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Methyl *tert*-butyl ether (MTBE) degradation by a microbial consortium

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Summary

The widespread use of methyl *tert*-butyl ether (MTBE) as a gasoline additive has resulted in a large number of cases of groundwater contamination. Bioremediation is often proposed as the most promising alternative after treatment. However, MTBE biodegradation appears to be quite different from the biodegradation of usual gasoline contaminants such as benzene, toluene, ethyl benzene and xylene (BTEX). In the present paper, the characteristics of a consortium degrading MTBE in liquid cultures are presented and discussed. MTBE degradation rate was fast and followed zero order kinetics when added at 100 mg l^{-1}. The residual MTBE concentration in batch degradation experiments ranged from below the detection limit (1 $\mu g\ l^{-1}$) to 50 $\mu g\ l^{-1}$.The specific activity of the consortium ranged from 7 to 52 $mg_{MTBE}\ g_{dw}^{-1}\ h^{-1}$ (i.e. 19–141 $mg_{COD}\ g_{dw}^{-1}\ h^{-1}$). Radioisotope experiments showed that 79% of the carbon-MTBE was converted to carbon-carbon dioxide. The consortium was also capable of degrading a variety of hydrocarbons, including *tert*-butyl alcohol (TBA), *tert*-amyl methyl ether (TAME) and gasoline constituents such as benzene, toluene, ethylbenzene and xylene (BTEX). The consortium was also characterized by a very slow growth rate (0.1 d^{-1}), a low overall biomass yield (0.11 $g_{dw}\ g^{-1}_{MTBE}$; i.e. 0.040 $g_{dw}\ g_{COD}{}^{-1}$), a high affinity for MTBE and a low affinity for oxygen, which may be a reason for the slow or absence of MTBE biodegradation *in situ*. Still, the results presented here show promising perspectives for engineering the *in situ* bioremediation of MTBE.

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Introduction

In an effort to reduce smog and comply with the strict federal clean air standards, American oil companies are mandated to add oxygenate to non-attainment areas. Methyl *tert*-butyl ether (MTBE) is the most used oxygenate in the US (Swain, 1999; Johnson *et al*., 2000). When accidentally released to the environment, the relative recalcitrance of MTBE to natural attenuation combined with its physico-chemical properties are a threat to underground water supplies and drinking water wells. Consequently, MTBE is currently the target of several US federal regulations (US Environmental Protection Agency, 2000), including the Leaking Underground Fuel Tank (LUFT) remediation programme. A recent USGS study has estimated that as many as 9000 community water wells in 31 states could be subject to MTBE contamination (Johnson *et al*., 2000). Hence, the US Environmental Protection Agency (US-EPA) has recommended, in March 2000, the phasing out of MTBE. When required, remediation actions are implemented at costs usually 10–30% higher than those at sites without MTBE contamination. The evaluation of innovative and cost-effective treatment such as bioremediation for MTBE spills is warranted.

The relatively recalcitrant nature of MTBE to oxidative and/or reductive microbial attack is inherent in its chemical structure, which contains a combination of two 'bio-recalcitrant' organic functional groups: the ether link and the branched moiety. MTBE hardly sustains microbial growth, and its biodegradation is characterized by a low-biomass yield (Salanitro *et al*., 1994; Eweis *et al*., 1998; Fortin and Deshusses, 1999a; Hanson *et al*., 1999). In addition to the steric hindrance of their structure, branched alkanes are sparsely represented in natural compounds and are more averse to enzymatic attack (Alexander, 1965; Pirnik, 1977). *Tert*-butyl alcohol (TBA) has long been recognized as being resistant to biodegradation by activated sludge (Ludzack and Ettinger, 1960). Similarly, aromatics with branched alkyl moieties are usually subject to slow biodegradation. On the contrary, the C-O-C molecular signature is quite ubiquitous: they are found in various natural compounds, such as syringic acid, vanillic acid and other lignin precursors, in cellulose as well as in synthetic ethoxylates (used as detergents) and pesticides. Still, only a relatively narrow

range of microorganisms has the ability to grow on ether compounds. Known biochemical mechanisms of ether scission have been reviewed (White *et al.*, 1996). Under aerobic conditions, enzymes with various ranges of substrate specificity (peroxidase, monoxygenases, dioxygenases, P450) can cleave the ether bond. Such reactions result in the incorporation of oxygen and subsequent production of a hemiacetal, which spontaneously dismutates in aqueous solution to the corresponding alcohol and aldehyde (White *et al.*, 1996). Interestingly, the peroxidase of *Phanerochaete chrysosporium*, known for its wide substrate specificity and its ability to degrade ether bonds, did not degrade MTBE (Kay-Shoemake and Watwood, 1996).

Under anaerobic conditions, the cleavage of ether bonds requires the presence of a hydroxyl group on the vicinal carbon and also yields a labile hemiacetal by a hydroxyl shift. Accordingly, MTBE is not subject to biodegradation under various low-redox potentials (Suflita and Mormile, 1993; Yeh and Novak, 1994; Borden *et al.*, 1997; Bradley *et al.*, 1999).

In spite of the apparent recalcitrance of MTBE in the environment, recent laboratory investigations have clearly shown that MTBE is biodegradable under aerobic conditions. For instance, short alkane-oxidizing bacteria fortuitously co-oxidize MTBE, with probable transient TBA accumulation. (Steffan *et al.*, 1997; Hyman *et al.*, 1998; Garnier *et al.*, 2000). Cytochrome P450 from liver cells and from some fungi was able to oxidize MTBE to TBA (Hardison *et al.*, 1997; Hong *et al.*, 1997). We described earlier the use of MTBE as sole organic carbon and energy source by a growing biofilm (Fortin and Deshusses, 1999a), with similar characteristics to those reported earlier (Salanitro *et al.*, 1994; Eweis *et al.*, 1998). A pure strain that degrades MTBE to CO_2 and cell mass has been described recently (Hanson *et al.*, 1999). Further, ethyl *tert*-butyl ether (ETBE), another short alkyl ether commonly used as a fuel additive, has also been shown to support aerobic growth of a mixed culture (Kharoune *et al.*, 1998) and of a pure strain that stoichiometrically releases TBA (Fayolle *et al.*, 1998).

The present paper reports on MTBE biodegradation by a microbial consortium cultivated in a liquid system, hereafter referred to as the F-consortium. Emphasis is given to the description of two indices of significant interest to biodegradation: the specific affinity (a°_A) and the energy discrepancy index (δ_e), both of which were found to be very high for MTBE. Potential uses of the F-consortium for field bioaugmentation are discussed.

Results and discussion

Bioremediation of recalcitrant compounds requires the enrichment of a suitable microbial culture followed by the determination of its metabolic and kinetic parameters. The F-consortium was enriched in laboratory-scale biological trickling filters treating MTBE vapours (Fortin and Deshusses, 1999a, 1999b). It is a stable mixed culture in the sense that its macroscopic characteristics do not change over time. The original inocula for the biotrickling filters where the F-consortium was enriched were samples from MTBE-contaminated sites. The basic characteristics of the consortium were determined.

MTBE removal, substrate specificity and biomass production

MTBE removal and mineralization A representative MTBE biodegradation profile with concurrent $^{14}CO_2$ release is shown in Fig. 1. The MTBE degradation rate followed zero order kinetics, $k_0 = 2.8$ mg l^{-1} h^{-1} (i.e. $k_0 = 3.18 \times 10^{-2}$ μM h^{-1}) for concentrations of MTBE of 100 mg l^{-1} or less (Fig. 1). Interestingly, the value of the rate constant is similar to that reported for the isolate PM1 (Church *et al.*, 2000). The residual MTBE concentration in the culture medium was routinely < 50 μg l^{-1} as determined by radioisotope experiments. Gas chromatography–mass spectrometry (GC-MS) using EPA method 8260 revealed that MTBE could be degraded down to below the detection limit (1 μg l^{-1}). Such low concentrations are close to or below the US-EPA drinking water advisory level fixed at 20–40 μg l^{-1}, as well as the odour and taste thresholds (15–180 μg l^{-1} and 24–135 μg l^{-1} respectively) (US Environmental Protection Agency, 1997). The low end-point concentration suggests that the F-consortium would be suitable for field applications such as bioaugmentation, *in situ* biological barriers or *ex situ* biotreatment. Altogether, 79.2 $\pm$ 0.7% of the carbon-MTBE was converted to carbon-CO_2 within 80 h of incubation, which is 1.7 times

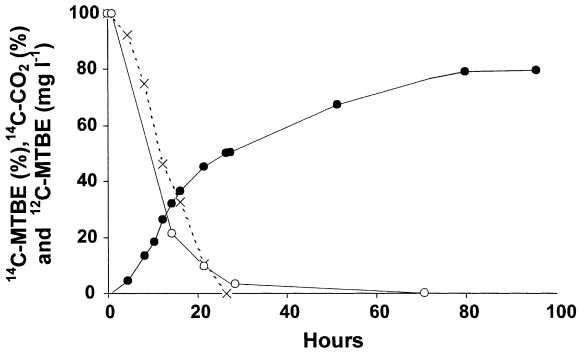

Fig. 1. MTBE mineralization. The F-consortium was cultivated on 100 mg l^{-1} of a mixture of [^{12}C]- and [^{14}C]-MTBE with an initial radioisotope specific activity of 1.07×10^{-3} mCi $mmol^{-1}$. The depletion of [^{14}C]-MTBE (open circles) in the medium, and the release of [^{14}C]-CO_2 (closed circles) trapped in the NaOH solution were followed over time. Another culture fed cold toluene only and MTBE depletion (crosses) was followed by GC-FID.

higher than the value recorded for the pure isolate PM1 (Hanson *et al*., 1999). TBA was found up to 65 $\mu g\ l^{-1}$; other metabolites were not detected by GC-flame ionization detection (FID). The specific activity of the F-consortium ranged from 7 to 18 $mg_{MTBE}\ g_{dw}^{-1}\ h^{-1}$ and reached values up to 52 $mg_{MTBE}\ g_{dw}^{-1}\ h^{-1}$ for smaller inoculum sizes (30-fold diluted; data not shown). This corresponds to 19–141 $mg_{COD}\ g_{dw}^{-1}\ h^{-1}$, which is in the middle to high range of usual values (3–150 $mg_{COD}\ g_{dw}^{-1}\ h^{-1}$) previously listed for common biodegradation processes (Lawrence and McCarthy, 1970). Further comparison reveals that the specific activity of the MTBE-degrading strain PM1 is higher, as it ranged between 25 and 890 $mg_{MTBE}\ g_{dw}^{-1}\ h^{-1}$ for an initial MTBE concentration of 50–500 mg l^{-1} (Hanson *et al*., 1999). Reports on co-metabolic MTBE degradation show comparable or lower specific activities: it was 26.7 $mg_{MTBE}\ g_{dw}^{-1}\ h^{-1}$ after propane induction of strain Env421 (Steffan *et al*., 1997) and 0.05 $mg_{MTBE}\ g_{dw}^{-1}\ h^{-1}$ after butane induction of *Graphium* sp. (Hardison *et al*., 1997). It is also worth noting that similar ranges of activities were reported for microbial cultures degrading ethyl *tert*-butyl ether (ETBE): 181 $mg_{ETBE}\ g_{dw}^{-1}\ h^{-1}$ for the isolate *Gordonia terrae* IFP 2001 (Fayolle *et al*., 1998) and 1.5–1.9 $mg_{ETBE}\ g_{dw}^{-1}\ h^{-1}$ for an enriched mixed culture (Kharoune *et al*., 1998).

Degradation of other relevant carbon sources The F-consortium could also use a wide range of hydrocarbons as sole carbon source. These included the branched alcohols: TBA and *tert*-amyl alcohol (TAA); other ether compounds. e.g. *tert*-amyl methyl ether (TAME), tetrahydrofuran (THF) and diethyl ether (DiEE) (Fig. 2A). Biodegradation rates of TBA and TAME were relatively similar to that of MTBE. The consortium also degraded gasoline co-pollutants: benzene, toluene, ethylbenzene and xylene (BTEX) compounds, and C_1 compounds such as methanol, methyl amine, dimethyl amine and trimethyl amine N-oxide. MTBE was well degraded over a wide range of pH: a marked decrease in the biodegradation rate was only observed for pH below 4 and above 8.

MTBE degradation with concomitant addition of alternative carbon sources As discussed later, biodegradation of MTBE was characterized by a very low overall biomass yield. Various attempts were made to isolate a pure culture capable of degrading MTBE, but none of the strains isolated from the F-consortium could degrade MTBE in liquid cultures after up to 6 weeks incubation. Production of more active biomass was investigated by supplementing the mixed culture with alternative carbon sources, such as ethyl acetate, ethanol, glucose and toluene. Figure 2 show typical results obtained with DiEE. The results were similar for all compounds tested and show that: (i) MTBE would only be degraded after complete depletion of the alternative

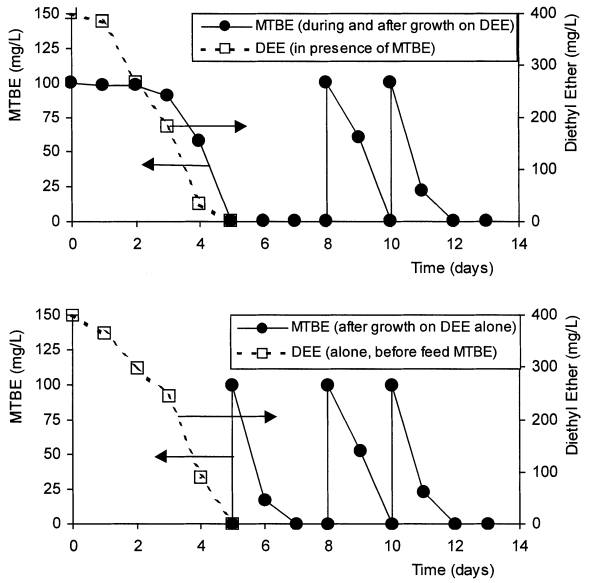

Fig. 2. MTBE biodegradation during and after growth on diethyl ether (DiEE) as an example of an alternative carbon source. Top graph: DiEE in the presence of MTBE before MTBE spikes on days 8 and 10. Bottom graph: DiEE alone before MTBE spikes on days 5, 8 and 10.

carbon source, which suggests that the pathway is subjected to catabolic repression or that MTBE biodegradation undergoes some kind of inhibition (Fig. 2A); (ii) the typical MTBE depletion rate resumed after depletion of the alternative substrate; (iii) a significant increase in biomass concentration was observed (biomass yields of 0.1–0.6 $g_{dw}\ g^{-1}{}_{substrate}$); but (iv) it was not accompanied by an increase in MTBE-degrading activity (Fig. 2A and B). The probable reason is that growth was from the ancillary strains, rather than from primary MTBE degraders.

Substrate kinetics and specific affinity

Apparent Monod constants for MTBE The half-saturation constant for MTBE oxidation was determined for washed suspended biofilm cells taken from an active biotrickling filter (Fortin and Deshusses, 1999a). MTBE-induced oxygen uptake rates (OUR) were recorded at a constant concentration of dissolved oxygen (7.54 mg l^{-1} final) and initiated by adding various amounts of MTBE from a 5% stock solution. The reaction reached saturation at low MTBE concentration (i.e. at concentrations < 10 mg l^{-1}), and the apparent half-saturation constant, Km_{MTBE}, is as low as 1.5 $mg_{MTBE}\ l^{-1}$ (i.e. 17 $\mu mol\ l^{-1}$). The maximum oxygen uptake rate Vm_{OUR} equalled 26.3 μmol of $O_2\ min^{-1}\ g_{dw}{}^{-1}$ (Fig. 3). Assuming that either 7.5 or 0.5 mol of O_2 are consumed mol^{-1} MTBE degraded enables one to determine the maximum oxidation rate of MTBE (Vm_{MTBE}). It ranged from 18 to 277 $mg_{MTBE}\ g_{dw}^{-1}\ h^{-1}$ (i.e. 0.2–3.2 $\mu mol_{MTBE}\ mg_{dw}^{-1}\ h^{-1}$) respectively (Table 1). This

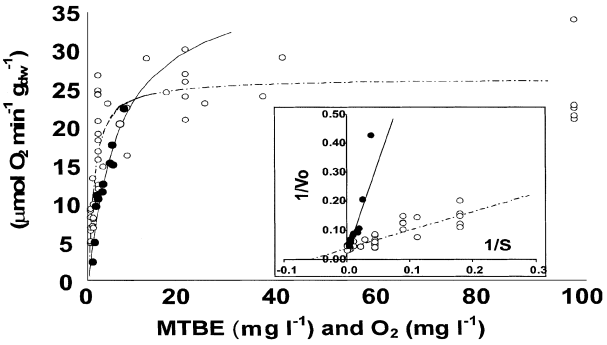

Fig. 3. Dissolved oxygen uptake rate of whole resting cells as a function of MTBE concentration (open circles) and as a function of dissolved oxygen concentration (closed circles). The inset shows the double reciprocal plot.

is in agreement with the specific activity we reported above, determined by GC analysis. As the biofilm biomass probably comprises a significant fraction of inactive biomass (as a result of the origin of the biofilm sample; see Fortin and Deshusses, 1999a), the above maximum specific biodegradation rate is underestimated.

Specific affinity for MTBE The determination of the specific affinity ($a°_A$), defined previously as V_m/K_m (Healey, 1980; Button, 1993), allows one to estimate the ability of the culture to sustain its growth at low substrate concentration. This index was introduced in marine microbiology to assess the ability of a strain to prosper under oligotrophy: the higher the specific affinity, the better the ability to compete or, simply, to feed in a limited substrate environment (Healey, 1980; Button, 1993). We found that the specific affinity of the F-consortium for MTBE biodegradation could be as much as 190 l h^{-1} g_{dw}^{-1} (Table 1). This is higher than average values reported earlier for eutrophic organisms (Button, 1985) but is similar to values reported for oligotrophic bacteria (Semenov, 1991; Schut *et al*., 1993). Compared with other microbial cultures degrading MTBE, the specific affinity of our consortium is significantly higher. This is probably a direct consequence of the enrichment procedure used for this consortium, as the reactors from which the culture originated were never exposed to MTBE concentrations >60 mg l^{-1}. Comparing $a°_{MTBE}$ with the specific affinity of soil microorganisms feeding on BTEX would provide a first approximation of which microorganisms would outgrow the others *in situ*.

Apparent Monod constants for oxygen The apparent half-saturation constant for oxygen (Km_{O2}) is also an important parameter for aerobic biodegradation, as oxygen acts as both a substrate and an electron acceptor. MTBE-induced OURs were therefore recorded at a constant MTBE concentration (35.5 mg l^{-1}, i.e. 0.4 mmol) and at various dissolved oxygen concentrations. The cell suspension was flushed with an air–nitrogen mixture until the desired oxygen concentration was obtained. Between 0% and 100% saturation with air, the reaction did not reach saturation (Fig. 3). Even so, the kinetic parameters were determined by non-linear regression using Michaelis–Menten kinetics. The apparent half-saturation constant Km_{O2} and the maximum uptake rate Vm_{O2} were 6.2 mg l^{-1} (193 µmol l^{-1}) and 39.2 µmol of O_2 min^{-1} g_{dw}^{-1} respectively. Km_{O2} values commonly range between 3.2 and 32 µg l^{-1} (i.e. 0.1–1 µmol l^{-1}) for conventional microorganisms for which oxygen only acts as a terminal electron acceptor (Button, 1985). However, when oxygen is also the substrate of dioxygenase, more comparable values (0.77–64 mg l^{-1}, i.e. 24–2000 µmol l^{-1}) are observed (Krooneman *et al*., 1998).

In the natural environment, where oxygen availability is often limited, aerobic microorganisms will only compete if they hold advantageous kinetic characteristics towards oxygen and if no other phenomena (i.e. inhibition, predation, etc.) interfere. For instance, it has been shown that the composition of a binary culture composed of organisms with different Km_{O2} values was significantly altered by the oxygen concentration at which the culture was grown (Laanbrock and Gerards, 1993; Laanbrock *et al*., 1994; Krooneman *et al*., 1998). Under aerobic or hypoxic conditions, possible cases of MTBE natural

Table 1. Specific affinity of various biological systems oxidizing MTBE.

Biological system	Km_{MTBE} (µmol)	Vm_{MTBE} (µmol mg_{dw}^{-1} h^{-1})	$a°_{MTBE}$ (l h^{-1} g_{dw}^{-1})	Reference
F-consortium[a]	17	0.2–3.2	12–190	This study
Strain PM1[b]	515–582	0.28–10.1	0.5–57	Hanson *et al*. (1999)
Mycobacterium vaccae[b,c]	950	2.5	2.63	Hyman *et al*. (1998)
Xanthobacter[b,c]	2400	5.56	2.32	Hyman *et al*. (1998)
Human liver cells	700–1400	ND	ND	Hong *et al*. (1997)

a. OUR experiment (Fig. 3). See text for details.
b. It is assumed that the protein weight equals 55% of the dry weight for normalization.
c. Co-metabolism.

attenuation have been reported (Borden *et al*., 1997; Schirmer and Barker, 1998; Bradley *et al*., 1999). But the observed MTBE decay rate constants (estimated as $k_1 = 0.0010\ d^{-1}$ and $0.0012\ d^{-1}$) were much lower than those of other gasoline contaminants (Borden *et al*., 1997; Schirmer and Barker, 1998). Interestingly, a side-by-side comparison of a biotrickling filter-degrading MTBE with a biotrickling filter-degrading toluene revealed very similar pollutant removal performance (Fortin and Deshusses, 1999a). The latter observation contrasts sharply with the large difference between the rate of *in situ* bioremediation of MTBE and that of toluene. Apparently, MTBE degraders do not have the capability to grow and express optimum catabolic activity *in situ*. One reason for this could be the diauxic biodegradation of MTBE in the presence of other hydrocarbons, as illustrated in Fig. 2. However, degradation of MTBE was not markedly different in plumes where MTBE was the sole contaminant. In view of the low specific affinity for oxygen of our MTBE-degrading consortium, another reason might be that MTBE degraders might not be able to compete for oxygen *in situ*, where low dissolved oxygen is the rule rather than the exception. This is consistent with a recent field-scale demonstration project showing that, when pure oxygen was sparged in the contaminated aquifer, MTBE biodegradation was observed even in the absence of bioaugmentation (Salanitro *et al*., 2000). The latter finding suggests that MTBE degraders may be more ubiquitous than commonly thought, but that they require high dissolved oxygen, the absence of co-contaminants and extended time to grow and express optimum activity.

Until now, many successful MTBE-degrading cultures have been obtained from well-aerated attached growth bioreactors in which co-contaminant interaction was limited, whereas cell retention, microbial diversity and promiscuity was promoted (Eweis *et al*., 1998; Fortin and Deshusses, 1999a). However, for such bioreactors, oxygen saturation is often accomplished, and the resulting cultures are not being selected for their high oxygen affinity. Further enrichments conducted at low oxygen tensions should be considered possibly to enrich for organisms with higher oxygen affinity, as demonstrated previously for another pollutant (Fritzsche, 1994).

Biomass yield and energy discrepancy

Achieving successful growth of the pollutant-degrading culture is a key to bioaugmentation. A review of recent studies on MTBE biodegradation reveals that MTBE is a poor growth substrate: regardless of the type of culture considered (consortium or pure strains), the experimental biomass yields on MTBE all appear to be lower than $0.2\ g_{dw}\ g_{MTBE}^{-1}$ (Salanitro, 1994; Eweis *et al*., 1998; Fortin and Deshusses, 1999a; Hanson *et al*., 1999). The value for the F-consortium in liquid batch cultures (100 mg l^{-1} MTBE) was $0.11\ g_{dw}\ g_{MTBE}^{-1}$.

Table 2 compares macroparameters pertaining to the metabolism of various classes of compounds. Theoretical biomass yield values (Y_m^{theo}) based on the amount of available electrons (Av. e^-), as proposed by Payne (1970), are listed and compared with experimental values. In Table 2, we introduce the energy discrepancy index (δ_e), defined as the ratio of the theoretical to the experimental biomass yield ($\delta_e = Y_m^{theo}/Y_m^{exp}$). This index is a global measure of the energy lost during a given metabolism. Values > 1 imply an energy loss. The advantage of such a global index is that it can be applied even in the absence of a detailed knowledge of the metabolic pathway and of the individual energy fluxes, which is the case for MTBE. A side-by-side comparison of MTBE with glucose, for instance, reveals that, although the energetic potential intrinsic to MTBE is 20% higher than that of glucose (based on the number of available electrons of the two compounds), its experimental yield is 88% lower. Consequently, the energy discrepancy index for MTBE is much greater than 1 (i.e. $\delta_e^{MTBE} = 10.47$). This is a clear indication that the MTBE metabolism is characterized by an inefficient carbon assimilation pathway, so that a large energy share is dissipated, not produced or rapidly consumed during MTBE metabolism. Each of these alternatives is discussed below.

Is MTBE an uncoupling agent of oxidative phosphorylation? The high mineralization degree and low yield could indicate that MTBE might be an uncoupler of oxidative phosphorylation. However, when the F-consortium was grown on MTBE and ethyl acetate together, a higher yield ($Yx/s = 0.50\ g_{dw}\ g^{-1}$) was obtained than when grown on ethyl acetate alone ($Yx/s = 0.43\ g_{dw}\ g^{-1}$). The small difference between these values corresponds to the yield value on MTBE, and this result disqualifies the hypothesis of an uncoupling effect.

Is the energy wasted during carbon ether bond cleavage? It has been shown recently that, during bacterial growth on different alcohol ethoxylates, the growth yield decreases as the number of ethoxylate units increases (Tidswell *et al*., 1996). In fact, part 3 of Table 2 shows that the greater the ethoxylate units, the greater the discrepancy index, reaching 3.7 for a compound containing eight ether bonds (OEGDE). We found that the discrepancy (δ_e) is even larger for MTBE, which only contains one ether bond. It is interesting to examine the case of MTBE and TBA, which only differ by the presence of an ether function in MTBE, remembering that TBA is the presumed first product of MTBE biodegradation. A comparative analysis of their Y_m^{exp} *and* δ_e values reveals that $\approx 50\%$ of MTBE energy is not converted to biomass. In view of these results and those of Tidswell *et al*. (1996),

Table 2. Comparison of experimental molar growth yield with theoretical molar growth yield predicted on the basis of available electrons for selected organic compounds.

Compounds	Organisms	Av. e^- [a] $e^-_{av.}$ mol^{-1}	Yields			δ_e [e]
			Y_m^{exp} [b] g_{dw} mol^{-1}	$Y_{av.\ e^-}^{exp}$ [c] g_{dw} $e^-_{av.}{}^{-1}$	Y_m^{theo} [d] g_{dw} mol^{-1}	
1: Carbohydrates and miscellaneous substrates						
Glucose	*Ps. aeruginosa*	24	78.1[f]	3.25[f]	73.68[g]	1.06
Benzoate	*Ps. C12B*	30	86.8[f]	2.89[f]	92.1[g]	0.94
Triethylene glycol	ND	30	103.1[f]	3.44[f]	92.1[g]	1.12
Dodecanol	*Ps. C12B*	72	217.0[f]	3.01[f]	221.04[g]	0.98
2: Aliphatic hydrocarbons						
Hexadecane	*Candida*	98	176.6[h]	1.80	203.84[i]	1.15
Heptadecane	*Candida*	106	177.9[h]	1.68	220.48[i]	1.24
Octadecane	ND	110	213.8[h]	1.94	228.80[i]	1.07
3: Ethoxy compounds						
MEGDE	*Ps.* sp. *SC25A*	82	191.8[j]	2.34	251.74[g]	1.31
DEGDE	*Ps.* sp. *SC25A*	90	164.8[j]	1.83	276.3[g]	1.68
TEGDE	*Ps.* sp. *SC25A*	102	162.0[j]	1.59	313.14[g]	1.93
OEGDE	*Ps.* sp. *SC25A*	152	126.0[j]	0.83	466.64[g]	3.70
4: Branched structures						
TBA	F-consortium	26	17.8[k]	0.68	79.82[g]	4.51
5: Branched ethoxy compounds						
MTBE	Isolate PM1	30	15.84[l]	0.53	92.1[g]	5.81
MTBE	F-consortium	30	8.8[k]	0.29	92.1[g]	10.47

a. Av. e^- is the number of available electrons mol^{-1} substrate.
b. Y_m^{exp} is the molar yield determined experimentally.
c. $Y_{av.e^-}^{exp}$ is the equivalent electron yield calculated according to the Payne relation ($Y_{av.e^-}^{exp} = Y_m^{exp}$/av. e^-). It is expressed as g of dry cell weight per available electron.
d. Y_m^{theo} is the theoretical molar yield determined using theoretical values of $Y_{av.e^-}$.
e. The energy discrepancy index (δ_e) is the ratio giving theoretical values to the experimental values ($\delta_e = Y_m^{theo}/Y_m^{exp} = Y_{av.\ e^-}/Y_{av.\ e^-}^{exp}$). A δ_e value > 1 indicates that energy is dissipated during the metabolism.
f. From Payne (1970).
g. For regular substrates, $Y_{av.e^-} = 3.07\ g_{dw}\ e^-_{av.}{}^{-1}$ (Payne and Wiebe, 1978).
h. From Johnson (1967).
i. For hydrocarbons, $Y_{av.e^-} = 2.08\ g_{dw}\ e^-_{av.}{}^{-1}$ (Payne, 1978).
j. From Tidswell *et al*. (1996).
k. This study.
l. From Hanson *et al*. (1999).
MEGDE, monoethylene glycol dodecyl ether; DEGDE, diethylene glycol dodecyl ether; TEGDE, triethylene glycol dodecyl ether; OEGDE, octaethylene glycol dodecyl ether. ND, not determined.

it appears that a large energy expenditure is to be associated with the cleavage of the ether bond. Nevertheless, $>40\%$ of the energy expenditure is unrelated to the ether bond, as illustrated by the low experimental yield value and the high discrepancy index of TBA ($\delta_e^{TBA} = 4.5$). Other phenomena influencing the biomass yield are thus involved; an attempt to identify them is pursued below.

Is the energy wasted during overall catabolism highly exigent in reducing equivalent? The catabolic enzymes may consume a large amount of reducing equivalents, wasting an important energy share. Such a phenomenon is known for enzymes such as monooxygenases, one of the proposed enzymes in the MTBE pathway. However, additions of millimolar amounts of reducing equivalents did not significantly increase the degradation rate of MTBE (Fig. 4), and hydroxylamine even acted as an inhibitor. Such results would be unequivocal for a pure culture. The existence of energy fluxes within the consortium and along the MTBE pathway are unknown for our mixed culture, and syntrophic interactions may well have biased the results presented in Fig. 4. Although these data appear to refute the above hypothesis, further investigations using an isolated strain, when such becomes available, are warranted.

Is the energy withdrawn the result of a highly exigent anabolism? The last possible scenario is that the anabolism of the strain(s) has large energy requirements. For instance, such a phenomenon is well documented for autotrophic nitrifiers that dissipate close to 80% of their energy through the Calvin cycle (Wood, 1986). In fact, we were able to derive a nitrifying–denitrifying culture from the F-consortium, which was able to co-metabolize MTBE while nitrifying ammonia, although other experiments revealed that nitrification was not a requirement for MTBE biodegradation by the F-consortium (data not shown).

At this time, the difference between the energy

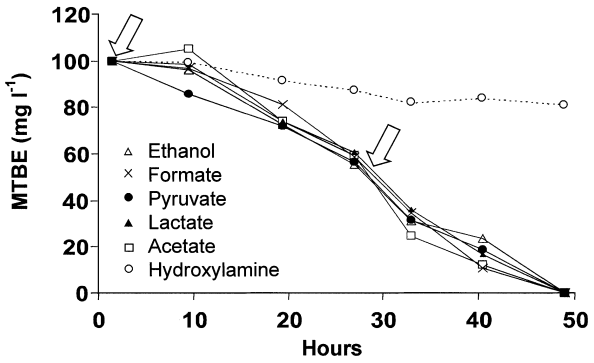

Fig. 4. MTBE depletion in response to millimolar amounts of electron donors. Additions of exogenous substrates were performed at 0 and 30 h (arrows). Not shown but exhibiting a similar pattern to ethanol were yeast extract (2.5 mg l^{-1}), β-hydroxybutyrate and (1:2 vol) methane.

discrepancy indexes of MTBE and TBA suggests that a large portion of the energy intrinsic to MTBE is dissipated during the cleavage of the ether bond. However, the high energy discrepancy index value for TBA suggests that further significant losses of energy occur downstream of the initial step in the MTBE pathway. Hence, the highly exigent anabolism hypothesis remains open.

In conclusion, the characteristics of the F-consortium are summarized in Table 3, which emphasizes that the biodegradation of MTBE is different in many respects from the biodegradation of BTEX or other gasoline constituents. Table 3 provides important data for developing effective MTBE bioremediation strategies. Although further challenges pertaining mostly to the low biomass yield and slow growth rate of the F-consortium persist, the results presented and discussed here show reasonably good prospects for engineering *in situ* bioremediation of MTBE.

Experimental procedures

Microbial culture

The MTBE-degrading consortium was obtained after 6 months enrichment in a biotrickling filter supplied with MTBE as the sole organic carbon and energy source (Fortin and Deshusses, 1999a). After three 1-week-long subcultures in shake flasks, the consortium used was simultaneously to reinoculate the trickling filters, and a fixed biofilm bioreactor was used as a source of the batch liquid cultures described here. Cells originating from the filter are referred to as biofilm biomass and were sampled directly from an 80- to 100-day-old biotrickling filter operated at a constant MTBE supply as described previously (Fortin and Deshusses, 1999a). The fixed film bioreactor consisted of a 2 l Bioflo reactor (New Brunswick) filled with 1.7 l of mineral salt medium (MSM1; see below), operated at room temperature under non-aseptic conditions and stirred at 400 r.p.m. About 1 ml of MTBE was added every week, and the medium was refreshed on a monthly basis. The culture was aerated by oxygen diffusion through 5 m of silicon tubing (outer diameter 8 mm; wall thickness 0.8 mm) coiled in the reactor, through which a gentle air flow was applied, so that MTBE stripping was limited. Biomass was simply retained by spontaneous attachment to the silicon tubing wall. No recording of MTBE degradation was performed in this system.

Mineral salts media and culture conditions

Catabolic activity and growth on MTBE were assessed under different nutritional conditions. The composition of MSM1 has been described previously (Fortin and Deshusses, 1999a). Batch cultures were supplemented with 20–800 mg l^{-1} MTBE (purity > 99%; Fisher) or other carbon sources as specified. Culture growth could not be monitored spectrophotometrically because the culture forms aggregates. The inoculum size was standardized by adding 0.3 g_{ww} of wet biomass to 50 ml of medium, so that the inoculum initially contained 286 mg_{dw} l^{-1}, unless otherwise stated.

Conversion factors and protein determination

Dry biomass weight was found to be 4.76 ± 0.12% of the wet weight by weighing wet and oven-dried (90°C) samples. The elemental composition of the consortium was: carbon, 40.96% by weight; hydrogen, 6.43%; nitrogen, 7.73%; and oxygen, 32.96% (determined by Desert Analytics). Cell samples were heated to 90°C in 0.25 M sodium hydroxide for 10 min before determining the protein content using a Bio-Rad protein assay and bovine serum albumin (BSA) as

Table 3. Relevant parameters of MTBE biodegradation by the F-consortium.

Degradation rate	0.5–2.8 mg l^{-1} h^{-1}
Specific activity	7–52 mg_{MTBE} g_{dw}^{-1} h^{-1} (i.e. 19–141 g_{COD} g_{dw}^{-1} h^{-1})
Residual concentration	< 50 μg l^{-1} ([^{14}C]-MTBE), < 1 μg l^{-1} (EPA method 8260)
Growth rate	0.106 day^{-1} (for 100 mg l^{-1} MTBE)
Biomass yield	0.11 g_{dw} g_{MTBE}^{-1} (i.e. Y_{COD} = 0.040 g_{dw} g_{COD}^{-1})
Energy discrepancy index	$\delta_e^{MTBE} = 10.47$
MTBE mineralization degree	79%
Metabolites	Below detection of 0.5 mg l^{-1} (GC-FID direct injection)
	Selected samples showed TBA (up to 0.06 mg l^{-1}) using EPA method 8260
Substrate affinity	Km_{MTBE} = 1.5 mg l^{-1} (17 μmol)
	Km_{O2} = 6.2 mg l^{-1} (193 μmol)
Specific affinity	a°_{MTBE} > 12–190 l h^{-1} g_{dw}^{-1}
Operating pH range	4–8 with a removal rate > 80% of the maximum rate

standard. To normalize data originating from other authors, it was assumed that cell protein weight equalled 55% of the dry cell weight.

Attempts to isolate a pure MTBE-degrading culture

Various attempts to isolate a pure strain capable of growing on MTBE from the consortium failed. Thirteen heterotrophic prokaryotes as well as four different fungi were isolated from the consortium by first plating on MSM1–agarose plates incubated in an MTBE atmosphere. Microscopic observation of the microcolonies (< 1 mm diameter) grown on MTBE–agarose plates were not significantly different in size, morphology and growth rate from those growing on agarose control plates. Using sterile needles, the prokaryotic microcolonies were transferred onto tryptic soy agar (TSA; 1:10 strength) or R2A agar medium. Similarly, fungi were isolated and spread on yeast nitrogen base (Difco) plates supplemented with 1 g l^{-1} glucose. Each isolate, pregrown on 10 times diluted Luria broth liquid medium, was incubated on 100 mg l^{-1} MTBE and analysed for MTBE depletion. The decrease in MTBE loss was not significantly different from the uninoculated control over a 4–6 week period of incubation. As no pure strain could degrade MTBE, no further characterization of these pure cultures was performed.

Liquid sample analysis

Liquid culture (300 μl) was collected and analysed immediately for MTBE and TBA. Cell-free aqueous sample (1 μl) was automatically injected into a Hewlett Packard model 6890 gas chromatograph fitted with a HP-FFAP column (50 m × 320 μm × 0.5 μm). The injection was pulsed splitless (4.8 atm for 1 min). The oven temperature was held at 80°C for 1 min and was ramped to 120°C at 50°C min^{-1} while the column pressure remained constant (0.7 atm; initial gas flow through the column was 1.2 ml min^{-1}). Detection was with a flame ionization detector (FID). The MTBE and TBA were quantified using standards of known concentrations; detection limits were 0.2 mg l^{-1}. To determine the residual MTBE concentration, analysis was done by a certified laboratory (Centrum) using EPA method 8260.

Oxygen uptake rate (OUR) of suspended whole cells

Cells from the MTBE biotrickling filters were harvested by centrifugation at 10 000 *g* for 5 min, washed twice, resuspended in 20 mM KH_2PO_4 (pH 7.2) and kept on ice before use. Oxygen uptake rate (OUR) was measured in a 2 ml thermostated vessel fitted with a Clark-type electrode (Yellow Springs Instruments) as described previously (Hernandez *et al.*, 1995). Assays were carried out at 30°C, at which temperature oxygen saturation with air corresponds to an aqueous concentration of 7.54 mg l^{-1}. Washed biofilm cells (1.4 g_{dw}) from the biotrickling filter were added to the 2 ml stirred chamber. Endogenous respiration was recorded for ≈ 5 min before adding the desired substrate solution through the cap's septum using a 5 μl Hamilton microsyringe. Substrate-induced oxygen consumption was then determined for 10–15 min and corrected for endogenous respiration. Some scattering was observed during OUR experiments, which was attributed to the variable proportion of MTBE-active and MTBE-inactive cells that constitute biofilm samples.

Radioisotope experiments, MTBE mineralization degree and residual MTBE concentration

Uniformly labelled [^{14}C]-MTBE in ethanol used in this study was custom synthesized to a purity of 99.5%, as determined by radiochromatography, with a specific activity of 5 mCi $mmol^{-1}$ (New England Nuclear Life Sciences). The experiment was performed in a 250 ml serum flask fitted with Teflon-lined septa. The culture volume was 50 ml of medium containing 1 g l^{-1} nitrate and 286 mg_{dw} l^{-1} MTBE pregrown cells. The initial radioactivity was 8.98×10^{-3} mCi g_{dw}^{-1}. Cold MTBE (100 mg l^{-1}) was also added, lowering the initial radioisotope specific activity to 2.10×10^{-3} mCi $mmol^{-1}$. Incubation was performed at 30°C on a rotary shaker at 250 r.p.m. The $^{14}CO_2$ released was trapped in a glass test tube filled with 4 ml of 4 M NaOH. Partition of MTBE in the NaOH would be < 8% of the total amount. Aliquots (500 μl) from the culture and from the NaOH solution were sampled at regular intervals. ^{14}C was quantified by liquid scintillation after fourfold dilutions in ScintiVerse II (Fisher Scientific) using a Triathler multilabel tester (Hidex). The apparatus counting efficiency coefficient equals 80%. For this set of experimental parameters, a count of detectable 1 c.p.m. corresponds to a total isotope concentration ([^{12}C]- and [^{14}C]-MTBE) of < 24 μg l^{-1}. A simultaneous experiment was performed with only cold MTBE, for which samples were analysed by GC-FID and GC-MS using method 8260 (see analysis above). A labelled control without microorganisms was performed in order to evaluate possible losses of radionucleide.

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