome P-450 catalyzed reactions that lead to the scission of the ether bond in this compound. Both *tert*-butyl formate (TBF) and TBA, MTBE degradation products, were detected, and the kinetics of the degradation pathway suggest that there is temporary TBF production preceding *tert*-butyl-alcohol accumulation; the TBF is converted to TBA, its further metabolism in this fungus still has not been elucidated.

However, the results of MTBE degradation assays with *Rhodococcus rhodochrous* 116, that produced two P-450 mono-oxygenases (Karlson *et al.*, 1993) but did not degrade MTBE, indicate that not all P-450 enzymes can oxidise it. Further studies regarding the MTBE degrading pathway, such as P-450 involvement, are needed.

MTBE BIOREMEDIATION

Microcosm experiments

Investigations carried out on stream-bed sediments showed that MTBE (initial concentration 150 μg/L) and TBA (initial concentration 400 μg/L) were degraded, up to 73% and 84% respectively, to CO_2 in microcosms (Bradley *et al.*, 1999). These results were obtained after 105 days of incubation for MTBE and 27 for TBA under aerobic conditions; no CH_4 production was detected. The same experiments carried out under anaerobic conditions showed no significant MTBE degradation.

Less satisfactory results were obtained by Church *et al*. (1999) with a series of model column aquifers where different groundwater samples spiked with approximately 100 μg/L MTBE were treated. MTBE was converted to TBA after 35 days, but in this case no evidence of subsequent TBA degradation was observed. Under anaerobic conditions there was no evidence of MTBE degradation, not even after 120 days. The same results were found with BTEX in the column, or when more favourable substrates were present (Church *et al*., 2000).

Instead Salanitro *et al*. (1999) observed the total removal of MTBE (10-12 mg/L) from oxygenated well water in a microcosm over a period of 4-9 weeks without bioaugmentation. In this case there was evidence of autochthonous ether-degrading bacteria in the contaminated water. The inoculation of a selected culture (50 mg cells/L) into an identical oxygenated microcosm enhanced the MTBE metabolism, that disappeared from the well water in two weeks. Similarly, with bioaugmentation, there was the total removal of MTBE (70-80 mg/kg) from contaminated soil treated in microcosm for 16 weeks.

Good results were also obtained in a microcosm where contaminated soil was mixed with oxygenated water. Also in this case the MTBE disappeared totally only after bioaugmentation, and over a 16 week period (Salanitro *et al*., 1999). The results confirm that if *in situ* biodegradation occurs, it is most likely under aerobic conditions.

In other experiments (Fortin and Deshusses, 1999a, b) MTBE vapours were treated under aerobic conditions; an enriched aerobic microbial consortium able to degrade MTBE was obtained in two waste-air bio-trickling filters after 6 months. After an acclimatisation phase the filters, working continuously, degraded a MTBE concentration (maintained between 0.65 and 0.85 g/m^3) at a rate of up to 50 g per cubic metre of reactor per hour. It was found that 97% MTBE was converted to carbon dioxide, and no metabolites were found in either the gas or the liquid phase.

Investigations carried out in microcosms containing 30 mL of surface-water sediments collected at eleven different sites throughout the USA demonstrated that the microbial consortia naturally present in the sediments removed MTBE. In fact, over 50 days, all the sediments mineralised 15 to 66% of the MTBE to carbon dioxide in oxygenated cultures. MTBE removal was evidenced also in sediments that did not have a history of MTBE contamination, the efficiency of the removal correlating with the silt and clay grain size distribution of the sediment samples, not with the number of microorganisms present. In fact, a strong inverse relation was observed between the final recovery of CO_2 and the percentage content of grains sized < 0.125 mm, and there was a strong positive relationship with grains of diameter 0.125-2.0 mm (Bradley *et al.*, 2001).

MTBE removal was also investigated in experiments using 9.95 L volume reactors with 4.2 days hydraulic retention time. In these conditions the MTBE removal was corresponding to 99.9% of influent MTBE. The addition of co-substrates into four bioreactors did not modify the removal efficiency (Pruden *et al*., 2001).

The five enriched cultures selected in the reactors were studied by denaturing gradient gel electrophoresis (DGGE). All five cultures were found to be mixed and most of the sequenced DGGE bands belonged to the *Cytophaga-Flexibacter-Bacteroides* phylum. This group was the only one found in all five reactors, suggesting that it could be the most important MTBE degrader; other authors have isolated pure culture degraders belonging to the *Methylococcus-Rhodococcus-Arthrobacter* genera and β-Proteobacteria. One uncultured microorganism attributable to *Nitrospira* was found in the reactor fed only with MTBE (Pruden *et al*., 2001).

Field experiments

Investigations carried out to verify natural attenuation in MTBE contaminated soil, water and groundwater proved not to be fruitful. Only recent field studies, reported by the US Environmental Protection Agency, have shown the possibility of obtaining effective results from *in situ* bioremediation treatments, applying sparged oxygen into MTBE contaminated groundwater. The studies have shown that this promising sparged oxygen technology, with and without microbial inoculation, can reduce the concentration of MTBE from over 1000 mg/L to less than 10 mg/L in less than 2 years (U.S. EPA, 1998).

The removal of MTBE in the field has also been confirmed in other tests that have demonstrated a higher rate of MTBE removal in the presence of a biobarrier in the contaminated soils (2-9 mg/L); such a barrier can be created by inoculating large quantities of selected MTBE-degrading cultures into the area and maintaining well-oxygenated conditions. Moreover oxygenation associated with

bioaugmentation resulted in greater biodegradation improvement than oxygenation alone, without any bioaugmentation. In fact in the former the concentration of MTBE started to decrease after 30 days, continuing throughout the 261 days of the experiment to decrease to up to 0.001-0.01 mg/L; in the latter (without bioaugmentation) the MTBE levels decreased to 0.01-0.1 mg/L after a period of 186-261 days (Salanitro *et al*., 2000).

Other field investigations have confirmed the importance of oxygenation to remove MTBE from the environment. Flowing groundwaters contaminated by an anaerobic MTBE plume were treated by dissolved oxygen that was released into the plume by polymeric tubing; the MTBE was removed ranged from several hundred micrograms to less than 10 μ/L during the passage through the oxygenated zone. The lag period was 2 months and the apparent pseudo-first-order degradation rate was 5.3/day. MTBE was added at subsequent steps of the experiment to a final concentration of 2.1 mg/L, and was degraded at rates of 4.4-8.6/day. These results suggest that MTBE can be removed *in situ* by biostimulation, performed by oxygen diffusion into the anaerobic plume (Wilson *et al*., 2002), confirming the results obtained by U.S. EPA (1998) and Salanitro *et al*. (2000).

Also Landemeyer *et al*. (2001) reported interesting results recently; it was found that a microbial community indigenous to groundwater degraded, *in situ*, 96% MTBE in a relatively oxic zone. The release of oxygen into the anoxic zone of the groundwater led to a decrease in MTBE content from 20 to 3 mg/L in less than 60 days. Under laboratory conditions samples of the natural microbial community present in the oxic zone degraded MTBE completely, without the accumulation of intermediates.

Other field experiments carried out to evaluate the possibility of removing MTBE in the vapour phase were performed using a lysimeter, representing a 2.3 thick sandy unsaturated zone over a gravel aquifer; 0.113 m^3 of excavated sand was contaminated with 0.79 L of a mixture of fuel compounds in the vapour phase, 5% MTBE being present. Monitoring the fuel vapour biodegradation revealed the disappearance of all the fuel compounds in 70 days, while the MTBE accumulated and volatilised from the unsaturated zone. Microbial population counts of the sand, showed no variation between time 0 and the 70^{th} day ($3 \pm 0.6x10^8$). A slight increase was evidenced at the 23^{rd} day ($6 \pm 1.2x10^8$ found at 1.1 and 2.05 m depth). First order biodegradation rates were estimated in the unsaturated zone from 0.05/day for MTBE up to 8.7/day for octane (Pasteris *et al*., 2002). These results show the recalcitrance of MTBE in the vapour phase, and are in contrast with results obtained in microcosm by Fortin and Deshusses (1999a, b). However these authors worked with selected cultures and under different experimental conditions.

CONCLUSIONS

Until a few years ago methyl *tert*-butyl ether was considered recalcitrant, particularly under anaerobic conditions where any attack by microorganisms appears to be very rare and where it is never mineralised.

However in recent years degradation possibilities have been demonstrated

(Prince, 2000; Fayolle *et al*., 2001), the biodegradation being slow when MTBE is the sole carbon and energy source: in some cases there is incomplete degradation, ranging from 8% (Mo *et al.*, 1997) to 50% (Hanson, 1999), and in others complete (Pruden *et al*., 2001); in fact in such conditions the growth rate of MTBE-degrader bacteria is very low.

The difficulty in degrading MTBE when present in the media as the sole carbon and energy source is presumably due to the fact that this compound is a poor substrate and energy source, or that some metabolites work as metabolic or electron-transport inhibitors. This latter hypothesis is supported by indications that the poor growth rate during MTBE degradation is the result of the formation of formaldehyde, a known sterilising agent used commercially to control microbial activity (Salanitro *et al*., 1998).

Other authors have attributed the scarce growth as being due to energetic metabolism (Fortin *et al*., 2001). From among the possible causes, such as the dissipation of a large quantity of energy, the lack of energy production and the rapid energy consumption during MTBE metabolism, the authors suggest the most probable as being the large quantities of intrinsic energy needed for MTBE ether bond cleavage and high energy consumption for the highly exigent anabolism. This latter characteristic is well known in autotrophic nitrifiers that dissipate about 80% energy through the Calvin-Benson cycle.

In several cases the addition of a co-substrate, such as *n*-alkane, and particularly pentane, improves MTBE degradation (Garnier *et al*., 1999; Steffan *et al*, 1997; Yang *et al*., 1998; Fayolle *et al*., 2001). Moreover mixed cultures perform better than pure cultures.

Recently, some potential to attack MTBE was found in microbial populations indigenous to groundwater not previously contaminated with MTBE, as a consequence there had been no adaptation by these populations to the substrate (Landemeyer *et al*., 2001; Fayolle *et al*., 2001).

Although long times are involved, MTBE can be removed under anaerobic conditions (Finneman and Lovley, 2001).

Included in the MTBE degrader bacterial groups are *Methylobacterium, Arthrobacter, Rhodococcus* (Mo *et al.*, 1997), *Sphyngomonas* (Hanson *et al*., 1999*), Pseudomonas putida* (Steffan *et al*., 1997), *Pseudomonas aeruginosa* (Garnier *et al*., 1999) and *Cytophaga-Flexibacter-Bacteroides* group (Pruden *et al*., 2001), these occurring the most frequently; among fungi, *Graphium* must be noted (Hardison *et al*., 1997).

The most satisfactory biodegradation occurred when another substrate (generally *n*-alkane) was added to MTBE. This suggests that pre-existing inducible enzymes are present, their synthesis being better induced by the presence of an adapt co-substrate. However the inducing of this enzyme (mono-oxygenase) also seems to be occur when MTBE is the only carbon and energy source, suggesting that the compound can act as the inducer of a probably not specific mono-oxygenase.

The involvement of mono-oxygenase activity in MTBE aerobic degradation has been described (Steffan *et al.*, 1997; Hardison *at al*., 1997; Fayolle *et al*., 2001).

It has been shown in field experiments that there is the possibility of removing MTBE completely, though the process is still slow. Well worth noting are the

treatments involving biostimulation, performed by oxygen diffusion in contaminated groundwaters (Salanitro *et al*., 2000; Landemeyer *et al*., 2001). Also bioaugmentation had been demonstrated to improve MTBE removal (Salanitro *et al*., 2000). Another factor that affects MTBE removal is soil composition, particularly the size of silt and clay grains (Bradley *et al*., 2001).

In conclusion, it is well known that decontamination of polluted sites may be obtained through natural bioremediation and bioaugmentation and microbiological and technological aspects are widely discussed and reported in literature (Anderson, 1995; Andreoni and Baggi, 1996). Also in the case of MTBE, with the progress of the investigations being made, the possibilities of removing this compound by *in situ* bioremediation seems to be becoming more and more promising (Prince, 2000; Fayolle *et al*., 2001).

Acknowledgements

The authors wish to thank ENI Technologies SpA for financial support and Barbara Carey for editing the manuscript.

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