NRC Publications Archive Archives des publications du CNRC

Use of *Mycobacterium austroafricanum* IFP 2012 in a MTBE-degrading bioreactor

Maciel, Helena; Mathis, Hugues; Lopes Ferreira, Nicolas; Lyew, Darwin; Guiot, Serge; Monot, Frédéric; Greer, Charles W.; Fayolle-Guichard, Françoise

This publication could be one of several versions: author's original, accepted manuscript or the publisher's version. / La version de cette publication peut être l'une des suivantes : la version prépublication de l'auteur, la version acceptée du manuscrit ou la version de l'éditeur.

For the publisher's version, please access the DOI link below. / Pour consulter la version de l'éditeur, utilisez le lien DOI ci-dessous.

Publisher's version / Version de l'éditeur:

<https://doi.org/10.1159/000121330>

Journal of Molecular Microbiology and Biotechnology, 15, 2-3, pp. 190-198, 2008-07-28

NRC Publications Record / Notice d'Archives des publications de CNRC:

<https://nrc-publications.canada.ca/eng/view/object/?id=f13b62f4-f344-45c1-ac2c-746aa6dee986>

<https://publications-cnrc.canada.ca/fra/voir/objet/?id=f13b62f4-f344-45c1-ac2c-746aa6dee986>

Access and use of this website and the material on it are subject to the Terms and Conditions set forth at

<https://nrc-publications.canada.ca/eng/copyright>

READ THESE TERMS AND CONDITIONS CAREFULLY BEFORE USING THIS WEBSITE.

L'accès à ce site Web et l'utilisation de son contenu sont assujettis aux conditions présentées dans le site

<https://publications-cnrc.canada.ca/fra/droits>

LISEZ CES CONDITIONS ATTENTIVEMENT AVANT D'UTILISER CE SITE WEB.

Questions? Contact the NRC Publications Archive team at

PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca. If you wish to email the authors directly, please see the first page of the publication for their contact information.

Vous avez des questions? Nous pouvons vous aider. Pour communiquer directement avec un auteur, consultez la première page de la revue dans laquelle son article a été publié afin de trouver ses coordonnées. Si vous n'arrivez pas à les repérer, communiquez avec nous à PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca.

Use of *Mycobacterium austroafricanum* IFP 2012 in a MTBE-Degrading Bioreactor

Helena Maciel^{a, b} Hugues Mathis^a Nicolas Lopes Ferreira^{a, b} Darwin Lyew^b
Serge Guiot^b Frédéric Monot^a Charles W. Greer^b Françoise Fayolle-Guichard^a

^aInstitut Français du Pétrole, Rueil-Malmaison, France; ^bBiotechnology Research Institute,
National Research Council of Canada, Montreal, Que., Canada

Key Words

Mycobacterium austroafricanum • Biodegradation •
Biofilter • Perlite • 16S rRNA gene primers

Abstract

Mycobacterium austroafricanum IFP 2012 is able to slowly grow on methyl *tert*-butyl ether (MTBE), a fuel oxygenate widely used as a gasoline additive. The potential of *M. austroafricanum* IFP 2012 for aerobic MTBE degradation was investigated in the presence of a secondary carbon source, isopropanol. The strain was then tested for MTBE biodegradation at the laboratory-scale in a fixed-bed reactor using perlite as the matrix, and isopropanol was injected once a week to maintain *M. austroafricanum* IFP 2012 biomass inside the perlite bed. The biofilter was operated for 85 days at an influent flow rate of 20 ml/h by varying the MTBE concentration from 10 to 20 mg/l. The hydraulic retention time was fixed at 5 days. The removal of MTBE depended on the inlet MTBE concentration and a MTBE removal efficiency higher than 99% was obtained for MTBE concentrations up to 15 mg/l. A set of 16S rRNA gene primers specific for *M. austroafricanum* species was used to analyze the DNA extracted from the biofilter effluent in order to detect the presence of *M. austroafricanum* IFP 2012 and to estimate the effect of periodic injections of isopropanol on the release of

the strain from the perlite bed. The results demonstrated that the injection of isopropanol served to maintain an active MTBE degrading biomass in the biofilter and that this system could be used to effectively treat MTBE contaminated groundwater.

Copyright © 2008 S. Karger AG, Basel

Introduction

Methyl *tert*-butyl ether (MTBE) is added to gasoline (up to 15%, v/v) to achieve a high octane rating and to reduce air pollution. Advantages of the use of MTBE include low production cost and its ability to mix well with other gasoline components. The massive use of MTBE, combined with its mobility in water and its low biodegradability, makes it an important and persistent pollutant of groundwater. Due to the frequent contamination of groundwater by MTBE reported in the USA and in Europe [Johnson et al., 2000; Schmidt et al., 2003; Thomson et al., 2003; USEPA, 1999] and to its organoleptic properties rendering water undrinkable even at very low concentrations, it is essential to develop efficient technologies to remediate MTBE-contaminated sites. Moreover, it has to be emphasized that there is a lack of reliable data on MTBE human toxicity and on MTBE ecotoxicity,

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2008 S. Karger AG, Basel
1464–1801/08/0153–0190\$24.50/0

Accessible online at:
www.karger.com/mmb

Françoise Fayolle-Guichard
Institut Français du Pétrole
1–4, avenue Bois-Préau
FR–92852 Rueil-Malmaison (France)
Tel. +33 1 4752 6864, Fax+33 1 4752 7001, E-Mail francoise.fayolle@ifp.fr

which makes these points still controversial. Recent reports on the effects of long-term exposure of fish to MTBE [Moreels et al., 2006b], or short-term exposure of fish to MTBE or its degradation intermediate, *tert*-butyl alcohol (TBA) [Moreels et al., 2006a] are quite alarming.

Even though there are reports of MTBE degradation in contaminated aquifers [for a review, see Moyer and Kosteki, 2003], it is generally considered persistent in the environment under both aerobic and anaerobic conditions. By studying the data available on MTBE degradation kinetics, Stringfellow et al. [2002] concluded that unfavorable MTBE biokinetics dominate environmental fate processes.

Cometabolism was shown to be involved in MTBE biodegradation [for a review, see Fayolle and Monot, 2005], possibly leading to the accumulation of degradation intermediates not available for further growth of the microorganisms. Steffan et al. [1997] observed that propane-oxidizing bacteria could oxidize MTBE and TBA but that the inability to efficiently utilize metabolites (e.g. hydroxyisobutyric acid or HIBA) probably prevented these microorganisms from growing on MTBE. In this case, the addition of a suitable co-substrate can facilitate MTBE removal by sustaining the microbial growth. Actually, the addition of co-substrates, like pentane, improved MTBE degradation in biofilters [Garnier et al., 1999]. Stringfellow and Oh [2002] carried out laboratory studies and determined that there were several compounds which could enrich MTBE metabolizing populations, and *iso*-pentane was found to be the most reliable among all the tested compounds.

In parallel, a few microorganisms are able to use MTBE as a sole carbon and energy source but with poor growth yield [for a review, see Fayolle and Monot, 2005]. Fortin and Deshusses [1999] calculated the ratio of the experimental growth yield on MTBE ($Y^{\text{exp}}_{\text{X/S}}$, expressed in g cell DW mol⁻¹) to the theoretical growth yield on MTBE ($Y^{\text{theo}}_{\text{X/S}}$, which was 92.1 g cell DW mol⁻¹), this value being equal to 1 for a readily assimilated carbon source. For strains growing on MTBE, *Methylibium petroleiphilum* PM1, *Hydrogenophaga flava* ENV735 and *Mycobacterium austroafricanum* IFP 2012, this value was 0.17, 0.38 and 0.35, respectively, during their growth on MTBE [Fayolle et al., 2003]. The advantage of this value is that it can be applied even in the absence of detailed knowledge of the metabolic pathway and of the individual energy/fluxes, and it provides a good estimate of the thermodynamic efficiency during growth on a given substrate. Lopes Ferreira [2005] calculated the theoretical production of ATP from MTBE degradation using established

data from the MTBE pathway of *M. austroafricanum* IFP 2012 ($Y^{\text{theo}}_{\text{ATP/S}} = 14$ mol ATP/mol MTBE). This low value, linked to the fact that the growth is slow, indicated a problem of maintenance energy for the bacteria during growth on MTBE. The issue of biomass production from MTBE was recently discussed by Müller et al. [2007] using a growth-related kinetic model. These authors emphasized the role of maintenance of biomass concentrations to sustain substantial environmental MTBE degradation.

Acuna-Askar et al. [2000] previously studied the biodegradation of MTBE in biofilm reactors and concluded that the presence of other easily assimilated carbon sources in the culture medium favorably influenced MTBE biodegradation. The critical effect of biomass of MTBE degraders was also observed in a suspended growth reactor by Wilson et al. [1999] where the MTBE removal efficiency was directly related to biomass concentrations and MTBE removal was efficient only when biomass exceeded 600 mg/l VSS. This positive effect of an unusually high biomass concentration in bioreactors was also shown by Vainberg et al. [2002] who used a biomass concentration greater than 10,000 mg of dry biomass solids per liter of expanded bed and by Zein et al. [2004] who developed a gravity flow, high biomass retaining bioreactor to efficiently remove MTBE from contaminated effluents.

To our knowledge, there are no reports of a MTBE-degrading biofilter seeded with a unique microorganism previously isolated for its capacity to grow on MTBE. The advantages of such a biofilter working with a well-known microorganism would be the reliability of the inoculum and the ability to track the fate of the microorganism by sampling inside the biofilter and in the effluent. In the case of in situ implementation, remediation performance can be evaluated by monitoring the microorganism in the different environmental compartments. Oligonucleotide primers based on the 16S rRNA gene sequence of *M. austroafricanum* species were specifically designed from the highly-variable regions of its 16S rRNA gene [Lopes Ferreira et al., 2006].

The purpose of this study was to determine the performance of *M. austroafricanum* IFP 2012 in a simple immersed biofilter suitable to examine the capacity for bioremediation of groundwater contaminated with MTBE under conditions that considered the limitations mentioned above, i.e. the low biomass yield on MTBE. A previous study [Lyew et al., 2006] determined that perlite was the most adequate support to immobilize *M. austroafricanum* IFP 2012. Isopropanol was a proposed in-

intermediate in the MTBE degradation pathway of *M. austroafricanum* IFP 2012 [Lopes Ferreira, 2005], and its use as a secondary carbon source, injected in a pulse-mode, was tested to help maintain the biomass concentration fixed on perlite in the bioreactor. Operational parameters of air flow rate, temperature and hydraulic retention time (HRT) were kept constant.

The set of oligonucleotide primers designed to specifically detect *M. austroafricanum* species was used to check for the presence of strain IFP 2012 in the effluent and to determine the effect of the weekly addition of isopropanol on the release of the strain from the perlite bed.

Results and Discussion

Growth of M. austroafricanum IFP 2012 on MTBE and/or Isopropanol

The effect of isopropanol on the growth of *M. austroafricanum* IFP 2012 in the absence or in the presence of MTBE was first tested in flasks by determining the residual concentrations of substrates and bacterial growth. The growth and the substrate concentration profiles of *M. austroafricanum* IFP 2012 on MTBE, isopropanol, and on MTBE plus isopropanol are presented in figures 1–3, respectively.

During growth on MTBE (at an initial concentration of 72 mg/l), TBA was transiently produced as previously observed [François et al., 2002] (fig. 1). At such a low MTBE concentration, we observed only a slight biomass increase. When *M. austroafricanum* IFP 2012 was cultivated on isopropanol (at an initial concentration of 385 mg/l), we observed a transient production of acetone (fig. 2). The production of biomass was significant ($OD_{600\text{ nm}}$ increased to 0.55). During the growth of *M. austroafricanum* IFP 2012 on MTBE (72 mg/l) in the presence of isopropanol (306 mg/l), we observed the production of the intermediates, acetone and TBA. MTBE was degraded completely and more quickly in the presence of isopropanol, reducing the time required for the degradation of a similar amount of MTBE to 200 h. The production of biomass was in the same range as that observed in the presence of isopropanol alone ($OD_{600\text{ nm}}$ increased up to 0.6).

As previously shown [François et al., 2002], *M. austroafricanum* IFP 2012 is able to grow on MTBE as a sole source of carbon and energy. However, the degradation is slow with a low biomass yield. We showed here that isopropanol was a good growth substrate and that it was

degraded via acetone. This mode of assimilation of isopropanol has been previously shown in *M. vaccae* JOB5 (reclassified as *M. austroafricanum*) during growth on propane [Perry, 1980].

The use of isopropanol was previously shown by Kuntz et al. [2003] to be a preferential inducer of vinyl chloride degradation by *Rhodococcus rhodochrous*, which is important as a secondary substrate since it is nontoxic, water soluble and relatively inexpensive.

Given the fact that isopropanol can be also a gasoline additive, as well as an MTBE degradation product [Lopes Ferreira, 2005; Steffan et al., 1997], interactions between MTBE and isopropanol are likely to be important in the fate of MTBE in contaminated environments. It was previously shown that the presence of other organic components of gasoline in groundwater plumes, especially the soluble monoaromatics, could have a detrimental effect on the biodegradation of MTBE [Deeb et al., 2001; Wang and Deshusses, 2007]. Thus, it was important to carefully check the effect of a potential secondary carbon source on the kinetics of MTBE degradation before considering its use in biofilter experiments or on site. From our results, it is clear that the addition of isopropanol was beneficial by allowing a faster degradation of MTBE. Therefore, we chose to use isopropanol as a secondary carbon source in our biofilter experiments.

Biofilter Monitoring

After seeding the perlite with *M. austroafricanum* IFP 2012, the biofilter was operated under the conditions described in 'Experimental Procedures'. The MTBE concentration in the influent varied from 10 to 20 mg/l and the MTBE removal efficiency by *M. austroafricanum* IFP 2012 was determined by measuring the MTBE concentration in the effluent. Isopropanol was used as a secondary carbon source to sustain growth and maintain the biomass concentration: it was injected at the bottom of the reactor in a pulse mode (an injection every 8 days) at varying concentrations (25 ml of either a 30, 40 or 50 mg/l isopropanol solution).

The biofilter aqueous effluent was regularly analyzed for the residual MTBE and isopropanol concentrations by GC. Spectrophotometric measurements ($OD_{600\text{ nm}}$) were used to evaluate the release of biomass from the perlite bed. Dissolved oxygen and CO_2 measurements were monitored continuously. The results are presented in figure 4.

The biofilter was operated continuously over 57 days, with a good MTBE removal efficiency ($\geq 99\%$, based on our MTBE detection limit of 0.07 mg/l) at inlet MTBE

Fig. 1. Growth profile of *M. austroafricanum* IFP 2012 on MTBE. During growth, OD_{600 nm} (◆) and the concentrations of MTBE (■) and TBA (□) were monitored.

Fig. 2. Growth profile of *M. austroafricanum* IFP 2012 on isopropanol. During growth, OD_{600 nm} (◆) and the concentrations of isopropanol (■) and acetone (□) were monitored.

Fig. 3. Growth profile of *M. austroafricanum* IFP 2012 on MTBE in the presence of isopropanol. During growth, OD_{600 nm} (◆) and the concentrations of isopropanol (■), acetone (□), MTBE (▲) and TBA (△) were monitored.

Fig. 4. MTBE degradation by *M. austroafricanum* IFP 2012 in an immersed biofilter at various MTBE concentrations. Inlet MTBE concentration (□), outlet MTBE concentration (■), OD_{600 nm} (△), dissolved oxygen (◆), CO₂ (*), MTBE removal efficiency (▲).

concentrations of 10 and 15 mg/l. After shifting to a MTBE concentration of 20 mg/l, the removal efficiency remained high (99.5%) for 9 days. Then, the efficiency decreased even after decreasing the influent MTBE concentration to 15 mg/l. No TBA was detected at any time throughout the experiment.

The first injections of isopropanol led to a dramatic decrease of the dissolved oxygen concentration which may have negatively affected MTBE degradation by limiting oxygen availability. Therefore, the concentration of the isopropanol solution was decreased from 50 to 30 g/l to avoid the decrease in dissolved oxygen following isopropanol injections. 25-ml injections of 30 g/l isopropanol maintained the biomass without affecting the DO or the loss of biomass from the bed (see below). Isopropanol and acetone were never detected in the effluent indicating that both compounds were immediately metabolized.

The fact that no substrates or metabolites (i.e. TBA) could be detected in the effluent provides a good basis to assess biofilter performance and efficiency.

As previously mentioned, MTBE is a poor growth substrate, but it is still necessary to produce sufficient degrading biomass to be useful in a bioprocess. Regardless of the type of culture (consortium or pure strains), the experimental biomass yields on MTBE appear to be lower than 0.2 g DW/g of MTBE in all the cases [Deeb et al., 2000; Eweis et al., 1998; Fortin and Deshusses, 1999; Hanson et al., 1999; Salanitro et al., 1994]. *M. austroafricanum* IFP 2012 is one of the few pure strains that is able to grow on MTBE as a sole carbon and energy source with a specific rate of MTBE degradation of 0.6 mmol/h/g DW of cells. The biomass yield for growth on MTBE was previously determined to be 0.44 g DW/g of MTBE [François et al., 2002].

During biofilter operation, when the inlet MTBE concentrations are lower, and more typical of concentrations found in the environment (10 mg/l), the amount of biomass can become critical, and the addition of a secondary carbon source is essential [Thomson et al., 2003].

Our initial attempts to operate a biofilter in the presence of *M. austroafricanum* IFP 2012 under similar low MTBE concentrations without a secondary carbon source (data not shown) led to a rapid decrease in the MTBE degradation efficiency following seeding. After determining the positive effect of isopropanol addition in flask experiments, we decided to ensure the maintenance of active biomass in the biofilter by supplementing *M. austroafricanum* IFP 2012 with isopropanol. With this biofilter, we were able to identify a balance between different concentrations of MTBE (10 and 15 mg/l) and the amount of isopropanol to ensure a MTBE removal efficiency higher than 99%. The importance of establishing this balance is shown in figure 4, as a too high concentration of isopropanol resulted in a detrimental oxygen limitation. It has been shown that during in situ MTBE bioremediation processes, oxygen availability is generally one of the most critical factors [Wilson, 2003].

Stringfellow [2004] argued that it is the poor kinetics of MTBE degradation that explains why natural populations of MTBE degrading bacteria, while possibly present at contaminated sites, are not necessarily able to attenuate MTBE plumes. The addition of a suitable secondary carbon source could be an important environmental variable controlling MTBE biodegradation rates in contaminated soils as well as in bioreactors.

This hypothesis is consistent with the effect observed in our lab-scale biofilter experiment.

Monitoring the Fate of *M. austroafricanum* IFP 2012

In our fixed-bed reactor, the successful immobilization of *M. austroafricanum* IFP 2012 to the perlite bed was critical. The biofilter was capable of degrading MTBE but it was important to verify whether *M. austroafricanum* IFP 2012 was being washed out of the perlite bed, especially following the isopropanol injections. The monitoring of OD_{600 nm} provided an indication, but since the biofilter material was not sterile to begin with, it merely provided a global estimate of the release of biomass. One of the advantages of working with a bioprocess having a well-defined strain is the ability to specifically detect the microorganism by using molecular methods.

The use of 16S rRNA gene sequences as a specific means to detect microorganisms, which does not rely on traditional culture-based microbiological methods, has

Fig. 5. PCR amplification of *M. austroafricanum* strains using specific 16S rRNA gene primers. Amplification conditions were as described in Experimental procedures. The expected PCR product size (331 bp) is indicated. Pure genomic DNA of strain *R. ruber* (lane 1), *Rhodococcus* sp. B-1 (lane 2), *P. resinovorans* CA10 (lane 3), *M. smegmatis* mc2 155 (lane 4), *E. coli* DH10B (lane 5), *M. austroafricanum* IFP 2012 (lane 6), *M. austroafricanum* IFP 2015 (lane 7) and samples from the middle of cassette 1 (lane 9) and the middle of cassette 2 (lane 10) of the MTBE-degrading pilot-biobarrier inoculated with *M. austroafricanum* IFP 2012 (see text) were used for the PCR reactions. GeneRuler™ 1 kb DNA ladder was used as a molecular marker (lane 8).

become a more definitive method to characterize natural microbial communities, and is now routinely applied in environmental engineering and microbial ecology [Muyzer and Ramsing, 1995]. Based on the 16S rRNA gene sequence of *M. austroafricanum* IFP 2012, a 24-bp forward oligomer (MaFV2) located in the V2 variable region of the prokaryotic SSU rRNA gene and a 24-bp reverse primer (MaRV6) located in the V6 variable region were designed [Lopes Ferreira et al., 2006]. The specific primer pair was tested for PCR amplification of genomic DNA from several strains: a *R. ruber* strain, *Rhodococcus* sp. B-1, *P. resinovorans* CA10, *M. smegmatis* mc2 155, *E. coli* DH10B and *M. austroafricanum* IFP 2015, isolated previously for its capacity to grow on MTBE [Lopes Ferreira et al., 2006] (fig. 5, lanes 1–5 and 7, respectively). *M. austroafricanum* IFP 2012 was used as a positive control to test PCR efficiency and the expected 331-bp band was observed (fig. 5, lane 6). A similar band was obtained using *M. austroafricanum* IFP 2015 while no PCR products could be detected using *R. ruber*, *Rhodococcus* sp. B-1, *P. resinovorans* CA10 or *E. coli* DH10B, clearly showing the specificity of the designed primers towards *M. austroafricanum* species. To assess primer specificity, we used less stringent PCR conditions, i.e. an excess of template DNA. A very weak band resulting from the PCR amplification of *M. smegmatis* mc2 155 was detected under these conditions.

To determine whether *M. austroafricanum* IFP 2012 was present in the effluent of the biofilter, samples were monitored regularly from days 42 to 79 by extracting DNA from the biofilter effluent and by specific PCR amplification using this primer pair. The results are presented in table 1. They showed that there was no correlation between the detection of *M. austroafricanum* IFP 2012 in the effluent and the injection of isopropanol.

The MaFV2 and MaRV6 primers were also used for PCR amplification of DNA extracted from a MTBE-degrading pilot-biobarrier with *M. austroafricanum* IFP 2012 fixed on a perlite bed (data not shown). Samples were taken in the middle of two different cassettes, total community DNA was extracted and PCR amplification was performed. In both cases (fig. 5, lanes 9 and 10), amplification of the expected 331-bp band was clearly obtained.

The results of this study demonstrate that MTBE was efficiently degraded by *M. austroafricanum* IFP 2012 in a biofilter with the pulsed-addition of a predetermined concentration of isopropanol as a secondary carbon source. The addition of isopropanol was necessary to maintain the biomass and a high MTBE degradation efficiency by the biofilter. The use of oligonucleotide primers designed to detect strains of *M. austroafricanum* allowed the biofilter to be effectively monitored for the presence of the seeded bacterium.

Experiments using fluorescently labeled primers are currently underway to evaluate their effectiveness for fluorescent in situ hybridization (FISH). The use of this technique will enable monitoring for the presence of *M. austroafricanum* strains in different environmental compartments during bioaugmentation assays or in biobarrier experiments on contaminated sites.

Experimental Procedures

Strains and Preservation

Stock cultures of *M. austroafricanum* IFP 2012 were kept frozen at -80°C in mineral medium, MM [François et al., 2002] containing 20% glycerol (v/v). A *Rhodococcus ruber* strain, *Rhodococcus* sp. B-1, *Pseudomonas resinovorans* CA10, *Mycobacterium smegmatis* mc2 155 and *Escherichia coli* DH10B were stored at -80°C in 10% TSB containing 15% glycerol (v/v).

Growth Medium and Culture Conditions in Flasks

Cultures were grown aerobically in tightly closed conical flasks after addition of the substrate to minimal medium at the required concentration, and incubated with shaking at 30°C . The headspace volume was sufficient to prevent any O_2 limitation during growth. Growth was followed by measuring the absor-

Table 1. Detection of *M. austroafricanum* IFP 2012 in the biofilter effluent by PCR amplification

Sample day	Influent MTBE concentration mg/l	PCR amplification result	Isopropanol injection
42	15	+	+
43	15	+	–
44	15	–	–
47	15	+	–
48	20	+	+
49	20	–	–
50	20	+	–
51	20	+	–
55	20	–	+
56	20	–	–
58	20	–	–
61	20	–	–
62	20	+	+
65	15	+	–
68	15	+	–
69	15	+	–
70	15	+	+
71	15	+	–
72	15	+	–

bance at 600 nm ($\text{OD}_{600\text{ nm}}$). The residual substrate or metabolite concentrations were measured on filtered samples using GC or HPLC.

Set-Up and Operation of the Immersed Biofilter

The schematic of the immersed biofilter is presented in figure 6. The total volume of the biofilter was 4.7 liters. It was filled with perlite, a mineral support previously shown to be the most efficient support for the immobilization of *M. austroafricanum* IFP 2012 [Lyew et al., in press]. The interstitial liquid volume was 2.35 liters.

An air flow rate of $0.12\text{ liter}^{-1}\text{ h}^{-1}$ was maintained throughout the experiment. The temperature was set at 20°C .

The biofilter was filled with MM medium.

MTBE, after dilution in the feeding medium, was injected at the bottom. The total feeding rate was maintained at 20 ml/h to get a hydraulic retention time of 5 days throughout the experiment.

Inoculation of the Biofilter

The inoculum of *M. austroafricanum* IFP 2012 for seeding the biofilter was prepared in 3 l of MM supplemented with 5 successive additions of TBA (1 g/l). The culture was harvested by centrifugation (23,000 g for 15 min) and the pellet (about 9 g DW of biomass) was resuspended in 100 ml and poured over the top of the perlite. The biofilter was closed and filled by injection from the bottom.

Fig. 6. Experimental set-up of the biofilter.

Analytical Procedures
MTBE, TBA, isopropanol and acetone, were quantified by flame ionization detection on a Varian 3300 gas chromatograph (Varian, France) equipped with a 0.32 mm × 25 m Porabond-Q capillary column (J&W Scientific, Chromoptic, Auxerre, France), using a two-step temperature gradient ranging from 105 to 200°C at 10°C/min. Helium (1.6 ml/min) was used as the carrier gas. Samples, filtered through 0.22 µm filters (Prolabo, Fontenay sous Bois, France), were injected without further treatment.

CO₂ and air were monitored in the gaseous effluent of the biofilter using a S710 apparatus (Maihak, Germany).

Residual dissolved oxygen was measured at the top of the biofilter using an O₂ probe (Ingold).

OD_{600 nm} was measured on a spectrophotometer UV-1601 (Shimadzu).

DNA Extraction and PCR Conditions

DNA was daily extracted from 120-ml-effluent biofilter samples from day 42 to day 79. Samples were centrifuged at 9,000 rpm, and DNA extracted and purified using a bead beater and a chloroform/isoamylalcohol (24:1) extraction procedure.

The oligonucleotide primer set was previously designed from the 16S rRNA gene sequence of *M. austroafricanum* IFP 2012 [Lopes Ferreira et al., 2006]. The sequences of the primers are: forward primer MaFV2 (5' GTCTAATACCGAATACACCCCTT-CT 3': nt 151–174) and reverse primer MaRV6 (5' GTAGTTGGC-CGGTCCTTCTTCTCC 3': nt 458–481). The expected size of the PCR product was 331 bp.

PCR conditions were as follows: each tube contained the following: 5 µl of bacterial genomic DNA extract from the effluent,

2.5 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech), 5 µl of 10× *Taq* DNA polymerase buffer (Amersham Pharmacia Biotech), 25 pmol of each primer, 4 µl of 2.5 mM deoxyribonucleoside triphosphates (200 µM each: dATP, dGTP, dCTP, and dTTP), 2 µl of 25 mM MgCl₂, and sterile distilled water to bring the final volume to 50 µl. The negative control contained the same mixture as described above except that the DNA was replaced with sterile water. Prior to the addition of the *Taq* DNA polymerase/10× buffer mixture, the samples were heated for 3 min at 95°C in the Bio-Rad Thermal iCycler machine (Bio-Rad, Mississauga, Ont., Canada), then the temperature was reduced to 80°C to add the enzyme mixture. In order to get specific amplification conditions we used the following amplification conditions: 30 s at 94°C, 1 min at 68°C, 1 min at 72°C for a total of 30 cycles and a final extension at 72°C for 7 min. PCR tubes were kept at 4°C until agarose gel electrophoresis was performed. 10 µl of each PCR sample was mixed with 2 µl of loading buffer (30% (v/v) glycerol, 0.15% (w/v) bromophenol blue, 0.15% (w/v) xylene cyanol), and loaded onto a 1% (w/v) TAE agarose gel together with the GeneRuler™ 1 kb DNA ladder (MBI Fermentas, Inc., Burlington, Ont., Canada). The gel was stained in ethidium bromide and observed by using a UV transilluminator at a wavelength of 254 nm. The gels were photographed using type 57 Polaroid film.

Chemicals

MTBE, TBA, and isopropanol were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). All chemicals were of the highest purity available.

References

- Acuna-Askar K, Engle AJ Jr, Hu C, Jin C: Methyl tertiary-butyl ether (MTBE) biodegradation in batch and continuous upflow fixed-biofilm reactors. *Water Sci Technol* 2000;42:153–161.
- Deeb RA, Hu HY, Hanson JR, Scow KM, Alvarez-Cohen L: Substrate interactions in BTEX and MTBE mixtures by an MTBE-degrading isolate. *Environ Sci Technol* 2001;35:312–317.
- Deeb RA, Nishino S, Spain J, Hu H-Y, Scow K, Alvarez-Cohen L: MTBE and benzene biodegradation by a bacterial isolate via two independent monooxygenase-initiated pathways. Preprints of extended abstracts. ACS National Meeting, AM Chem Soc. Div Environ Chem 2000;40:280–282.
- Eweis JB, Schroeder ED, Chang DPI, Scow KM: Biodegradation of MTBE in a pilot-scale biofilter; in Wickramanayake GB, Hinchey RE (eds): *Natural Attenuation of Chlorinated and Recalcitrant Compounds*. Columbus, Battelle, 1998, pp 341–346.
- Fayolle F, Francois A, Garnier L, Godefroy D, Mathis H, Piveteau P, Monot F: Limitations in MTBE biodegradation. *Oil Gas Sci Technol* 2003;58:497–504.
- Fayolle F, Monot F: Biodegradation of fuel ethers; in Magot M, Ollivier B (eds): *Petroleum Microbiology*. Washington, ASM Press, 2005, pp 301–316.
- Fortin, NY, Deshusses MA: Treatment of methyl *tert*-butyl ether vapors in biotrickling filters. 1. Reactor start up, steady-state performance, and culture characteristics. *Environ Sci Technol* 1999;33:2980–2986.
- François A, Mathis H, Godefroy D, Piveteau P, Fayolle F, Monot F: Biodegradation of methyl *tert*-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. *Appl Environ Microbiol* 2002;68:2754–2762.
- Garnier PM, Auria R, Augur C, Revah S: Cometabolic biodegradation of methyl *t*-butyl ether by *Pseudomonas aeruginosa* grown on pentane. *Appl Microbiol Biotechnol* 1999;51:498–503.
- Hanson JR, Ackerman CE, Scow KM: Biodegradation of methyl *tert*-butyl ether by a bacterial pure culture. *Appl Environ Microbiol* 1999;65:4788–4792.
- Johnson R, Pankow J, Bender D, Price C, Zogorsky J: MTBE. To what extent will past releases contaminate community water supply wells? *Environ Sci Technol* 2000;34:210A–217A.
- Kuntz RL, Brown LR, Zappi ME, French WT: Isopropanol and acetone induces vinyl chloride degradation in *Rhodococcus rhodochrous*. *J Ind Microbiol Biotechnol* 2003;30:651–655.
- Lopes Ferreira N: Catabolisme du méthyl *tert*-butyl éther (MTBE): caractérisation des enzymes impliquées dans la dégradation du MTBE chez *Mycobacterium austroafricanum* IFP 2012; PhD thesis, INA-PG, France, 2005.
- Lopes Ferreira N, Maciel H, Mathis H, Monot F, Fayolle-Guichard F, Greer CW: Isolation of a new *Mycobacterium austroafricanum*, IFP 2015, growing on MTBE. *Appl Microbiol Biotechnol* 2006;70:358–365.
- Lyew D, Guiot S, Monot F, Fayolle-Guichard F: Comparison of different support materials for their capacity to immobilize *Mycobacterium austroafricanum* IFP 2012 and to adsorb MTBE. *Enzyme Microb Technol* 2006 DOI:10.1016/j.enzmictec.2006.10.040.
- Moreels D, Lodewijks P, Zegers H, Rurangwa E, Vromant N, Bastiaens L, Diels L, Springael D, Merckx R, Ollevier F: Effect of short-time exposure to methyl *tert*-butyl ether and *tert*-butyl alcohol on the hatch rate and development of the African catfish, *Clarias gariepinus*. *Environ Toxicol Chem* 2006a;25:514–519.
- Moreels D, Van Cauwenberghe K, Debaere B, Rurangwa E, Vromant N, Bastiaens L, Diels L, Springael D, Merckx R, Ollevier F: Long-term exposure to environmentally relevant doses of methyl *tert*-butyl ether causes significant reproductive dysfunction in the zebrafish (*Danio rerio*). *Environ Toxicol Chem* 2006b;25:2388–2393.
- Moyer EE, Kostecki PT: MTBE Remediation Handbook. Amherst, Amherst Scientific Publishers, 2003.
- Muyzer G, Ramsing NB: Molecular methods to study the organization of microbial communities. *Water Sci Technol* 1995;32:1–9.
- Müller RH, Rohwerder T, Harms H: Carbon conversion efficiency and limits of productive bacterial degradation of methyl *tert*-butyl ether (MTBE) and related compounds. *Appl Environ Microbiol* 2007;73:1783–1791.
- Perry JJ: Propane utilization by micro-organisms. *Adv Appl Microbiol* 1980;26:89–115.
- Salanitro JP, Diaz LA, Williams MP, Wisniewski HL: Isolation of a bacterial culture that degrades methyl *t*-butyl ether. *Appl Environ Microbiol* 1994;60:2593–2596.
- Schmidt TC, Bittens M, Arp HP, Haderlein SB: Transfer pathways of MTBE into groundwater: the role of diffuse vs. point sources; in Bilitewski B, Werner P (eds): *Proceedings of the First European Conference on MTBE*. Dresden, Dresden University of Technology, 2003, pp 8–15.
- Steffan RJ, Mac Clay K, Vainberg S, Condee CW, Zhang D: Biodegradation of the gasoline oxygenates methyl *tert*-butyl ether, ethyl *tert*-butyl ether and *tert*-amyl methyl ether by propane-oxidizing bacteria. *Appl Environ Microbiol* 1997;65:4788–4792.
- Stringfellow WT: Unfavorable MTBE biokinetics dominate environmental fate processes; in Barcelo D, Petrovic M (eds): *Second European Conference on MTBE, IIQAB-CSIC*, Barcelona, 2004, pp 44–46.
- Stringfellow WT, Oh K-C: Initiation of MTBE biotreatment in fluidized-bed bioreactors. *J Environ Eng* 2002;128:852–861.
- Thomson JAM, McKinley JW, Harris RC, Hart HJ, Hicks P, Ramsden DK, Wilson B: MTBE occurrence in surface and ground water; in Moyer EE, Kostecki PT (eds): *MTBE Remediation Handbook*. Amherst, Amherst Scientific Publishers, 2003, pp 63–72.
- USEPA: Achieving Clean Air and Clean Water: The report of the blue ribbon panel on oxygenates in gasoline. EPA 420-R-99-021, US Environmental Protection Agency, 1999.
- Vainberg S, Togna AP, Suttton PM, Steffan RJ: Treatment of MTBE-contaminated water in fluidized bed reactor. *J Environ Eng* 2002; 128:842–851.
- Wang X, Deshusses MA: Biotreatment of groundwater contaminated with MTBE: interaction of common environmental co-contaminants. *Biodegradation* 2007;18:37–50.
- Wilson JT: Fate and transport of MTBE and other gasoline components; in Moyer EE, Kostecki PT (eds): *MTBE Remediation Handbook*. Amherst, Amherst Scientific Publishers, 2003, pp 19–61.
- Wilson BH, Shen H, Cho J, Vardy J: Use of BIO-SCREEN to evaluate natural attenuation of MTBE; in Alleman BC, Leeson A (eds): *Natural Attenuation of Chlorinated Solvents, Petroleum Hydrocarbons and Other Organic Compounds*. Proceedings 5th International In-Situ and On-Site Bioremediation Symposium, San Diego, 1999, pp 115–120.
- Zein MM, Suidan MT, Venosa AD: MTBE biodegradation in a gravity flow, high-biomass retaining bioreactor. *Environ Sci Technol* 2004;38:3449–3456.