DEPARTAMENTO DE MICROBIOLOGÍA. INSTITUTO DEL AGUA
UNIVERSIDAD DE GRANADA

Desarrollo y estudio biológico de un sistema de bajo costo para el tratamiento de aguas subterráneas contaminadas con compuestos oxigenantes de gasolinas: MTBE, ETBE y TAME

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Tesis Doctoral

Granada, 2011

Editor: Editorial de la Universidad de Granada
Autor: Jessica Purswani
D.L.: GR 670-2012
ISBN: 978-84-694-6370-3

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MEMORIA PARA OBTENER EL DOCTORADO EUROPEO

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Este trabajo ha sido financiado por el Ministerio de Ciencia y Tecnología, Dirección General de Investigación, para realizar el proyecto: “Tratamiento de aguas subterráneas contaminadas con compuestos oxigenantes de gasolinas (MTBE, ETBE y TAME) mediante reactores de lechos sumergidos” Nº de referencia: **CTM2006-02297.**

Me siento muy afortunada de haber tenido la capacidad de trabajar y aprender de mis dos directores de tesis, Jesus y Clemen, y les agradezco su continua ayuda durante estos años, y en especial estos últimos meses, empujándome siempre con cariño para conseguir esta meta.

Me gustaría agradecerle a Clemen su paciencia y sus innumerables consejos durante todo este trayecto, tanto profesionales como personales. Me llevo conmigo para siempre su amistad y sus enseñanzas, una de las cuales incluye como escribir las palabras agudas, llanas y esdrújulas. Una persona digna de admiracion.

Al gran jefe Jesús agradecerle el haber acogido a esta llanita en su grupo, aportándome siempre mucha confianza y animo durante los momentos difíciles. Por tener siempre un momento para hablar conmigo aun estando muy ocupado.

También me gustaría extender mi agradecimiento a todos los doctores que me han enseñado a traves de diversas formas las "*wonderful techniques of microbiology, biotechnology and genetics*": Belén Rodelas, Belén Juárez, Maximino Manzanera, Mª Victoria Martínez y Concepción Calvo.

Sin duda, de mis compañeros he aprendido mucho (los que están y los que estuvieron), ya que una tesis no se puede hacer sin la ayuda de un gran equipo, como lo son: Nacho, Fede, Imane, Paula, Maria, Patri, Kadiya, Juan Jesús, Almu, Rafa, Paqui, Marisa, Isa, Alex, Cinta, Mar, Estefanía, Sergio, Cristina. A ellos les agradezco muchos momentos divertidos, tanto en el laboratorio, como en el Maury, el Trébol, o…..

Claro esta que no me he olvidado de Andrea y de Lucia, pero para mi no solo son compañeras ni amigas, la amistad que me une a estas dos grandes personas trasciende el trabajo y la amistad. Ellas han limado mi camino rocoso, cuando se lo pedía, y especialmente cuando no. Os quiero chicas!

A mi familia granadina les agradezco esos viernes de tapas, esos fines de semana juntos, y estos años de apoyo: Teresa, Fadwa, Arantxa, Juan, Carmen, Ruben P., Ruben C., Juanma, Manolo, David, Fernando, Mati, Marina, Marta, Pedro.

A mi familia de Nerja, en especial a Eva y José por acompañarme durante estos meses. A Rocio agradecerle el sacarme muchas sonrisas cuando menos quería.

A mi padre, a Ram y a Romi les agradezco el intentar entender este mundo extraño para ellos, por quererme y por entusiasmarse cuando mis "*bichos crecían*". A tita Indra y a Sunil por quererme y mimarme siempre que lo he necesitado. Y aunque no estén aquí, se lo dedico a mi tio Lolo que lucho contra la adversidad, y a mi madre que fue la que me instalo el deseo de conocer, saber y aprender, y sin ella, no estaría aquí y ahora.

A mi Antonio, agradecérselo todo.

Caminante, no hay camino,
se hace camino al andar
Antonio Machado

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ABSTRACT

The evaluation of a biological aerated filter for the bioremediation of fuel oxygenates MTBE, ETBE and TAME, was the goal of the study at hand. In order to comply with the study, a set of pre-steps were needed, thus the steps followed were: selection of fuel ether biodegrading strains, set-up and design of a pilot-biofilter, and finally, the evaluation of seeded biofilters with theoretically recent contaminated groundwater. We selected a total of 9 bacterial strains, and tested their biotransformation capacities on individual ethers, and assayed their microbial count up to 21 days. Good growth curves were not obtained, but we chose *Acinetobacter calcoaceticus* M10, *Rhodococcus ruber* E10 and *Gordonia amicalis* T3 for further studies.

An evaluation of the GC method used was performed, since conventional methods (purge and trap or headspace autosamplers), were unavailable in our lab. We concluded from a storage test with abiotic and biotic samples, that the ether was hydrolysed more quickly in abiotic samples than their biotic counterparts. We were also able to obtained LODs and MDLs similar and in some cases better than those in the literature with headspace autosamplers.

In addition, we also evaluated the most adequate DNA extraction method from 0.22 μm nitrocellulose filter after filtration of groundwater. Varying DNA extraction protocols were tested in order to understand the different outcomes and reproducibility in samples examined through TGGE. Mechanical lysis with the FastDNA kit was one of the most reproducible protocols, exclusive bands appeared in with this method, which did not appear in the others, and thus was this protocol was used for biofilm and filter samples.

The three chosen strains were evaluated further for growth and biotransformation of metabolites TBA and TAA. Their physiological activity was also tested using flow cytometric analysis in order to evaluate whether they were viable and active, even when no growth or poor growth had been observed. The highest activity was observed with strain E10 in the presence of MTBE for 24 h.

EPS production was assessed on all three strains, however, only M10 produced a significant amount of EPS, therefore we decided on performing attachment assays on the biofilter designed with the individual strains, and the dual strain consortia M10-E10 and M10-T3, since strain M10 was the only observed to attach with several layers to the support material. The consortia M10-E10 attached successfully to the support material (Bioflow 9®),

therefore we decided on using strain M10 and consortium M10-E10 further to study their ability in removing MTBE, using the designed downflow pilot-scale biofilter.

Three biofilters were tested at the same time in order to evaluate the oxygenate removal with different inocula, but with the same influent. Biofilter D was seeded with *A. calcoaceticus* M10, biofilter E with the consortium M10-E10, and a control biofilter (F) was mounted to evaluate the performance of a non-seeded biofilter. Among the biofilters, only biofilter E showed oxygenate removal tendency. Toxicity levels were also lowered when removal of MTBE was shown, thus no toxic metabolites were being accumulated.

The functionality of the biofilter in removing MTBE was sought by amplifying the genomic DNA of all biofilters with primers specific to *alkB* and *ethB*, previously found to biotransform MTBE. Though no *ethB* genes were found, *alkB* amplification was positive in all biofilters and in strains M10 and E10. Bacterial isolates from the biofilms of the biofilters were cultivated, identified taxonomically and tested for the *alkB* gene. Seven of the 13 isolates were found to contain the gene, however, none of the isolates were found among the TGGE bands from RNA extracts except for strains E10 and M10. We believe the consortium *A. calcoaceticus* M10 and *R. ruber* E10 was the only bacterium biodegrading MTBE in biofilter E, and should therefore be used further, after seeking optimization of the environmental conditions for growth and MTBE degradation to improve its function.

CAPÍTULO I. INTRODUCCIÓN

La sensibilización social por el medioambiente ha incrementado en las últimas décadas debido tanto al aumento de la producción de sustancias xenobióticas como a los derrames y/o vertidos de las mismas, tanto accidentales como premeditados. En respuesta a este requerimiento de la sociedad, los gobiernos y organizaciones no gubernamentales se han puesto en marcha para prevenir rigurosamente cualquier accidente que pueda surgir y en los casos en los que se hayan producido, establecer los mecanismos adecuados para el tratamiento de las zonas afectadas.

El incremento de la contaminación mundial se debe, entre otros motivos, a la producción masiva de nuevos compuestos que existen a bajas concentraciones en la naturaleza. Un concepto amplio de ***compuesto xenobiótico*** cubre todos los compuestos que son extraños al organismo estudiado, aunque a veces se utiliza este término para incluir también a compuestos naturales que se presentan a altas concentraciones.

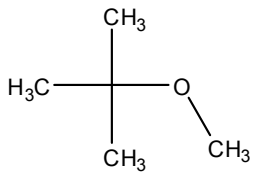
Los compuestos xenobióticos pueden ser de difícil degradación, y aunque algunos existan en la naturaleza, la presencia ó vertido de éstos a concentraciones y/o cantidades elevadas pueden generar procesos de contaminación. Por esta razón, hay cada vez más leyes preventivas contra la posible contaminación de estas sustancias, considerando además que el transporte de compuestos tóxicos previamente inexistente, ahora es un hecho habitual aumentando las posibilidades de accidentes.

1. Los oxigenantes. Historia y estado actual

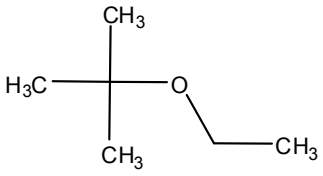
En 1979 y como consecuencia de los alarmantes niveles de monóxido de carbono (CO) y otras emisiones a la atmósfera que favorecen el efecto invernadero, la Agencia de Protección del Medio Ambiente de Estados Unidos (USEPA) estableció la disminución e incluso la eliminación del plomo en la formulación de las gasolinas en aquel país. Este hecho condujo al uso de nuevos compuestos llamados **“aditivos oxigenantes”,** los cuales poseían una serie de características que los convertían en idóneos candidatos para favorecer la combustión de las gasolinas reduciendo de esta forma las emisiones de CO a la atmósfera. Estos compuestos contienen un elevado grado octánico, se producen a bajo coste, se mezclan bien con los hidrocarburos, incrementan el contenido de oxígeno de los combustibles y son fáciles de transportar. No obstante, la sustitución del plomo por estos compuestos oxigenantes, favoreció tanto a la industria petroquímica como a la automovilística ya que significaba la

producción de nuevos compuestos, y en este último caso, la longevidad de los catalizadores utilizados en los tubos de escape de los automóviles.

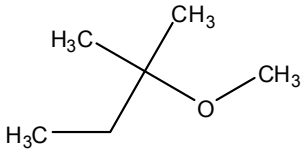
De todos los oxigenantes adicionados a las gasolinas (metil *tert*-butil éter: MTBE, etil *tert*-butil éter: ETBE, *tert*-amyl metil éter: TAME, *tert*-butil alcohol: TBA, di-isopropil éter: DIPE, metanol y etanol), el MTBE ha sido el compuesto más utilizado.



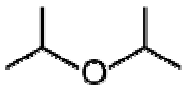
Metill *tert*-butil eter



Etil *tert*-butil eter



Tert-amil metil eter



di-isopropil éter: DIPE

Figura 1.1 Estructura química de MTBE, ETBE, TAME y DIPE.

Al inicio de la década de los 90, el 61% de la gasolina que se consumía en EEUU contenía MTBE en su formulación (Andrews, 1998).

El consumo de gasolina sin plomo en España y por consiguiente adicionada de oxigenantes, se inició en 1995. En 1996, el porcentaje de consumo de gasolina sin plomo con respecto al total era de un 34,7% (el más bajo de Europa). En el año 2001, la Unión Europea aprobó una normativa para retirar totalmente las gasolinas con plomo del mercado (EEA, 2002). A España le concedieron una prórroga hasta el año 2002, año en el que el porcentaje de gasolina sin plomo era del 63,5%. Sin embargo, se produjo una sustitución con un aditivo basado en potasio, el cuál también se retiró a finales del año 2005.

La concentración media de MTBE en las gasolinas europeas oscila alrededor del 2% (v/v), aunque este valor varía enormemente entre países. En España, por ejemplo, el porcentaje fluctúa entre un 4% en gasolinas sin plomo de 95 octanos, y un 10% en gasolinas sin plomo de 98 octanos. Además, y debido a las rígidas leyes de Protección Medioambiental

existentes en Europa con respecto a la emisión de gases, se estima un porcentaje aún mayor (alrededor de un 12%) en un plazo mínimo de tiempo.

Actualmente en España, el MTBE ha dejado paso al ETBE, incrementándose en gran medida la producción de este último, acompañada también por el incremento de la producción de bioetanol.

El uso de estos oxigenantes ha provocado una intensa discusión sobre los efectos positivos y negativos que conllevan. Esta discusión iniciada hace tiempo en Estados Unidos se ha trasladado, con varios años de retraso, a Europa y las conclusiones obtenidas en la 1ª, 2ª y 3ª Conferencia Europea sobre MTBE y otros oxigenantes mantenidas en Dresden (2003), Barcelona (2004) y Amberes (2007) respectivamente, así lo atestiguan.

A mediados de los años 90, el oxigenante MTBE se detectó en numerosos acuíferos de Estados Unidos, lo cuál provocó que su Agencia de Protección del Medio Ambiente (EPA) estableciera un rango de niveles máximos en aguas potables el cual osciló entre 20-40 µg/l. En 1999, el estado de California redujo el límite máximo de concentración en aguas potables a sólo 13 µg/l y en el 2003 prohibió su uso. En determinados países de Europa (Francia, Bélgica, Alemania, Reino Unido y España) el MTBE se muestra como un emergente contaminante de los recursos hídricos junto con el ETBE y el TAME. (Barceló, 2007; Klinger y colaboradores, 2002; Schmidt y colaboradores, 2002).

2. Características fisicoquímicas de los oxigenantes

El MTBE es un compuesto químico, producido a partir de la reacción entre metanol e isobutileno.

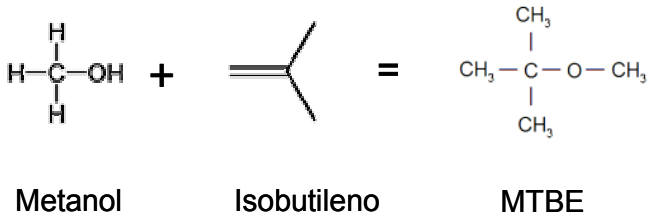


Figura 2.1. Reacción de síntesis de MTBE

El ETBE se obtiene de la reacción entre isobutileno y etanol. El TAME es producido mediante la reacción entre el iso-amil y el metanol. Estos tres compuestos son incoloros, pero tienen un olor desagradable, típico de los éteres.

De acuerdo con las propiedades presentadas en la Tabla 2.1, los tres éteres son altamente volátiles, se disuelven bien en agua y en hidrocarburos. Debido a su baja hidrofobicidad (log K_{ow},) y su coeficiente de adsorción a la matriz del suelo (log K_{oc}), no es de esperar que estos compuestos se adsorban a la materia orgánica del suelo o de los sedimentos. Además, debido a la baja densidad y poca viscosidad, grandes volúmenes de estos éteres son capaces de lixiviarse por el suelo, alcanzando las masas de agua subterránea. Basándose en la constante de la ley de Henry, el MTBE es más volátil que el TAME y tiende a evaporarse fácilmente a la atmósfera desde soluciones acuosas (Huttenen y colaboradores, 1997).

3. Problemas derivados del uso de MTBE, ETBE y TAME

3.1. Contaminación de las masas de aguas.

Debido a que el MTBE ha sido y es el compuesto químico más ampliamente e intensamente utilizado como sustituto del plomo en la formulación de gasolinas, la mayor parte de la información que existe sobre los efectos del uso de éteres sobre el medio ambiente está referida a este compuesto.

Como se ha expuesto anteriormente, el MTBE se ha detectado en numerosos acuíferos de Estados Unidos y concretamente en 1999, el Estado de California redujo el límite máximo de concentración en aguas potables sólo a 13 µg/l tras detectarse la presencia de 600 mg/l de MTBE en la reserva de agua municipal de la ciudad de Santa Mónica. La concentración detectada fue 30.000 veces mayor a la permitida. El MTBE también fue detectado en aguas superficiales y subterráneas, así como en pozos privados y públicos. Squillace y colaboradores (1996), identificaron entre el año 1985 y 1995 al MTBE como el segundo compuesto orgánico volátil más frecuentemente detectado en pozos de zonas urbanas de USA. Desde el año 2003, el estado de California ha prohibido su uso.

Actualmente, la producción de ETBE es más común en Europa debido principalmente a los incentivos gubernamentales que se reciben, rebajando las tasas sobre aquellos que utilicen bioetanol para producir el grupo *etilo* del ETBE (Directiva Europea 2003/30/EC). Aún así, se han detectado cantidades de MTBE en agua de lluvia, en aguas subterráneas, aguas superficiales, aguas residuales, agua potable, y en muestras de agua marina (Rosell y colaboradores, 2007).

Tabla 2.1 Propiedades fisico-químicas de MTBE, ETBE y TAME.

Nombre del producto químico	Metil *ter*-butil éter	Etil *ter*-butil éter	*Ter*-amil metil éter
Abreviatura	MTBE	ETBE	TAME
Peso molecular	88,15	102,18	102,18
Fórmula estructural	$CH_3OC(CH_3)_3$	$CH_3CH_2OC(CH_3)_3$	$CH_3OC(CH_3)_2\,CH_2CH_3$
Punto de ebullición (1atm)	55,2 °C	72,2 °C	86,3 °C
Densidad kg/l ([a] 20°C)	0,74	0,74	0,77
Solubilidad (g/l) en agua	48	12	12
[c]Log Koc	[b]1,6	-	[b]1,8
[d]Log Kow	[b]1,2	-	[b]1,6
Cte Ley de Henry	57 Pa/m3/mol 20°C)	-	[b]90 Pa/m3/mol (20°C)
Nivel de olor en aire	(0,24 µg/l, 0.05 mg/l)	-	-
Nivel de gusto en agua	680 µg/l	-	-
Otro	Inestable en condiciones ácidas	-	-
[e]RON	118	118	115

[a]Fayolle y colaboradores, 2001. [b]Huttenen y colaboradores, 1997. [c]Coeficiente de adsorción del carbono orgánico en suelos. [d] Coeficiente de partición octanol/agua. [e]Research octane number (RON) define la calidad de combustión de la gasolina. Actualmente en Europa se requiere para las gasolinas un valor RON de 95.

La principal característica del ciclo hidrográfico en España es su estacionalidad, observándose un marcado déficit estival, que es el principal factor limitante, no sólo para el desarrollo de la vegetación y de la agricultura, sino para la eliminación de los usos y vertidos urbano-industriales. Para paliar dicho déficit se debe recurrir al agua embalsada, superficial o subterránea, al objeto de compensar la estacionalidad de la pluviometría y los consumos de agua.

Las aguas subterráneas son una de las principales fuentes de suministro para uso doméstico y para el riego en muchas partes de España (especialmente en Andalucía). En España, alrededor de la tercera parte del agua que se usa en las ciudades y en la industria y la cuarta parte de la que se usa en agricultura procede de las aguas subterráneas.

En nuestro país, el problema de contaminación de acuíferos por oxigenantes de gasolinas es un problema reciente, con niveles de contaminación detectados entre 20 y 670 µg/l (Rosell y colaboradores, 2003). Además se ha concluido que el MTBE es mucho más

persistente que el índice BTEX, por lo que podría considerarse como un inmejorable indicador de contaminación por gasolinas.

La ruta predominante por la cual las aguas subterráneas son contaminadas por los oxigenantes de gasolinas se muestra en la Figura 3.1. Generalmente estos productos proceden de fugas de tanques de gasolineras, fugas en los sistemas de conducción ó como consecuencia de derrames accidentales desde los contenedores de almacenamiento, arrastrándose estos compuestos por la lluvia hasta el subsuelo o hasta las masas de agua superficial. Además, y en algunos casos, los barcos depositan ciertas cantidades de gasolina en el agua aunque no haya ningún caso de derrame.

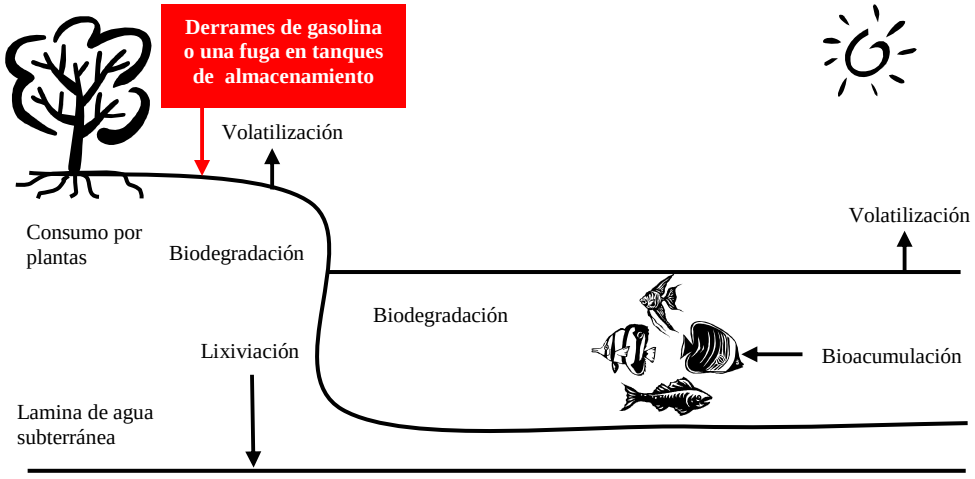


Figura 3.1. Ruta seguida por los compuestos oxigenantes de las gasolinas hasta aguas superficiales y subterráneas.

La poca afinidad del MTBE por la matriz del suelo y su alta volatilidad ha provocado que este compuesto químico no cause demasiados problemas ambientales con respecto al suelo. Desafortunadamente, su tendencia a lixiviarse y
su alta solubilidad en agua, hace que llegue a contaminar las aguas subterráneas. Las características físico-químicas del MTBE combinadas con su estructura inerte y su carácter recalcitrante, son las responsables de la contaminación de grandes masas de aguas subterráneas frente a la contaminación producida por otros componentes de las gasolinas. Además, estas masas contaminadas migran rápidamente a otras zonas, manteniéndose durante mucho tiempo (Stupp, 2007).

3.2. Efectos ecológicos y patológicos en animales y humanos

Tal y como hemos comentado anteriormente, la mayoría de los estudios existentes sobre los efectos de los oxigenantes han sido realizados con el MTBE. Sin embargo, a medida que el resto de oxigenantes sean utilizados intensamente, es de prever que los mismos problemas irán surgiendo en el tiempo. Debido a las características físico-químicas de estos compuestos, el medio acuático es el medio más sensible a la presencia de estas sustancias.

Rousch y Sommerfeld (1998) observaron la toxicidad del MTBE en el fitoplancton de varias lagunas, utilizando para ello tres especies de algas unicelulares. Concluyeron que aunque la composición de la comunidad de algas fue modificada, la concentración utilizada no era letal.

Werner y colaboradores, (2001) estudiaron la incidencia de altas concentraciones de MTBE y TAME sobre *Daphnia magna*, observando unas altísimas concentraciones letales al 50% (LC50 542 mg/l y > 100 mg/l respectivamente).

Los estudios realizados con peces también muestran los elevados valores de LC50 para el MTBE. Así, Hockett (1997) y Longo (1995) estudiando el efecto de dosis crecientes de MTBE sobre larvas de *Pimephales promelas* de un día, y con embriones de un día de *Oryzias latipes*, observaron una reducción del crecimiento a los 388 mg/l de MTBE y significativas lesiones oculares y en el sistema cardiovascular a 2600 mg/l de MTBE, respectivamente.

La bioacumulación de los oxigenantes en determinados organismos ha sido también estudiada. Fujiwara y colaboradores (1984) evaluaron la acumulación del MTBE en la carpa Japonesa sometida a niveles de oxigenante (MTBE) entre 10 y 80 mg/l. Después de cuatro semanas, el tejido de la carpa contenía una concentración de MTBE 1,5 veces mayor que la del entorno, acumulándose más en el hígado y en la branquia.

Los estudios realizados con mamíferos han concluido que la ruta de absorción del MTBE por vía oral y respiratoria es bastante rápida, mientras que por vía cutánea sólo un 39% era absorbido. El oxigenante tardó 15 minutos en alcanzar los máximos valores en el cuerpo, registrándose las más altas concentraciones en el hígado y en los riñones. En las secreciones después de su administración oral, sólo un 46% de MTBE fue eliminado y un 11% fue secretado en la orina. Después de la inhalación, la mayor parte de MTBE y sus metabolitos fueron secretados en la orina (53-72%). En todos los casos estudiados, los principales

metabolitos fueron el TBA y el formaldehído. En 1998, Lee y colaboradores, informaron de daños en el ADN de linfocitos en ratas tras la exposición con MTBE.

La toxicidad de los compuestos oxigenantes de las gasolinas en los humanos también se ha estudiado. Se sabe que el MTBE es utilizado terapéuticamente en cantidades pequeñas, para disolver piedras en el riñón (Hellstern y colaboradores, 1998). El MTBE a dosis de 5 mg MTBE/m^3 durante una hora puede provocar dolores agudos de cabeza, irritaciones nasales y oculares, tos y mareos. También puede causar inflamaciones oculares, en vías respiratorias y cambios en la conducta del sujeto.

Aunque no se ha podido determinar que los oxigenantes MTBE, ETBE y TAME sean cancerígenos, tampoco se descarta el hecho de que puedan serlo. En el año 2002, el profesor M.A. Mehlman, criticó duramente al *National Toxicology Program* por haber denegado la inclusión del MTBE en su lista de posibles sustancias cancerígenas.

4. Degradación microbiana

El relativo carácter recalcitrante de los oxigenantes a ser oxidados y/o atacados por los microorganismos es inherente a su estructura química, la cual contiene una combinación de dos grupos funcionales bastante especiales: un enlace éter y una molécula ramificada (Figura 1.1.)

En la naturaleza existen numerosos compuestos químicos que contienen enlaces éter en su molécula, como por ejemplo la lignina que contiene enlaces que incrementan el ya carácter recalcitrante del polímero. La presencia de estos compuestos explica la existencia de microorganismos capaces de degradar estos enlaces. White y colaboradores (1996), estudiaron los mecanismos bioquímicos en la escisión del enlace éter, haciendo su investigación relevante en la biodegradación de los oxigenantes. La principal reacción es la hidroxilación por oxigenasas en los carbonos de los grupos CH_2 ó CH_3 adyacentes al enlace éter, convirtiéndolo en un compuesto hemiacetal inestable (ver primer paso Figura 4.3.).

La biodegradación del MTBE, ETBE y TAME y la remediación de ecosistemas contaminados han incrementando la demanda de investigaciones básicas y aplicadas en este tema (Kharoune y colaboradores, 2001).

4.1. Cultivos bacterianos puros y mixtos.

La primera investigación que informó acerca de la degradación bacteriana de oxigenantes (concretamente de MTBE) fue descrita por Salanitro (1994) utilizando un cultivo bacteriano mixto, extraído de fangos activos de una planta depuradora para tratar efluentes industriales. El consorcio bacteriano demostró degradar hasta 200 mg/l a una tasa de 34 mg/g celular/h. No obstante, aunque el cultivo pudo utilizar MTBE como única fuente de carbono, la tasa de crecimiento y el rendimiento celular eran muy bajos. Se especuló que la baja tasa de crecimiento fue debida a una posible deficiencia de carbono o que el MTBE actuaba como inhibidor metabólico.

En 1997, Mo y colaboradores aislaron tres cultivos puros, *Methylobacterium mesophilicum* (ATCC 700107), *Rhodococcus sp*. (ATCC 700108) y *Arthrobacter ilicis* (ATCC 700109) los cuales eran capaces de crecer en MTBE como única fuente de carbono y energía. Las tres cepas degradaron un 29% MTBE (de 200 mg/l) en dos semanas. La tasa de degradación decayó significantemente en la presencia de otras fuentes de carbono (*tert*-butil alcohol: TBA, ácido *tert*-butil fórmico : TBF, iso-propanol, acetona y ácido pirúvico).

Hardison y colaboradores, (1997) identificaron al TBF (ácido *tert*-butil fórmico) como el primer intermediario metabólico de la biodegradación del MTBE usando la cepa *Graphium sp*. bajo condiciones aeróbicas. La degradación de MTBE a TBA fue bloqueada con el uso de enzimas inhibidoras sugiriendo que tanto el MTBE como los *n*-alcanos son oxidados por la misma enzima: el citocromo *P-450.*

Hyman y colaboradores en 1998, mostraron que en la biodegradación co-metabólica del MTBE se necesitaban compuestos aromáticos ó alcanos para el crecimiento de cepas tales como *Mycobacterium vaccae* y su consiguiente degradación a TBA ó CO_2,

Deeb y colaboradores (2000a, 2000b), mostraron en sus estudios con la cepa *Rubrivivax gelatinosus* PM1 que ésta degradaba MTBE a tasas muy altas (50 mg/g celular/h), siendo la concentración de oxigenante en el medio de cultivo de 500 mg/l. PM1 también era capaz de crecer en TBA como única fuente de carbono y energía. La ausencia de TBA en el medio de cultivo indicó que la degradación de este compuesto era más rápida que la de MTBE y ETBE.

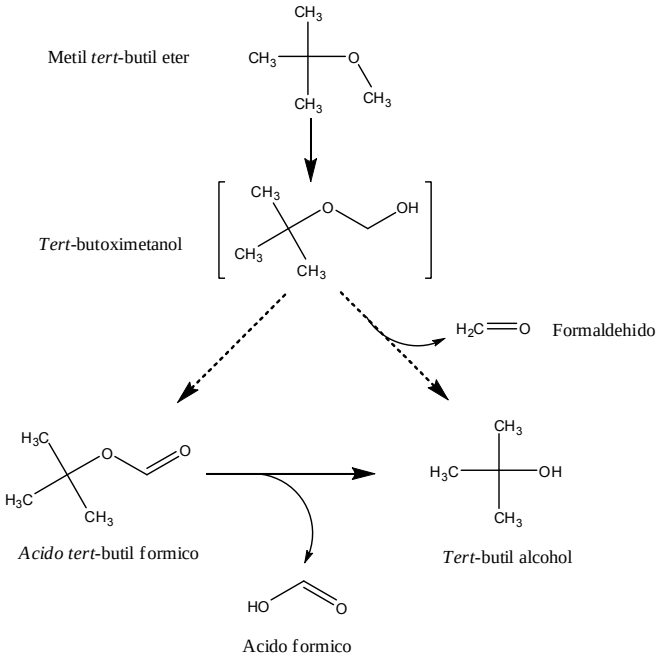


Figura 4.2.1. Primer paso en la degradación de MTBE.

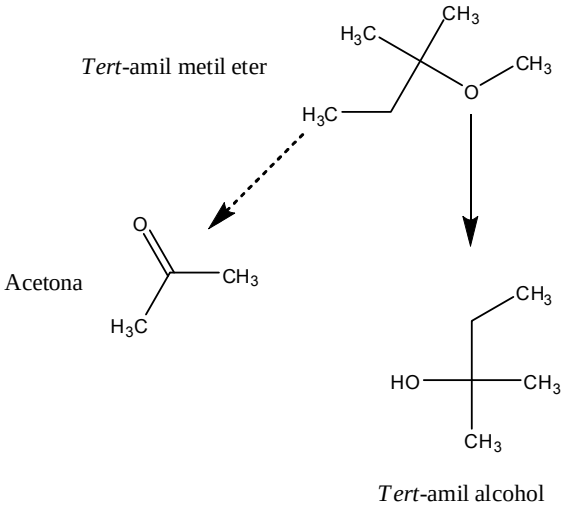


Figura 4.2.2. Primer paso en la degradación de TAME.

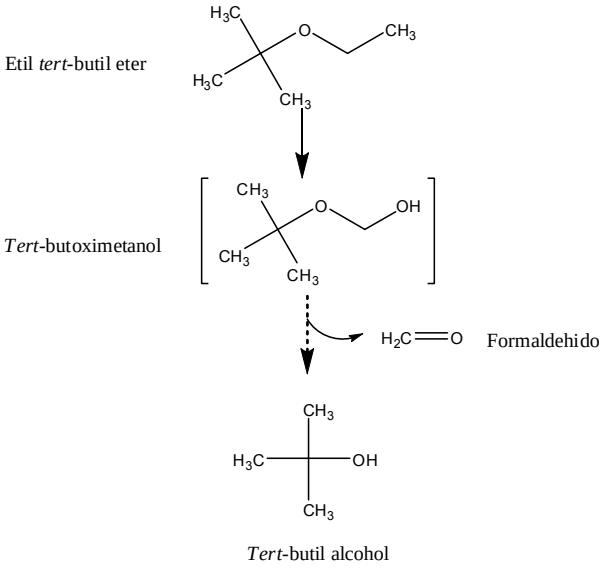


Figura 4.2.3. Primer paso en la degradación de ETBE**.**

4.2. Mecanismos de degradación

Las rutas de degradación del MTBE, ETBE y TAME aún no se conocen en su totalidad; no obstante, las Figuras 4.2.1., 4.2.2. y 4.2.3. muestran las primeras fases en las rutas propuestas de degradación de estos compuestos.

Como se puede observar en la Figura 4.2.1, el MTBE se convierte en un compuesto hemiacetal inestable llamado *tert*-butoximetanol mediante el concurso de una monoxigenasa (por ejemplo, un citocromo *P-450*).

Los citocromos *P-450* son comunes en células eucariotas y procariotas y forman parte de las proteínas de membrana. Estas monoxigenasas son dependientes de hierro, por lo general, incorporan un átomo de oxígeno en un compuesto orgánico: la reacción suele resultar en una hidroxilación. Estas enzimas se utilizan el la industria para biotransformación de hormonas, fermentación, formación de ácidos di-carboxílicos de alcanos y la hidroxilación de oleofinas aromáticas entre muchos otros (Urlacher y colaboradores, 2004). En la naturaleza, son enzimas involucradas en la biosíntesis de prostaglandinas, esteroides, y en la limpieza de compuestos xenobióticos en aguas.

Hay dos posibles rutas que explican la transformación de MTBE a TBA. En la primera de ellas, se transforma el hemiacetal inestable en *tert*-butil-alcohol (TBA) mediante oxidación, eliminando ácido fórmico. En la segunda ruta, el compuesto TBF (ácido *tert*-butil fórmico) se transforma en TBA eliminándose una molécula de formaldehído; este proceso no se sabe si es liderado ó no por una enzima.

Algunos estudios indican que el TBF es el primer metabolito en la degradación del MTBE (Hardison y colaboradores, 1997). Sin embargo, este metabolito se hidroliza rápidamente a TBA, y por consiguiente es difícil que se acumule a concentraciones detectables (Church y colaboradores, 1997).

No existen muchas investigaciones sobre la degradación de TAME por microorganismos; la ruta propuesta se muestra en la Figura 4.2.2, siendo el compuesto intermediario producido el t*ert*-amil alcohol (TAA).

La ruta de degradación propuesta para el ETBE (Figura 4.2.3) es bastante parecida a la del MTBE, generándose en ambas rutas el mismo intermediario: TBA.

La Figura 4.2.4 muestra una posible ruta de degradación del intermediario TBA, la cuál conllevaría la completa mineralización de los oxigenantes MTBE y ETBE. En esta ruta aún no se conocen todas las enzimas o factores involucrados en el metabolismo de estos compuestos. Church y colaboradores, (2000) indicaron en sus estudios, que las cepas que degradaban TBA crecían en todos los intermediarios de las rutas de degradación de oxigenantes, aunque más lentamente.

No siempre se produce la mineralización completa de los oxigenantes (especialmente MTBE y ETBE), detectándose una importante acumulación de TBA en los medios de cultivo. Este compuesto es altamente carcinógeno y presenta problema mayores que el compuesto inicial (Cirvello y colaboradores, 1995). Originariamente se pensaba que el TBA era un compuesto inerte en tejidos animales y se utilizaba mucho como disolvente, pero estudios posteriores revelaron que este compuesto tenía efectos secundarios muy dañinos ya que se metabolizaba a acetona, tras haber liberado formaldehído en el paso del hemiacetal a TBA.

La biodegradación anaeróbica de los oxigenantes MTBE, ETBE y TAME también ha sido estudiada (Pearce y Heydemann, 1980). En este sentido, diferentes sustancias (nitrato, sulfato, Fe^{3+}) han sido propuestas como aceptores finales de electrones en ausencia de oxígeno.

Para los mecanismos que no requieren la incorporación de un átomo de oxígeno, convertir el enlace éter a la inestable estructura hemiacetal requiere la presencia de un grupo hidroxilo de un carbono próximo al oxígeno del éter.

4.3. Genes de degradación de MTBE, ETBE y TAME

Hasta el año 2001 no se conocía ningún mecanismo enzimático involucrado en la degradación de los éteres presentes en la gasolina. Chauvaux y colaboradores (2001) fueron los primeros autores en corroborar que una monoxigenasa estaba implicada en el primer paso de la ruta metabólica de degradación MTBE/ETBE. Dichos autores obtuvieron un mutante ETBE negativo (*Rhodococcus ruber* IFP2007) tras sub-cultivar durante 60 generaciones en un medio rico, y compararon este mutante con la cepa silvestre (*R. ruber* IFP2001) a nivel genómico y proteómico. El cultivo creciendo en distintas fuentes de carbono (EtOH y ETBE) produjo expresiones proteicas diferentes y, mediante electroforesis en campo pulsante, pusieron de manifiesto la deleción de un fragmento en el mutante que contenía el operón *ethRABCD* (Figura 4.3.1.).

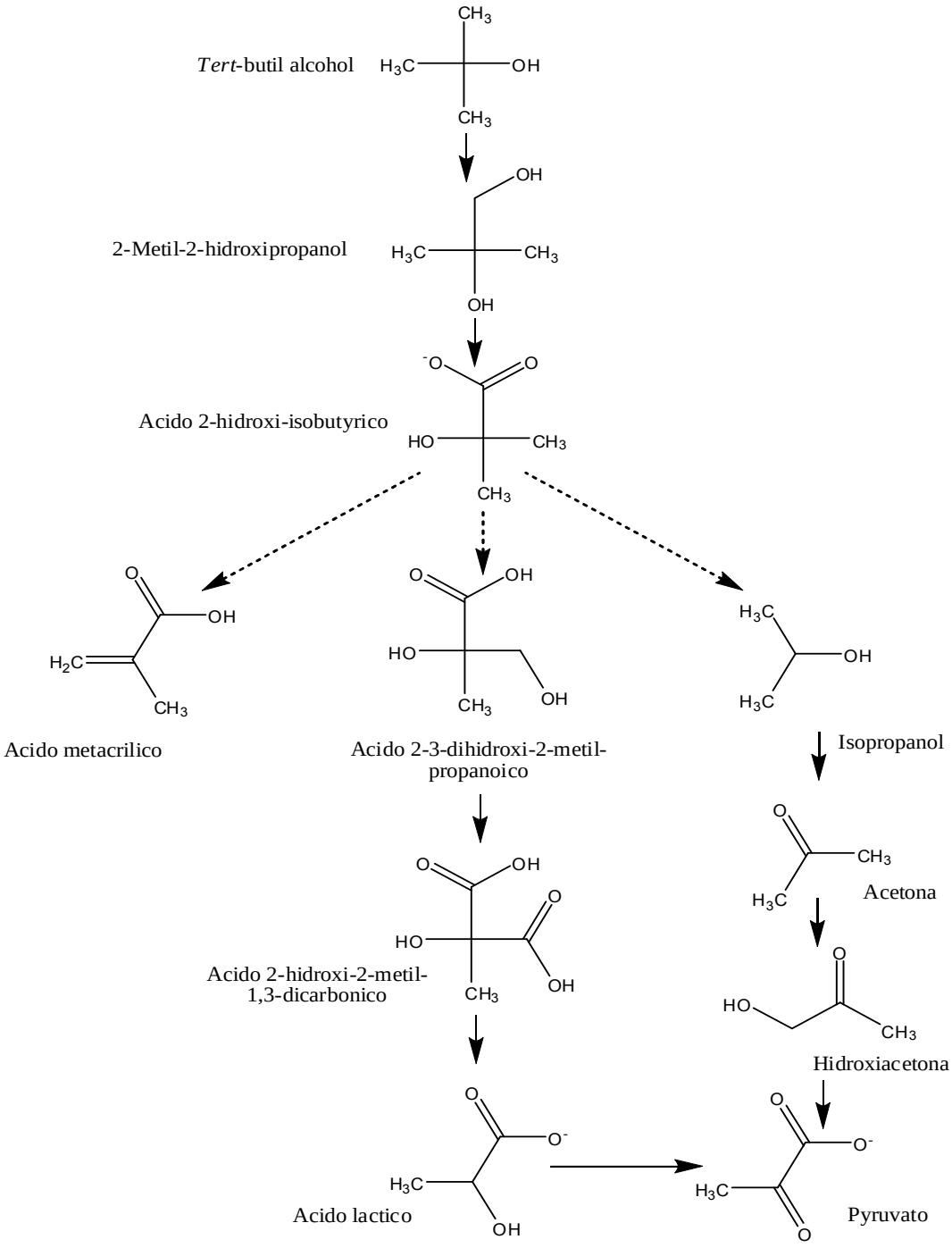


Figura 4.2.4 Ruta de degradación propuesta para TBA. (Ferreira y colaboradores, 2006b)

En este mismo artículo también describieron el mecanismo de delección del operón *ethRABCD*. Este mecanismo consiste en una recombinación homóloga entre dos transposones idénticos situados a ambos lados del operón y orientados en la misma dirección. El resultado es una deleción del operón *ethRABCD*, como se observa en la Figura 4.3.1.B

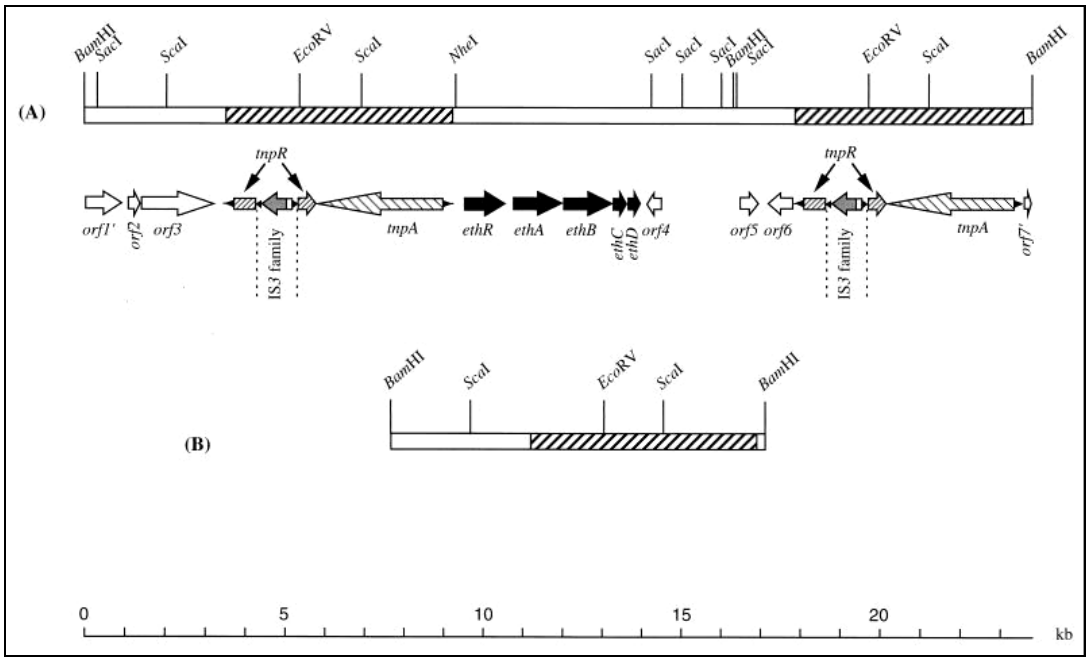


Figura 4.3.1 (A y B). **Organización de las regiones adyacentes al operón *ethRABCD* en *Rhodococcus ruber* IFP2001 antes (A) y después (B) de la supresión por recombinación homóloga**

(Chauvaux y colaboradores, 2001).

En la Tabla 4.3.1. se reflejan las propiedades y funciones de cada proteína codificada por el operón *ethRABCD*.

Chen y colaboradores (1996) aislaron, tras crecimiento en octano, una cepa (*Pseudomonas putida* GPo1) capaz de degradar MTBE. Más tarde, Smith y Hyman (2006) curaron esta cepa de plásmidos, lo que les permitió asociar la incapacidad de degradar MTBE con la eliminación de los operones *alkBFGHJKL* y *alkST*, ambos incluidos en el plásmido OCT (Egglink 1987 y 1988). De estos operones, es el gen *alkB*, codificante de una alcano-monoxigenasa, el señalado como responsable de la degradación de MTBE a TBA.

Tabla 4.3.1. Descripción del operón *ethRABCD*.

Gen	Descripción	nº de aa
ethR	Regulador transcripcional de la familia *Arac*	332
ethA	Ferrodoxina reductasa	413
ethB	Monoxigenasa citocromo P450	401
ethC	Ferrodoxina	107
ethD	Función desconocida	104

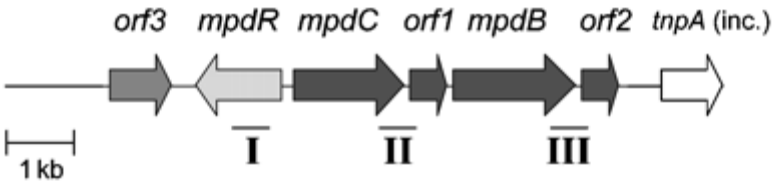


Figura 4.3.2. Organización y orientación de los genes involucrados en la ruta de 2MPD a 2-HIBA (Ferreira y colaboradores, 2006ª).

En el año 2006 se publicaron más resultados sobre los genes implicados en las rutas de degradación de MTBE. Lopes Ferreira y colaboradores (2006) aislaron una cepa bacteriana, *Mycobacterium austroafricanum* IFP2012, capaz de crecer en MTBE como única fuente de carbono, y, consecuentemente, degradarlo. Un análisis de las proteínas sintetizadas en distintas fuentes de carbono (MTBE y glucosa) mostró la existencia de tres proteínas involucradas en la ruta metabólica desde 2-metil-2-hidroxipropanol (2-M1,2-PD) al ácido 2-hidroxi-isobutírico (HIBA). Los genes codificantes de estas proteínas se organizan en el operón *mpdRCB*, el cual contiene un transposón (Figura 4.3.2.).

Rohwerder y colaboradores (2006) describieron un mutasa dependiente de cobalamina para la degradación de 2-HIBA al 3-Hidroxi butiril CoA. Sus dos subunidades, IcmA y IcmB, son similares a las correspondientes de *Methylibium petroeliphilum* PM1, bacteria también ampliamente conocida por su capacidad de crecer en MTBE como única fuente de carbono y cuyo genoma está publicado en la base de datos del NCBI (Kane y colaboradores 2007).

Tabla 4.3.2. Oligonucleotidos descritos en la literatura para amplificar los genes *ethB* y *alkB*

Gen (número de acceso en NCBI)	Secuencia del cebador	Tamaño esperado (pares de bases)
ethB (FJ607040)	*ethB*-F2: 5'-CACGCGCTCGGCGACTGGCAGACGTTCAGT-3' *ethB*-R2: 5'-TCCGACGCACATGTGCGGGCCGTACCCGAA-3'	881
alkB (FJ590423)	*alk*-H1F: 5'-CIG IIC ACG AII TIG GIC ACA AGA AGG-3' *alk*-H3R: 5'-IGC ITG ITG ATC III GTG ICG CTG IAG-3'	549

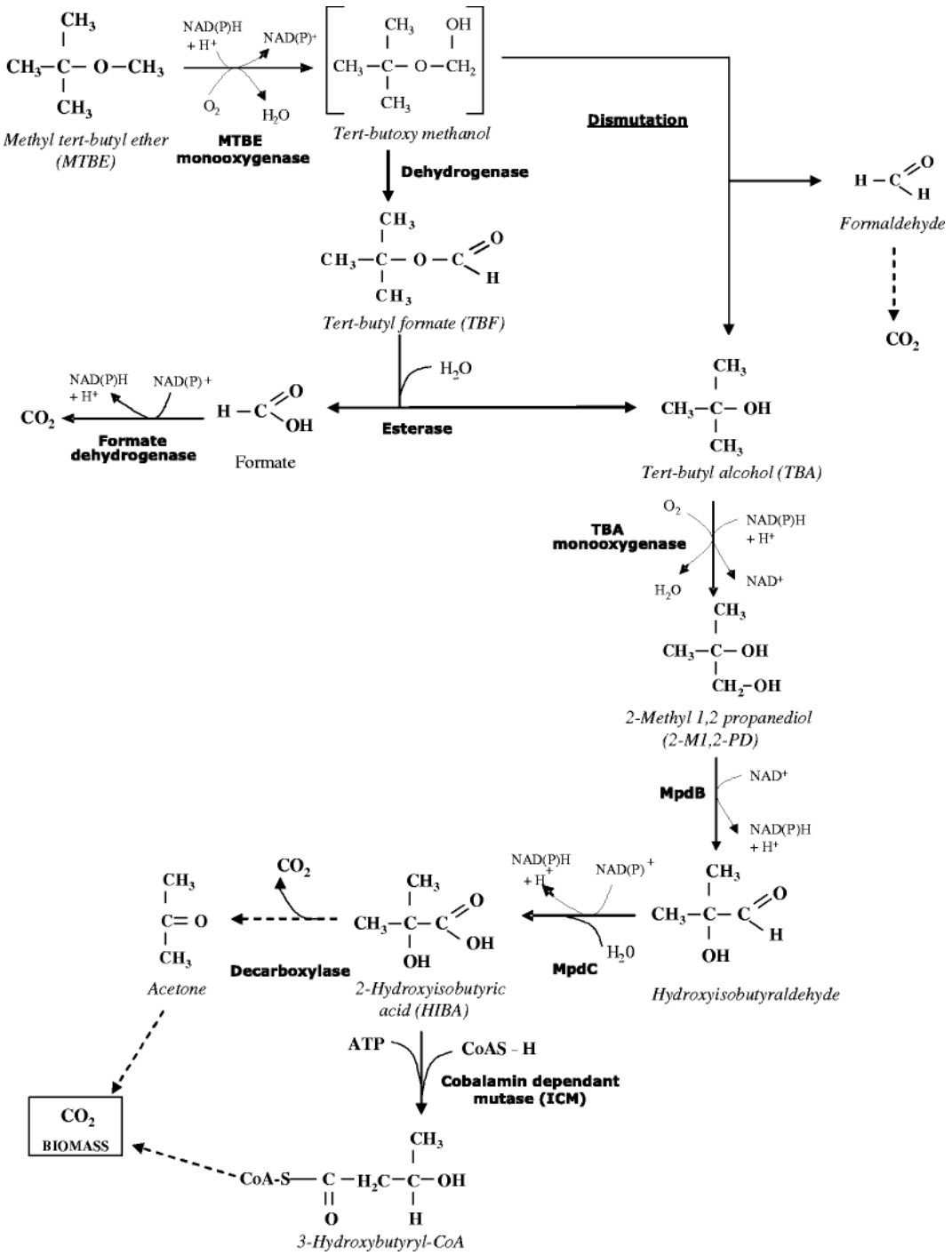


Figure 4.3.3. Enzimas involucradas de la ruta de degradación de MTBE y su posición de actuación. (Ferreira y colaboradores, 2006[b])

En el año 2007, Schafer y colaboradores encuentran una posible enzima que degrada el TBA a 2-HIBA, la cual se asemeja a una ftalato dioxigenasa. Sin embargo, debido a la

reacción que cataliza esta enzima, ahora se describe como una monoxigenasa (Rohwerder, 2009. Comunicación personal).

Tras la secuenciación completa del genoma de *M. petroleiphilum* PM1 (Kane y colaboradores 2007), la ruta completa de degradación de MTBE se infirió mediante inducción de la ruta y posterior análisis de los transcritos a través de "*microarrays*" diseñados para este genoma (Hristova y colaboradores, 2007). Aunque hasta la fecha únicamente se ha conseguido demostrar la actividad de la monoxigenasa MdpA en la degradación de MTBE a TBA (Schmidt y colaboradores, 2008), sin duda el trabajo presenta la predicción más completa de la ruta de degradación de MTBE por un microorganismo capaz de utilizar MTBE como única fuente de carbono.

Auffret y colaboradores (2009) describieron cebadores para la búsqueda de la enzima que cataliza el paso inicial de degradación de los éteres, utilizando como base, los genes *alkB* y *ethB*. En la Tabla 4.3.2. se muestran las secuencias de los oligonucleótidos utilizados para este fin.

Recientemente, Eixarch y Constante (2010) han detectado genes "sobre expresados" en la degradación del compuesto MTBE; teóricamente codifican para una ATP sintasa, una proteína ribosómica, una proteína periplásmica de unión a aminoácidos y una cuarta proteína, de función desconocida. Se ha postulado que estas cuatro proteínas disminuyen la síntesis proteica para sobrevivir en ambientes hostiles. Por su parte, la proteína de unión a aminoácidos podría ser una bomba implicada en expulsar los compuestos orgánicos, de forma similar a lo descrito en con las proteínas de la familia TolC (Anderesen y colaboradores, 2000).

A continuación se muestra una figura (Figura 4.3.3.) resumen de los genes de degradación descritos y verificados en la ruta de degradación del MTBE.

5. Métodos para la determinación de oxigenantes de las gasolinas

Existen diferentes métodos para determinar la concentración de éteres de gasolina desde fases sólidas y líquidas. Todos estos métodos utilizan la técnica de cromatografía de gases y/o la espectrometría de masas, ya que los compuestos analizados son compuestos orgánicos polares volátiles. En la Tabla 5.1., se describen los métodos más comúnmente utilizados.

La purga y trampa (*Purge & Tramp*) es la metodología empleada por la USEPA para determinar los oxigenantes de gasolinas, incorporando MTBE en sus protocolos de detección de sustancias peligrosas en acuíferos. La elección de la metodología más idónea para determinar los oxigenantes de gasolina, dependerá del motivo de la determinación, y a su vez, del rango de concentración y precisión requerida.

Tabla 5.1. Métodos más comúnmente utilizados para la detección y cuantificación de compuestos oxigenantes de las gasolinas. Caso de MTBE.

	Método	**Descripción breve**	**Límite Detección MTBE (µg/l)**
DAI	Inyección directa desde fase acuosa	Las muestras, no requieren de un pre-tratamiento exceptuando una filtración o centrifugación. La muestra se inyecta desde su fase acuosa.	0.1
HS	Espacio de cabeza (*Headspace*)	Este método utiliza la partición de los analitos desde fase acuosa/sólida a gaseosa en un sistema cerrado. Las muestras se inyectan desde la fase gaseosa.	0.2
P&T	Purga y trampa	La muestra se inyecta en fase liquida acuosa, previamente filtrada. Una vez inyectada, el gas inerte utilizado en el GC, se burbujea a través de la muestra, arrastrando los analitos en fase gaseosa. Estos después, se concentran en sobre un matriz, y a continuación, se separa en el GC.	0.001
ME	Micro extracción	La muestra se somete a una fibra adsorbente. Esta fibra se transfiere al GC, donde el metabolito se libera en forma de gas, para su posterior separación.	0.007

6. Remediación de aguas subterráneas contaminadas con oxigenantes de gasolinas

Debido a su estructura química y características, los compuestos oxigenantes de las gasolinas MTBE, ETBE y TAME, se comportan de forma distinta a los hidrocarburos del petróleo cuando alcanzan las aguas subterráneas, por lo que ha sido necesario y aún están en fase de experimentación, diversas tecnologías específicas para su eliminación.

Aunque a menudo es difícil y laborioso, la eliminación de estos oxigenantes en aguas contaminadas es posible gracias a la aplicación de procesos físicos, químicos y biológicos. A continuación vamos a realizar un rápido repaso de las tecnologías más utilizadas, extendiéndonos en los procesos en los cuales los microorganismos tienen una especial contribución.

6.1. Procesos físicos-químicos

Hay distintos sistemas físicos y/o químicos descritos para remediar acuíferos contaminados con éteres de gasolina. Uno de estos sistemas es el denominado "*air sparging*", cuyo proceso se reduce a inyectar aire directamente al acuífero contaminado para volatilizar los contaminantes *in situ*. Estudios realizados con el MTBE muestran que se llegan a obtener altas reducciones (desde 1000 µg/l hasta 10 µg/l). Sin embargo, la adición de oxígeno disuelto no ha mostrado, en ningún caso, incrementos significativos en la biodegradación del oxigenante (USEPA, 1998).

A diferencia de las técnicas generalmente utilizadas para el tratamiento de acuíferos contaminados con petróleo, el bombeo de agua y su posterior tratamiento es la técnica más eficaz para la eliminación del MTBE y otros oxigenantes. En estas condiciones, el agua subterránea es sometida a distintos tratamientos, como por ejemplo, el uso del carbón activo. Este sistema consiste en hacer pasar el agua contaminada a través de un lecho de carbono activo, al objeto de retener los compuestos orgánicos. El inconveniente que tiene esta técnica es que debido a la baja afinidad de los oxigenantes por el carbono, es necesario pasar repetidas veces, grandes volúmenes de agua contaminada antes de que toda la contaminación sea eliminada (USEPA, 1999).

En la 3ª Conferencia sobre oxigenantes (Amberes, Bélgica, 2007) se describió un proceso utilizando ozono como catalizador para la ruptura de esta molécula tras una inyección

directa en el acuífero contaminado. Sin embargo, esta claro que este tipo de acción química sobre ecosistemas contaminados conllevaría desastres más sutiles al cabo del tiempo (como puede ser una precipitación de minerales, ó una disminución en la microbiota acuática), lo que provocaría una desestabilización en el equilibrio ambiental (Ross y colaboradores, 2007).

6.2. Biorremediación

En ecosistemas naturales (suelos y acuíferos), la atenuación natural de cualquier contaminante está fundamentalmente controlada por la migración y la biodegradación. La cuestión de la atenuación natural de MTBE se ha abordado en estudios de campo y en experimentos de laboratorio (Landmeyer y colaboradores, 1998).

Los estudios de campo están de acuerdo en la conclusión de que la atenuación natural es lenta y en algunos casos no detectable. De acuerdo con Johnson y colaboradores (2000), basándose en el número de casos documentados de contaminación en la actualidad, la degradación abiótica del MTBE es considerada insignificante. Schirmer y colaboradores (1998), pusieron de manifiesto que debido a la lentitud de la degradación del MTBE, la atenuación es probablemente insuficiente en la protección de acuíferos, una vez que este compuesto oxigenante alcanza una zona.

Aunque las investigaciones iniciales mostraron que los oxigenantes, y especialmente el MTBE, eran resistentes a la biodegradación, diversos estudios i*n batch*, a escala de laboratorio, (Fortin y Deshusses, 1999; Kharoune y colaboradores, 2001; Moreels y colaboradores, 2004) han indicado elevadas tasas de eliminación de estos compuestos de aguas subterráneas contaminadas, usando tanto cultivos puros (solos ó en consorcio) como cultivos mixtos. En este sentido, Corcho y colaboradores (2000), seleccionaron un cultivo mixto capaz de crecer en un hidrocarburo recalcitrante, ciclohexano, como única fuente de carbono y energía. Después del crecimiento en el ciclohexano, este cultivo fue capaz de metabolizar MTBE, ETBE, TAME y TBA. El benceno y el tolueno también se degradaron, una propiedad muy interesante en vista del contenido de hidrocarburos solubles de los acuíferos contaminados. El citocromo *P-450* parecía estar involucrado en la degradación del MTBE y TBA.

Entre las actuaciones realizadas para la biorremediación de aguas subterráneas contaminadas con oxigenantes de las gasolinas, la aplicación de diversas tecnologías tales como reactores de lecho fluidificado, biorreactores de biopelícula fija ó biorreactores de

membrana sumergida ha cobrado especial interés en la última década. Las siguientes tablas extraídas del libro “Fuel Oxygenates (Barceló, 2007), recogen las características así como los resultados obtenidos mediante la aplicación de estas tecnologías a aguas contaminadas con oxigenantes y compuestos aromáticos (caso de BTEX).

De estos datos, se deduce que diferentes materiales se han utilizado como relleno en estos sistemas. El carbono activo granulado y el material de relleno Filtralite® adsorben los compuestos. Aquellos rellenos que presuntamente se comportan como rellenos inertes son las bolas de vidrio, arena, polietileno, cerámica y la fibra microporosa. La mayoría de los sistemas necesitaron de un tiempo inicial de 10 a 200 días para que el rendimiento del sistema fuera de un 99%. Este amplio rango de duración de la puesta en marcha de los sistemas, responde a diversos factores tales como la concentración inicial de contaminante, la microbiota presente, el flujo de aire inyectado causando “*air stripping*”, el volumen de agua, el relleno utilizado y las propiedades del mismo, tiempo de retención hidráulica así como la temperatura de operación.

En este contexto, los procesos biológicos aplicados mediante sistemas de biopelícula fija (sistemas de filtros sumergidos) aparecen como una alternativa viable en la biorremediación de aguas subterráneas contaminadas con diversas sustancias, como puede ser el uso para la desnitrificación (Moreno y colaboradores, 2005, Gómez y colaboradores, 2009), el uso para la eliminación de metales pesados (Vilchez y colaboradores, 2007), o el uso para la eliminación de compuestos fenólicos (Pozo y colaboradores, 2007)

Los sistemas de biofiltros sumergidos (sistema de biopelícula fija) están constituidos por biopelículas (comunidades microbianas localizadas en la matriz de polisacárido extracelular, EPS, con el que desarrollan fuertes relaciones) adheridas a un sustrato (partículas rocosas, plásticos o de otro origen), a través del cuál se hace circular el agua a tratar. Este sistema de tratamiento ofrece una serie de ventajas frente a otros procesos, como por ejemplo: menor superficie para la instalación, menor sensibilidad a los tóxicos y menor producción de ruidos y olores. Por otro lado la cantidad de biomasa activa es superior en estos procesos de células inmovilizadas, ventaja que sumada a las anteriores hace de este sistema, un sistema muy atractivo desde diversas vertientes para la eliminación de diversas sustancias de aguas contaminadas.

Diversos autores han realizado experimentos usando sistemas de biopelícula fija para el tratamiento de aguas subterráneas contaminadas con oxigenantes de las gasolinas. Fortin y

Deshusses, (1999) trabajando con sistemas de biopelícula fija, mostraron una mineralización del MTBE del 97% sin la aparición de ningún metabolito ni en la fase líquida ni en la fase gaseosa. La tasa de degradación del MTBE fue de 5-10 mg MTBE/g células/h, siendo el rendimiento celular parecido a aquel ya comunicado en otros estudios *in batch* usando cultivos puros y mixtos (Salanitro y colaboradores, 1994; Piveteua y colaboradores, 2001).

Kharoune y colaboradores (2001), demostraron una eliminación del 99% de MTBE, ETBE y TAME de un influente cargado con 100 mg/l, usando un reactor con biopelícula fija, siendo el flujo ascendente y el tiempo de retención hidráulica de 24 h. El biorreactor fue inoculado con un cultivo puro, aislado de un suelo contaminado con gasolina sin plomo, después de 12 semanas de selección, utilizando los tres oxigenantes como única fuente de carbono y energía en condiciones aeróbicas.

Aplicaciónes de reactores: Lechos con relleno

Descripción del reactor	Características del influente	Datos operativos	Eficiencia del tratamiento	Tiempo de inicio (dias)	Comentarios
Tipo: Lecho ascendente Vol.: 2 l Material: anillos de vidrio	Oxigenante: MTBE, ETBE, TAME Concentración: 10-100 mg/l each Recirculación: 650 l/d	TRH: 13 h VSS: ~ 1 g/l Temperatura: 28±1 ºC O_2: >2 mg/l Recirculación: si	Eliminación: >99 % for MTBE, TAME and Carga de ETBE a 135-140 mg/l.d Eficiencia: 1-2.2 µg/l	40	Reactor sembrado con biomasa de degradadadoras de éteres. A las 13 h TRH, la tasa de eliminación fue de entre 133 y 170 mg/l.d para todos los éteres. La eliminación de ETBE fue la mas rapida.
Tipo: Lecho ascendente Vol.: 0.5 l Material: Filtralite®	Oxigenante: MTBE, TBA Concentración: 3.2 mg/l MTBE Carga: 258 mg/(l.d)	TRH: 9.8 min Temperatura: 19±1 ºC O2: > 2 mg/l (outlet)	Eficiencia:30 µg/l	~120	Maxima eliminación de MTBE despues de 3 meses fue de 19 mg/(l.d)
Tipo: Lecho ascendente Vol.: 1.2 l Material: Esferas de vidrio	Oxigenante: MTBE Concentración: 150 mg/l	TRH: 1 day O2: 14.4 mg/l Recirc: non	Eliminación: 70 %		Reactor sembrado con fango activo de una planta petroquimica. El genero dominante fue Micrococcus: La eliminación de MTBE fue baja en comparación.
Tipo: Lecho ascendente Dimension: 100 x 5 cm Relleno: Cuarzo	Oxigenante: MTBE Concentración: ~ 160 mg/l Flujo: 500 ml/d O2: > 4 mg/l	TRH: 80 h Temperatura: ~25 ºC Recirc: no	Eliminación: 50 %		La eliminación fue baja en comparación con sistemas parecidos. El tiempo de operación dell sistema solamente fue de 33 dias.
Tipo: Lecho descendente Material: Antracita y arena Area: 80 m2	Oxigenante: MTBE Concentración: 10-55 µg/l Flujo: 4-28 m3/h	TRH: 10-72 min Temperatura: >10 ºC Recirc: non	Eliminación: 95-100 % Eficiencia: < 5 µg/l		Estudios sobre filtros para aguas potables
Tipo: Trickling filter Vol.: 0.7 l Material: Tierra	Oxigenante: MTBE Concentración: 13 mg/l Carga: 0.1-2.5 mg/(l.h)	TRH: 4.8-84 h Recirculación: non	Eliminación: 100 % upto Carga 2.5 mg/(l.h)		Nitrificación simultanea
Tipo: Trickling filter	Oxigenante: MTBE Conoc.: 0.1-25 mg/l Flujo: 1-35 m3/h Carga: 3-5 g/(m3.h)	TRH: 0.1 h Temperatura: >14 ºC	Eliminación: >90 % Eficiencia: 10 µg/l		Estudios ejecutados en 15 parcelas. El tratamiento cuesta alrededor de $0.3/m3 de agua subterránea

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Aplicaciónes de reactores: Lecho sumergido

Descripción del reactor	Características del influente	Datos operativos	Eficiencia del tratamiento	Tiempo de inicio (dias)	Comentarios
Tipo: Lecho fluidificado Vol.: ~900 l Material: Carbon activo	Oxigenante: MTBE, BTEX Concentración: ~9.6 mg/l MTBE Flujo: 15 l/min Recirculación: 121 l/min	Temperatura: 10.6-23.8ºC O2: 2.5mg/l (outlet) Recirculación: si	Eliminación: 96 % MTBE	30-40	Reactor sembrado con carbon activado "bio-active". El tiempo de inicio fue mayor que otro reactor parecido.
Lecho fluidificado Vol.: 1.56 l Material: Carbon activo	Oxigenante: MTBE Concentración: 10-50 mg/l Recirculación: 840 l/d Flujo: 5-20 l/d	TRH: 1.7-10.8 h Temperatura: 27-29 ºC O2: 4 mg/l Recirculación: si	Eliminación: hasta >98 % Cargas de 700 mg/(l.d)	30-50	Iso-pentano puede que haya ayudado en el tiempo de inicio, debido al cometabolismo.
Lecho fluidificado Vol.: 7.88 l Material: Carbon activo	Oxigenante: MTBE, BTEX Concentración: 7.8-8.8 mg /l MTBE Concentración: 2 mg/l BTEX Recirculación: 150 % (Bed vol.) Flujo: 22.7-36.41 l/d	TRH: 1 h (empty Bed) Temperatura: 20 ºC O2: > 2 mg/l Recirculación: si	Eliminación: 99.9 % MTBE and BTEX Eficiencia: 18-20 µg/l MTBE Eficiencia: 1-2.2 µg/l BTEX	30	BTEX añadido al influente despues de 225 dias. Eliminación instantanea de BTEX. Reactor sembrado con una cepa tipo PM1 de un reactor de membrana.
Lecho fluidificado Vol.: 4.5l Material: Carbon activo	Oxigenante: MTBE Concentración: 10 mg/l Flujo: 0.1 and 0.34 l/ h	TRH: 3 and 1 h Expansion: 125 % Recirculación: si O2: 2 mg/l	Eliminación: 90 y 99 % a 1 y 3 g TRH respectivamente Eficiencia: 100 µg/l a 3h TRH	~ 30	Reactor sembrado con una cepa degradadora de MTBE, de in bioreactor de membrana.
Lecho fluidificado Material: arena	Oxigenante: MTBE Concentración:1.7 mg/l (max) Flujo: 40 l/min	Recirculación: si O2: ~8 mg/l	Eficiencia: <1 µg/l	~ 150	Reactor sembrado con cepa PM1
Lecho fluidificado Vol.: 3.53m3 Material: arena	Oxigenante: MTBE, TBA Flujo: 60 l/min Concentración: 12 mg/l MTBE Concentración: 300 µg/l TBA Recirculación: 180 l/min	Recirculación: si TRH: 1 h	Eficiencia: <1 µg/l MTBE and TBA		Reactor sembrado con cultivo de PM1. Niveles altos de oxigeno disuelto incrementaron la tasa de eliminación de MTBE
Lecho fluidificado Vol.: 4.5l Material: Carbon activo	Oxigenante: MTBE, TBA Concentración: 350 mg/l MTBE Concentración: 170 mg/l TBA Recirculación: ~ 20 l h	TRH: 7.5 h Temperatura: 25-30 ºC Tss.: >10 g/l Expansión: ~127 % Recirculación: si O2: >1 mg/l	Eficiencia: 1±15 µg/l MTBE Eficiencia: 3±3 µg/l TBA	~ 20	Reactor sembrado con carbon activo "bio-active".

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Aplicaciónes de reactores: Membrana

Descripción del reactor	Características del influente	Datos operativos	Eficiencia del tratamiento	Tiempo de inicio (dias)	Comentarios
Tipo: Membrana Vol.: 9.95 l Membrana: polietileno	Oxigenante: MTBE Carga: 370 mg/ (l.d) Flujo: 2.37 l/d	TRH: 4.2 days Temperatura: 20 ºC	Eliminación: 99.9 % Eficiencia: ~ 1 µg/l	100-200	BTEX, DIPE, DEE y ethanolfueron degradados en reactores similares y no tuvieron eficiencia en la eliminación de
Tipo: Membrana Membrana: polietileno	Oxigenante: MTBE Concentración: 150 mg/l Flujo: 2.37 l/d	TRH: 4.2 days SRT: > 20 days VSS: ~ 1 g/l (max) Temperatura: 20 ºC O2: 3 mg/l	Eliminación: 99.99 % Eficiencia: ~ 1 µg/l	100-200	Reactor inoculado con biomasa adaptada a MTBE; concentración de solidos suspendidos volátiles alcanzo un maximo de 2.5 g/l
Tipo: Membrana Vol.: 5.9 l Membrana: ultrafiltración con ceramica	Oxigenante: MTBE Concentración: 5 mg/l Flujo: 142 l/d	TRH: 1 h SRT: 150-400 days VSS: ~3.5 g/l (max) Temperatura: 18-20 ºC O2: 3 mg/l	Eliminación: 99.99 % Eficiencia: 0.32± 39 µg/l	~ 150	Ensuciamiento de la membrana hizo necesaria el incremento paulativo en la presion transnmembrana, en el tiempo
Tipo. Membrana Vol.: 6 m3 Membrana: polietileno	Oxigenante: MTBE, BTEX Concentración: 2.9 mg/l MTBE Flujo: 19 l h O2: >8 mg/l	TRH: 6 h VSS: 2.5 g/l Temperatura: 13-26 ºC	Eliminación: 99.91 % MTBE Eliminación: 99.98 % BTEX Eficiencia: 2.62 µg/l MTBE	70-90	Reactor sembrado con cultivos enriquecidos en MTBE y BTEX. No hubo que aplicar presion para pasar el flujo por la membrana.
Tipo: Membrana Vol.: 85 l Membrana: fibra hueca microporoso	Oxigenante: MTBE Concentración: 1 g/l Flujo: 1.2 l/h	TRH: 3 days TSS: 12 g/l O2: 2 mg/l	Eliminación: 99.99 % Eficiencia.. 0.1 mg/l	10-20	Reactor iniciado con un cultivo degradador de MTBE. Tasa de eliminación fue de 1008 mg/(l d) a TRH de un dia.
Tipo: Membrana Vol.: 1m3 Membrana: polietileno	Oxigenante: MTBE Concentración: 5 mg/l Flujo: 104.17 l/h O2: >3 mg/l	TRH: 4 h SRT: >100 days VSS: ~ 1 g/l Temperatura: 10-25 ºC Recirculación: si	Eliminación: 97.93 % Eficiencia: <1 µg/l	20-50	Reactor sembrado con cultivos enriquecidos en MTBE y BTEX. No hubo que aplicar presion para pasar el flujo por la membrana.

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7. Importancia de las biopelículas y su estudio

El estilo de vida bacteriana más frecuente en ambientes naturales y artificiales, es aquel en el que las bacterias viven adheridas a una superficie, formando una estructura conocida como *biopelícula* (Costerton y colaboradores, 1995; Danese y colaboradores, 2000; Decho, 2000; Watnick y Kolter, 2000) (Figura 7.1.), donde se encuentran los requisitos necesarios para su desarrollo. Las biopelículas pueden formarse en sustratos sólidos con humedad, en superficies de tejidos blandos de los organismos vivos y en las interfases aire-líquido, líquido-sólido y sólido-sólido. Su estructura morfológica y fisiológica es diferente a la exhibida por las células bacterianas en estado planctónico.

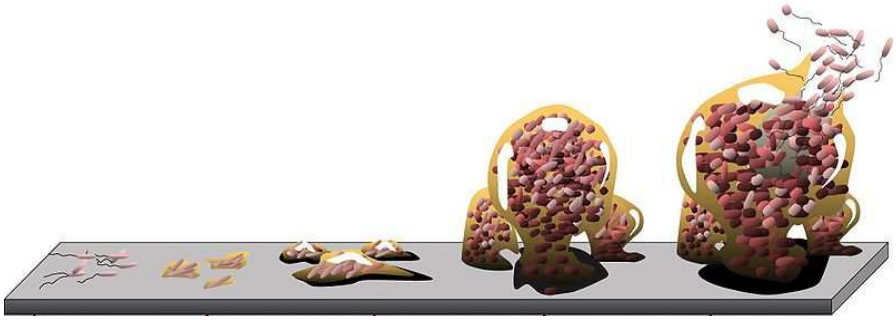

Figura 7.1. Proceso de formación de una biopelícula desde una fase reversible de adhesión, hasta una fase irreversible de adhesión a una interfase física entre fase aire, liquido y sólido. ***Fuente: American Society for Microbiology***

Una biopelícula puede definirse como " *una estructura compleja formada por agregados de células embebidas en una matriz polimérica de naturaleza orgánica y origen microbiano, EPS, adherida a un material o interfase que puede ser de naturaleza abiótica (rocas, metales, vidrios, plásticos, etc.) o biótica (mucosa intestinal, plantas, etc.,) las cuales exhiben un estado metabólico alterado comparadas con crecimiento planctónico correspondiente, especialmente con respecto a la trascripción y las interacciones entre las células*" (Davies y colaboradores, 1998;. Geesey y Jang, 1989; Costerton, 1999; Decho, 2000; Watnick y Kolter, 2000).

El estudio sobre las biopelículas se ha incrementado en los últimos años debido a un mayor conocimiento de su impacto sobre los sistemas naturales y artificiales, así como en la salud humana. Sin embargo, las biopelículas pueden ser de enorme ayuda para la biorremediación. En este sentido, son la estructura base de diversos reactores aerobios y

anaerobios (biofiltros percoladores, biofiltros sumergidos, discos biológicos rotativos...) para el tratamiento de aguas residuales municipales e industriales ó en forma de "biobarreras" *in situ* para proteger suelos y aguas subterráneas frente a sustancias contaminantes (Cao y colaboradores, 1992; Hunter y Shaner, 2009; Ross y colaboradores, 2001). La complejidad de la actividad de una biopelícula y su rendimiento en la biorremediación requiere la cooperación de muchas disciplinas, tales como la microbiología, la bioquímica, la ingeniería o la física. Numerosos trabajos de investigación se publican con frecuencia sobre biopelículas y los resultados hacen referencia a aspectos tales como la estructura, composición y características de estos ecosistemas especiales. En consecuencia, la aplicación de técnicas independientes de cultivo para el estudio de la microbiología de los sistemas para la descontaminación de las aguas (superficiales y/o subterráneas) ha supuesto un importante avance en la comprensión de la ecología microbiana de estos sistemas.

7.1. Visualización de la estructura microbiana

El aumento de la resolución proporcionada por la microscopía electrónica ha permitido la visualización detallada por ejemplo, de la interacción entre los microorganismos, la formación de EPS, los microtúbulos entre las células que pueden conferir la conjugación, ó las moléculas superficiales aptas para la adhesión a matrices sólidas. La estructura y los cambios estructurales también pueden ser observados a través de microscopía electrónica de barrido (SEM), permitiendo el análisis de difracción de rayos X (EDX), la ubicación real de metales pesados en estructuras y/o organismos (González-Muñoz y colaboradores, 1997; Vílchez y colaboradores, 2007).

Uno de los inconvenientes de la utilización de métodos basados en la amplificación de ADN/ARN mediante PCR en la toma de "*fingerprinting*" es que casi no da ninguna información en cuanto a número de microorganismos y su distribución espacial. Este último aspecto ha sido resuelto mediante el uso de técnicas como la hibridación *in situ* fluorescente (*Fluorescence in situ Hibrydation*, FISH*)*. La distribución espacial de bacterias individuales puede ser observada en las biopelículas utilizando microscopios de fluorescencia. La microscopía láser confocal de barrido (*Confocal Scanning Laser Microscopy*, CSLM) permite obtener imágenes en tres dimensiones en comparación con las imágenes de dos dimensiones obtenidas con microscopios de fluorescencia convencionales. Esta primera nos da la posición exacta de los grupos bacterianos con respecto a otros grupos, y, por consiguiente, se puede determinar la relación entre los grupos clasificando éstas en tres categorías: mutualismo, antagonismo o la distribución aleatoria de las poblaciones en la biopelícula estudiada.

Diferentes grupos taxonómicos se pueden diferenciar mediante el uso de sondas fluorescentes, correspondientes a regiones conservadas de orden/género/especie utilizando el ARN ribosómico (ARNr) como indicador taxonómico.

Las plataformas en la red tales como ProBase (www.microbial-ecology.net/probase) contienen un gran número de sondas distintas de ARNr para distintos grupos taxonómicos (Loy y colaboradores, 2003), especificando las condiciones de hibridación *in situ* adecuadas, lo que lo convierte en una herramienta esencial para ecólogos microbianos.

7.2. Metodología para el estudio de la diversidad microbiana

7.2.1. Métodos de *fingerprinting*

La taxonomía microbiana clásica, basada en el cultivo de microorganismos aislados y sus pruebas morfológicas y bioquímicas, se ha sustituido o complementado con el análisis de macromoléculas (ADN, ARN y proteínas) de estos microorganismos a través del uso de herramientas de biología molecular. Las especies microbianas se definen mediante enfoques polifásicos que toman en cuenta propiedades genotípicas y fenotípicas, ya que el uso exclusivo de técnicas clásicas o moleculares por sí solas nos proporcionan una información sesgada (Martín-Platero y colaboradores, 2009). La comparación genómica es el principal criterio para distinguir las especies; sin embargo, las comparaciones del genoma completo para la definición de las especies puede parecer prematura, teniendo en cuenta que sólo aproximadamente 1500 genomas completos de bacterias y arqueas están disponibles (base de datos NCBI, 2010), y que la diversidad de los microorganismos no cultivables en el ambiente es alta. La secuenciación de los genes ribosómicos ha mostrado su utilidad para la identificación de microorganismos por métodos dependientes e independientes de cultivo. Así, la identificación de los microorganismos dentro de una biopelícula, puede ser evaluada por técnicas dependientes e independientes de cultivo.

7.2.2. Técnicas dependientes de cultivo

Las técnicas utilizadas para identificar los microorganismos cultivables y aislados, incluyen diversas técnicas específicas. Entre otros, mencionar los sistemas API, BIO-LOG$_{TM}$ o Phene Plate$_{TM}$ basados en las características fenotípicas y metabólicas de los microorganismos. Las pruebas basadas en fenotipos proteicos (análisis en gel desnaturalizante de acrilamida) y el perfil lipídico (*Fatty Acids Methyl Ester*, *FAME*) (Slabbinck y colaboradores, 2010) también se utilizan para clasificar las cepas microbianas. Los métodos mencionados son tediosos, costosos, y no siempre son reproducibles.

Los métodos de identificación basados en ADN o ARN son rápidos y fácilmente reproducibles. La hibridación ADN-ADN de genomas, es primordial para una precisa identificación de cepas; sin embargo este método también es tedioso y costoso. Los siguientes métodos están basados en la reacción en cadena de la polimerasa (PCR): RISA-PCR (*rRNA gene Internal Spacer Analysis*), la secuenciación del gen 16S ARNr, ARDRA-PCR (*Amplification Ribosomal DNA Restriction Analysis*), ITS *(Internal Transcribed Spacer*) y la amplificación de genes específicos de una especie. Estos métodos, en su mayoría, se centran en el estudio de los genes que codifican el ARN de los ribosomas bacterianos (5S, 16S y 23S). Las bases de datos en la red de acceso libre, han hecho que se utilice estas herramientas para la identificación de un gran número de aislados. Las biopelículas microbianas pueden ser muy diversas, o limitadas a sólo unos pocos microorganismos, por lo que el método seleccionado para determinar la composición microbiana se debe elegir dependiendo del nivel de confianza que la investigación requiera y los objetivos que se pretendan cubrir.

Los métodos denominados *"fingerprinting"* también se utilizan debido a su simplicidad y viabilidad en un corto período de tiempo. El objetivo de este tipo de técnicas es la creación de "huellas genéticas" de las especies e incluso interpretarlas como un código de barras, lo que permite la identificación a través de la tipificación microbiana (Vandamme y colaboradores, 1996). Dentro de estos métodos, existen algunos que no requieren conocimientos previos de las secuencias de ADN específicas (caso de la amplificación aleatoria de ADN polimórfico (RAPD-PCR *Randomly Amplified Polymorphic DNA*, ó la AP-PCR *Arbitrarily Primed PCR*). Sin embargo, si la secuencia objetivo es conocida, la definición y cebadores utilizados se definen y las condiciones de alineamiento son más restrictivas; por ejemplo, la REP-PCR (*Repetitive Extragenic Palindromic PCR*) que se basa en la técnica de RAPD-PCR, pero sólo requiere de un oligonucleótido.

Otras técnicas de códigos de barras requieren la amplificación de ADN (gen 16S ARNr principalmente o en la región intergénica entre los genes 16S y 23S ARNr) y la posterior digestión con enzimas de restricción, tales como *Amplification Ribosomal DNA Restriction Analysis*, ARDRA (Vaneechoutte y colaboradores, 1992). El método AFLP (*Amplified Fragment Length Polymorphism*) también podría ser utilizado; sin embargo todos los métodos de tipificación sólo pueden ser comparados con las cepas de referencia contenidas en cada laboratorio, ya que la magnitud de las combinaciones (cebadores y cepas) es indefinida, y no se conocen bases de datos en red lo bastante amplia para facilitar la identificación por *"fingerprinting*".

En última instancia, el uso exclusivo de técnicas dependientes de cultivo sobre la

diversidad microbiana en cualquier hábitat proporcionará una información muy sesgada, ya que la estimación de las bacterias cultivables está entorno al 1% del total de la población bacteriana, y sólo el 1-5% de las poblaciones bacterianas de aguas potables y subterráneas son cultivables en medios de cultivo (Byrd y colaboradores, 1991).

7.2.3. Técnicas independientes de cultivo

Una gran ventaja de la utilización de las técnicas independientes de cultivo es que la biodiversidad de diferentes ecosistemas se puede determinar en el espacio y en el tiempo, sin la necesidad de aislar los microorganismos. Las limitaciones que exhiben están generalmente asociadas con los aspectos técnicos, tales como la pureza del ADN/ARN o los errores de amplificación del ADN Stach y colaboradores, 2001; Forney y colaboradores, 2004), entre otros. Al igual que con las técnicas dependientes de cultivo, para el estudio de la diversidad en una biopelícula es necesario secuenciar ampliamente el gen ARNr 16S. Además de las numerosas ventajas sobre el uso de este gen como marcador taxonómico, hay también varios inconvenientes, por ejemplo, la escasa discriminación entre especies estrechamente relacionadas. Podría pensarse en el uso de otros genes funcionales que resolvieran esta realidad pero su uso como marcadores está limitado principalmente por la falta de regiones conservadas en primer lugar y la falta de secuencias disponibles. Como se describió anteriormente, las técnicas de "*fingerprinting*" generan los resultados como código de barras, aunque, en este caso, el perfil observado en el ADN/ARN de muestras ambientales se corresponde con múltiples microorganismos. La técnica de electroforesis en gel con gradiente de temperatura (TGGE, *Temperature Gel Gradient Electrophoresis*) separa fragmentos de tamaños idénticos o parecidos debido al contenido G+C. Esta técnica, utilizada junto con la amplificación y secuenciación parcial del gen ARNr 16S, genera bandas que representan las poblaciones dominantes dentro de un perfil de una comunidad bacteriana compleja. Esta técnica, junto con otras basadas igualmente en la desnaturalización en base al contenido en G+C como el DGGE (*Denaturing Gradient Gel Electrophoresis*), han sido ampliamente utilizadas en los últimos años para examinar la diversidad biológica de bacterias en las biopelículas desarrolladas en ecosistemas artificiales y reales, incluyendo plantas de tratamiento de fangos y los sistemas de tratamiento de aguas residuales urbanas y agrícolas (Wagner y colaboradores, 2002; Gómez-Villalba y colaboradores, 2006; Pozo y colaboradores, 2007; Vílchez y colaboradores, 2007). Para el estudio de una comunidad microbiana utilizando TGGE, generalmente se asume que cada banda corresponde a una sola especie y su densidad se corresponde con la abundancia relativa. Sin embargo, tenemos que

tener en cuenta que esta declaración no es del todo cierta, debido a que la técnica TGGE basada en la PCR, puede crear quimeras (Wang y Wang, 1996). Esta técnica no es cuantitativa, sino cualitativa en relación porcentual en aquellas poblaciones que representen al menos el 1% del total de la comunidad bacteriana (Muyzer y Smalla, 1998). El análisis de SSCP (*Single-Stranded Conformational Polymorphism*) también se basa en la amplificación del gen ARNr 16S, y su separación electroforética sobre la base de la diferencia en la movilidad de ADN de cadena sencilla (Schwieger y Tebbe, 1998).

Otras técnicas empleadas utilizan ADN marcado con fluoróforos a través de una electroforesis capilar, como la técnica del Polimorfismo Terminal de la Longitud del Fragmento de la Restricción (T-RFLP, *Terminal Restriction Fragment Length Polymorphism*) y la LH-PCR (*Length Hetorogeneity-PCR*). Las técnicas ARDRA-PCR y RISA-PCR también se pueden utilizar para determinar la población no cultivable (Vaneechoutte y colaboradores, 1992; Liu y colaboradores, 1997; Suzuki y colaboradores, 1998).

Aparte de las técnicas de "*fingerprinting*", otras técnicas como técnicas *in situ*, los "*microarrays*" y la citometría de flujo tienen una importante aplicación en el estudio de estos sistemas. En este sentido, técnicas como los *"microarrays"* (basados en la metodología de hibridación) se han aplicado para determinar la presencia y el número relativo de especies bacterianas. Una aplicación comercial de esta técnica lo constituye el microarray "PhyloChip®". La miniaturización y la automatización le confieren un atractivo especial a esta técnica, sin embargo las secuencias de ADNr 16S no presentes en el chip, pasarían por alto.

7.2.4. La diversidad funcional dentro de las comunidades microbianas

La función llevada a cabo por los microorganismos dentro de una biopelícula está determinada por las condiciones físico-químicas y la composición genética de los microorganismos que la componen. Al objeto de describir la actividad microbiana en un determinado sistema, se suelen realizar búsquedas específicas de genes.

En este sentido y como ejemplo, las funciones específicas como la oxidación de amonio y las actividades oxidantes y desnitrificantes en sistemas de biopelícula son determinadas genéticamente mediante la amplificación de los genes *amoA* y *nosZ*, respectivamente (Gómez-Villalba y colaboradores, 2006; Nakano y colaboradores, 2008;).

Sin embargo, una de las principales desventajas del uso de cebadores degenerados para la amplificación de genes metabólicos es la sobreestimación de la diversidad genética a través de los perfiles de bandas como consecuencia de la aparición de secuencias redundantes.

Un enfoque proteómico también se puede utilizar para observar los cambios en el

perfil de genes funcionales en el tiempo, ya que ofrece una vista única de la dinámica en la función celular. Inicialmente, la técnica SDS-PAGE (electroforesis en gel de poliacrilamida, 1D) se utilizó para generar las huellas digitales de proteínas de diversas comunidades, sin ningún intento de identificar las proteínas (Macrae y Smit, 1991; Francisco y colaboradores, 2002.). Más tarde, Kan y colaboradores (2005) utilizaron la técnica de electroforesis en dos dimensiones (2D) para separar las proteínas de una comunidad microbiana.

7.2.5. Los problemas actuales y las tendencias futuras en la evaluación de la población microbiana

Las especies microbianas están definidas por un enfoque polifásico basado en propiedades genotípicas y fenotípicas. El uso exclusivo de técnicas clásicas o moleculares sólo puede conllevar a un resultado sesgado; sin embargo optar por usar los dos enfoques es laborioso y requiere mucho tiempo.

El uso exclusivo de técnicas dependientes de cultivo sólo informa de un pequeño porcentaje de las especies microbianas presentes en las estructuras de biopelículas complejas, e incluso las cepas aisladas pueden ni siquiera llegar al 1% de las especies dominantes dentro de esa comunidad. Las técnicas independiente de cultivo usadas para la identificación de la comunidad microbiana en las biopelículas establecidas y en su evolución, tienen diferentes grados de errores y en diferentes etapas: producidos en a) la extracción de ADN/ARN, b) la amplificación por PCR y c) el grado de separación. El número elevado de bacterias Gram negativas detectado por métodos independientes de cultivo en muestras de aguas residuales puede deberse tanto a una selección natural en las biopelículas de aguas residuales como a la preferente detección de este grupo debido a su menor dificultad en la extracción de ADN. Para ello, una de las principales soluciones es la ruptura mecánica de las muestras. Durante la amplificación por PCR muchos errores pueden ocurrir, por ejemplo el diseño de cebadores universales se basa sólo en las secuencias conocidas actualmente, resultando en la amplificación de géneros ya conocidos o por conocer y géneros muy parecidos a los ya descritos. Las condiciones de alineamiento en la PCR no siempre son las condiciones óptimas para todos los microorganismos presentes, por lo que un protocolo *"touchdown"* de PCR puede funcionar mejor. La cuantificación por TGGE estará sesgada debido a la eficiencia del alineamiento del cebador: si el alineamiento es bueno, se observará una mayor amplificación e implicará una mayor representación en la biopelícula. La amplificación de las regiones sin sentido (*nonsense regions*) y artefactos quiméricos son producidos con frecuencia en la amplificación del gen ARNr. Los perfiles y agrupamientos determinados por *"fingerprinting"*

(por ejemplo, TGGE, DGGE) pueden dar información estadística errónea debido a la cantidad de bandas de artefactos o regiones sin sentido producidos (Cowell y colaboradores, 2010).

La recomendación de comparar genomas completos para la definición de las especies puede parecer prematura, teniendo en cuenta que solo ~ 1500 genomas de bacterias y *archaea* están disponibles actualmente y que la diversidad de los microorganismos sin cultivar en el medio ambiente es alta. A pesar de esto último, más y más genomas de bacterias están siendo secuenciados, y el rápido desarrollo de las tecnologías de secuenciación (*next generation sequencing*) pronto aumentará el número de secuencias genómicas disponibles en varios órdenes de magnitud (Bentley, 2006). En este sentido, los análisis de secuencias *multilocus* (MLSA), basados en el análisis filogenético de múltiples genes codificadores de genes constitutivos (*house-keeping genes*), se han sugerido para limitar los criterios que definen las especies microbianas (Hanage y colaboradores, 2006).

Las tendencias futuras para definir los grupos funcionales microbianos en las biopelículas podrían basarse en la utilización de la proteómica, tal y como Ram y colaboradores, (2005) reflejaron en el estudio de una comunidad microbiana procedente de una biopelícula de drenaje ácido de minas naturales. Aproximadamente 2000 proteínas se identificaron mediante el uso de una base de datos creada a partir de la secuenciación de una comunidad microbiana de muestreo de la misma mina, pero en un lugar y tiempo diferentes.

La funcionalidad de una biopelícula es aplicable sólo a las condiciones que la rodean y la adaptación a los recursos disponibles es la clave del éxito de la biotecnología microbiana. La manipulación de cepas microbianas a través del uso de la ingeniería genética para fines humanos, tales como tratamientos de aguas residuales, sólo puede ser eficaz cuando la mayoría de los parámetros que afectan a la población microbiana en las biopelículas son conocidos, incluyendo la acumulación de la expresión génica global al objeto de conseguir un funcionamiento correcto de las biotecnologías empleadas en aguas contaminadas.

CAPÍTULO II. OBJETIVOS

La contaminación de las aguas subterráneas es una realidad cada vez más frecuente en los países desarrollados, y casi exclusivamente por acción antropogénica. En los países industrializados, las contaminaciones originadas por productos derivados del petróleo, incluidas las gasolinas, constituyen una amenaza real y creciente para la calidad de este recurso. Los éteres MTBE, ETBE y TAME, químicos que reemplazaron al plomo en la formulación de las gasolinas, son emergentes contaminantes de las masas de agua subterránea, por lo que la sociedad ha demandado y demanda nuevas tecnologías para la eliminación de estos compuestos xenobióticos del agua, permitiendo de nuevo su uso y el restablecimiento del equilibrio de los ecosistemas.

En este contexto, y como objetivo principal de esta investigación, se ha realizado un estudio biológico de un biofiltro aireado con biopelícula fija diseñado a escala de laboratorio para eliminar ó biotransformar compuestos oxigenantes de las gasolinas (MTBE, ETBE y TAME) presentes en aguas subterráneas.

Para alcanzar este objetivo general, se han desarrollado las siguientes actividades:

1. Selección y aislamiento de cepas bacterianas con capacidad para utilizar MTBE, ETBE y TAME como única fuente de carbono y energía

2. Evaluación y modificación de metodologías "*Headspace*" (espacio de cabeza) para la detección de éteres de la gasolina y extracción de ADN genómico total de muestras de agua subterránea.

3. Estudio del proceso de formación de biopelícula y de la actividad de las cepas bacterianas con probada capacidad para biotransformar oxigenantes de las gasolinas

Estudio químico y biológico de la eficiencia de la biopelícula establecida en los biofiltros aireados para la eliminación de MTBE, ETBE y TAME.

Estos objetivos han sido desarrollados en el ámbito del proyecto de investigación titulado: "Tratamiento de aguas subterráneas contaminadas con oxigenantes de gasolinas (MTBE, ETBE y TAME) mediante reactores de lechos sumergidos". Programa Nacional de I+D, MEC (CTM2006-02297/TECNO).

CAPÍTULO III. RESULTADOS

SECTION III.1

SELECTION AND IDENTIFICATION OF BACTERIAL STRAINS WITH methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE) AND *t*-amyl methyl ether (TAME) DEGRADING CAPACITIES.

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Published at ***Environmental Toxicology and Chemistry*** **(2008). 27 (11) 2296-2303.**

ABSTRACT

Nine bacterial strains isolated from two hydrocarbons contaminated soils were selected due to their capacity of growing in culture media amended with 200 mg/L of one of the following gasoline oxygenates: methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE) and *t*-amyl methyl ether (TAME). These strains were identified by amplification of their 16S-rRNA gene, using fD1 and rD1 primers, and tested for their capacity to grow and biotransform these oxygenates both in mineral and cometabolic media. The isolates were classified as *Bacillus simplex*, *Bacillus drentensis*, *Arthrobacter* sp., *Acinetobacter calcoaceticus*, *Acinetobacter* sp., *Gordonia amicalis* (two strains), *Nocardioides* sp. and *Rhodococcus ruber*. *Arthrobacter sp.* (strain MG) and *Acinetobacter calcoaceticus* (strain M10) consumed 100 mg/L and 82 mg/L of oxygenate TAME in 21 days grown in cometabolic and mineral media respectively under aerobic conditions. *Rhodococcus ruber* (strain E10) was observed to use MTBE and ETBE as sole carbon and energy source, while *Gordonia amicalis* (strain T3) used TAME as sole carbon and energy source for growth. The results showed that all the bacterial strains transformed oxygenates better in the presence of an alternative carbon source (ethanol) with the exception of *Acinetobacter calcoaceticus*, strain M10. Finally, the capacity of the selected strains to remove MTBE, ETBE and TAME look promising for its application in bioremediation technologies.

Keywords: Soil microorganisms, Methyl *tert*-butyl ether, Ethyl *tert*-butyl ether, *Tert*-amyl methyl ether, Gasoline oxygenate- degrading microorganisms

INTRODUCTION

Around the early 1970s, the Environmental Protection Agency (EPA) of the United States recommended the phasedown of tetraethyl lead in the formulation of gasoline due to the alarming lead concentrations found in air and blood. In 1975, car manufacturers opted for lead free catalytic converters, which favored the move from leaded petrol to unleaded petrol. Amongst the tetraethyl lead substitutives proposed to enhance combustion and reduce exhaust emissions, were three oxygen-containing substances (oxygenates) named methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME) (http://www.epa.gov/history/topics/lead/03.htm).

In accidental gasoline spills, large volumes of these ethers leach through the soil and reach the groundwater table due to their low organic carbon adsorption coefficient and high solubility in water. The log Koc values varies between 1.09 for MTBE and 2.2 for ETBE y TAME [1] while 0.54 mol MTBE/L, 0.12 mol ETBE/L and 0.12 mol TAME/L are the values for solubility in water of these oxygenates [2].

The growing detection of these oxygenates in drinking water firstly alarmed the state of California [3], which echoed across the country eventually reaching Europe, where 75 % of drinking and irrigation water is derived from groundwater, and the other 25 % comes from surface or desalinated water (http://www.grid.unep.ch/product/publication/freshwater_europe/ecosys.php). In the 1997, the EPA established maximum levels of MTBE in drinking water to 20 µg/L, a concentration below taste and odor threshold.

The ecological and human health effects have only recently been studied and information on toxicity to freshwater organisms is even scarcer. In human experiments, 5 mg MTBE/m^3 were administered during 1 h leading to the following symptoms: acute headaches; irritations and inflammations in the optical and respiratory systems; and nausea [4]. The chronic effects of MTBE, only studied in animals were inconclusive. Methyl *tert*-butyl ether has not been determined as a possible health hazard by the National Toxicology Program, and some investigational groups criticise this severely [5], as a consequence of reported DNA damage in rat lymphocytes following MTBE exposure [6] and increased DNA damage to the human HL-60 cell line exposed for 1 h to 88 to 2645 mg MTBE/L [7]. Studies in rats and mice showed increased cancer incidence when given 250 and 1000 mg MTBE/kg body weight, 4 d per week during the 104 week trial [8].

The vast amount of information found on oxygenate biodegradation involves MTBE

since it was the first oxygenate to be used and detected in drinking water.

Although initial investigations showed MTBE to be resistant to biodegradation, increasing studies have demonstrated that MTBE is biodegradable under various aerobic controlled environments, but the biodegradation rate of this xenobiotic is low mainly due to its branched structure.

Few reports of microorganisms in either pure or mixed cultures capable of biodegrading gasoline oxygenates have been described. The first report concerning MTBE bacterial degradation was described by Salanitro et al. [9] using a mixed bacterial culture from an activated sludge unit treating industrial chemicals. In 1997, Mo et al. [10] isolated three pure cultures, *Methylobacterium mesophilicum* (ATCC 700107), *Rhodococcus* sp. (ATCC 700108) and *Arthrobacter ilicis* (ATCC 700109) those degraded up to 29 % of an initial concentration of 200 mg MTBE/L in two weeks; however, complete degradation of the oxygenate was not observed. MTBE degradation rates decreased significantly in the presence of other carbon sources including TBA (*t*-butyl alcohol, the first intermediate in the MTBE degradation), *t*-butyl formate (TBF), iso-propanol, acetone and pyruvate. Slow growth rates were attributed to either poor growth substrate or inhibition by the presence of a metabolite.

Hatzinger et al. [11] investigated MTBE and TBA degradation using *Hydrogenophaga flava* where TBA (*t*-butyl alcohol) was found to accumulate even though the strain could degrade TBA. MTBE and TBA degrading genes were found to belong to separate loci. *Burkholderia cepacia* [12] was found to metabolise TBA but not MTBE, whereas *Mycobacterium austroafricanum* [13] was found to grow on MTBE and TBA, where TBA was accumulated and was only degradable at low MTBE concentrations.

In the present study, we described the isolation and identification of several bacterial strains capable of using MTBE, ETBE or TAME as carbon and energy source. The growth characteristics of these bacterial strains as well as the consumption of these oxygenates in mineral and cometabolic media and their identification by analysis of the sequence of the gene encoding 16S-rRNA gene are reported.

MATERIALS AND METHODS

Soil samples

Soil surface samples (0-15 cm) taken from two contaminated hydrocarbon sites at Puertollano (Ciudad Real, Spain) were used in the present study. The texture of soil sample named as I was: silt (23 %), sand (50 %) and clay (27 %). The texture of soil sample named as

II was: silt (15 %), sand (50 %) and clay (35 %). The soil samples were collected in plastic bags, air dried at room temperature, homogenized and sieved (2 mm mesh).

Chemicals

Unless indicated otherwise, all chemicals were of reagent grade or the highest purity available and were purchased from commercial sources. MTBE (99.9 % purity) was purchased from Fluka (Milwaukee, WI, USA), ETBE (99 % purity) and TAME (97 % purity) were purchased from Sigma-Aldrich Chemical (Milwaukee, WI, USA).

Growth medium

The growth media used in the experiments was a mineral salts medium [14] with the following (g/L) composition: KH_2PO_4, 0.225; K_2HPO_4, 0.225; $(NH_4)_2SO_4$, 0.225; $MgSO_4.7H_2O$, 0.050; $CaCO_3$, 0.005; $FeCl_2.4H_2O$, 0.005; and 1ml trace elements solution. Trace elements solution comprised of the following (g/L): $ZnSO_4.7H_2O$, 0.1; $MnCl_2.4H_2O$, 0.03; H_3BO_3, 0.3; $CoCl_2.6H_2O$, 0.2; $CuCl_2.2H_2O$, 0.01; $NiCl_2.6H_2O$, 0.02; and $NaMoO_4.2H_2O$, 0.03.

Mineral salts-plus medium was comprised of mineral salts medium with the addition of 0.2 g/L yeast extract and 0.3 g/L of ethanol.

Isolation of oxygenate-degrading microorganisms

The enrichment phase of microorganisms with oxygenate-degrading capacities was performed using the method described by Okeke and Frankenberger [15] which involved the incubation of eight grams of each soil in Erlenmeyer flasks (500 ml total volume) with 125 ml of mineral salts medium amended with 200 mg/L of each oxygenate (MTBE, ETBE, or TAME). These trials were done in triplicate. The Erlenmeyer flasks were incubated in the dark during 4 weeks at 30 °C in an orbital shaker (rotation speed of 150 rpm). Subsequently, 1 ml of the culture was transferred onto a fresh sterile mineral salts medium with 200 mg/L of the corresponding oxygenate and was incubated for a further two weeks.

Following completion of the enrichment phase, 1 ml aliquots of each culture were harvested (10,000 rpm, 10 min), and resuspended in 100 µl of sterile mineral salts medium. Aliquots of this suspension were plated onto mineral salts agar medium amended with MTBE, ETBE or TAME, and further selection was performed by plating on the same medium containing washed agar to eliminate carbon impurities. Washed agar was prepared as follows: 10 g of bacteriological agar were resuspended in 500 ml mineral medium and allowed to stand during 15 min. The supernatant was carefully decanted and this procedure repeated twice,

followed by the addition of 400 ml mineral medium. Nine strains grew in this phase between 3 to 4 days, and were used for the following experiments.

Microbial growth in presence of MTBE, ETBE or TAME

To test for the utilization of the oxygenates MTBE, ETBE or TAME by the isolated strains as sole carbon and energy source for growth, 1 ml of a preinoculate grown in mineral salts-plus medium, centrifuged and washed with sterile saline solution (0.9 % w/v NaCl), was transferred to 125 ml glass vials with 25 ml fresh mineral salts medium amended with 100 mg/L of MTBE, ETBE or TAME, and incubated for 21 d at 30 °C under aerobic conditions and controlled agitation (rotation speed, 100 rpm). The glass vials were sealed with Teflon septa. Platable cell counts were estimated by the dilution plate technique using tripticase soy agar (TSA, Difco®, Detroit, MS, USA) medium. All the data are average of three independent assays and a control glass vial (without oxygenate) was included for each strain tested. Plates were incubated between 1 to 4 d at 30 ºC and growth was expressed as Log CFU/ml. One-way analysis of variance (ANOVA) using the software package STATGRAPHICS 3.0 Plus version (STSC Inc., Rockville, MD, USA) was performed to identify significant differences between the treatments. A significance level of 95% *(p<0.05)* was selected.

Genetic identification of gasoline oxygenate-degrading strains

Selected bacterial isolates were identified by sequence analysis of the gene encoding 16S rRNA (16S rDNA). Primers fD1(5´-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3´) and rD1 (5´-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3´) [16] and synthesized by Sigma Genosys (Dorset, UK), were used to amplify the 16S-rRNA gene partially and sequenced as previously described by Pozo et al. [17].

DNA sequences were processed, assembled and edited using software programs Chromas lite v. 2.0. and GeneDoc. Closely matching sequences were found in the GenBank Database using the BLASTn algorithm [18]. A phylogenetic tree was constructed using Clustal X v.1.81 [19] for sequence alignment and MEGA3 v.3.0 for the construction of a phylogenetic tree by the neighbour-joining method [20, 21], with the Kimura [22] correction. The stability of relationships was assessed by means of a bootstrap analysis of 1000 datasets.

The partial 16S rRNA sequences of the isolates identified in the present study (*Bacillus drentensis* strain MD, *Arthrobacter* sp. strain MG, *Acinetobacter* sp. strain M9, *Acinetobacter calcoaceticus* strain M10, *Gordonia amicalis* strain EA, *Nocardioides* sp. strain

E7, *Rhodococcus rubber* strain E10, *Bacillus simplex* strain T2 and *Gordonia amicalis* strain T3) have been deposited in the GenBank nucleotide sequence database under accession numbers EU427313, EU427314, EU427315, EU427316, EU427317, EU427318, EU427319 EU427320 and EU427321, respectively.

Rep-PCR of Gordonia amicalis strains

Repetitive extragenic palindromic-PCR (polymerase chain reaction) was performed to assess the diversity of the two isolates identified as *Gordonia amicalis* (EA and T3 strains). The method included a DNA preparation, PCR amplification, agarose gel electrophoresis and computer assisted data processing as described elsewhere [23]. The primer used to obtain the rep-PCR profiles was 5´-GTGGTGGTGGTGGTG-3´.

Biodegradation assays

Inoculated and control (lacking bacteria) vials with mineral salts or cometabolic media (mineral salts medium containing one fifth of mineral-plus medium) amended with 100 mg/L of selected oxygenate were used in the biodegradation assays. The bacterial inoculums were grown in mineral salts-plus medium at 30 ºC, harvested by centrifugation (6000 rpm, 15 min), washed and resuspended in 5 ml of sterile mineral salts medium. The bacterial suspension was placed in 125 ml vials which contained 25 ml of mineral salts medium and sealed with Teflon septa. The vials were incubated at 30 °C under aerobic conditions (100 rpm) for 21 days. For biodegradation assays in cometabolic media, 5 ml of the total mineral salts medium was replaced by 5 ml of mineral salts-plus medium.

Oxygenate analysis

MTBE, ETBE and TAME utilization by selected strains were assayed by gas chromatography mass spectrometry (GC/MS) technique. After 21 d incubation, the inoculated and control samples were filtered (0.45 μm) and diluted 50 fold with ultrapure water, followed by the injection of 5 ml of each diluted sample into the purge and trap equipment (Tekmar Dohrmann 3100). The trapped compounds were auto-injected into a Hewlett Packard model 6890 gas chromatograph coupled to a mass spectrometer (Hewlett Packard 5973 Mass Selective Detector). The analysis was performed on an Agilent Technologies capillary column (DB-624, 60 m x 0.25 mm x 1.4 μm). The temperature program was: 40 ºC (3 min), 10 ºC/min to 235 ºC and held for 5 minutes. Helium was used as the carrier gas at a flow rate of 1 ml/min. Control losses by volatilization or by abiotic reaction of each oxygenate, were subtracted from all corresponding samples.

RESULTS

Table 1. Number and type of bacterial colonies from enrichment phase isolated in mineral agar medium and mineral medium with washed agar.

	Isolated colonies from enrichment phase in mineral agar medium		Colony types in mineral agar medium		Isolated colonies in mineral medium with washed agar[a]	
Oxygenate	SI[b]	SII[c]	SI	SII	SI	SII
MTBE	124	175	12	11	1	3
ETBE	21	42	11	12	1	2
TAME	16	2	3	1	1	1

[a] The bacterial isolates were isolated from one representative colony of each type obtained from enrichment media.
[b]SI – Soil sample 1. [c]SII – Soil sample 2
MTBE: Methyl *t*-butyl ether, ETBE: ethyl *t*-butyl ether, TAME: *t*-amyl methyl ether

Isolate selection from hydrocarbon-contaminated soils.

Table 1. shows the plate counts from enrichment cultures in mineral salts agar plates and mineral salts washed agar plates supplemented with MTBE, ETBE or TAME, as well as the number of morphological distinct colonies from the enrichment phase obtained for soil samples I and II. The selected isolates were plated on solid mineral medium supplemented with the corresponding oxygenate, with previously washed agar to remove any carbon traces in the agar. The criterion used to select the isolates was the different colony morphologies within the oxygenate group, taking into account size, color, form, elevation and margin.

Our results indicated that the number of colonies observed in solid media supplemented with MTBE were much higher than that observed in media supplemented with ETBE or TAME. This behaviour was observed for both soils. However, only four colonies were isolated when the plate counts were performed in solid media with washed agar and MTBE as sole carbon source. These isolates were named as MD, MG, M9 and M10. In mineral salts media with washed agar amended with ETBE, only three isolates

grew, named EA, E7 and E10. Finally, in media with washed agar amended with TAME, only two isolates grew: named as T2 and T3.

The nine selected isolates (MD, MG, M9, M10, EA, E7, E10, T2, and T3) were used for further studies.

Genetic identification of isolated strains

The results comparing the 16S-rRNA gene sequences of the isolated strains and sequences of GeneBank nucleotide sequence database are shown in **Figure 1**

The three main clusters observed are grouped by the genus *Bacillus*, genus *Acinetobacter* and Order *Actinomycetales.* This last cluster contains five of the nine isolated strains, which are contained in three sub-clusters. In the MTBE enrichment cultures, both Gram positive (belonging to genus *Bacillus* and *Arthrobacter*) and negative bacteria (belonging to genus *Acinetobacter*) were observed, nevertheless, in ETBE and TAME enrichment cultures, only Gram positive bacteria were isolated (i.e. *Bacillus, Gordonia*, *Nocardioides* and *Rhodococcus*). The rep-PCR patterns obtained for strains EA (isolated from ETBE enrichment culture) and T3 (isolated from TAME enrichment culture) which have the same 16S DNA sequence, were 96 % similar in the dendrogram **(Fig. 2)** obtained after using the Pearson correlation coefficient. Most bands appeared at the same level but some of them showed different bright intensities, inferring that these isolates identified as *Gordonia amicalis* were not identical.

Bacterial growth and oxygenates biotransformation studies

Figures 3, 4, 5 and 6 show the growth curves of strains M10, E7, E10 and T3 in mineral salts medium amended individually with 100 mg/L of MTBE, ETBE or TAME as sole carbon source. The growth curves of MG and EA strains in mineral media amended with the oxygenates have not been included because no significant differences *($p<0.05$)* versus control (growth media without oxygenates and inoculated with the corresponding strain), were observed during the incubation time. In addition, preliminary experiments showed that strains MD, M9 and T2 did not grow or cometabolise the oxygenate in liquid medium (data not shown), so these strains were not included further.

The utilization of MTBE, ETBE and TAME (as mg/L oxygenate consumed) by isolated strains MG, M10, EA, E7, E10 and T3 grown both in mineral and cometabolic media amended with 100 mg/L of each oxygenate and incubated under controlled temperature (30 ºC) and agitation (100 rpm) for 21 days is shown in **Figure 7.**

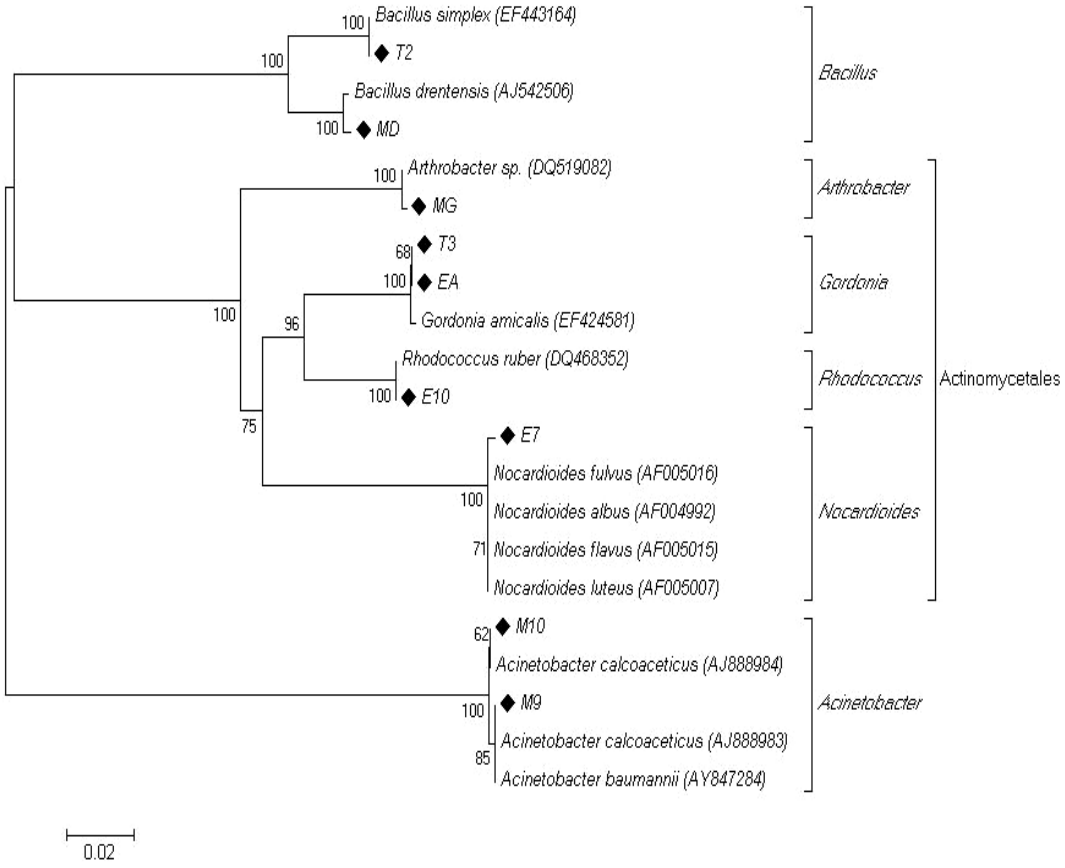

Figure 1. Phylogenetic Neighbour-Joining tree of the 16S-rRNA gene sequences from the bacterial isolates. Sequences retrieved from the European Molecular Biology Laboratory database are indicated with their corresponding accession numbers. Bootstrap values below 50 are not shown. ♦ - Bacterial strains isolated in the present study

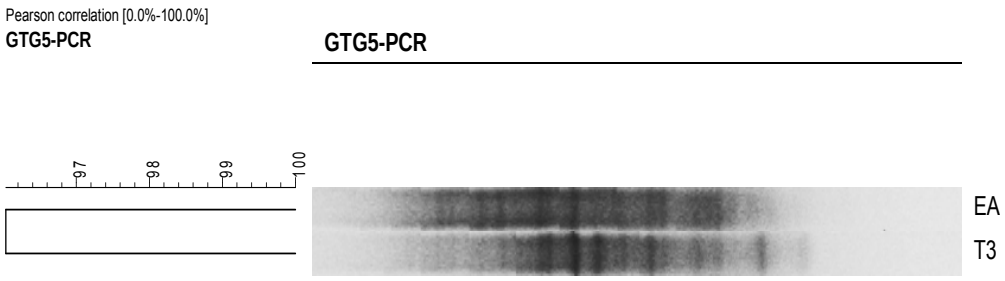

Figure 2. Cluster analysis of digitised banding patterns, generated by rep-PCR using the $(GTG)^5$ primer of *Gordonia amicalis* strains (EA and T3). The dendrogram was constructed using the unweighted pairgroup method using arithmetic averages with correlation levels expressed as percentage values of the Pearson correlation coefficient.

Although no significant differences *(p*<0.05*)* versus control were observed when *Arthrobacter* sp. (strain MG) was grown in mineral medium amended with MTBE, ETBE or TAME, the strain was capable of consuming the three oxygenates in cometabolic medium (Fig. 7), e.g. TAME was totally consumed after 21 d of incubation. *Acinetobacter calcoaceticus* (strain M10) was able to grow in mineral salts medium amended with ETBE and TAME as sole carbon and energy source (Fig. 3). However, the growth of the strain decreased after 7 d of incubation. The highest consumption detected by the bacterial strain in mineral medium was in TAME (82 mg/L in 21 d of incubation).

No growth was observed in mineral media inoculated with *Gordonia amicalis* strain EA in the presence of oxygenates and the MTBE, ETBE and TAME consumptions were recorded only in cometabolic media **(Fig. 7).**

Growth was observed for strain E7 **(Fig. 4)** when was grown in mineral medium amended with ETBE after 14 d of incubation and this growth was accomplished by oxygenate consumption in mineral media. Strain E7 was also observed to utilise 31 mg/L MTBE in 21 d but no growth was observed. Nevertheless this bacterial strain used all three oxygenates when cultured in cometabolic medium **(Fig. 7).**

Growth of strain E10 in mineral media amended with MTBE and ETBE was significantly higher *(p*<0.05*)* than the control for the first few days **(Fig. 5).** Additionally, a decrease of the three gasoline oxygenates was observed in mineral media; MTBE biotransformation was the most effective **(Fig. 7).**

Although no significant growth *(p*<0.05*)* was observed for strain T3 in mineral salts medium amended with MTBE when compared versus control **(Fig. 6)** the strain was capable of using 53 mg/L of oxygenate in 21 d of incubation **(Fig. 7).** No growth or consumption was observed when the strain was cultured in medium amended with ETBE, however, the growth of strain T3 was 1.0 logarithmic units higher than the control in medium supplemented with TAME, accompanied by a consumption of 14.8 mg/L of this oxygenate after 21 d of incubation **(Fig. 6).**

DISCUSSION

The increasing importance given to bioremediation of gasoline oxygenates such as MTBE, ETBE and TAME, is owed to the growing detection of these compounds in ground

and surface water. The first step in the design of new bio-treatment systems for the palliation of contaminated water is the selection of bacterial strains with the capacity to degrade or remove these oxygenates and their subsequent metabolites, individually or in consortia.

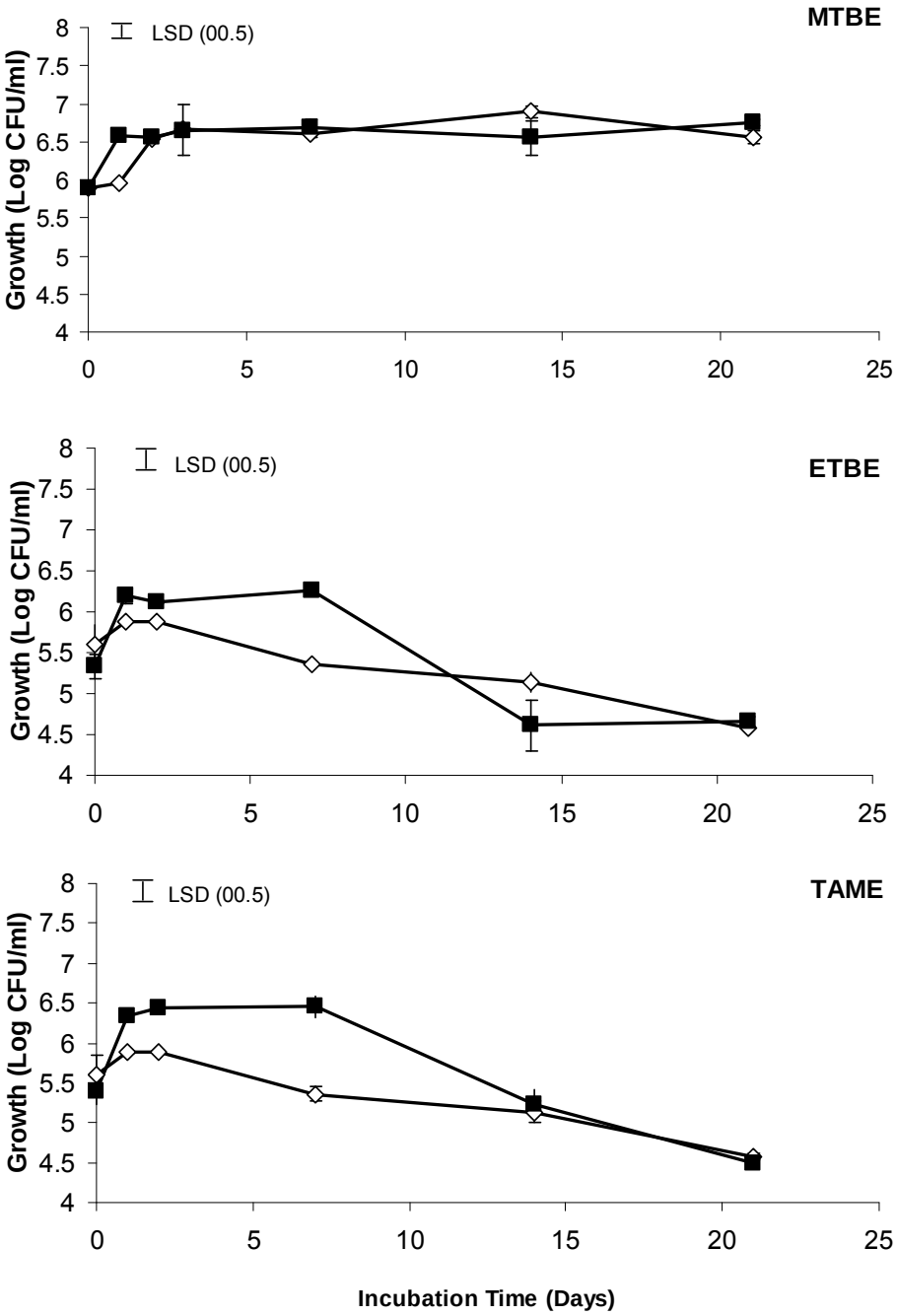

Figure 3. Growth as Log colony-forming units (CFU)/ml of strain M10 in the presence of methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE) and *t*-amyl methyl ether (TAME). ■ - 100 mg/L, ◇ - Control without gasoline oxygenate. Values are an average of three experiments *(p<0.05)*

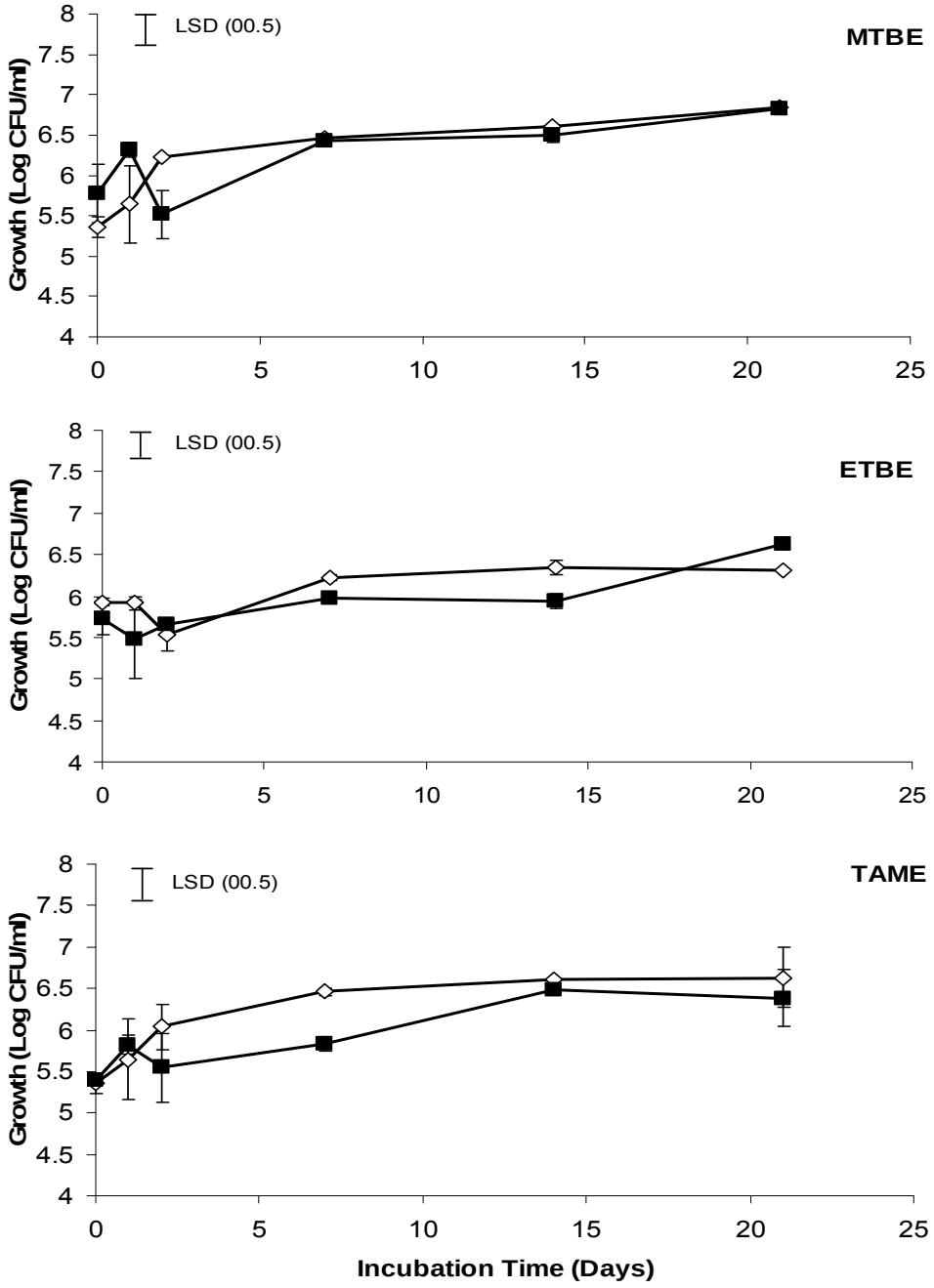

Figure 4. Growth as Log colony-forming units (CFU)/ml of strain E7 in the presence of methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE) and *t*-amyl methyl ether (TAME). ■ - 100 mg/L, ◇ - Control without gasoline oxygenate. Values are an average of three experiments *(p<0.05)*

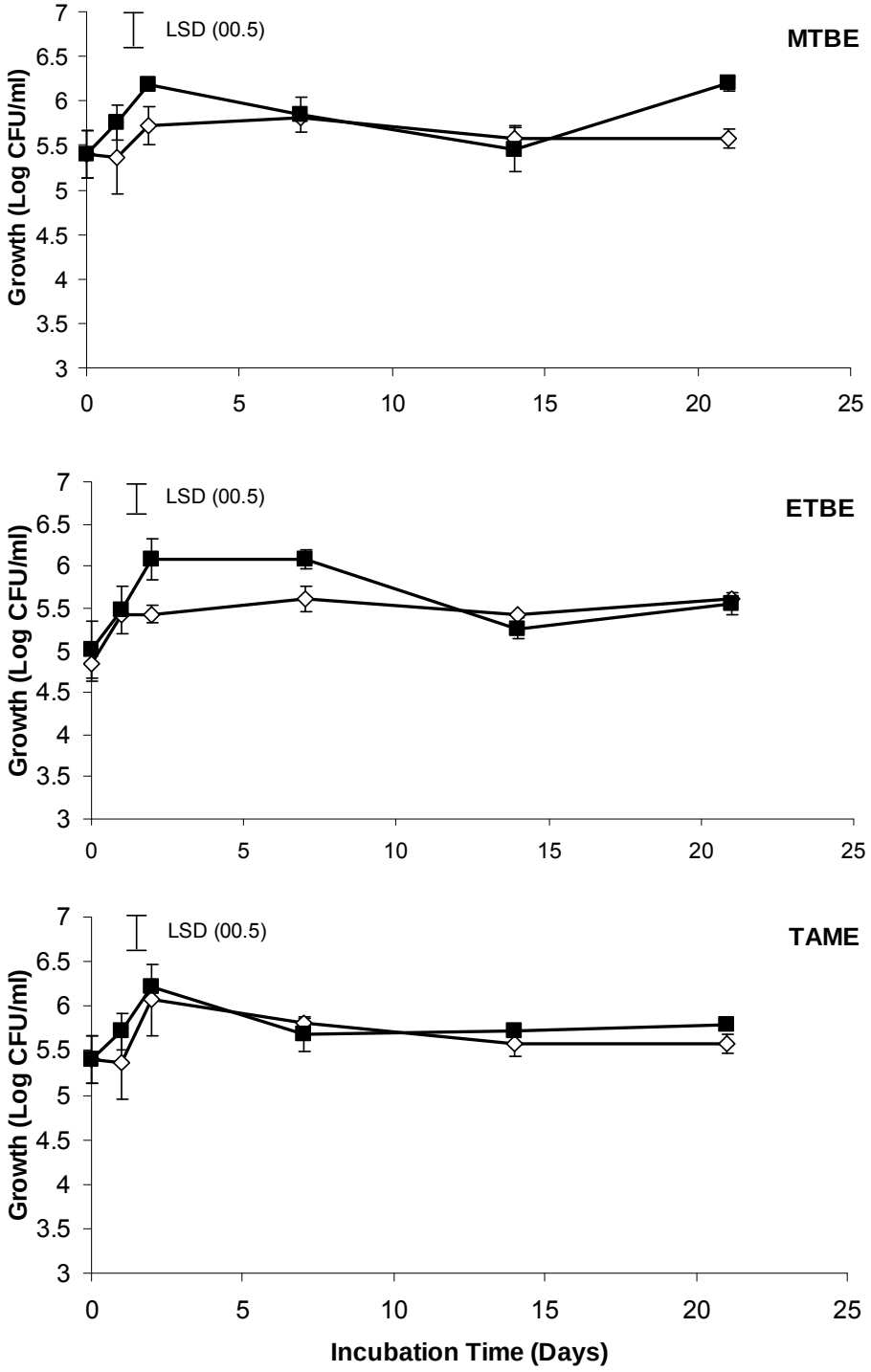

Figure 5. Growth as Log colony-forming units (CFU)/ml of strain E10 in the presence of: methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE) and *t*-amyl methyl ether (TAME). ■ - 100 mg/L, ◇ - Control without gasoline oxygenate. Values are an average of three experiments *(p<0.05)*

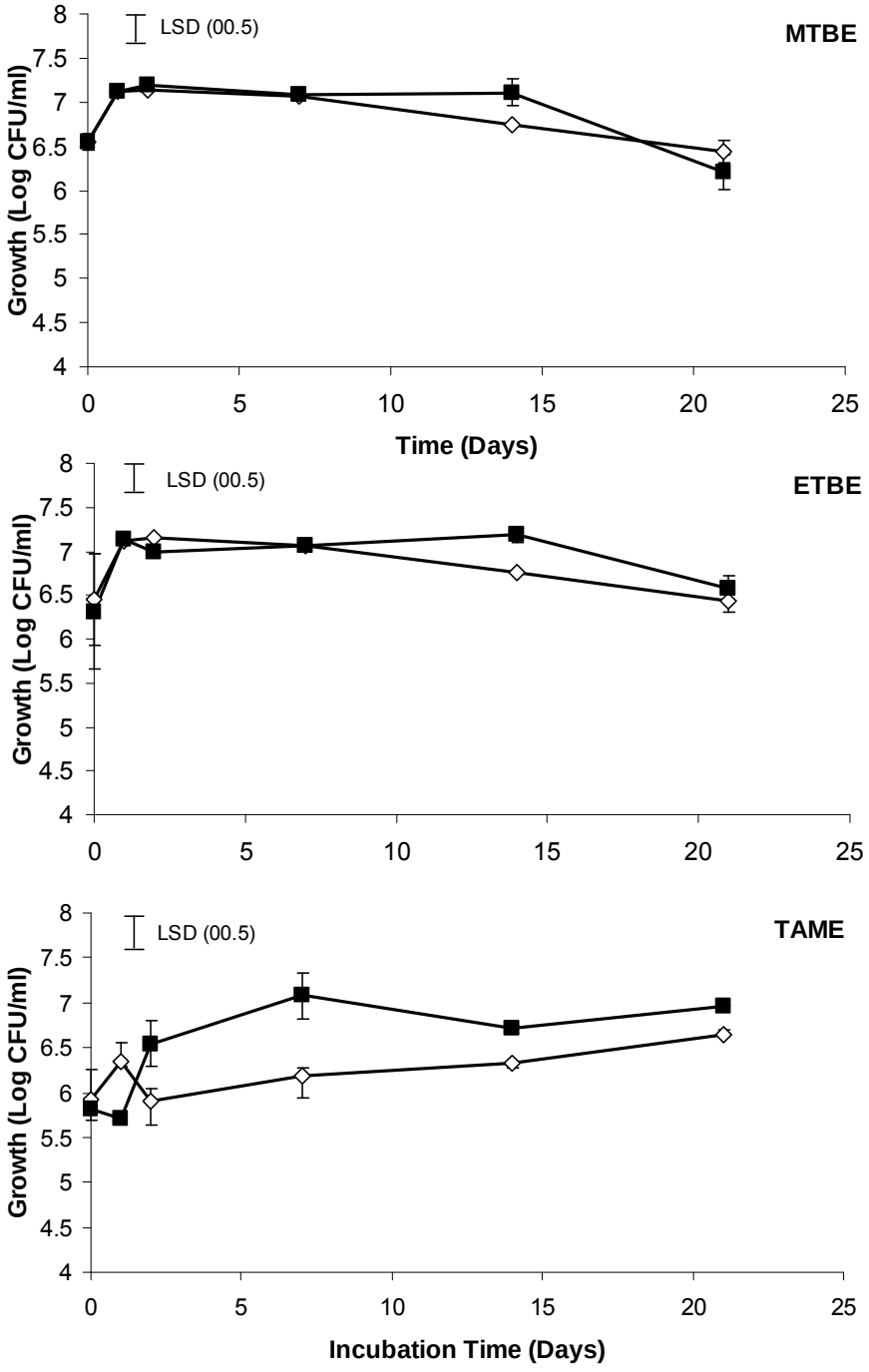

Figure 6. Growth as Log colony-forming units (CFU)/ml of strain T3 in the presence of methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE) and *t*-amyl methyl ether (TAME). ■ - 100 mg/L, ◇ - Control without gasoline oxygenate. Values are an average of three experiments *(p*<0.05*)*

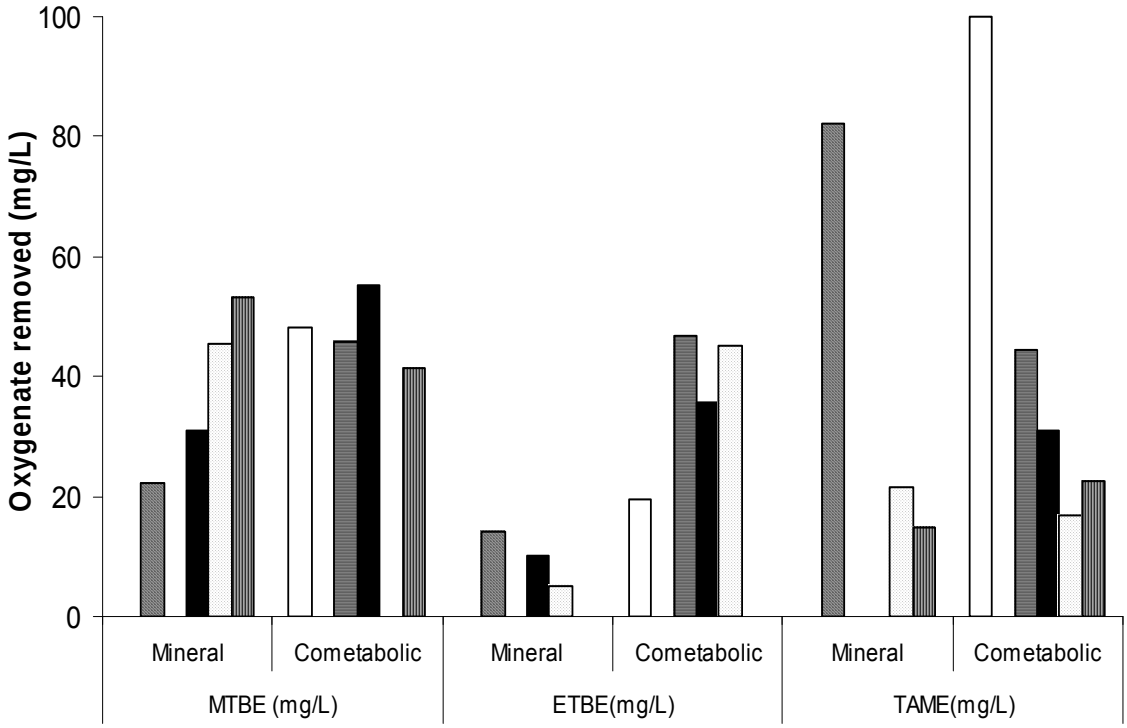

Figure 7. Methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE) and *t*-amyl methyl ether (TAME) removal (as mg/L) by the bacterial strains (MG, M10, EA, E7, E10, and T3) in mineral and cometabolic media amended with the gasoline oxygenates after 21 d of incubation at controlled conditions (30 ºC, 100 rpm). □ Strain MG; ▨ Strain M10; ▤ Strain EA: ■ Strain E7; ⁝⁝ Strain E10; ▥ Strain T3.

The present study started out with the selection of nine bacterial strains grown in solid mineral media amended with MTBE, ETBE or TAME as sole carbon and energy source. *Arthrobacter sp.* (MG strain) was only capable of using MTBE, ETBE and TAME in cometabolic media (supplemented with yeast extract and ethanol), where TAME was completely (100 mg/L) eliminated after 21 d of incubation, although the bacterial strain was incapable of using it as sole carbon and energy source in mineral medium. The fact that oxygenate consumption was only observed in cometabolic media suggests that certain co-factors or inducers could be necessary for efficient growth on the oxygenate and consequently a major degradation will be achieved.

Members of the *Acinetobacter* family have shown to biodegrade a wide range of xenobiotics, including ortho-nitrophenol (o-NP) and pentachlorophenol (PCP) [24]. Several *Acinetobacter calcoaceticus* strains were capable of degrading branched alkanes such as iso-

butane and iso-pentane both abundant in the gasoline formulation and good structural analogues of MTBE [25]. In the present study *A. calcoaceticus* (M10 strain) showed the capacity of biotransforming the three oxygenates (MTBE, ETBE and TAME) in mineral salts media, with the highest removal capacity (up to 82 mg/L) in mineral media amended with TAME. However, after a week, the strain did not maintain itself, and entered death phase (Fig. 3). The presence of several intermediates (such as TAA) could be the reason of the growth inhibition observed.

Hatzinger et al., [11] reported that the growth inefficiency observed in bacterial strains growing in MTBE as sole carbon source could be due to the lack of enzymes to complete degradation, or the inhibitory presence of intermediate by-products (such as TBA) in the biodegradation of these oxygenates. These authors indicated that products from cellular metabolism and those present in the culture medium could inhibit degradation of TBA. Moreover, Müller et al. [26] proposed that low growth observed in bacterial strains growing in MTBE was due to slow kinetic degradation rates which confer slow energy release, which was mainly employed for growth maintenance.

On the other hand, the inability to biodegrade the components in mineral medium supplemented with yeast extract and ethanol was also observed, inferring inhibition by the preferential consumption of ethanol and/or yeast extract, or the direct inhibition by the latter two components

Several authors [27, 28] showed that *Rhodococcus ruber* (formerly *Gordonia terrae*) IFP2001 and a constitutive strain *R. ruber* IFP2007 derived from strain IFP 2001, were capable of growing in ETBE and not in MTBE or TAME, even though the last two oxygenates were degraded to TBA (*t*-butyl alcohol) and TAA (*t*-amyl alcohol) in the presence of another carbon source (i.e. ethanol). These strains were capable of using ETBE for growth because they used the two-carbon compounds (e.g. acetaldehyde) derived from degradation of ETBE, better than TBA or one-carbon compounds (e.g. formaldehyde) derived from MTBE and TAME degradation. In our study, both *Nocardiodes* sp. (strain E7) and *R. ruber* (E10 strain) used the oxygenate ETBE as sole carbon and energy source for growth and biotransformed the three oxygenates in mineral salts media with the exception of strain E7 when grown in media amended with MTBE.

Biodegradation of gasoline oxygenates by *Rhodococcus* and *Nocardioides* sp. have been reported previously and in this sense, several plasmids containing genes involved in the metabolism of xenobiotics have been detected in *Rhodococcus* species [29]. However, to our knowledge few investigations have been reported in the scientific literature on the influence

of gasoline oxygenates on *Gordonia* spp.

Gordonia amicalis strains (EA and T3 strain) were not identical as previously stated, and behaved differently towards the different oxygenates. EA strain only biodegraded ETBE in the presence of another carbon source, while T3 strain was incapable of using this oxygenate neither in mineral nor cometabolic media. However this strain was capable of biotransforming MTBE and TAME in mineral and cometabolic media showing a significant growth *($p<0.05$)* in mineral medium amended with TAME.

The results obtained in the present study have shown that all the bacterial strains transformed oxygenates better in the presence of an alternative carbon source such as ethanol with the exception of *A. calcoaceticus*, strain M10.

The description of new bacterial strains with the ability to degrade MTBE, ETBE and TAME could be of significance in the development of new bioremediation technologies. In turn, the selection of bacterial strains with specific degradation properties and the transforming property of xenobiotic metabolic genes may be important for the biological treatment of water contaminated with gasoline oxygenates such as MTBE, ETBE and TAME. Thus, experimental procedures using aerated submerged biofilters inoculated with selected strains isolated in the present study are in progress as well as the identification of biodegradation products such as TBA (*t*-butyl alcohol), TAA *(t*-amyl alcohol) and 2-hydroxy isobutyric acid, (HIBA).

ACKNOWLEDGEMENTS

This work was funded by the Spanish Ministerio de Ciencia y Tecnología (MEC), through the Programa Nacional de I+D (project reference CTM2006-02297). Oxygenate analysis were performed by EMASA (Empresa Municipal de Aguas, Málaga, Spain).

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SECTION III.2

DEVELOPMENT OF CHEMICAL AND BIOLOGICAL METHODS FOR THE STUDY OF AN AERATED SUBMERGED BIOFILTER FOR FUTURE OXYGENATE BIOREMEDIATION

III.2-1. EVALUATION OF A HEATED STATIC HEADSPACE METHOD FOR FUEL ETHER DETECTION AND QUANTIFICATION

III.2-2. COMPARATIVE ANALYSIS OF MICROBIAL DNA EXTRACTION PROTOCOLS FOR GROUNDWATER SAMPLES

III.2-1. EVALUATION OF A HEATED STATIC HEADSPACE METHOD FOR FUEL ETHER DETECTION AND QUANTIFICATION

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Prepared for submission to the

International Journal of Environmental Analytical Chemistry

ABSTRACT

A manually controlled heating headspace method was assessed for limit detections as well as replication under different conditions for oxygenates MTBE, ETBE and TAME. The method consisted in reducing the headspace volume in relation to the volume sample. Limit of detection of the three oxygenates was 0.01 ppb, and method detection limits for the three were 10 ppb ($p<0.05$). These limits fall within the limits of headspace autosampler methods and as shown in this study, oxygenate determination may be quantified in the absence of a headspace autosampler. Different sample heating times were tested, showing increasing linearity of TAME area from 10 to 50 min, however, constant counts were only observed as from 80-90 min heating time. Reversible equilibrium of TAME in the dissolved and gaseous phase occurs between 50 and 80 min heating time and thus oxygenate concentration should not be measured between these heating times. Surprisingly, sample storage tests revealed that abiotic samples at 4ºC were transforming TAME more rapidly than biotic samples containing degrading strain *Acinetobacter calcoaceticus* M10, thus we recommend a maximum of 2 weeks storage time at 4 ºC in the dark to reduce transformation of ether compounds. This method is recommended for broad range fuel ether detection above 10 ppb, and the use of the split-scan mode for the lowest variability in a sample.

INTRODUCTION

Lead substitution for oxygenate compounds in gasoline formulation are being used worldly to enhance combustion and reduce harmful fume pollutants. Among the oxygenates used, methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME) are highly produced and used. In spite of the benefits gained from toxic fume reduction, their occurrence in water environments is high due to their high solubility in water [1], low soil adsorption and recalcitrant structure. Worldwide groundwater ether occurrence ranges from 0.01 ppb to 16,000 ppm, in surface water occurrence ranges from 0.01 ppb to 5 ppb and in sea water from 0.02 ppb to 1.8 ppm [2]. The contamination of these pollutants in drinking water is hazardous and thus detection and quantification of these compounds are essential. Gas chromatography (GC) is one of the techniques most widely employed to quantify mixtures of organic compounds. In the analysis of fuel oxygenates and BTEX in water, gas chromatography is generally combined with mass spectrometry (MS) [3, 4] or flame ionization detection (FID) [5, 6]. Analytical methods include purge and trap (P&T) [7], headspace solid-phase dynamic extraction (HS-SPDE) [8], headspace solid-phase microextraction (HS-SPME) [5, 6, 8, 9, 10], headspace solvent microextraction (HS-ME) [11], headspace (HS) [12, 13] or direct aqueous injection (DAI) [14, 15]. Recently, a new alternative to improve sensitivity, maintaining the simple headspace (HS) instrumentation, has been proposed. It consists of the use of a programmed temperature vaporizer inlet (PTV) to inject the samples into the chromatographic column [13, 16, 17]. P&T-GC/MS as well as HS-GC/MS are the most commonly used methods, the first for its low LODs, and the latter for its wide range of detection. P&T-GC/MS concentrates the sample with no need for pre-treatment, therefore the lowest LODs are detected (lowest LOD detected was with a MS detector – 0.001 ppb MTBE [18]). Disadvantages of P&T enrichment are that the method should be used on clean water samples, and that adsorption to particulated samples will not be taken into account due to the need for prior filtration for this method. Different HS methods have been used for ether quantification in water samples, i.e. static HS, HS-SPME, SPDME, and LPME. The advantages to headspace analysis is its robustness for any type of sample (solid, liquid, gaseous, either clean or highly contaminated samples), thus making it ideal for unknown environmental samples. The main disadvantage of headspace methods are the higher method detection limits (MDLs) for MTBE, however, these tend to be lower than EPA's MTBE concentrations limits in drinking water (20-40 ppb).

The work performance of a modified HS-GC/MS method with no added salt, manually controlled heating and robotically controlled injection system was assessed. Heating and storage times were also analysed. The modified HS-GC/MS method consists in reducing the headspace volume, thus concentrating the oxygenates for further analysis. Most headspace (heated and non-heated) methods require a volume of 1-10 ml sample and with an equal volume headspace. The headspace concentration we suggest is at least 2 fold, and the volume injected is 50 µl gas phase.

MATERIAL AND METHODS

Headspace GC/MS Method

Different concentrations of methyl *tert*-butyl ether, ETBE and TAME in bidistilled water were assayed by gas chromatography/mass spectrometry headspace technique. A volume of 1.5 ml unfiltered samples were transferred with a micropipette into 2 ml vials and quickly capped and crimped with a Teflon coated septa. The samples were heated at 90 °C in a thermoblock during 90 min (unless stated otherwise), and quickly transferred with forceps to an autosampler (HP6890 series). An injection volume of 50 µl of the gas phase was programmed for all samples, using a gastight 100 µl syringe (Agilent). Separation was performed on a Quadrex capillary column (007-1, Dimethylpolysiloxane-PHAT Phase, 20 m x 0.18 mm x 6.0 µm), within the GC/MS equipment (Hewlett-Packard 6890 gas chromatograph coupled to a mass spectrometer Hewlett-Packard 5973 mass selective detector, Palo Alto, CA, U.S). The temperature program was: 40 ºC (1.5 min hold), 10 ºC/min to 120ºC. Helium was used as the carrier gas at a flow rate of 0.4 ml/min. All samples were taken in triplicate (unless stated otherwise). Room temperature was kept constant at 24 ºC. Quantification of MTBE, ETBE, and TAME was performed using external standard calibration.

Determination of GC/MS conditions

Different GC/MS conditions were tested in order to achieve the most reproducible conditions for quantifying MTBE, ETBE and TAME using the equipment described above. A combination of the injection modes split and splitless, and the detection modes SIM and scan were tested with 50 ppm TAME due to the fact that this compound was observed to evaporate more rapidly from its dissolved state. Heating time was kept constant at 90 min. Statistical analysis using boxed diagrams were performed in order to evaluate reproducibility. Replicate

number was 10 for each condition. The split ratio was 29.9:1 and the ions (mz) used for SIM mode were 41, 43, 55, 73 and 87. Scan mode detected ions from 40-110 mz.

Detection and quantifiable range

All compounds were diluted to different concentrations which ranged from 0.001 to 1000 ppm from a stock solution of 10,000 ppm. Heating time was 90 min in split/SIM mode.

Method detection limits (MDL), limit of detection (LOD) and saturation limits were determined when the R squared was ≤ 98%.

Reproducibility at different heating times

The variance was tested for quantification of 50 ppm TAME samples at different heating times. Replicate number was 5 for each condition.

Storage

Abiotic and biotic (*Acinetobacter calcoaceticus* M10) samples were kept at 4 ºC in the dark, and analysed to observe concentration loss from an initial 50 ppm TAME. Samples were tested at different storage times, up to 28 days without prior filtration, nor addition of a strong acid/base nor salt.

Statistical analysis

The program SSPS v.15.0.1 was used as a statistical analyser, as well as to represent the data in box-plot graphs. The graphs show a series of symbols: the circle represents the outliers; the line inside the box indicates the median value; the box indicates 50% of the data around the mean and the limits cover 95% of the analyzed data.

RESULTS AND DISCUSSION

Method evaluation began with the replicate analysis of a combination of injection (split and splitless) and detection (SIM and scan) modes. As shown in **Figure 1**, combined splitless-scan, split-scan and split-SIM have a similar behavior, with a mean of 2.95×10^7 and a deviation of 1.50×10^6 (RSD – 5%). These values differ from the combination splitless-SIM, which was the most variable at a deviation 8.9×10^6, however its area was a third higher than the other three combination modes. On the other hand the split-scan combination shows the

best clustering results, the difference between the maximum and minimum value is 2.6 x10^6, which is the smallest value compared with other combinations.We suggest that the split-scan mode be used to quantify the ether compounds under the conditions described.

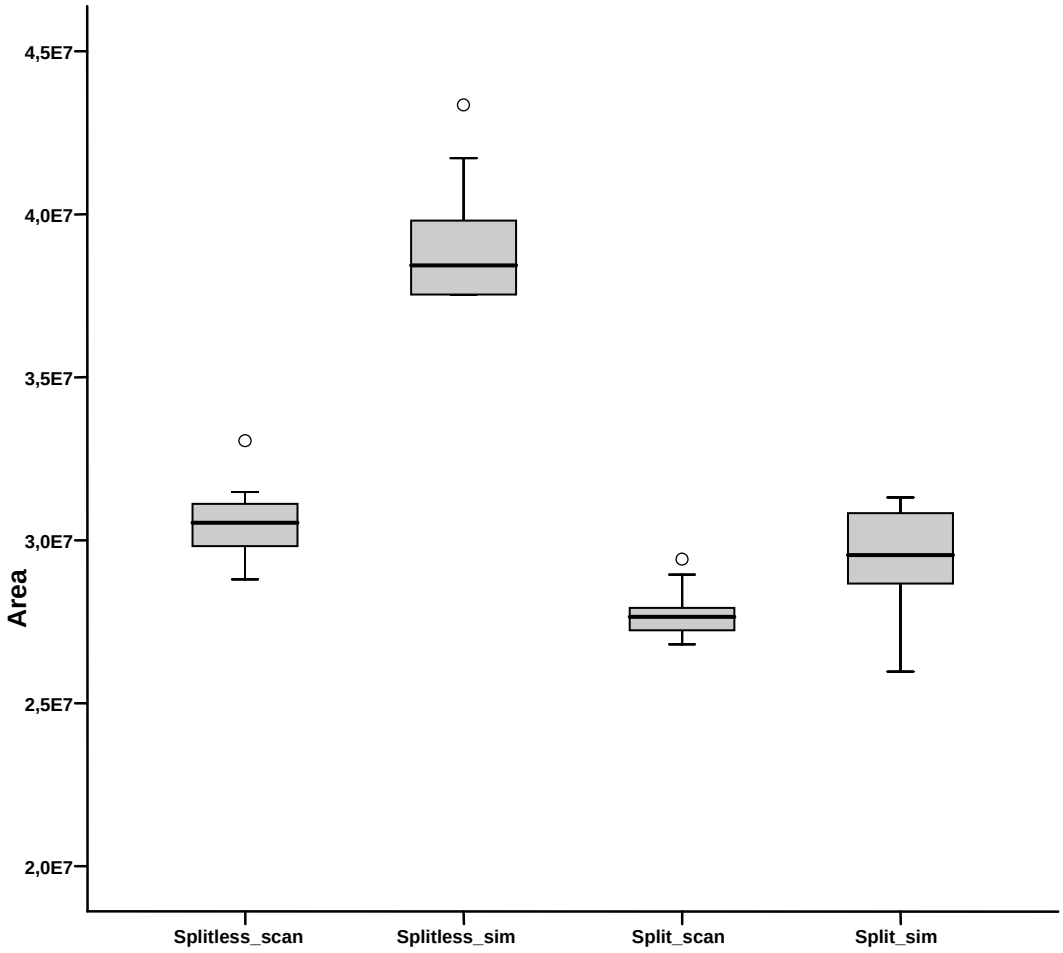

Figure 1. Variance of injection and detection modes combined shown in a box-plot diagram. All samples contained 50ppm TAME.

Calibrations curves performed from 0.001 ppb until 1,000 ppm were used to evaluate the method detection limit and saturation point thus allowing for a range ideal for groundwater ether detection of MTBE (R>0.99), ETBE (R>0.99) and TAME (R>0.99). As can be observed in **Table 1**, the LOD of all three compounds is low and the MDL is comparable to other methods as can be observed in Table 2, even though a smaller injection volume and smaller sample volume are used. Saturation points do differ, nevertheless, in the unlikely event of a highly contaminated aquatic site, the samples could be diluted to fall within the MDL and saturation point.

Table 1. Fuel ether calibration characteristics performed in split-SIM mode.

	MTBE	*ETBE*	*TAME*
Method detection limit (MDL) (ppb)	10	10	10
Limit of detection (LOD) (ppb)	0.01	0.01	0.01
Peak time (min)	6.06	7.33	8.74
Saturation point (ppm)	>1,000	~ 600	~ 400

Table 2. Comparison of our C-HS-GC/MS method with others HS methods for MTBE. a) Manual heating, b) headspace autosampler, c) manual injection, d) robotically controlled injection, e)SIM, f) Scan, g) GC/MS, h) GC/FID, i) splitless, k) split (ratio). * 12h equilibration time at 4ºC. NR – Not reported

Method	*Salt (g/l)*	*Injection volume*	*Sample volume (ml)*	*Headpsace volume (ml)*	*Heating time (min)*	*Heating temp (ºC)*	*MDL (ppb)*	*LOD (ppb)*	*Range (ppb)*	*Ref*
b, d, h	0	NR	10	10	60	60	NR	50	50 - 1000	22
b, d, h	0	1 ml	10	12	12	70	NR	5.7	>5.7-15000	18
a, d, e, k (2:1)	250	50 µl	1	1	0 *	25	1.2	NR	NR	23
b, d, f, k (1:13)	200	1 ml	10	10	30	80	0.21	NR	0.5 - 1000	12
a, d,	0	50 µl	1.5	<0.5	90	90	10	0.01	10 - $>10^5$	This study

Heating times as observed in **Table 2** vary from 10 to 90 min, with a temperature range of 25-90 ºC. The results shown in **Figure 2,** evaluate the time required to preheat the samples to determine the concentration of the oxygenate in ppb, from a stock solution prepared to 50 ppm TAME. During the first 50 minutes of heating, the concentration increases linearly with a rate of change of 607.95 ppb/min. Between 50 and 80 minutes (non-inclusive) the results show variations, indicating the beginning of the balance between the two phases of the oxygenate. To use this method, it is important to obtain a balance between the gas and liquid phases and have a constant partition coefficient, i.e. controlling these conditions in order to achieve sensitivity and reproducibility for this method. Thus shown in **Figure 2,** this partial constant lies between 80 and 90 minutes of preheating, with a variation of 120 ppb/min, which is hardly significant since the deviation observed for each heating time (+/- 853 ppb) is higher than the variation among both conditions. We can say that the 90-minute heating time for the sole oxygenate mole fraction solution is proportional to the vapour pressure according to Raoult `s law.

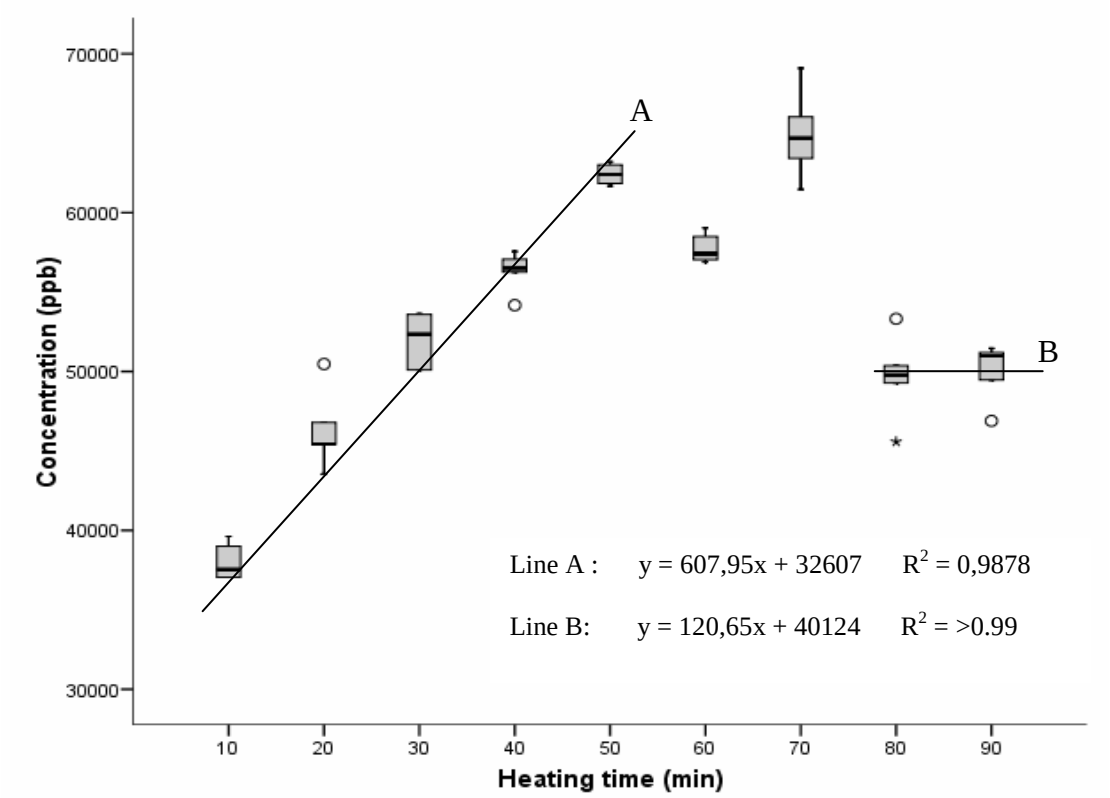

Figure 2. Box-plot representations of replicate TAME samples at different heating times at 90 ºC.

A non-heating static headspace used to determine 50 ppm TAME would have given a lower GC area, and in turn, the MDL would be higher. Overall, 50 min heating time gives the best combination of highest area with low variance, and would therefore give the lowest MDL with this method.

Higher heating times (80-90 min) show less TAME area than at 50 min, and may be due to hydroxylation of TAME, but the area is constant. Lin et al [12] used a HS autosampler, heated the sample during 30 min at 80 ºC with 200 g/l of NaCl in a 10 ml sample, within a 20 ml vial. The injection volume was 1 ml. They used the scan mode to analyse the samples. Hydrolysis at 80 ºC was determined at pH 2 and resulted in minimum to maximum hydrolysis in the order MTBE, ETBE and TAME.

Storage time tests of 50 ppm TAME were performed on an abiotic and biotic samples and the results are shown in **Figure 3.** The TAME concentration of the abiotic control decreased at a higher rate than the biotic equivalents, even when the biotic sample contained a TAME degrading strain (*Acinetobacter calcoaceticus* M10). The latter may be due to TAME adsorption by the strain. Manual clamping of the septum can lead to leaks through micropores, however the constant loss in TAME observed is probably due to some type of transformation.

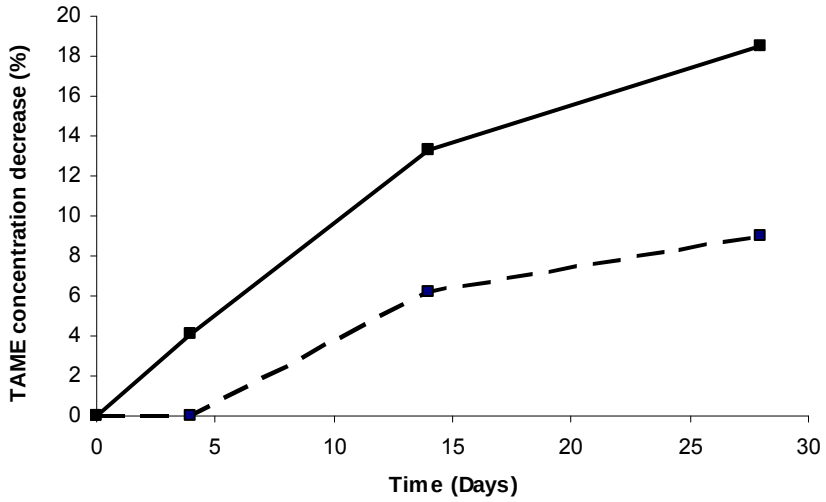

Figure 3. Influence of storage time on abiotic biotic samples throughout 28 days at 4ºC in the dark.

———— Abiotic ---------- Biotic

Hydrolysis of TAME to TAA under acidic conditions has been tested and proved at 26 ºC [19], however, TAA was not detected in our storage assay. In another study [20], samples stored at 4 ºC were acidified ("preserved") and not acidified ("unpreserved"), and TBA was not observed either in this study when determining MTBE storage. However they do not observe a constant decrease in MTBE during 4 weeks, and could probably be due to the lack of sample repeats, for there was a tendency of MTBE concentration decrease. A difference in pH for sample storage from 1 to 2 accounted for a decreased rate of hydrolysis during storage at room temperature [21]. Thus, hydroxylation is likely even though samples were stored in the dark at 4 ºC and not acidified.

Oxygenate transformation occurred abiotically at a rate of 0.36 ppm per day. At this constant rate, the TAME present in the vial will have been transformed completely in 5 months. The rate of change - 0.36 ppm, has a value lower than the deviation obtained by averaging the results of repetitions performed (4.72ppm). We recommended samples to be stored without further treatment in the dark at 4 ºC, no more than 2 weeks prior to GC analysis in order to limit oxygenate transformation to less than 5 %. Addition of trisodium phosphate to a pH of 11-12 is recommended by different authors [12, 21].

These same sample vials were analysed in order to evaluate whether one could use the same vial more than once for future analysis, however there was no pattern observed as to the number of times one could inject the sample into the GC, thus once injected, the vial could no

longer be used again. If headspace autosamplers used this method with a higher injection volume (e.g. 500 μl), they would be able to lower the MDL probably 10 fold. Thus range of gastight syringe volumes is a limiting factor.

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III.2-2. COMPARATIVE ANALYSIS OF MICROBIAL DNA EXTRACTION PROTOCOLS FOR GROUNDWATER SAMPLES

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Accepted for publication

Analytical Biochemistry DOI: 10.1016/j.ab.2011.05.024

ABSTRACT

A comparative analysis of four different DNA extraction protocols are performed to determine the best choice for groundwater microbial diversity studies using TGGE analysis. The methods used are: a chelex-based method; salting out procedure (MSOP); and commercial kits Epicentre®; and a FastDNA® kit. Both commercial kits exhibited greatest reproducibility in their methods, however their band patterns were very different. The protocol that showed the highest diversity was the chelex-based method, and lowest was the FastDNA® kit.

Keywords

MSOP, Chelex, Epicentre, FastDNA, Fastprep, DNA extraction, TGGE, groundwater

Groundwater constitutes one of the main sources of drinking water [1], therefore its quality is of great importance. This oligotrophic environment dominated by microorganisms [2], sustains relative small amount of cells (ranging from 10^3 to 10^6 cfu/ml) where most are non culturable [1, 3]. Due to this dominance of uncultured microorganisms in natural microbial ecosystems [4, 5] the study of microbial communities and its functionality should include culture independent approaches. Consequently, recent studies of these environments are based on molecular techniques based on DNA analysis. Several studies are interested in correlating microbial diversity in these kind of environments to different contaminant factors [6], outstanding the importance of an appropriate DNA extraction method to obtain more accurate correlations. There are several commercial kits and laboratory protocols available for DNA extraction in these environments. After cell concentration, one of the most popular methods uses a combination of chemical and mechanical lysis [7]. Other methods ease the chemical lysis with a previous enzymatic digestion [8], while others are exclusively lysed chemically [7]. Alternatively, there are protocols made *ad hoc* for microbial environments of low amounts of microorganism [9]. In this comparative work, we have shown the different bias, in terms of diversity and abundance, in the analysis of a groundwater microbial community depending on the DNA extraction method used.

Groundwater was collected in a 25 l sterile tank. A volume of 2 l of groundwater was filtered through each 0.22 μm nitrocellulose filter (EZGSWG474 - Millipore). Each filter was stored in a sterile 15 ml falcon tube, where 2 ml of sterile bidistilled water was added to each tube. The filter was crushed into the water with the use of a sterile tip, and vortexed for 10 s. The samples were then placed in an ultrasound bath for 10 min, and vortexed again for another 10 s. The supernatant was transferred to a 2 ml tube and centrifuged during 15 min at 14,000 rpm. The pellet was then stored for further analysis. Four different DNA extractions protocols were used on the pelleted samples. A chelex-based method [9], a modified salting out procedure method (MSOP) [8], and two commercial kits were used: "MasterPure™ Complete DNA & RNA Purification Kit" (Epicentre Biotechnologies®, Madison, Wisconsin, USA); and "FastDNA®SPIN Kit for Soil", including the use of the FastPrep® Instrument (MP Biomedical, Santa Ana, CA, USA). All samples were resuspended in 100 μl dH_2O or TE, as recommended. DNA concentrations and quality were tested using a NanoDrop 2000 (Thermo Scientific) (**Table 1**). For each method, three filters were used. The DNA from each filter was processed individually until TGGE analysis, thus replicates of TGGE analysis belong to a single filter sample. Protocol comparison was performed using TGGE analysis described by [10], with the first primer set being fD1 (5´ -

CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG - 3´) and rD1 (5´ - CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC - 3´) for 16S rDNA amplification, and the second primer set being GC-P1 (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG) and P2 (ATTACCGCGGCTGCTGG) for V3 rDNA amplification.

Pattern differences and similarities were analysed with GelCompar®II program using both Pearson and Dice statistical algorithms. Range weighted richness (Rr) and functional organization (Fo) were calculated with the indications described in [11].

The program SSPS v.15.0.1 was used as a statistical analyser, as well as to represent the data in box-plot graphs.

The DNA extraction methods compared in this study are very different from each other: the chelex-based method has been described apt for an environment with a low bacterial count; the MSOP method uses an enzymatic lysis prior to chemical lysis; the Epicentre® kit exclusively uses a chemical lysis; and the FastDNA® kit combines both chemical and mechanical lysis by bead beating.

Table 1. Genomic DNA quality and amplification of 16S rDNA and V3 rDNA from different DNA extractions protocols.

		DNA extraction method			
		Chelex	MSOP	Epicentre®	FastDNA®
	Minimum method time per sample (min)	120	76	63	36
Genomic DNA	Concentration (ng/µl)	75.27±6.96	4.80±1.25	11.40±2.25	9.43±0.57
	260/230nm	0.27±0.00	0.11±0.03	0.21±0.09	0.04±0.03
	260/280nm	0.93±0.01	5.81±2.74	2.04±0.22	1.91±0.20
16S rDNA	Concentration (ng/µl)	531.67±68.93	249.10±45.11	231.87±30.50	413.87±125.28
V3 rDNA	Concentration (ng/µl)	291.93±165.89	188.57±146.89	280.93±62.17	138.00±46.95
	16S amplification	+	-	+	+
	V3 amplification	+	+	+	+

As can be observed from **Table 1,** variations in genomic DNA extractions are detected among the different methods; the chelex-based method obtained the highest DNA concentration; however, the variability in the chelex TGGE profiles was highest in this method. TGGE Neighbour-Joining cluster analysis of four DNA extraction methods from a sole groundwater sample, using the Pearson correlation coefficient **(Figure 1a)** showed that FastDNA® and Epicentre® methods clustered at >95% of similarity. The MSOP was shown to be less reproducible (clustered at 93% similarity), and the chelex-based method was clustered with a 84% similarity. The average similarity of each method against the rest, resulted in Epicentre® and the chelex-based method being the most similar (~75%), and the FastDNA® kit being the most dissimilar (52%).

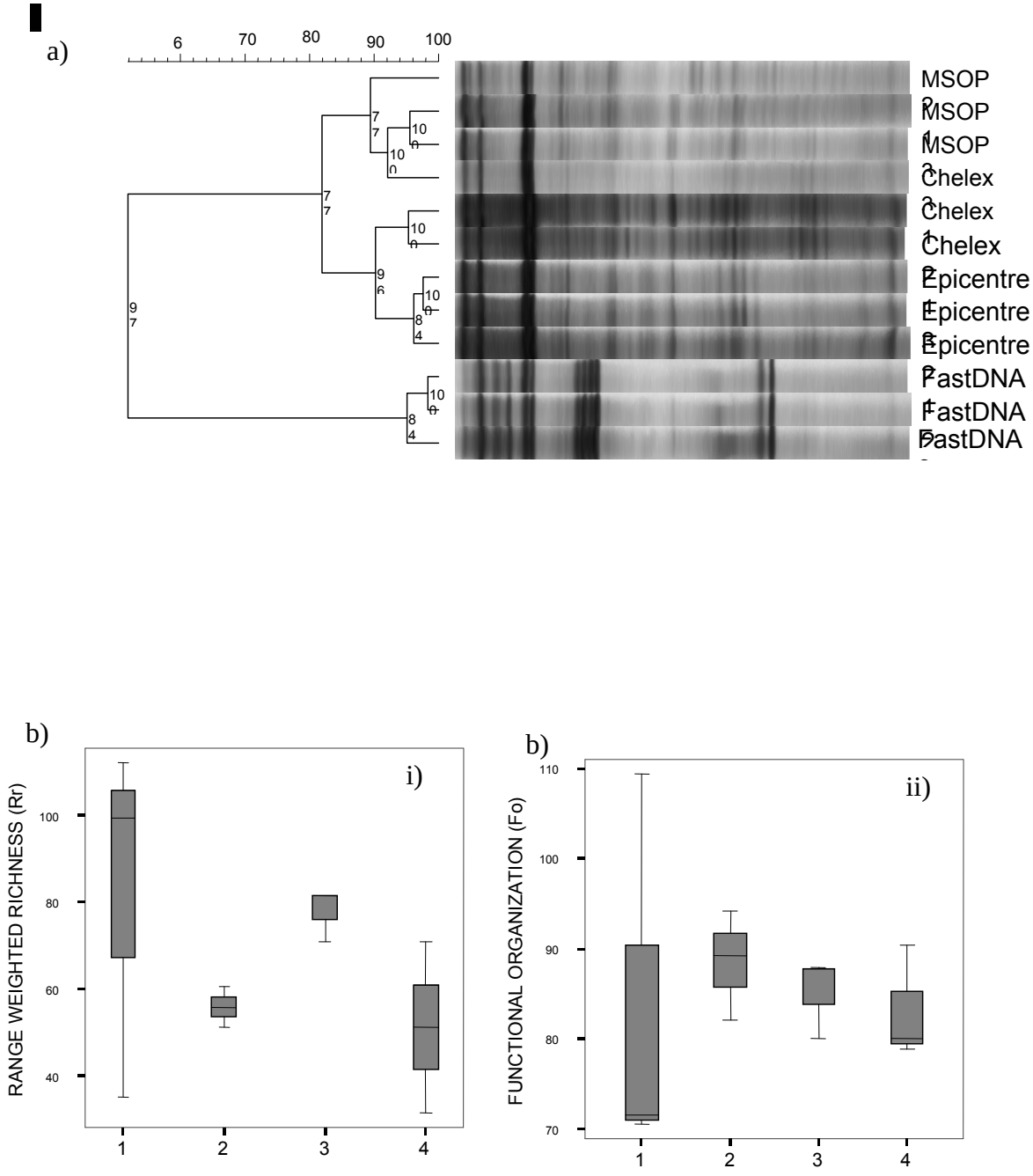

Figure 1. a) TGGE Neighbour-Joining cluster analysis of four DNA extraction methods from a sole groundwater sample, using the Pearson correlation coefficient. The circle represents the outliers; the line inside the box indicates the median value; the box indicates 50% of the data around the mean and the limits cover 95% of the analyzed data. The cophonetic correlation is shown in the dendogram. b) Box-plot diagrams showing the variation within each DNA extraction method with the following coefficients: i) Range weighted richness and ii) Functional organization). 1 – Chelex, 2 – MSOP, 3 – Epicentre®, 4 – FastDNA®.

MSOP 16S rDNA PCR products were not "visibly" amplified, however, when 1 µl was used for the clamped PCR (V3 rDNA), these were "visibly" amplified and showed high similarities in the TGGE profiles with Epicentre® and chelex-based extraction methods.

Surprisingly, the FastDNA® method was clustered in a separate group to the other three methods since its band patterns were very different to the rest. Thus, why is the FastDNA method so reproducible, yet does not show the same microbial diversity? The genomic DNA concentration of FastDNA® and Epicentre® were similar, thus concentration of DNA is not the motive for the difference in band amplification. There are two clear observations: bands amplified exclusively in the FastDNA® method; and those bands present in the other three methods (Chelex-based, MSOP and Epicentre® - *CME*), but ghostly in the FastDNA®. The band sequences were sequenced and clustered along with closely related strains as can be observed in **Figure SM1.** The motive of taxon grouping was discarded since bands in FastDNA® belonged to different suborders, as did those in the *CME* methods. Band G+C% content was also dismissed since there was no clear pattern or difference between those sequences in the FastDNA®, and those in the *CME*. In **Table SM1,** differences and similarities among the methods at a glance are shown, and the only significant differences of the FastDNA® method, are that the method does not contain an enzymatic treatment with proteinase K, and it lyses the cells mechanically. Exclusive bands in FastDNA® methods, could be due to a physical reason, i.e. strains are attached to other particles, and do not lyse under chemical and heat conditions. Those bands amplified in *CME* and present in ghostly traces in FastDNA® may be accounted by *CME* extraction procedures probably damage the extracted DNA less than the bead beating used in the FastDNA®, thus maximizing the potential detection sensitivity [12].

There are different ways of describing microbial communities through molecular biological techniques, as described previously [11], 3 different parameters are reported: range weighted richness (Rr), dynamics (Dy) and functional organization (Fo). These parameters can be given a numerical number, which can then be compared with other studies using e.g. DGGE, TGGE or tRFLP, even when different conditions apply such as temperature or urea concentration. Thus the use of these coefficients need not change too much within a given sample. In Figure 2, we show the variability within each method when Rr (the richness in diversity of a community) **(Figure 1b-i)** and Fo (the specificity of a community and its possibility to adapt to external changes) **(Figure 1b-ii)** coefficients are used in a study. The least variable were MSOP and Epicentre in both coefficient box-plots. Microbial diversity was highest with the chelex-based method, and the lowest was observed with FastDNA.

Among all the methods, the commercial kit Epicentre®, was the least variable method which also represented a high number of species within the TGGE analysis. It was also the most similar to the other methods, thus we recommend its use when a comparison is performed with other microbial diversity studies which have been carried out by different methods. Both Epicentre® and FastDNA® can be used when a high reproducibility is wanted from a method. The FastDNA® protocol will also allow for amplification of cells which are difficult to lyse. However, when the aim is to observe the widest diversity within a community, the chelex-based method could be used since the highest number of bands was observed with this protocol.

ACKNOWLEDGEMENTS

This work has been funded by the Spanish Ministerio de Ciencia y Tecnología, through the Programa Nacional de I+D (project reference CTM2006-02297).

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SUPLEMENTARY MATERIAL

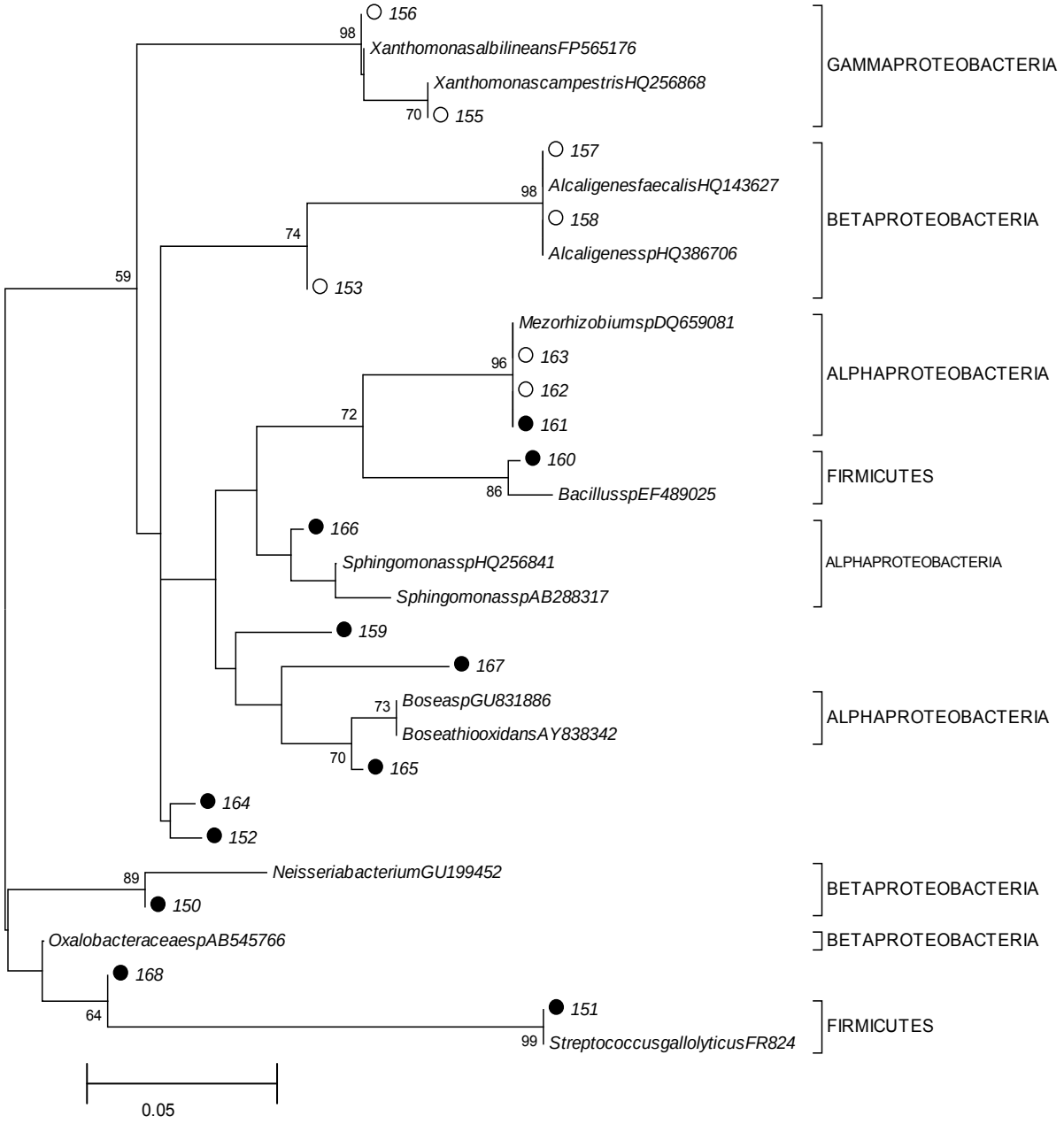

Figure SM1. Phylogenetic tree of band sequence differences in FastDNA® protocols, and the rest of methods. Open circle – band sequence present in FastDNA® protocol and not the rest. Closed circle – band sequence in chelex, MSOP and Epicentre® protocols, and not present in FastDNA®.

Table SM1. Summary of the similarities and differences among the DNA extraction protocols.

	Chelex	MSOP	Epicentre®	FastDNA®
Lyzozyme treatment	Yes	Yes	No	No
Proteinase K treatment	Yes	Yes	Yes	No
Other lytic enzymatic treatment	No	Mutanolysin	No	No
RNase treatment	No	Yes	No	No
Lysis	Heat and Vortex	Chemical, Heat and Vortex	Chemical and Vortex	Mechanical
Protein precipitation	No	Yes	Yes	Yes
Elution	Bidistilled water	0.5x TE	TE	DNase free pyrogen water

SECTION III-3

STUDY OF SET-UP AND DEVELOPMENT OF AN AERATED SUBMERGED BIOFILTER TECHNOLOGY FOR BIOREMEDIATION OF FUEL OXYGENATES CONTAMINATED GROUNDWATER

III.3-1. BIOFILM FORMATION AND MICROBIAL ACTIVITY IN A BIOFILTER SYSTEM IN THE PRESENCE OF MTBE, ETBE AND TAME

III.3-2. BIOLOGICAL AND CHEMICAL ANALYSIS OF A LAB-SCALE BIOFILTER FOR OXYGENATE BIOREMEDIATION IN HYPOTHETICAL RECENT GROUNDWATER SPILLS.

III.3-1. BIOFILM FORMATION AND MICROBIAL ACTIVITY IN A BIOFILTER SYSTEM IN THE PRESENCE OF MTBE, ETBE AND TAME

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Submitted to *Chemosphere* **(Under revision)**

ABSTRACT

Emerging water contaminants derived from unleaded gasoline such as methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME), are in need of effective bioremediation technologies for restoring water resources. In order to design the conditions of a future groundwater bioremediating biofilter, this work assesses the potential use of *Acinetobacter calcoaceticus* M10, *Rhodococcus ruber* E10 and *Gordonia amicalis* T3 for the removal of MTBE, ETBE and TAME in consortia or as individual strains. Biofilm formation on an inert polyethylene support material was assessed with scanning electron microscopy, and consortia were also analysed with fluorescent *in situ* hybridisation to examine the relation between the strains. *A. calcoaceticus* M10 was the best colonizer, followed by *G. amicalis* T3, however, biofilm formation of pair consortia favoured consortium M10-E10 both in formation and activity. However, degradation batch studies determined that neither consortium exhibited higher degradation than individual strain degradation. The physiological state of the three strains were also determined through flow cytometry technique thus gathering information on their viability and activity with the three oxygenates since previous microbial counts revealed slow growth. Strain E10 was observed to have the highest physiological activity in the presence of MTBE, and strain M10 activity with TAME was only maintained for 24 h, thus we believe that biotransformation of MTBE occurs within the active periods established by the cytometry analyses. Viable cell counts and oxygenate removal were determined in the presence of the metabolites *tert*-butyl alcohol (TBA) and *tert*-amyl alcohol (TAA), resulting in TBA biotransformation by M10 and E10, and TAA by M10. Our results show that *A. calcoaceticus* M10 and the consortium M10-E10 could be adequate inocula in MTBE and TAME bioremediating technologies.

Keywords: MTBE, ETBE, TAME, biofilter, biofilm, biodegradation

INTRODUCTION

Semi-volatile organic compounds, methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME), are three common fuel oxygenates used as tetraethyl lead substitutes in gasoline. Their physicochemical properties enhance the combustion of fuels, and decrease the emissions otherwise released into the atmosphere. Nevertheless, the contamination of groundwaters with ether compounds is common as a consequence of gasoline spillage, storage tank leaks and road runoff water (Barceló, 2007). MTBE levels in groundwater have been detected up to 830 mg L^{-1} in Europe, and up to 16 g L^{-1} in the US (Barceló, 2007).

Increasing basic and applied microbial bioremediation studies are being performed in order to evaluate the best approach for gasoline oxygenate bioremediation. Bioaugmentation, and biofilter technologies have been described using non-selective and selective inocula from varying sources. In direct MTBE degradation, only two systems have been described using a single bacterial strain as inoculum: a perlite biofilter system with *Mycobacterium austroafricanum* IFP2012 (Maciel et al., 2008) and an alginate immobilized *Methylibium petroliphium* PM1 (Chen et al., 2008) biofilter. Isolated degrading strains have recently been tested in consortia, all capable of biodegrading/biotransforming MTBE, ETBE, TAME, TAA (*tert*-amyl alcohol) and/or TBA (*tert*-butyl alcohol) (Auffret et al., 2009).

Poor growth is generally observed from oxygenate-degrading microorganisms when the oxygenate is the only carbon and energy source. Müller et al. (2007) stated in their theoretical study that the energy needed to degrade the oxygenates might only be sufficient for cell maintenance, thus generating uncertainty as to the physiological state of oxygenate-degrading bacteria. However, the addition of co-metabolic compounds such as isopropoanol, ethanol and alkanes, can increase growth and degradation rates (Smith et al., 2003). So far, aerobic degradation has proved to render higher energy efficiencies, favouring degradation rates.

Efficient biofilm formation on a support material is essential for biofilter technologies and the porosity of the support material as its surface area is an important factor for microbial attachment. The production of exopolysaccharide (EPS) or adhesion appendages from the microorganisms can indicate good biofilm formation. Biofilm thickness usually varies from tens of μm to more than 1 cm. However, activity with increasing thickness is lowered, because nutrient diffusion becomes a limiting factor.

In a previous study (Purswani et al., 2008), three bacterial strains were isolated from a hydrocarbon contaminated soil site, enriched with 100 mg L^{-1} of MTBE, ETBE and TAME: *Acinetobacter calcoaceticus* M10 (CECT 7739), *Rhodococcus ruber* E10 (CECT 7740) and *Gordonia amicalis* T3 (CECT 7741). The degradation levels observed after 21 d incubation in a mineral salts medium amended with the fuel oxygenates MTBE, ETBE and TAME were: 22, 14 and 82 mg L^{-1} for strain M10; 45, 5 and 21 mg L^{-1} for strain E10; and 53, 0 and 14 mg L^{-1} for strain T3, respectively.

In the present study, we performed individual and consortium tests on *A. calcoaceticus* M10, *R. ruber* E10 and *G. amicalis* T3 to determine the an inoculum capable of forming a biofilm on an inert support material in a submerged aerated biofilter at laboratory scale, for future fuel ether removal from contaminated groundwater.

MATERIALS AND METHODS

Chemicals

All chemicals purchased were of reagent grade or of the highest purity available. The fuel ethers MTBE (99.9% purity) and fluorobenzene (99.7%) were purchased from Fluka (Milwaukee, WI, USA), and ETBE (99% purity), TAME (97% purity), TBA (99%) and TAA (99%) were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

Growth medium

The growth media used in all experiments (unless stated) was a mineral salts medium described by Herman and Frankenberger (1999), and previously used in Purswani et al., 2008. The mineral salts-plus medium was comprised of mineral salts medium with the addition of 0.2 g L^{-1} yeast extract and 0.3 g L^{-1} of ethanol.

For biofilm formation on the biofilter and EPS production experiments, MY medium (Moraine and Rogovin, 1966) was used. The composition of MY medium was the following (g L^{-1}): yeast extract, 3.0; malt extract, 3.0; protease peptone, 5.0; and glucose, 5.0.

Bacterial strains used

The bacterial strains used in this study were: *A. calcoaceticus* M10 (*Colección Española de Cultivos Tipo* - CECT 7739), *R. ruber* E10 (CECT 7740) and *G. amicalis* T3 (CECT 7741), previously isolated from a hydrocarbon-contaminated soil site (Purswani et al.,

2008). Strains were stored at -80 °C in mineral medium and trypticase soya broth (Difco, Detroit, MS, USA), in 30% glycerol (v/v).

Microbial cell count in presence of MTBE, ETBE, TAME, TBA or TAA

To test the utilization of the oxygenates MTBE, ETBE, TAME, and the intermediates TBA or TAA by the strains individually or in consortia as sole carbon and energy source, 1 mL of each bacterial strain, pre-grown in mineral salts-plus medium previously centrifuged and washed with sterile saline solution (0.9 % w/v NaCl), was transferred to 125 mL glass vials with 25 mL fresh mineral salts medium amended with 100 mg L^{-1} of MTBE, ETBE, TAME, TBA or TAA, and incubated for 21 d at 30 °C under aerobic conditions and controlled agitation (100 rpm). The glass vials were sealed with Teflon septa. Viable cell counts were estimated by the dilution plate technique using trypticase soy agar (Difco, Detroit, MS, USA) medium. All the data were an average of three independent assays and a control glass vial (without oxygenate) was included for each strain or consortium tested. Plates were incubated between 1 to 4 d at 30 ºC and viable cell number was expressed as log cfu mL^{-1}.

Physiological studies by flow cytometry

The physiological state of the bacterium individual cells was characterized using multi-parameter flow cytometry in different conditions. Presence of both an intact polarized cytoplasmic membrane and active transport systems, essential for a fully functional cell, was tested by the addition of propidium iodide (PI) and 3,3-dihexylocarbocyanine iodide ($DiOC_6$). PI binds to DNA, but cannot cross an intact cytoplasmic membrane, and $DiOC_6$ accumulates intracellularly when membranes are polarized or hyperpolarized (Müller et al., 1999; Shapiro, 2003). Thus, using the two dyes together, it is possible to verify the physiological state of individual bacterial cells submitted to various growth conditions. The method followed was previously described by Reis et al., (2005).

A volume of 1 mL of each bacterial strain (approx 10^7-10^8 cfu mL^{-1}) was transferred to 125 mL glass vials with 25 mL fresh mineral salts medium amended with 100 mg L^{-1} of MTBE, ETBE, TAME, TBA or TAA, and incubated for 21 d as previously described. Periodically, cells were harvested and resuspended in phosphate buffer saline solution (PBS) and stained with fluorophores at final concentration of 1 μg mL^{-1} PI and 5 ng mL^{-1} $DIOC_6$. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), a well known ionophore, was used as control (working solution 10 μg mL^{-1}) (Reis et al., 2005) and sodium dodecyl sulphate (SDS) and mercaptoethanol were added to obtain a negative control. Flow cytometry was performed

using a FACS Vantage from Becton Dickinson (Becton Dickinson Immunocytometry System. San José, CA. USA). The software used for the acquisition and the analysis of the data was Cell Quest™ v.3.1 from Becton Dickinson. Bacterial fluorescence was detected using the FL1 and FL2 detectors respectively, using a BP filter of 530 ± 30 nm for FL1 and a BP filter of 585 ± 25 nm for FL2. The laser was used at 488 nm, regulated at 30 mW, with a flow of 500 cells s^{-1} up to 10000 cells. Viability was expressed as percentage of total viable cells by total cells gated. Cell activity was expressed as fluorescent units, calculated by the product of the mean fluorescence and the number of cell counts in the quadrant stipulated as active and viable. This was performed in order to prevent false results from samples with very few bacterial counts in the viable and active quadrant with high mean fluorescence.

Headspace fuel ether determination

MTBE, ETBE, TAME, TBA and TAA utilization by selected strains were assayed by gas chromatography/mass spectrometry headspace technique. Periodically, 1.5 mL of the inoculated and control samples were placed (not filtered) into 2 mL vials and clamped. In the MTBE samples, a 10 μL 1:200 fluorobenzene:methanol mix was added as an internal standard. The samples were heated at 90 °C during 90 min, followed by the injection of 50 μL of the gas phase into the GC/MS equipment (Hewlett-Packard 6890 GC coupled to a MS Hewlett-Packard 5973 mass selective detector, Palo Alto, CA, U.S). The analysis was performed on a Quadrex capillary column (007-1, Dimethylpolysiloxane-PHAT Phase, 20 M x 0.18 mm x 6.0 μm). The temperature program was: 40 ºC (3.5 min), 10 ºC min^{-1} to 85 ºC, 7 °C min^{-1} to 235 °C. Helium was used as the carrier gas at a flow rate of 0.4 mL min^{-1}. Losses by volatilization or by abiotic reaction of each oxygenate were monitored with control samples. All samples were taken in triplicate, and the mean and standard deviations calculated.

Statistical analysis

Multi-factor analysis of variance using the software package STATGRAPHICS 3.0 Plus v.4.1 (STSC, Rockville, MD, USA) was performed to identify significant differences between the treatments. A significance level of 95% *($p < 0.05$)* was selected.

Lab-scale EPS production

For lab-scale EPS production, the methodology described by Quesada et al. (1993) was used. The bacterial strains were grown in MY medium and incubated at 30 °C up to 7 d.

After, cells were harvested by centrifugation at 9000 rpm, for 45 min at 5 °C. The supernatant was mixed with 2 volumes of cold (-80 °C) ethanol and left standing during 24 h at 4 °C. The EPS precipitate was collected after centrifugation (7500 rpm, 15 min at 5 °C). The pellet was resuspended in bi-distilled water, dialyzed 24 h to get rid of salts, and freeze-dried. Both the cellular pellet and the EPS pellet were weighed. Protein content in the EPS was determined using the Bradford method.

Set-up of the lab-scale biofilter

The lab-scale biofilter (**Figure 1**) consisted of a glass cylindrical column (50 cm high and 6 cm diameter) filled with a polyethylene support material (Bioflow® 9) with a surface area of 800 $m^2\,m^{-3}$. The total biofilter volume was 1.2 L, with an interstitial liquid volume of 0.8 L, and a headspace of 200 mL. The aerated biofilter working conditions were: 0.1 L min^{-1} for airflow, recirculation at 1.2 L d^{-1}, and a constant temperature of 20 °C. The inert material was manually packed and a mesh was located at the top of the glass cylinder to prevent support movement.

The biofilter and all its components were autoclaved and mounted under sterile conditions. Both air influent and effluent contained a sterile 0.22µm filter, in order to avoid contamination.

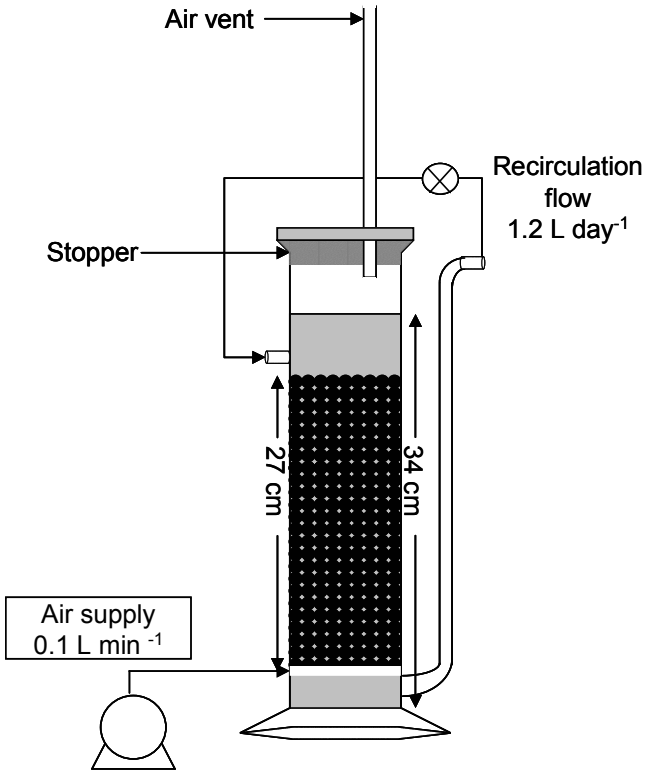

Figure1. Schematic diagram of the down flow biofilter used in the recirculation system. Constant temperature (20 °C), aeration (0.1 L min^{-1} air) and recirculation during the 5 d was established for biofilm formation.

Bioflow® 9 adsorption

In order to evaluate the adsorption of the oxygenates to (Bioflow® 9) grids, several tests were performed at controlled temperature (20 ºC) and agitation. Oxygenate concentration was determined throughout 7 d, and the data were compared to controls (without inert material). The inert material did not adsorb any of the oxygenates (MTBE, ETBE and TAME).

Observation of biofilm formation by Field-Emission Scanning Electron Microscope (FESEM) technique

For biofilm assessment in the biofilter, the bacterial strains (M10, E10 and T3) were pregrown in MY medium supplemented with the oxygenates at 30 °C for 1 or 4 d. Evaluation of biofilm formation up to 5 d recirculation period in MY medium was achieved by extracting Bioflow® 9 support samples at different column heights (top, middle and bottom) after 1, 3 and 5 d of recirculation. The units were fixed in 2.5% glutaraldehyde in PBS pH 7.4 for 24 h at 4 °C. The samples were washed three times in PBS for 20 min. Post fixation followed with addition of 1% osmium tetroxide, kept in the dark during 1 h at room temperature and washed with distilled water 5 min, three times. Samples were dehydrated in increasing ethanol concentrations, 15 min each: 50, 70, 90 and 100 % (twice). Desiccation was performed using the Anderson method (1951) with CO_2 in a critical point dryer CPD7501. The samples were covered by the evaporation of carbon with a HITACHI evaporator. Samples were observed using a LEO 1530 FESEM. A control was included for comparison obtained by adding drops of bacterial culture (*A. calcoaceticus* M10 at 10^8 mL^{-1}) onto support material units, and processing the units as described above to disregard attachment through fixation.

Biofilm formation and bacterial activity by in situ hybridization

Biofilm extraction from Bioflow® 9 units was performed by resuspending and washing the units in PBS. Samples were fixed by mixing 1 volume sample with 1 volume ice-cold 96% (v/v) ethanol. Fixed 10 µL aliquots of the ethanol samples were dried in Teflon-coated microscope slides at 46 °C for 15 min, were sequentially dehydrated in 50, 80 and 96% (v/v)

ethanol for 3 min each, and dried at 46 °C for 2 min. Fluorescence *in situ* hybridization (FISH) was performed as described previously (Wagner et al., 2003). The hybridisation buffer stringency was high, and contained the following composition: 180 μL 5 M NaCl, 20 μL 1 M Tris/HCl, 35% formaldehyde, 1 μL 10% SDS, up to 1 mL dH_2O. Fluorescent probe mix for eubacteria contained the following oligonucleotides labelled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS): EUB338 – GCTGCCTCCCGTAGGAGT; EUB338-II – GCAGCCACCCGTAGGTGT; and EUB338-III – GCTGCCACCCGTAGGTGT (Daims et al., 1999). The following gammaproteobacteria probe was labelled with Cy3: GAM42a – GCCTTCCCACATCGTTT (Manz et al., 1992). An additional non-fluorescent probe was added as a gammaproteobacterium probe competitor: BET42a – GCCTTCCCACTTCGTTT (Manz et al., 1992) Eubacteria mix, GAM42a and BET42a probes were added (1 μL each) to 10 μL of hybridization buffer and applied onto the dehydrated sample. The remaining buffer was poured onto tissue paper inside a falcon tube, the slide was placed inside and sealed to keep in moisture. Hybridisation took place at 46 °C for 90 min in the dark. The slide was placed in 50 mL washing buffer (700 μL 5 M NaCl, 1 mL 1 M Tris/HCl, 0.5 M EDTA, up to 50 mL dH_2O, preheated in a water bath at 48 °C) in a fume hood and incubated for 10 min at 48 °C. The slide was then dipped in ice-cold bi-distilled water and quickly air-dried and stored at -20 °C. AF1 (Citifluor) was used as the antifadent, and the samples were viewed in a Carl Zeiss Axio Imager A1 fluorescent microscope. Overlapped images were made with the Carl Zeiss AxioVision Rel. 4.6 software. The filters used were “Pinkel 1 set” (Chroma) for the FLUOS fluorophore, and “Filter set 20” (Zeiss) for Cy3. The program DAIME (Daims et al., 2005) was used to calculate the bioarea percentage of the individual strains in the consortium relative to the total population, as well as to calculate the spatial distribution.

RESULTS

Viable cell number and TBA/TAA removal in batch cultures

The results shown in **Figure 2** represent the viable cell count of the bacterial strains in mineral salts media amended with the metabolites TBA and TAA, as well as the concentration removal after 21 d incubation. M10 cell counts in TAA were favourable in comparison to the control, as was E10 in TBA and TAA, while T3 viable counts in the metabolites were not significantly different to the control. TBA and TAA concentration removal after 21 d incubation were recorded for the three strains **(Figure 2).** Biotransformation of TBA by

strains M10 and E10 was up to 50 mg L^{-1}, and TAA was only biotransformed by the strain M10 to 80 mg L^{-1}.

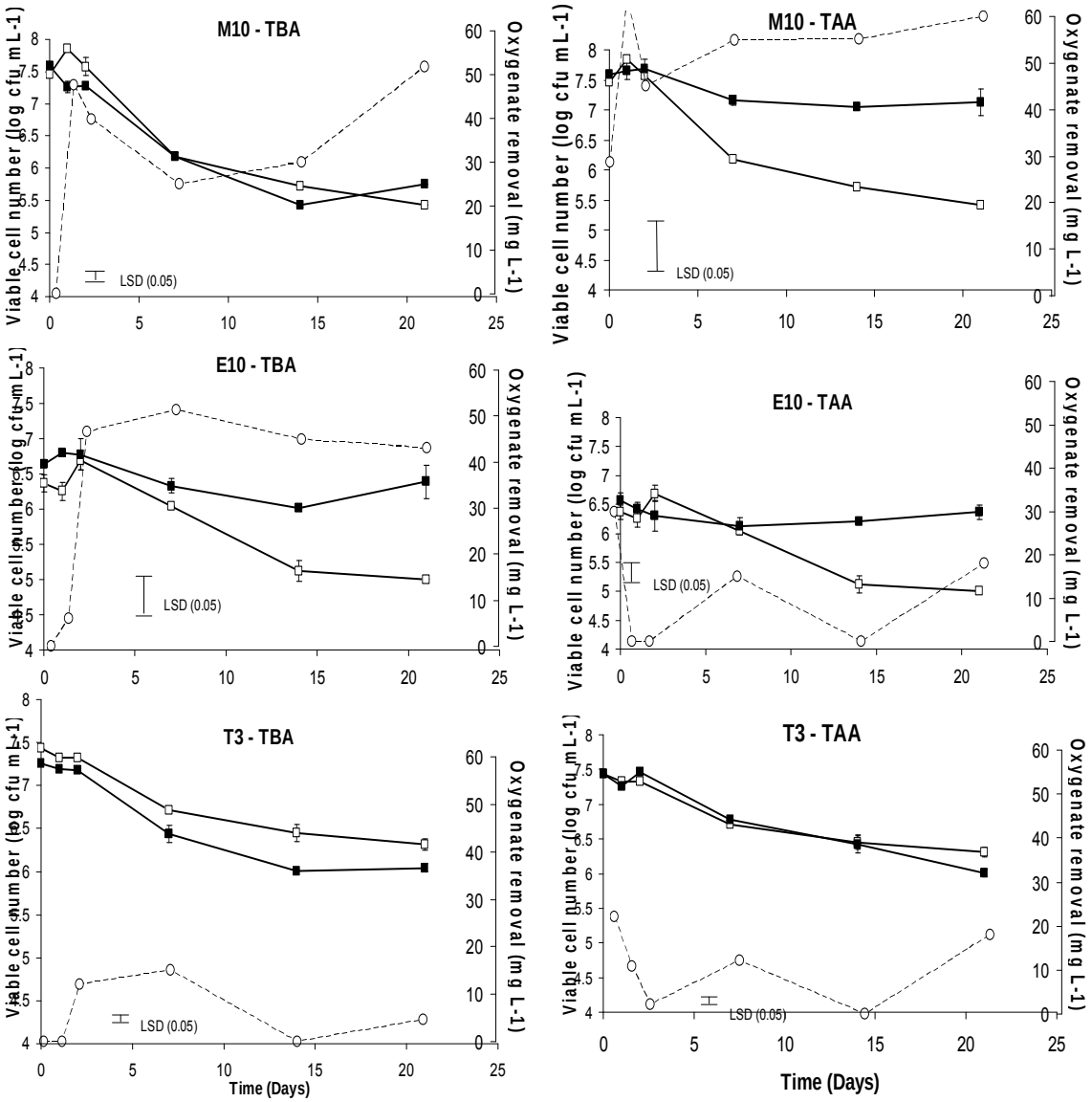

Figure 2. Growth and degradation of strains M10, E10 and T3 on TBA and TAA. Values are an average of three experiments (p<0.05). LSD – Least Significant Difference for viable counts. ■ – Growth with and □ – without compound. ○ – TBA/TAA removal

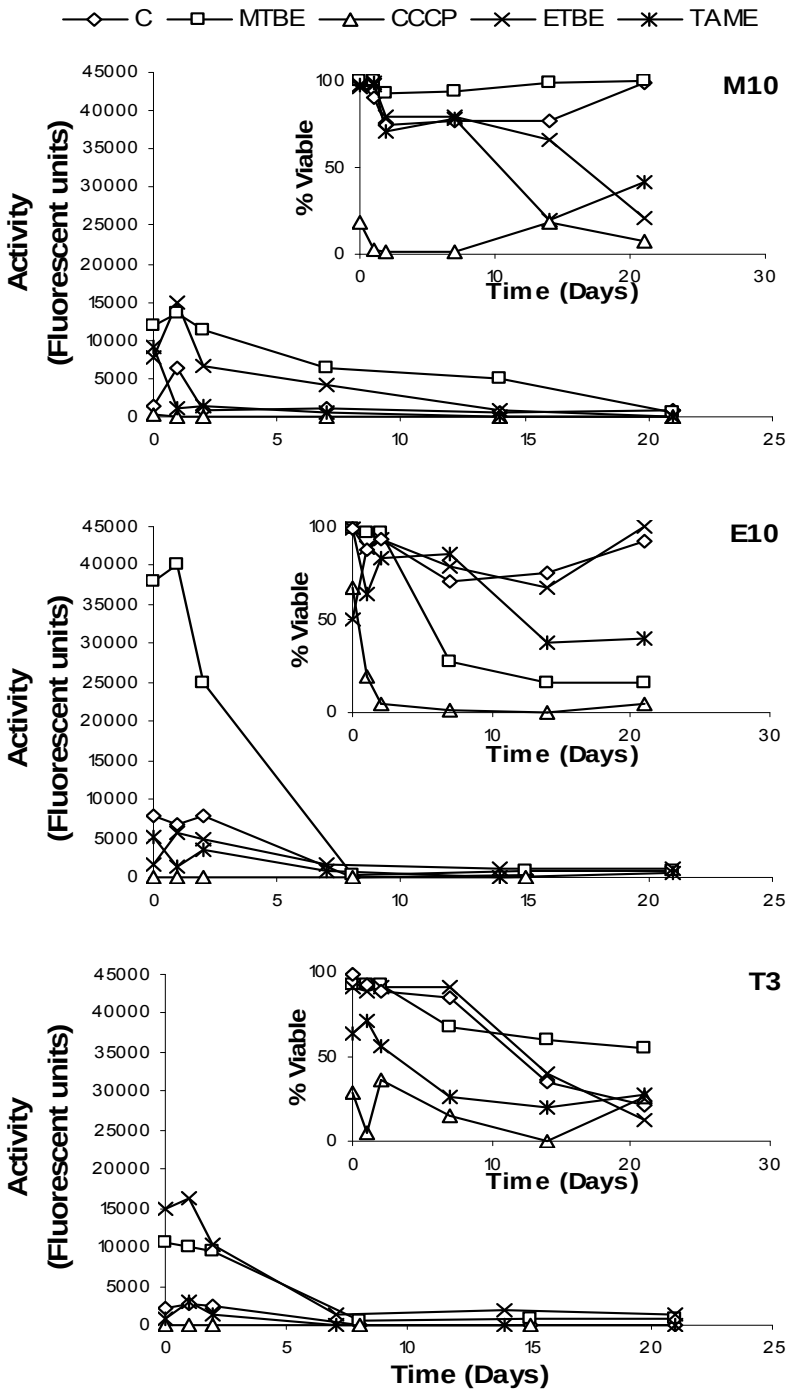

Figure 3. Cytometric pattern of activity and viability of the bacterial strains grown in a mineral salts medium amended with oxygenates. Fluorescent unit calculated by multiplying the mean fluorescence by the number of cells in the quadrant stipulated as active and viable. CCCP - Carbonyl cyanide m-chlorophenylhydrazone

Oxygenate influence on bacterial viability and activity

Poor growth was generally observed when the strains were grown in mineral salts medium amended with fuel ethers, thus generating interest into their physiological state. Cytometric analyses using fluorescent dyes PI and $DIOC_6$ were used to determine strain viability and physiological activity throughout 21 d incubation with MTBE, ETBE and TAME. The influence of the ether compounds on the strains was different in each case as can be observed in **Figure 3.**

A. calcoaceticus M10 was viable throughout 21 d with MTBE, 14 d with ETBE and 7 d with TAME before a reduction of 50% was observed. The activity recorded with MTBE was observed within the first 14 d, unlike with ETBE which was active 7 d, and in the presence of TAME, active only the first 24 h.

R. ruber E10 was viable 21 d with ETBE, 7 d with TAME and 2 d with MTBE before a reduction of 50% was observed. The highest activity was observed with E10 in the presence of MTBE, although only lasted 2 d. In the presence of ETBE and TAME, activity was observed in the first 2 d, though much lower.

G. amicalis T3 was viable during 21 d in MTBE and 7 d in ETBE. In the presence of TAME, although it was viable throughout the incubation period, the viability decreased ~ 75% after 2 d. The activity shown in the presence of MTBE and ETBE lasted 2 d, and with hardly any activity in the presence of TAME.

Consortia cell count and removal of fuel oxygenates and intermediates in batch cultures

Growth tests of the bacterial consortia M10-E10 and M10-T3 against all the oxygenates and intermediates TBA and TAA in batch systems were performed **(Figure 4).** Bacterial counts for each strain in the consortium since both strain colonies were different in colour, among other bacterial colony characteristics. Overall, joint growth of M10 and T3 did not favour any of the strains in the presence of the oxygenates or metabolites. The response of E10 in consortium M10-E10, was always favoured in the presence of the oxygenate, compared to its control, while M10 in the consortium M10-E10 was favoured or maintained its cell number. However, all curves show a loss in biomass with time. Degradation tests were also performed on the consortia with the MTBE, ETBE and TAME, however oxygenate removal was only observed in consortia M10-T3 with TAME **(Figure 5).**

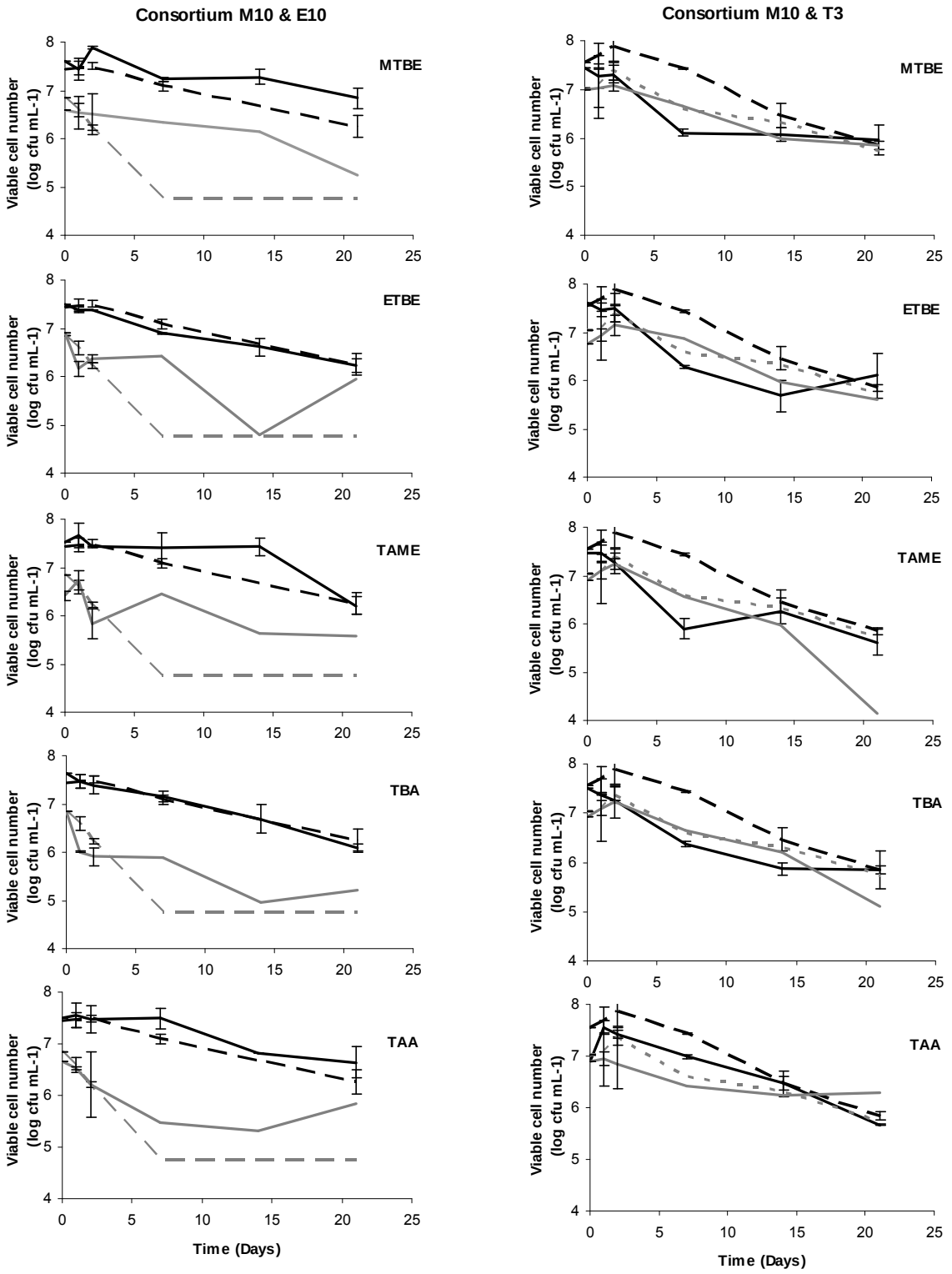

Figure 4. Batch consortium growth. Dotted lines are the control vials without oxygenates. ▬ M10 ▬ E10 or T3 in their respective columns

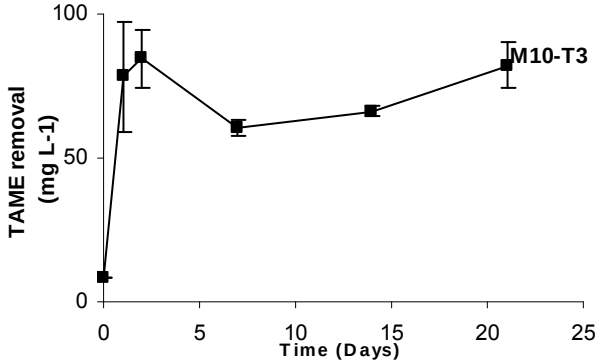

Figure 5. Batch consortium degradation. LSD – Least Significant Difference, for viable counts. ■ – M10-T3 consortium, □ – Abiotic control.

EPS production by the bacterial strains

The production of EPS was tested for each strain in order to assess initial attachment for biofilm formation, since EPS production and biofilm formation are factors known to be linked

(Bobrov et al., 2008 ; Branda et al., 2005). *A. calcoaceticus* M10 produced the most EPS after 1 d incubation (143 mg g^{-1} dry weight cell). No EPS production was detected under the conditions assayed for *R. ruber* E10 and *G. amicalis* T3. Although EPS production was performed after 1, 3, 5 and 7 d incubation with MY medium, no correlation was observed between EPS concentration against time (data not shown). The protein content of M10 EPS was 2% after a day's incubation. The EPS production results matched biofilm formation micrographs obtained by FESEM technique from Bioflow® 9 grids taken from the bottom of the biofilter after 5 d recirculation using MY medium.

Biofilm formation on polyethylene units

The washings and fixation steps in the SEM pre-treatment procedure have "destructive" effects, however units at different heights were used in order to evaluate biofilm formation, and the units were consistent with the representative images shown in **Figure 6**. *A. calcoaceticus* M10 was the most efficient in adhering to the support material **(Fig. 6aB),** followed by *G. amicalis* T3 **(Fig. 6aD).** *R. ruber* E10 was not successful in attaching or forming a biofilm on the Bioflow® 9 support (Fig. 6aC). *A. calcoaceticus* M10 was also recirculated in the biofilter with minimum mineral medium, nevertheless, the results viewed by the electronic microscope showed less efficiency in attaching to the material support.

Foaming was only observed in the biofilter inoculated with M10 and at a lesser degree with T3. A decrease in the air volume rate to 5 mL min^{-1} air decreased foaming considerably.

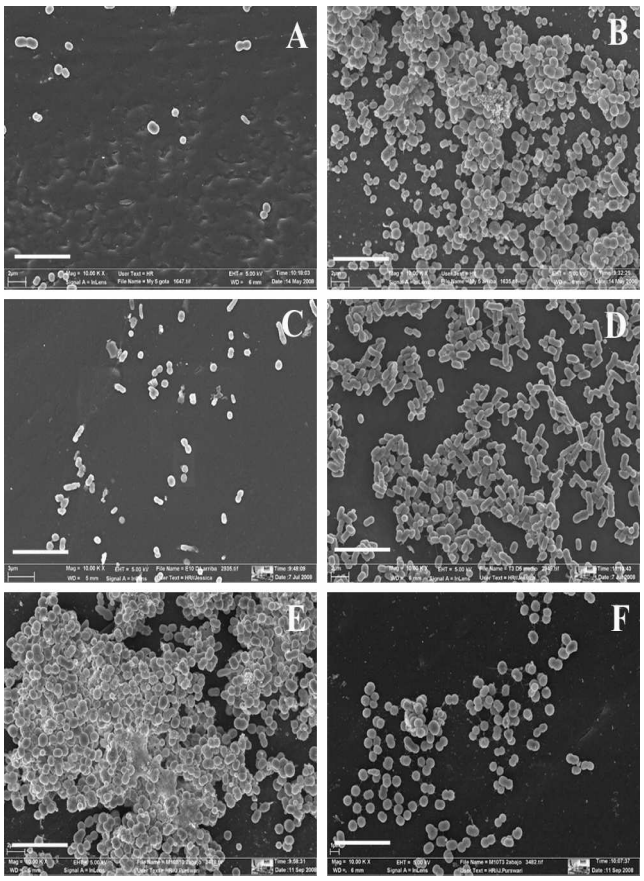

Figure 6a. Scanning electron microscope images of biofilm formation on the Bioflow 9 support. Samples shown were taken from the bottom of the biofilter after 5 d recirculation. A – Control (drop of *Acientobacter calcoaceticus* M10), B – M10, C – E10, D – T3, E – Consortia M10 – E10, F – Consortia M10 – T3. Scale bars represent 5 μm

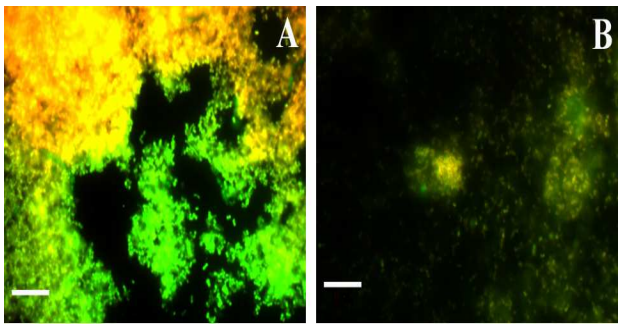

Figure 6b. Biofilter consortia *in situ* hybridisation with fluorescent probes for eubacteria (false coloured green) and gammaproteobacteria (false coloured red) observed with an epifluorescent microscope with the 100× optical lens. Controls were performed with individual strains. *A. calcoaceticus* M10 combined fluorescence is observed yellow. A – Consortia M10 – E10, B – Consortia M10 – T3. Scale bars represent 10 μm

Consortium biofilm formation on lab-scale biofilter

FESEM and FISH analysis were performed in order to evaluate successful colonisation and activity of the joint population. The capability of *A. calcoaceticus* M10 to attach successfully and form a biofilm on the surface of the polyethylene grids, made us consider two consortia: firstly *A. calcoaceticus* M10 and *R. ruber* E10 (M10-E10); and secondly *A. calcoaceticus* M10 and *G. amicalis* T3 (M10-T3). Biofilters were recirculated with a consortium for 5 d with MY medium, were stopped at different times, and samples were taken from different heights of the biofilter. Both consortia exhibited foaming. As can be clearly observed in representative SEM micrographs **Fig. 6aE** and Fig. **6aF,** biofilm formation was favoured for consortium M10-E10, and was thicker than biofilm M10 on its own (**Fig. 6aB).**

The viability and activity of the consortia were also tested, under an epifluorescent microscope after biofilm samples were processed using the FISH technique described **(Fig. 6b).** The consortium M10-E10 **(Fig. 6bA)** was much more dense and active, than consortium M10-T3 **(Fig. 6bB).** Controls were performed with individual isolates; overlapped falsely coloured images of strain M10 emitted yellow fluorescence, and both strains E10 and T3 emitted green fluorescence. The program DAIME was used to calculate a rough estimate of bioarea percentage of *A. calcoaceticus* against the total population, giving us also the rest percentile of either *R. ruber* E10 or *G. amicalis* T3. A total of 30 random snapshots were taken for each column in order to decrease the biased nature of the count. In both columns, *A. calcoaceticus* was represented in a median bioarea percentage of 75-80% of the total population. The spatial distribution for both strains in column M10-T3 was that of uniformity due to the poor aggregation observed, unlike column M10-E10, where *R. ruber* E10 was observed in most micrographs to be on the periphery of the *A. calcoaceticus* M10 agglomerate, observing mutual avoidance.

DISCUSSION

The aim of this study was to find a bacterial inoculum (individual strain or in consortium) that would be able to attach successfully to a support material in an oxygenate-remediating biofilter as well as maintain itself active in the presence of MTBE, ETBE and TAME. Due to the different concentrations found in groundwater, we established the initial

contamination concentration to be 100 mg $L^{-1.}$ Selective consortia have been previously used in numerous microbial bioremediation technologies both in terrestrial and aquatic contaminated environments (Chirnside et al., 2007; Luo et al., 2009; Mishra et al., 2001), each contributing to the same or different metabolic routes. Previously, established consortia have been used to evaluate ether degradation in bioreactors (Hu et al., 2004), and recently joint individual degrading strains have been evaluated in batch cultures (Auffret et al., 2009).

In this study, *A. calcoaceticus* M10 was the best biofilter colonizer from the three strains assayed, and thus both consortia tested contained this bacterium. Attachment and growth with the oxygenates mainly favoured strain E10 in consortium M10-E10, however, oxygenate analysis in batch experiments proved that the consortia actually biotransformed the oxygenates less than the strains on their own. Although great activity can be observed with this consortium as the FISH images show **(Figure 6bA),** spatial arrangement analysis of the individual strains determined mutual avoidance. As described by Wimpenny et al., (2000), "Two microcolonies might compete, cooperate, or be independent", and these two strains were definitely not cooperating under batch conditions. However, the fact that strain E10 was favoured in the growth and attachment experiments even though degradation was not taking place, could probably be caused by nutrient and adhesive properties of the EPS produced by M10. The area observed in **Figure 6bA** containing M10 was much higher than E10, confirming the importance of M10 for E10 attachment.

A. calcoaceticus M10 biotransformed TAME (Purswani et al., 2008) and TAA **(Figure 2)**. Flow cytometry tests show that in less than 24 h, physiological activity of strain M10 in the presence of TAME decreases, thus TAME and TAA concentration decrease should occur within this period. The attachment experiments also favoured strain M10 against the others, although this was to be expected due to the EPS production in liquid media. The only slight problem with M10 biofilm formation was the foaming produced.

R. ruber E10 previously shown to biotransform MTBE (Purswani et al., 2008), and in this study to biotransform TBA, is potentially a good bioremediating bacterium. The incapacity to attach to the polyethylene support could be compensated with the attachment in consortia with M10. Previously, a *R. ruber* strain (IFP2001) has also been described as a MTBE and ETBE biodegrader (Chauvaux et al., 2001). The physiological activity shown with MTBE biotransformation **(Figure 3)** suggests that strain E10 could be the focus of a genetic study, comparing this with the known MTBE/ETBE degrading genes (Chauvaux et al., 2001; Schmidt et al., 2008).

The successful attachment of *G. amicalis* T3, with poor EPS production, is probably

due to adhesin molecules. *G. amicalis* is a recently described species (Kim et al., 2000) and very little is known of its characteristics. Attachment of consortium M10-T3 was not favoured as shown by the SEM image **(Fig. 6aF)**. Poor aggregation and low fluorescent signals observed in FISH images along with negative growth shown in batch studies **(Figure 4)** allow us to believe that the consortium makes for a poor active biofilm **(Fig. 6bB).** The spatial analysis indicated that neither population avoided each other, nor were they arranged randomly. Moreover, M10 and T3 populations in batch growth assays in the presence of the oxygenates showed a decrease in population with respect to control assays, suggesting competition of nutrients. Biotransformation of TAME observed by consortium M10-T3 **(Figure 5)** mimicked that of M10 alone (Purswani et al., 2008), therefore most likely the consortium M10-T3 compete for the nutrients released after M10 has biotransformed TAME, decreasing the chances of energy available for growth.

G. amicalis T3 could also be used for MTBE bioremediation in a biofilter, although help from the indigenous microbial population from the water source would allow complete degradation to CO_2 and water, as it was not capable of biotransforming TBA. A previous study on nitrate removal from groundwater using a selective *Pseudomonas* denitrifying strain deduced that although the strain maintained itself through the bioreactor experiment, its biofilm count was reduced gradually to ~1%, however, maintaining nitrate removal efficiency throughout. This could only be accounted by the attachment of other microbial populations with higher denitrifying activity (de la Rua, 2007), thus initial inoculation aided attachment of other microorganisms. *R. ruber* E10 eliminated MTBE and TBA, and was very active in the presence of MTBE in batch physiological studies. However, E10 was not a good Bioflow® 9 colonizer, although this may be overcome in the future by immobilizing the cells in calcium alginate beads as previously described by Chen et al. (2008), or could be tested along with M10 in biofilter experiments. Although in batch studies, MTBE degradation was not efficient, this may be overcome in a biofilter due to the mutual avoidance observed in the latter system. Growth rates of the three bacterial strains should be improved through induction with other compounds to achieve higher degradation rates.

The addition of co-substrates such as iso-pentane and iso-butane might help increase the bacterial population number, and consequently increase oxygenate biodegradation, because as described by Lopes Ferreira et al. (2006) the induction of the alkane monoxygenase (MTBE/ETBE degrading protein) leads to MTBE and ETBE biotransformation to TBA. The use of ethanol as a co-substrate was previously evaluated (Purswani et al., 2008), although the addition of yeast extract may have inhibited the

degradation of the fuel ethers as did occur in some cases. The use of isopropanol in the biodegradation has been very successful in the bioremediation of MTBE in a biofilter inoculated with *Mycobacterium austroafricanum* IFP2012 in a working period of 25 d (Maciel et al., 2008). The efficiency of isopropanol against ethanol may be more interesting due to isopropanol being a metabolite of the MTBE degradation pathway.

The degradation pathway and the genes involved in TAME degradation have not been described; therefore a genetic study of the TAME degradation in *A. calcoaceticus* M10 could be of great use.

ACKNOWLEDGEMENTS

We would like to acknowledge the technical work performed by Jaime Lazuen Alcon, Juan de Dios Bueno Pérez, and Alicia González Segura from the flow cytometry, preparation of biological samples and scanning electron microscopy departments in the *Centro de Instrumentación Científica* from the University of Granada, Spain. This work was funded by the Spanish Ministerio de Ciencia y Tecnología, through the Programa Nacional de I+D (project reference CTM2006-02297).

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III.3-2. BIOLOGICAL AND CHEMICAL ANALYSIS OF A LAB-SCALE BIOFILTER FOR OXYGENATE BIOREMEDIATION IN HYPOTHETICAL RECENT GROUNDWATER SPILLS.

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Prepared for submission to the

Applied Microbiology and Biotechnology

ABSTRACT

Unleaded gasoline additives such as methyl *tert*-butyl ether (MTBE) are being substituted for others (e.g. ethanol, ETBE) due to the growing market for bioethanol. Nevertheless, MTBE is still widely found in aquifers and arise from: accidental spills directly or indirectly through soil lixiviation: leaking storage tanks; highway runoff; and gaseous MTBE trapped and brought down in the form of precipitation.

Microbial remediation of contaminated aquifers has been widely studied due that their relative efficiency/cost is low compared to other remediating techniques. In the light of previous studies, we assessed a control (without prior inoculation) and two inocula for MTBE bioremediation in a pilot-scale biofilter: *Acinetobacter calcoaceticus* M10 and a consortium between strain M10 and *Rhodococcus ruber* E10. Temporary bioremediation of MTBE (up to 80% removal) from the biofilter inoculated with the consortium was observed in 44 days. In addition, RNA and DNA TGGE profiles of the V3 region of 16srDNA of this biofilter was observed to contain more active strains than in the other two systems. The presence of *R. ruber* E10 (and absence of strain M10) in DNA and RNA extracts, along with the amplification of the *alkB* gene in E10, lead us to conclude that it was responsible for the bioremediation of MTBE in the biofilter with the consortium inoculum.

INTRODUCTION

Groundwater is the main drinking and irrigating source of water in most countries around the world. In several southern European countries such as Spain, Portugal and Italy, the existing groundwater abstraction is equal to or greater than the groundwater availability in the country. In addition, desertification is on the increase too [UNEP], thus, accidental spills or unnoticed leakages of unleaded gasoline, will quite often contaminate essential aquifers and endanger this natural resource. Fuel oxygenates such as methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME), substitutes of lead, reduce harmful emissions in motorised vehicles, however, they are also the second most found contaminant detected in aquifers in the USA (de Lacy Costello et al., 2005). The European Environmental Agency's acknowledgement of the problems arisen by fuel oxygenate contamination have been given less importance than the American counterparts, even though oxygenate spills have been detected throughout Europe (Germany, Austria, Belgium, Switzerland, Spain, Italy) in different environmental water bodies (Rosell, 2007). Presence of tert-butyl alcohol is also monitored, since this MTBE metabolite is more toxic than the initial contaminant.

Remediation technologies to palliate MTBE contaminated groundwater include adsorption via activated carbon and other synthetic sorbents, air sparging, membrane technology, phytoremediation and biological technologies among others.

Biological technologies have been tested with varying inoculants and at different operational conditions for MTBE removal, all with start-up times ranging from 10 to >150 days. (Waul et al., 2007).

In this paper, we describe the start-up and the effectiveness of a selected inoculated aerated submerged biofilter working under controlled conditions during two months and designed for removal or transformation of gasoline oxygenates from spiked unfiltered groundwater samples. This scenario would represent the use of a biofilter in a recently contaminated site.

Moreover, we studied the microbial diversity present in the system and the presence/absence of two monooxygenases -*Eth*B and *Alk*B-, depicted by Chauvaux et al., (2001) and Smith et al., (2003) respectively, using gene specific primers described recently by Auffret et al., (2009).

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade or the highest purity available and were purchased from commercial sources. Methyl *t*-butyl ether (99.9 % purity) was purchased from Fluka (Milwaukee, WI, USA) and TAME (97 % purity) were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

Groundwater samples

Unchlorinated groundwater samples (25 L) from a well located near Granada city (*Los Vados*, Granada, Spain), was taken and transferred to the laboratory in sterile tanks. Groundwater samples were tested for presence of oxygenates and BTEX, however, the groundwater was free of all of these compounds. Filtered (0.22μm nitrocellulose filter, EZGSWG474 - Millipore®) and unfiltered groundwater were transferred to sterile glass bottles, where they were amended with 100 mg/L of fuel ether, and used in continuous flow mode as influent in the biofilter. The glass bottles were not contained within a thermostat, and were changed daily with fresh amended groundwater.

Bacterial strains

The bacterial strains used in this study, *Acinetobacter calcoaceticus* M10 (*Colección Española de Cultivos Tipo* - CECT 7739) and *Rhodococcus ruber* E10 (CECT 7740), were isolated from a hydrocarbon-contaminated soil site (Purswani et al., 2008) and pre-selected due to their ability to biotransform gasoline oxygenates, keep up their physiological activity, and to form a biofilm on an inert support material (i.e. Bioflow® 9) in the presence of oxygenates MTBE and ETBE respectively (see section III.2).

Setup of biofilters

The laboratory-scale biofilter consisted in a glass cylindrical column (50 cm high and 6 cm diameter) filled with a polyethylene support material (Bioflow® 9) with a surface area of $800 m^2/m^3$. The glass cylindrical column and all its components were autoclaved and mounted under sterile conditions. Both air influent and effluent tubes contained a sterile 0.22 μm filter (Millipore®) in order to avoid contamination. Teflon (PFTE) tubing was used throughout the system, except at connections and at the pump region. Silicon and Marprene (Watson Marlow, Wilmington, MA, USA) tubing were tested, and the latter material was chosen because less oxygenate diffusion was observed since the porosity of Marprene (gas permeability, 5.8

O_2cc.cm x 10-8 / cm2.sec.atm) was smaller than that of the silicone tubing (gas permeability, 400 O_2cc.cm x 10-8 / cm2.sec.atm). Air stripping in the biofilters was assessed under different hydraulic retention times and air flow rates for MTBE, ETBE and TAME. Air stripping was assessed by subtracting the effluent ether concentration from the influent ether concentration a day (12 h and 24 h HRT) or two days before (48 h).

Three biofilters were setup at the same time and were named D, E and F. Biofilter D was selectively inoculated with bacterial strain *A. calcoaceticus* M10. The bacterial consortium formed by *A. calcoaceticus* M10 and *R. ruber* E10 was used as selective inoculum in biofilter E, and no strain was recirculated in biofilter F (a control biofilter).

The aerated inoculated biofilters were incubated under controlled temperature (20ºC) for 8 weeks in a continuous flow mode. The working conditions were the same for all the experiments: 5 ml/min air, recirculating (1.2 L/day flow) pre-grown inoculum in MY medium (Moraine and Rogovin, 1966) during 5 days amended with 100 mg/L oxygenate. To ensure an adequate starter biomass in the biofilter, a further recirculation period was established, recirculating filtered groundwater amended with 0.1 g/L glucose and 100 mg/L of MTBE for another 5 days. The control columns were not inoculated; however, uninoculated media was recirculated too.

After this initial period of biofilm formation on the support material, unfiltered groundwater amended with 100/150 mg/L of MTBE was used as influent in the system at a flow rate of 800 ml/d and HRT (hydraulic retention time) of 24 h, being the aeration and temperature conditions as previously referred.

Oxygenate analysis

The oxygenate concentrations of the groundwater samples were determined by GC/MS headspace technique described previously (see section III.2), heating the vials at 90 ºC for 90 min, with the scan mode. Quantification of MTBE and TAME was performed using an external standard calibration (R >0.99).

Biofilm sampling

Bioflow 9 ® grids with established biofilm were only taken at weeks 2 and 8 of each experimental period. Meanwhile, biofilm samples at 2 weeks belong to polyethylene units on the upper level of the biofilter, biofilm samples at 8 weeks were to those present in the whole system. Bioflow 9® units of each biofilter were resuspended in 1 x PBS (phosphate buffer saline solution) through vortexing and sonication during 30 s. The biomass was harvested and

treated according to the requirements of further procedures.

Observation of biofilm formation by Field-Emission Scanning Electron Microscope (FESEM) technique

Bioflow® 9 support samples were extracted at different column heights after 5 days recirculation period in MY medium. These were fixed in 2.5% glutaraldehyde in 1×PBS solution pH 7.4 during 24 hrs at 4°C. The samples were washed three times in 1×PBS during 20 min. Postfixed with 1% osmium tetroxide in the dark during 1 hr at room temperature and washed 3 times during 5 min with distilled water. Samples were dehydrated during 15 min in increasing ethanol concentrations: 50%, 70%, 90% and 100% (twice). Desiccation using the Anderson method (1951) with CO_2 in a critical point dryer CPD7501. The samples were covered by the evaporation of carbon with a HITACHI evaporator. Samples were observed using a LEO 1530 Field-Emission Scanning Electron Microscope.

DNA extraction

Genomic DNA extracted from filter samples and biofilm samples were performed using the "FastDNA®SPIN Kit for Soil" and the FastPrep® Instrument (MP Biomedical, Santa Ana, CA, USA). Genomic DNA extracted from each cultivable strain was performed using the method described by Martin-Platero et al., 2007.

Weekly, 2 L groundwater influent samples were filtered through 0.22 μm nitrocellulose filter (EZGSWG474 - Millipore). Each filter was stored in a sterile 15 ml falcon tube, and processed as described previously (see Section III.3.1. Purswani et al., 2011, *in press*)

Temperature gradient gel electrophoresis (TGGE) technique

Temperature gradient gel electrophoresis (TGGE) was performed according to Calderon et al., (2011), after amplification of 16S rDNA with the primer set fD1 (5´ - CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG - 3´) and rD1 (5´ - CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC - 3´), and the anchored amplification of the V3 region in the 16S rDNA with the primer set GC-P1 (5´ - CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCA GCAG - 3´) and P2 (5´ - ATTACCGCGGCTGCTGG - 3´). Pattern differences and similarities were analysed with GelComparII program using Pearson correlation coefficient.

EthB and AlkB PCR of biofilm samples

Polymerase chain reaction (PCR) amplification of known ether degrading monoxygenases such as *ethB* and *alkB* were tested on the total genomic DNA extracted from biofilm samples developed on Bioflow 9 ® grids at weeks 2 and 8, as well as from bacterial strains isolated from the biofilm samples, and strains M10 and E10. The PCR conditions for the *alkB* gene were: 96ºC for 7 min; a 30 cycle of 96 ºC for 30 s, 40 ºC for 40s, and 72 ºC for 1 min; and an extension of 10min at 72 ºC. The PCR conditions for the *ethB* gene were: 96ºC for 7 min; a 30 cycle of 96 ºC for 30 s, 40-60 ºC gradient for 40s, and 72 ºC for 1 min; and an extension of 10min at 72 ºC. The primers for *ethB* amplification were: ethB-F2: 5'-CACGCGCTCGGCGACTGGCAGACGTTCAGT-3' and ethB-R2: 5'-TCCGACGCACATGTGCGGGCCGTACCCGAA-3'.

The primers for *alkB* were: alk-H1F – CIGIICACGAIITIGGICACAAGAAGG and alk-H3R – IGCITGITGATCIIIGTGICGCTGIAG (Auffret et al., 2009).

These were first tested on the genomic DNA of the biofilms at weeks 2 and 8. *eth*B gene did not amplify under any conditions tested, and thus were not tested on cultivable strains.

Toxicity analysis

Microtox® system was used to measure acute toxicity on effluent samples from the biofilters at weeks 0, 2, 4, 6 and 8. The bioluminescent *Vibrio fischeri* was grown against the effluent samples, where toxicity was expressed as the reduction of 50% luminescence from its initial luminescence (EC_{50}) (Onorati F. et al., 2004).

RNA extraction and retrotranscription of biofilter samples

Total RNA was extracted biofilm samples using the FASTRNA® PRO KIT with the the FastPrep® Instrument (MP Biomedical, Santa Ana, CA, USA), followed by a DNase digest. Samples were purified with Qiagen RNAeasy Total RNA purification kit. We then performed reverse transcription on the RNA samples using the Superscript II (Invitrogen) as decribed in the manual, and used the cDNA to amplify the 16S rDNA, followed by the V3 region (with the primers described above).

RESULTS AND DISCUSSION

Microbial bioremediation of fuel ethers have been described previously (Waul et al, 2007), however the approach offered in this study, evaluates the chemical and biological aspects implied in the bioremediation of ether contaminated groundwater using an aerated submerged biofilter at lab-scale, with selected bacterial inocula. The groundwater used was tested for ether presence before starting the assays; no fuel ethers were detected (i.e. 0.01 ppb or higher). Thus the case scenario is the use of the biofilter in a recent groundwater ether-contaminated site, assuming that the bacteria in the incoming groundwater, have not been pre-selected by the presence of fuel ethers.

In order to evaluate the biofilter performance, we ventured into addressing the most difficult aspect of designing a biofilter for these purposes, i.e. air stripping. As **Figure 1** shows, two clear patterns exist: higher rate of air and higher HRT resulted in a higher concentration of ether removal through air stripping. However, the conditions whereby least fuel ethers were removed were not chosen, since a HRT of 12 h would be insufficient for ether degradation due to the slow rates of degrading bacteria, and no air would have meant insufficient oxygen for correct degrading conditions. Thus, a HRT of 24 h and a 5 ml/min air flow rate was chosen for all assays performed.

As described previously, three biofilters were setup at the same time and were named D, E and F. Biofilter named D was selectively inoculated with bacterial strain *A. calcoaceticus* M10. The bacterial consortium formed by *A. calcoaceticus* and *R. ruber* E10 was used also used as a selective inoculum in biofilter E, and no strain was recirculated in biofilter F (a control biofilter). To confirm biofilm formation on Bioflow 9 ® grids, SEM micrographs (**Figure 2**) were obtained after the recirculation periods with MY medium and the filtered groundwater amended with glucose, and before the entry of groundwater amended with MTBE.

MTBE concentrations, external temperature and toxicity units, monitored throughout the 8 week period, are shown in **Figure 3**. The influent MTBE concentrations were kept constant (**Figure 3a**) throughout the experimental period; this could have been different since the influent was not stored under a controlled temperature. The difference at day 1 between the influent and the effluent after the treatments, is mainly due to air stripping of the ether compound. The temperature value (Figure 3b) showed slight fluctuations since the seasonal period was July to September.

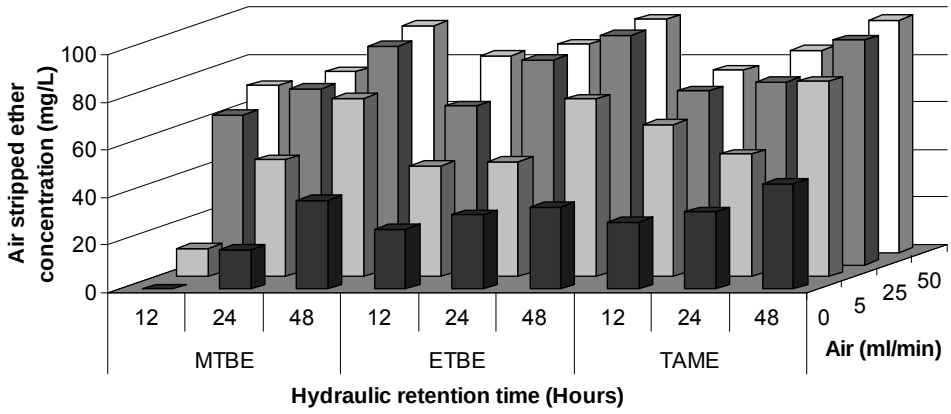


Figure 1. *Air stripping* of ether compounds at different hydraulic retention time and volume of infused air. Air pressure was constant at

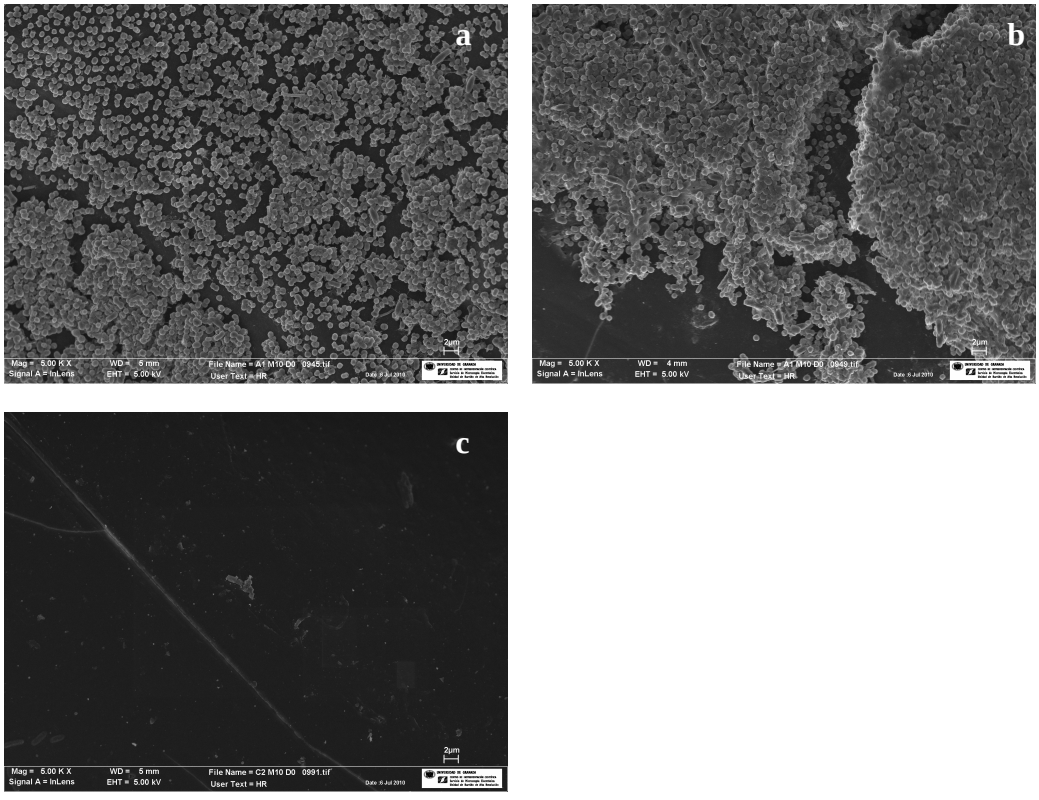


Figure 2. SEM micropgraphs on day 0 from the support material in biofilter columns treating MTBE. a) Inoculated biofilter with strain M10, b) inoculated biofilter with strain M10 and E10, c) uninoculated biofilter). Magnification: 5000x.

With regard to effluent MTBE concentrations, biofilters D and F, showed a common tendency and this was different to that of biofilter E. Although at specific points, biofilter F was significantly lower than biofilter D, they both had increasing tendencies, which could be accounted by a decrease in external temperature and therefore decreasing air stripping. Biofilter E has a negative tendency, implying removal of MTBE, reaching its lowest at day 44. *Tert*-butyl alcohol was not detected throughout the sampling period. The lowest toxicity units (**Fig 3c**) for biofilter E was found around the time of the lowest effluent concentration. The comparison between the decrease in MTBE concentration, toxicity, and decreasing dynamics in the influent at the same time (**Figure 4b**), can only be explained by an external factor: the presence of groundwater MTBE degrading-strains, which were being established in all biofilters. However, we believe that strain E10 seems to play a large part, since in the other two biofilters, there was no significant MTBE concentration decrease.

Another possibility could be that the stabilization of the microbial community in the influent helps stabilize the degrading microbial population by reducing competition. The latter theory could be explained through the TGGE profile similarities observed among the populations of the influent and the biofilms (**Figure 4a**). The biofilm communities present in biofilters D, E and F at week 2, are clustered with the communities present in the influent during weeks 1, 2 and 3 (clustered at ~45% similarity). The same pattern was observed with biofilter profiles in week 8 (clustered at ~65% similarity); however, these were more similar than those in week 2. Thus the changes in biofilm were directly due to the microbial influent from the previous two weeks.

The DNA and RNA profiles of the biological community at the end of the experiment (week 8) were drawn in two Venn diagrams after processing the data of TGGE profiles as observed in Figure 7c and SM1a for DNA and RNA extracts respectively. Venn Diagrams were drawn with the statistical package Venn Diagrams for R (Chen and Boutros, 2011) (Figure 5) showing the band similarities and differences between the biodiversity among the three biofilters. The biofilm community detected through DNA extracts, were not entirely active even though present. Clearly indicated via number of RNA bands, the microbial community present in biofilter E was more active compared to those in biofilters D and F. The percentage of active strains vs. total number of strains (as accounted by the TGGE profiles) were 0%, 46% and 18% in the biofilters D, E and F, respectively.

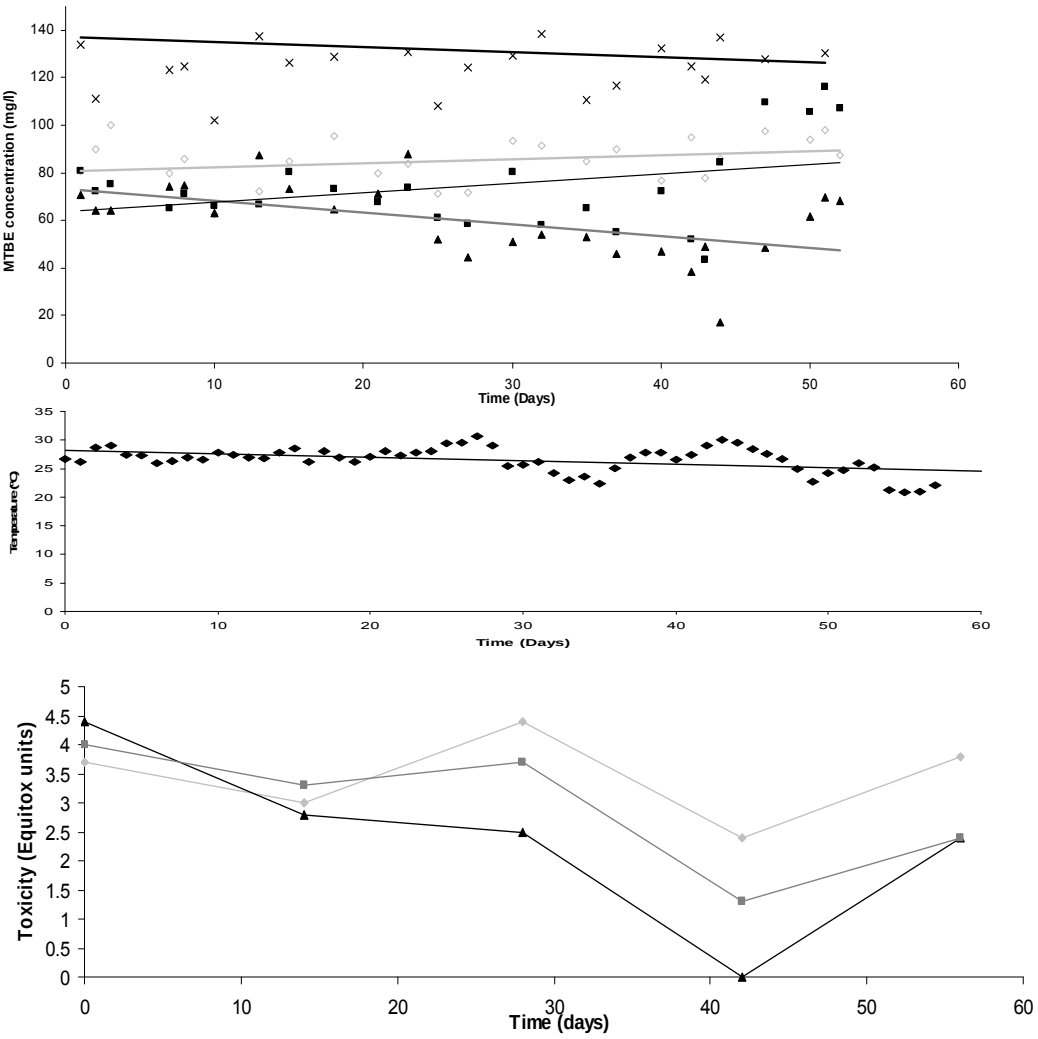
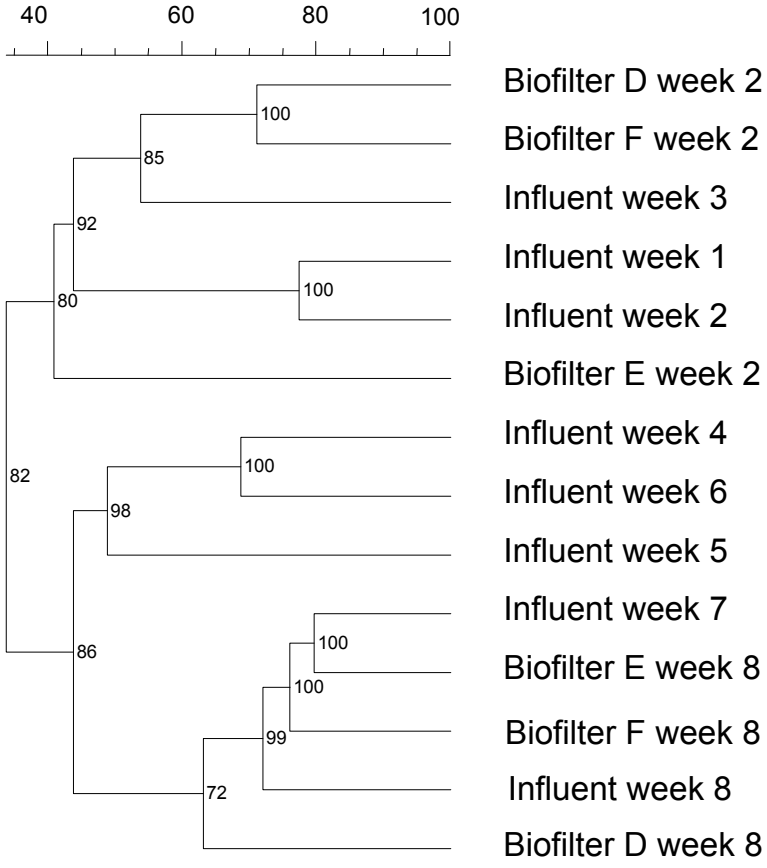
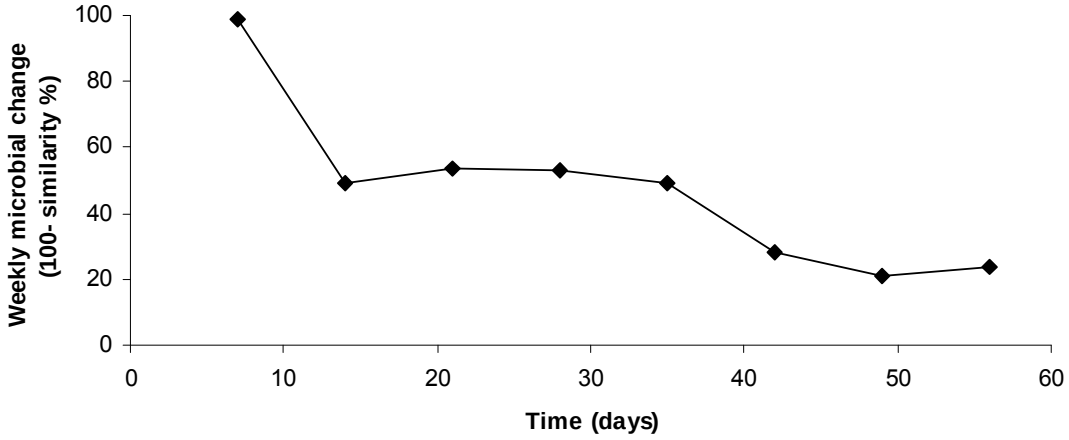


Figure 3. a) MTBE influent and effluent concentrations from biofilters b) External temperature during the experiment period. c) Microtox evaluation of the effluent of columns D, E and F. × – influent, ♦ – Acinetobacter calcoaceticus M10 inoculated biofilter.▲ – Consortia M10–E10 inoculated biofilter■ – uninoculated biofilter

The TGGE bands from DNA and RNA biofilm extracts were re-amplified, sequenced, and compared to those present in the NCBI database (Figure 7b, Figure SM1a and b). The relative band intensities were used along with the corresponding taxonomic assignations to compile qualitative pie charts for the DNA present in the biofilters at weeks 2 and 8 (Figure 6). Little community changes from the taxonomically identified bands, occurred from weeks 2 to 8. So if the biofilm present in week 8 are linked to the community present in the influent, we can only assume that the changes from week 2 to 8 have occurred mainly within the population established as SUOS (sequenced bands, unlike other strains).

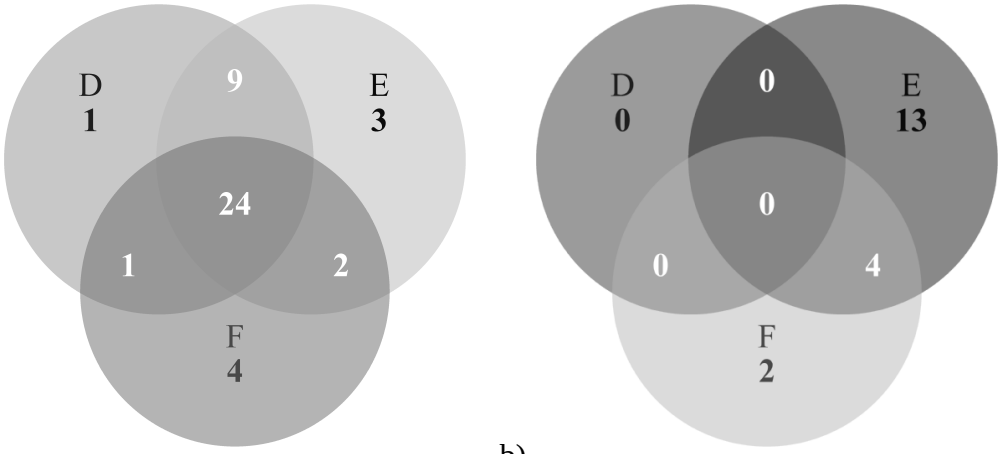


a)



b)

Figure 4. a) Cluster analysis of the biofilter columns treating MTBE, using the Pearson correlation (position tolerance - 0.26). D – Inoculated biofilter with strain M10, E – inoculated biofilter with strains M10 and E10, and F – uninoculated biofilter b) Microbial dyanamics of the influent,of biofilters treating MTBE during the experimental period as described in Mazorati et al(2008).



a) b)

Figure 5. Venn diagram showing V3 band sharing among the three biofilters at the end of the 56 day period with MTBE from a) whole genomic DNA extracted samples and b) whole genomic RNA extracted samples. D – Inoculated biofilter with strain M10, E – inoculated biofilter with strains M10 and E10, and F – uninoculated biofilter.

Bacterial isolates from biofilm samples at week 8 were grown on solid mineral medium in a MTBE gas chamber. The phylogenetic tree shown in **Figure 7a**, shows the 16S rDNA sequences of the cultivable strains, along with highly similar strains from the NCBI database. Cultivable strains were numbered according to the biofilter from which they were chosen (DD1 belong to the biofilm in biofilter D, etc). Amplification of the gene *alkB* was performed on all isolated strains, and those which were positive, are indicated with a closed circle. Only *alkB* amplification is shown since preliminary *alkB* and EthB amplification of the genomic DNA of each biofilter at week 2 and 8 was performed, resulting in no *ethB* amplification, and *alkB* amplification in all conditions (with different intensities).

It is worth mentioning, that no cultivable strain or TGGE band was identified as *A. calcoaceticus* in either week 2 or 8 in DNA extracts (Figure SM1b), nor among the cultivable strains (**Figure 7a**), however, RNA TGGE band 415 from biofilter E was identified as *A. calcoaceticus* M10.

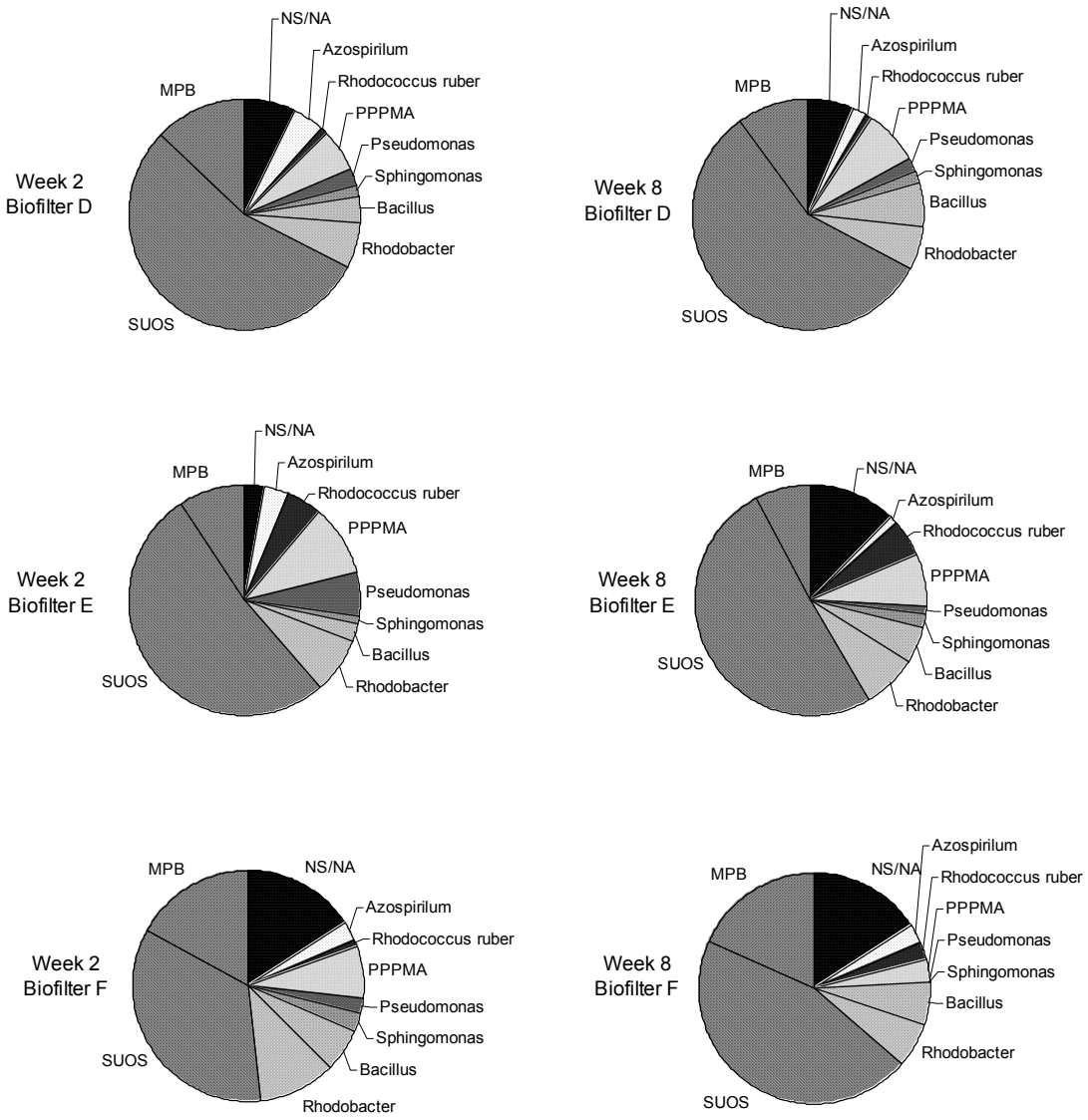


Figure 6. Relative DNA quantitative band intensity at weeks 2 and 8. NS/NA – Not sequenced/not amplified. SUOS – Sequence, unlike other species (85% or lower similarity). MPB – Mixed population in a single band. PPMA – Sequence identified with the following species: *Pseudoxanthobacter, Parvibaculum, Paracoccus, Maritaleas, Albidovulum*

The absence of this strain in biofilter D in all microbial extracts and microbial isolates imply that the individual strain could not maintain stability within the biofilm and was washed away quickly. High populations of *R. ruber* were found in all biofilter communities with TGGE analysis of DNA extracts, but were highest in biofilter E (**Figure 6**). In TGGE analysis of RNA extracts, presence of strain E10 is observed in biofilter E (**Figure 7b**).

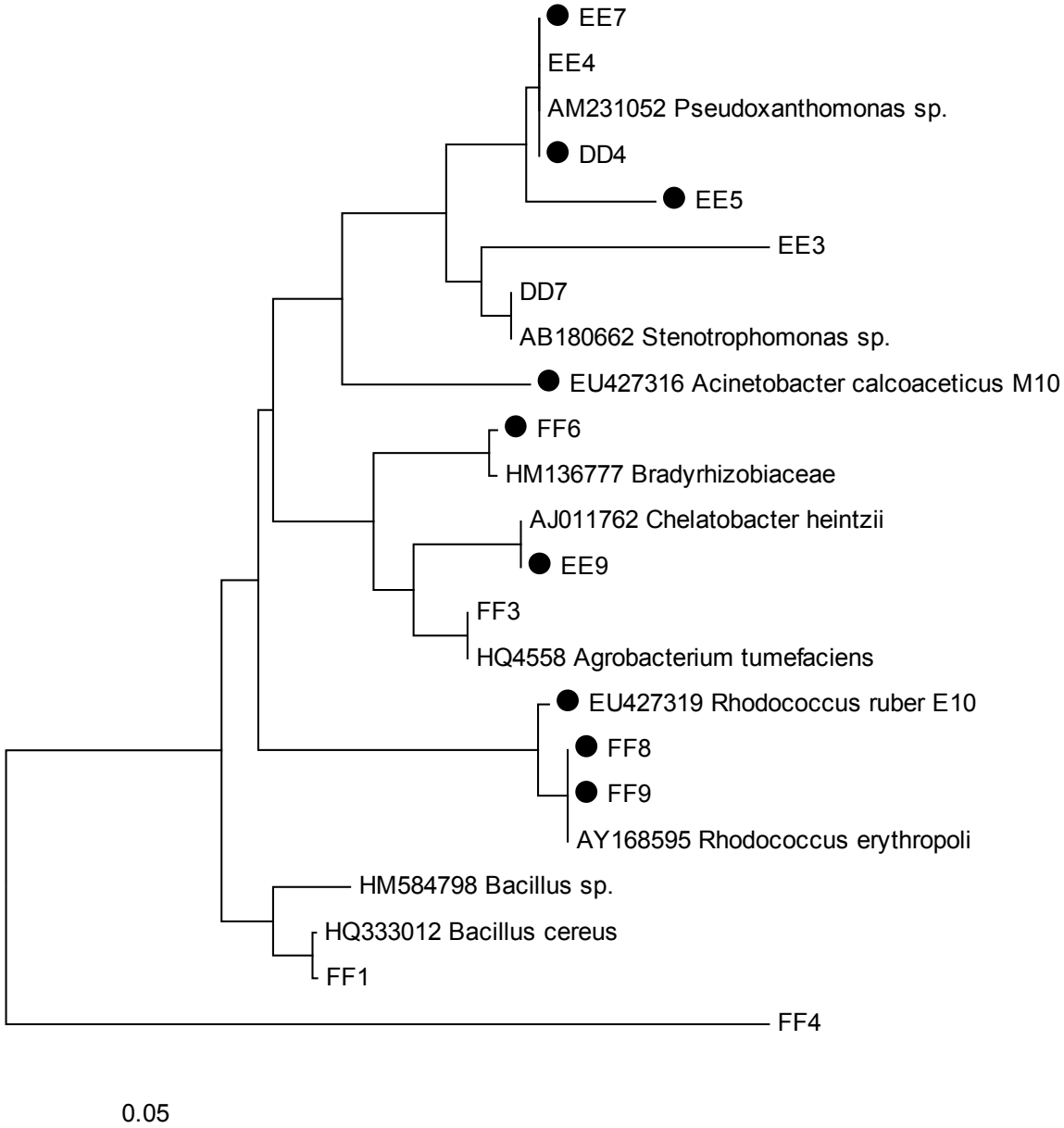


Figure 7a. Phylogenetic neighbor-joining tree of the 16S rDNA sequences of cultivable bacteria isolated from biofilm biofilter samples. Isolate strains are named after their corresponding biofilter. ● - Positive *alkB* amplification

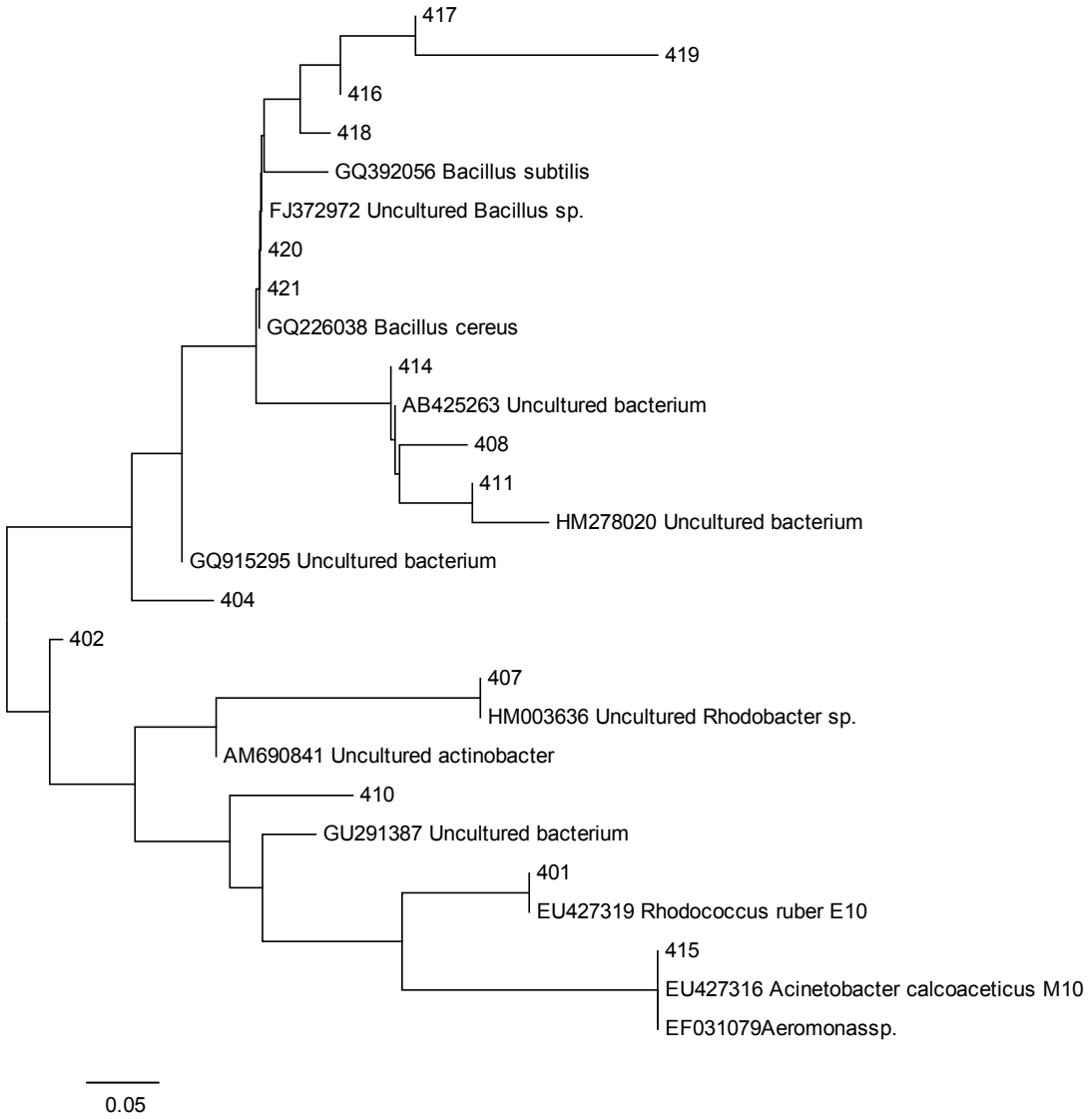


Figure 7b. Phylogenetic tree of V3 rDNA TGGE band isolates belonging to biofilm biofilter RNA extracted samples. Band isolates 401-415 belong to biofilter E. Band isolates 416-421 belong to biofilter F.

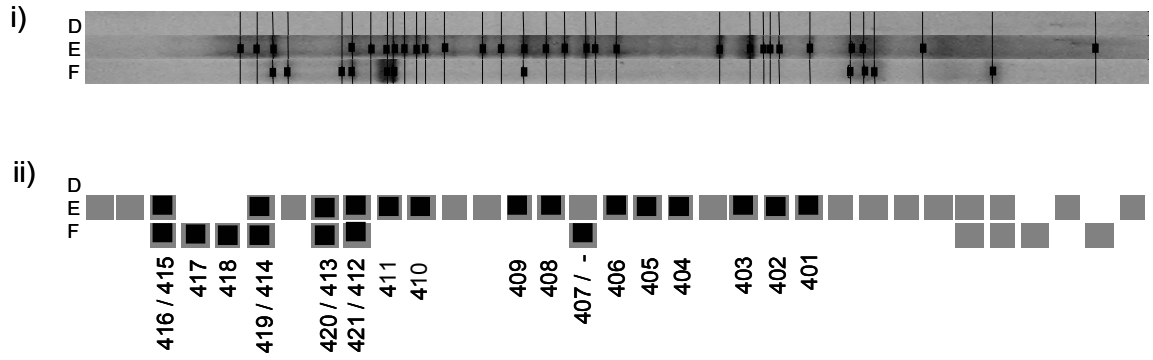


Figure 7c. i) TGGE image of V3 rDNA from RNA extracted biofilm samples. c.ii) Presence (grey box)/absence (no box) of band. Black boxes represent the position of bands isolated. Sequences retrieved from the NCBI database are indicated with their corresponding accession number.

Thus we can state that the initial seeded M10-E10 consortium was still present and active after 8 weeks, but that the concentration of E10 is probably higher than M10 since strain M10 was not detected among the TGGE band isolates. We also tested for *alkB* amplification of the bacterial strains *R. ruber* E10 and *A. calcoaceticus* M10, and both resulted positive, therefore we infer that consortium M10-E10 is the primary reason for MTBE removal in biofilter E.

Strangely, we were not able to retrieve back strains M10 or E10 among the cultivable bacterial isolates. A biofilter study (de la Rua, 2007) treating nitrate rich groundwater, observed that the selective denitrifying inoculum was not working, and was substituted with other groundwater autochthonous strains which were decreasing nitrate concentrations. Although we don't believe this to be what is happening with our seeded consortia, we have observed that the microbial changes in the influent, might alter the stabilization of the inoculum in the biofilm.

As a whole, we did not find any other cultivable strain present among the TGGE RNA bands, where the *alkB* gene positively amplified, even though we did find many cultivable bacteria that contained the gene in all three biofilters (1 strain in biofilter D, 3 strains in biofilter E and 3 strains in biofilter F). As a consequence, we must state that amplification of the *alkB* gene of biological samples in biotechnologies does not imply exclusively MTBE degradation.

A further assay was performed in order to see if TAME could be degraded with *A. calcoaceticus* M10, however, as can be observed in Figure 8, the effluents maintained the same pattern as the influent, suggesting that its gradual decrease was due to a gradual increase in air stripping. No further data was analysed since no reduction on effluent TAME concentrations was observed.

In previous studies, strain E10 was found to be physiologically very active in the presence of MTBE and the exclusive use of this strain may be sufficient for MTBE bioremediation. However, its inability to attach the Bioflow 9 units require for a consortium inoculum in the biofilter design presented. Further studies need to be implemented in order to establish *R. ruber* E10 as individual or consortium inoculum apt for scale-up, along with longer experimental time since other studies have found start-up time to be over 100 days (Arvin et al., 2003; O'Connell, 2001).

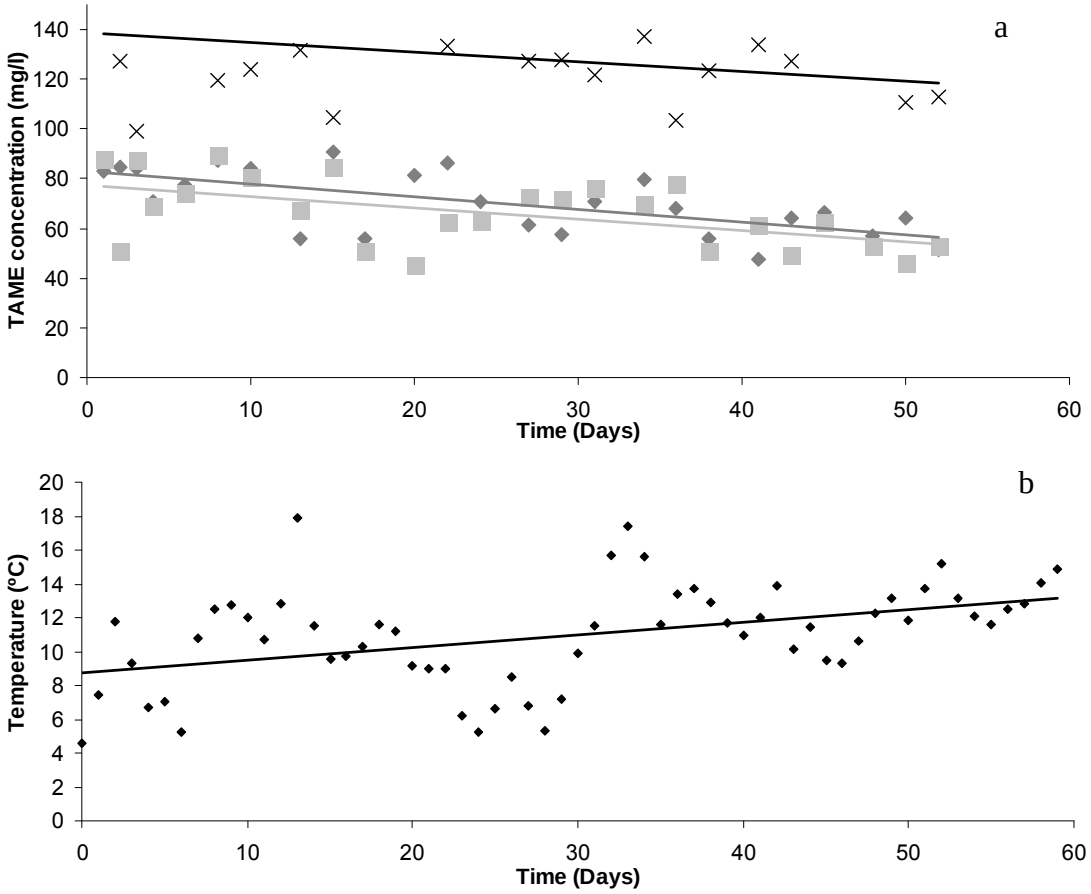


Figure 8. a) TAME influent and effluent concentration from biofilter. × - influent, ■ – uninoculated biofilter, ♦ - Acinetobacter calcoaceticus M10 inoculated biofilter. b) External temperature during the experiment period.

A denser biofilm and probably continuous consortium inoculation may help stabilise the biofilm. Reactors that use biofilms can maintain high biomass concentrations and are meant to be more resistant to external environmental changes (Bryers and Characklis, 1989), however, we did not observe high biomass concentrations at the end of the experimental period (data not shown). Co-metabolism such as alcohol injections observed in the work of Maciel et al (2008), or of alkanes, could help the microbial community grow better and further induce the genes responsible for MTBE degradation, such as *alkB*. Higher biomass concentrations were described to efficiently increase MTBE removal (Read :Wilson et al 1999). The use of an MTBE adsorption material described by Baus and Brauch (2007) for a packed biofilter such as the one described in this study, may also favour microbial attachment and growth, as well as removing high removal levels of MTBE from the liquid flow. Entrapment of bacterial cells such as the "water-in.oil-in-water" double emulsion described in Pimentel-Gonzalez et al. (2008) could also be used as a way to prevent loss of lab-selective degrading microbial

population in bioreactors.

ACKNOWLEDGEMENT

We would like to acknowledge the technical work performed by Juan de Dios Bueno Perez, Alicia Gonzalez Segura and David Porcel Muñoz from the preparation of biological samples, scanning electron microscopy and confocal laser microscope departments in the *Centro de Instrumentación Científica* from the University of Granada, Spain. This work was funded by the Spanish Ministerio de Ciencia y Tecnología, through the Programa Nacional de I+D (project reference CTM2006-02297).

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SUPPLEMENTAL MATERIAL

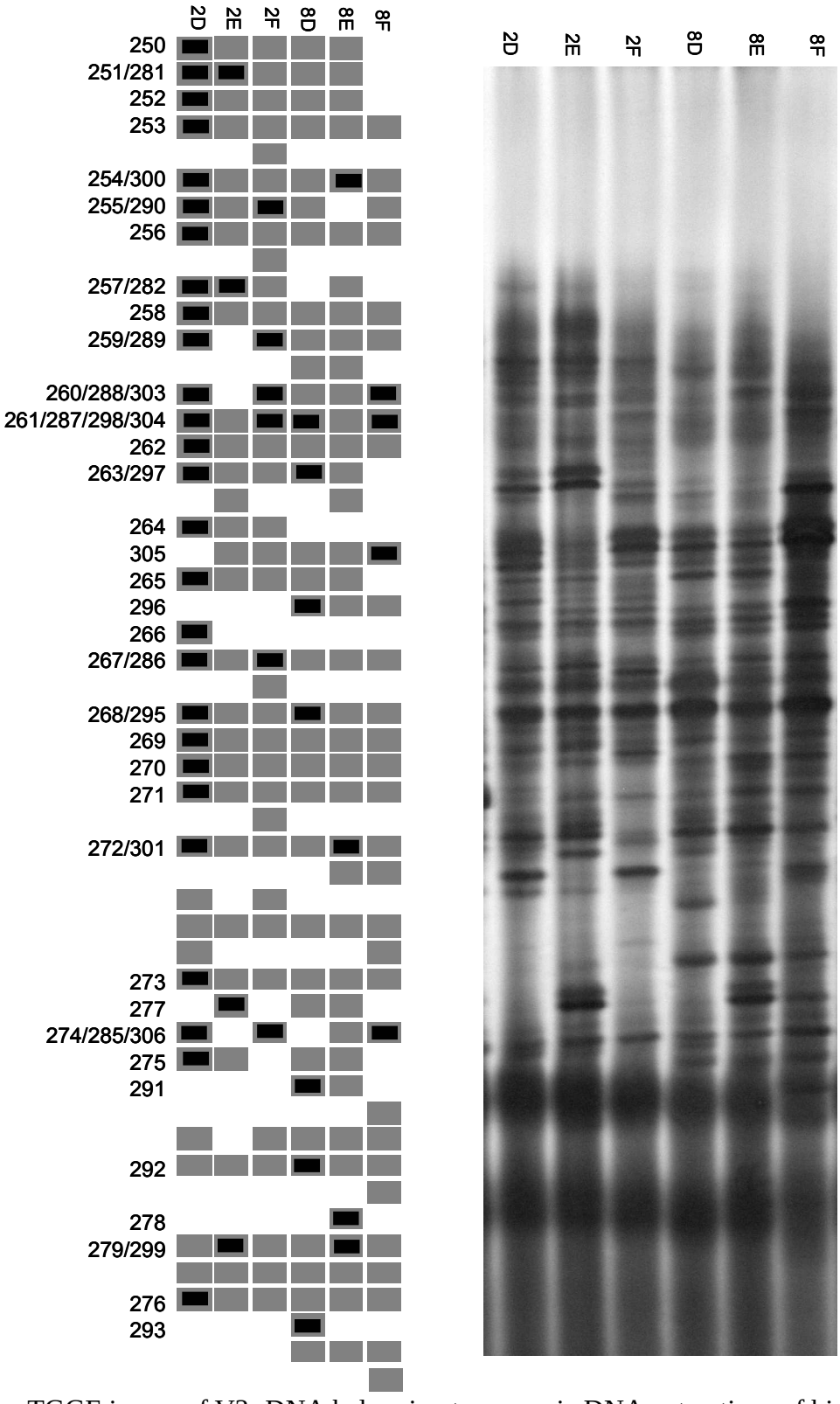

Figure SM1a. TGGE image of V3 rDNA belonging to genomic DNA extractions of biofilm biofilter samples. Presence (grey box)/absence (no box) of band. Black boxes represent the position of bands isolated

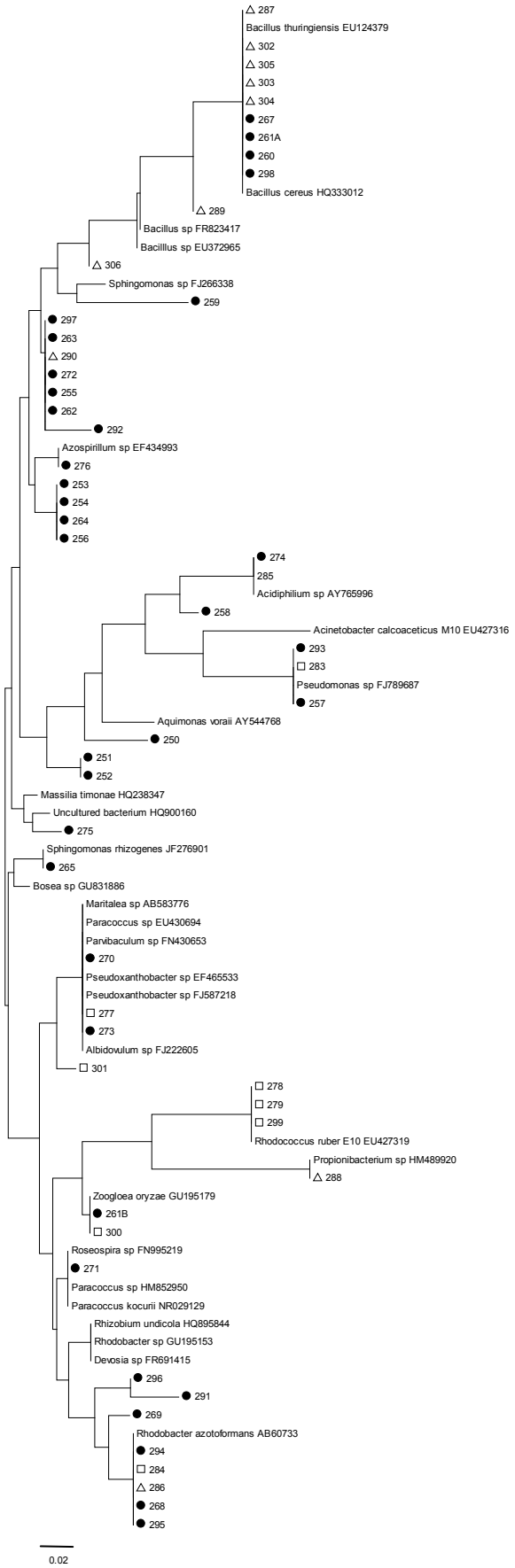

Figure SM1b. Phylogenetic neighbor-joining tree of the V3 rDNA sequences of TGGE band isolates from biofilm biofilter samples.

CAPÍTULO IV. OVERALL DISCUSSION

As described in the introductory section, MTBE, ETBE and TAME are semi-volatile man-made compounds produced mainly for the substitution of lead in gasoline. The utilization of these compounds have helped in the reduction of many toxic fumes from motor vehicles, however, their physicochemical properties have also made them readily present in aquatic sources due to poor detainment of storage fuel tanks, or the accidental spills. Human consumption tests of these ethers are not available; nonetheless, inhalations of the ethers and long term exposure could cause more serious chronic health problems like those observed in rat assays (Lee et al., 1998).

Palliation of contaminated groundwater has been addressed by many scientists with different backgrounds resulting in remediation technologies such as: air sparging, adsorption, chemical oxidation, and biological technologies among others. Different biological technologies have been proposed in the past: packed bed reactors (Kharoune et al., 2001), membrane bioreactor (Pruden et al., 2001), fluidized bed reactor (Pruden et al., 2003), and aerobic upflow sludge bed reactor (Acuna-askar et al., 2000)

The evaluation of a biological aerated filter for the bioremediation of fuel oxygenates MTBE, ETBE and TAME, was the goal of the studies described in the previous chapters. In order to comply with the study, a set of pre-steps were needed, thus the order of study followed was: selection of fuel ether biodegrading strains, set-up and design of a pilot-biofilter, and finally, the evaluation of seeded biofilters with theoretically recent contaminated groundwater.

MTBE, the most studied ether compound since it was the most produced and used, was considered recalcitrant to microbial attack, until in 1994, Salanitro et al, found a consortium which degraded the oxygenate, and as from this date, different mixed cultures and strains have been described to degrade MTBE (Debor and Bastiens, 2007). During the selection of microbial degrading strains we encountered a set-back, i.e. many of the chosen strains did not survive a second round of plate selection. One of the main reasons could be the co-dependence of an “uncultivable” colony to the excreting factors produced by its neighbouring colony (D'Onofrio et al., 2010), and thus the reason for observing many different colonies on a first round, and a more limited selection after the second round of plate selection. In addition, we also found that more bacterial isolates grew on MTBE than on ETBE or TAME (Chapter III.1; Table 1). We managed to select a total of 9 bacterial strains, of which 7 were Gram positive belonging to the order *Actinomycetales* and genus *Bacillus*. Similar results were found by Bartling et al., (2011), i.e. increasing predominance of Gram positive bacteria was observed when evaluating the microbial community changes in the

presence of ETBE and TAME in soil.

In our study, the strains were then tested for their biotransformation capacities on individual ethers (Chapter III.1; Figure 7), along with the microbial count up to 21 days. Good growth curves were not obtained when grown on the ethers as sole carbon and energy source. Müller et al. (2007) revealed that slow growth on MTBE is due to slow kinetic degradation rates, conferring slow energy release. Co-metabolic studies had been described with alkanes, BTEX, and ethanol (Hyman et al., 200), and thus we ventured into growing our strains too in co-metabolic media with ethanol and very low yeast extract amounts to see the outcome. Growth was favoured highly in all strains, but oxygenate removal was not in most cases (except TAME removal with strain *Arthrobacter* sp. MG). In a study by Hu et al. (2004), yeast extract concentrations in a bioremediating biofilter inhibited MTBE removal, and thus co-metabolic media was discarded. We chose strains that could biotransform the ether compounds as sole carbon and energy source, thus selecting *Acinetobacter calcoaceticus* M10, *Rhodococcus ruber* E10 and *Gordonia amicalis* T3 for further studies. Previous *Rhodococcus* strains (Mo et al., 1997; Chauvaux et al., 2001) had been described to biodegrade MTBE, with TBA accumulation since it did not contain the following enzymatic machinery for the following degrading mechanisms. Another important MTBE degrading Actinomycete described in the literature is *Mycobacterium austrafricanum* (strains IFP2012 and IFP2015) (Ferreira et al., 2006). Another taxonomic group which hold many other MTBE degrading bacteria is Burkholderaceae; *Methylibium petroleiphilum* PM1 (Hanson et al., 1999) and *Aquincola tertiaricarbonaris* L108 (Rohwerder et al., 2006) have been described to biodegrade MTBE completely.

The three chosen strains were evaluated further for growth and biotransformation of metabolites TBA and TAA, since MTBE, ETBE or TAME removal did not exactly imply complete degradation, and these metabolites could also be water contaminants if accumulated, especially TBA. From our results shown on Chapter III.3.1; Figure 2, TBA was removed by strains M10 and E10, and TAA was only removed by strain M10, therefore M10 was degrading MTBE and TAME, and E10 was degrading MTBE, further than their first metabolites. Their physiological activity was also tested using flow cytometric analysis in order to evaluate whether they were viable and active, even when no growth or poor growth had been observed. A lot of information was obtained from this assay; however, many results seemed to be inconclusive. For example, many strains were viable during the 21 day experiment in the presence of certain oxygenates, but did not show much activity. The latter is probably due to defence mechanisms, which do not entail high membrane activity.

Pseudomonas putida DOT-T1E contains efflux pumps (Ramos et al., 1998; Mosqueda and Ramos., 2000) which can tolerate high levels of toluene and other toxic organic compounds, however, the use of this mechanism would entail a high membrane activity, and so we do not believe that these strains contain such pump systems since their physiological activity was low with fluorochrome DIOC6. The conditions whereby activity values were significant, did not last more than 7 days, and in most cases, resulted in decreasing viability after loss of activity e.g. in strain E10 with oxygenate MTBE. Under other conditions, activity was maintained for 14 to 21 days, with a corresponding viability (as in the case of M10 with MTBE and ETBE), but did not correspond with the poor MTBE removal observed in Chapter III.1. Indeed, the highest activity was observed with strain E10 in the presence of MTBE for 24 h, after this, a downfall of activity and viability is observed until day 7 where activity is practically 0, and viability is <25% (Chapter III.3.1; Figure 3). We believe that the use of cytometric analysis gives some insight into the state of microbial strains to the oxygenate present, and thus could be used to monitor degradation in free-moving strains in bioreactor studies. This analysis may be more difficult to study in biofilm structures, but ways of dealing with aggregates have been extensively described. (Harnisch et al., 2011).

An evaluation of the GC method used was performed, since conventional methods (*purge and trap* or *headspace* autosamplers), were unavailable in our lab. Purge and trap enrichment from aqueous samples is the method chosen by the USA Environmetal Agency (USEPA) to determine ether concentration since it has the lowest limits of detection and quantification (0.001 μg/L LOD, Lacorte et al., 2002). However, this method does has certain setbacks: high concentrations of the ether cannot be determined without diluting the samples, contamination of the equipment due to high concentrations of ethers could cause cross contamination between samples, and pre-filtration of the sample is needed in order to remove solid particles from the samples, thus stripping from manipulation can cause detection at a lower concentration. Other methods such as direct aqueous injection and microextraction techniques have been used less in the literature. The former method is advantageous because no pre-treatment is required, but the views that the GC/MS equipment will be harmed by the presence of water vapour by analysts make this method less popular. Microextraction techniques have been used with varying fibre materials, and have resulted in low LODs similar to those found in *purge and trap* methods (Fang et al., 2003). *Headspace* techniques in general have higher detection limits, but the robustness of the method make it ideal detection of a wide range of volatile/semi-volatile substances, at very wide concentration ranges. In Chapter III.1, the oxygenate analysis of our samples were determined in an external analytical

laboratory, but the necessity to determine oxygenates *on site* without extended storage times was necessary. As a consequence, a *headspace* GC/MS method was thought through, and was put into practice in Chapters III.3.1 and III.3.2 The evaluation of the method described in Chapter III.2.1 allows us to comprehend the rate of oxygenate hydrolysis occurring in control GC/MS vials at different storage times, after storing samples at 4ºC in the dark, without further pre-treatment (no acidic o basic compounds were added). Manual heating and handling of the samples were always performed with the utmost care with respect to timings and heating temperatures, and statistical variability was computed. Limit of detection (LOD) were low, however, the method detection limits (MDL) were not but were in accordance with other headspace methods (Jochmann and Schmidt, 2007). Also beneficial was the conclusion drawn from an abiotic and biotic storage test (Chapter III.2.1; Figure 3), whereby oxygenates were hydrolysed at a higher rate in abiotic samples than their biotic counterparts, implying that degradation analysis may be higher if biotic degradation is compared to abiotic control samples, especially if samples are stored for long periods.

After determining the most fundamental method in the study, aspects of the biofilter design were questioned, such as the hydraulic retention time we would use, the support material adsorption, microbial attachment on the support material, amount of oxygenate lost through air stripping and materials used within the biofilter such as tubing. One of the main difficulties experienced in defining microbial degradation, was the air stripping phenomena when air was applied to ensure aerobic degradation. Minimization of air agitation was achieved by introducing air pressure regulators at minimal air flow rates. Air stripping from tubing were tested in order to reduce ether escape from the liquid media, and thus Teflon (PFTE) and Marprene tubing were used. High air stripping values were detected (Chapter III.3.1; Figure 1), even then, air flow conditions were decided on 5 ml/min air flow, with a hydraulic retention time (HRT) of 24 h, to allow enough time for oxygenate biotransformation. Other studies have also relayed the amounts of air stripping in biofilter studies e.g. at an oxygen flow rate of 1.6-2.9 ml/min, and 24 h HRT, 0% of volatilization was observed, but at an oxygen flow rate of 46-150 ml/min, and 24 h HRT, 20-70% of MTBE volatilization was observed (Hu et al., 2004).

Biological technologies use microbial cultures in suspended biomass or as immobilized microorganisms in a biofilm. The use of suspended biomass would be apt for bacteria with good doubling times; however, most fuel ether degrading strains have been described to have poor growth. Microorganisms in a biofilm can attain high microbial densities and be protected from shearing from liquid flow and therefore we chose this type of

biotechnology. Biofilm structures need a support material on which to attach and grow on, and these can be of a mineral nature (e.g. clay schists), can be adsorbent to the contaminant in question (granular activated carbon) or inert (of synthetic nature). Polyethylene Bioflow® 9 units were chosen for microbial attachment since adsorption was negative and therefore the inert material would not be a factor to consider when assessing the degrading ability of a microbial inoculum in the biofilter, and also due to their high surface area.

Selective inocula for MTBE degradation biotechnologies have been used previously with unknown and known microbial degrading consortia (Pruden et al., 2003; Acuna-askar et al., 2000) and individual strains (Maciel et al., 2008). The work described in the previous chapters, was initiated with the study of EPS production by strains M10, E10 and T3. Only M10 produced a significant amount of EPS, therefore we decided on performing attachment assays on the biofilter designed (Chapter III.3.1; Figure 1) with the three individual strains, and the dual strain consortia M10-E10 and M10-T3; since strain M10 was the only strain observed to attach with several layers to the support material (Chapter III.3.1; Figure 6a). The consortia M10-E10 attached successfully to the Bioflow® 9 units, and as a consequence, we decided on using strain M10 and consortium M10-E10 further to study their ability in removing MTBE in the designed downflow pilot-scale biofilter.

Viable counts and degradation of consortia M10-E10 and M10-T3 were assayed in batch conditions, however, the results were no better than those observed from individual strains. Consortia growth and degradation described in Auffret et al. (2009) did favour degradation of not just MTBE, but also BTEX compounds under batch conditions.

The lab-scale biofilter designed for bioremediation of MTBE, was ready to be tested and evaluated with the inocula M10 and M10-E10. Three biofilters were tested at the same time in order to evaluate the oxygenate removal with different inocula, but with the same influent. Biofilter D was seeded with *A. calcoaceticus* M10, biofilter E with the consortium M10-E10, and a control biofilter (F) was mounted to evaluate the performance of a non-seeded biofilter. The incoming groundwater was previously tested for ether content, and this was negative (LOD of 0.01 μg/L), thus, we intentionally spiked the groundwater, making the experiment ideal to study bioremediation in recently contaminated groundwater. Among the biofilters, only biofilter E showed oxygenate removal tendency (Chapter III.3.2; Figure 3a). After 21 days of operational time, a general decrease of MTBE concentration in the effluent was observed. The latter also coincided with a downfall of toxicity levels (Chapter III.3.2; Figure 3c), and the reduction and stabilization of dynamics changes in the influent (Chapter III.3.2; Figure 4b).

Before initiating biofilter studies through biological analysis, we evaluated the most adequate DNA extraction method for microbiota trapped in 0.22 μm nitrocellulose filters after filtration of groundwater. This was tested since we would be comparing the microbial community in the influent and in the biofilm, and the pre-treatment of these communities would have to be performed with the same DNA extraction protocol. Varying DNA extraction protocols were tested in order to understand the different outcomes and reproducibility in samples examined through TGGE. Mechanical lysis with the FastDNA kit was the most different protocol, and although there were less visible bands (Chapter III.2.2; Figure 1a), the protocol was reproducible and exclusive bands appeared in with this method, which did not appear in the others. Thus we implemented this method on the influent samples collected weekly on 0.22 μm filters, as well as on biofilm samples extracted from the biofilters.

The link between microbial biofilm diversity and microbial diversity in the influent was clearly observed (Chapter III.3.2; Figure 4a), and only at >40 days did the influent changes reduce significantly, allowing for maximum removal of MTBE from the biofilm in biofilter E. The comparison of TGGE bands from DNA and RNA extracts also corroborate that the activity in biofilter E was higher than the other two biofilters.

The microbial community present in a petroleum-hydrocarbon contaminated site (Kao et al., 2010) were tested for the presence of different genes [phenol hydroxylase (PHE), ring-hydroxylating toluene monooxygenase (RMO), 31 naphthalene dioxygenase (NAH), toluene monooxygenase (TOL), toluene 32 dioxygenase (TOD), and biphenyl dioxygenase (BPH4)]. Monitoring of natural attenuation was the aim of the study, and the analysis of the microbial community at different point sources through DNA extraction DGGE analysis revealed strains such as *Bacillus* spp. *Sphingomonas* spp, and *Pseudomonas* spp. to be in common with DNA extraction TGGE band isolates found in our studies (Chapter III.3.2; Figure SM1b). Among the RNA extraction TGGE band isolates, only *Bacillus* spp. was also present, thus the presence of this species may contain genes involved in the degradation of the oxygenates. Our study as well as Kao et al. (2010) and Barbera et al. (2011) were able to isolate *Bacillus* strains since they are well growing cultivable bacteria.

The microbial community of MTBE degrading biofilter and the effect of the dynamics of the microbial community in the influent on a biological reactor have not been previously described. Our study evaluated the influence of the microbial community present in the influent (i.e. autochthonous aquatic microorganisms) on the biofilm community. The results of the Pearson correlation coefficient of the influent and biofilm samples, and the dynamics of the influent from week to week shown in Chapter III.3.2; Figure 4, show that the biofilm were

very much influenced by the incoming microbial community. Most likely, degradation was not observed until the biofilm structure was relieved from the initial microbial nutrient and site competition.

The functionality of the biofilter in removing MTBE was sought by amplifying the genomic DNA of all biofilters with primers specific for *alkB* and *ethB* genes, previously found to biotransform MTBE (Chauvaux et al., 2001; Smith et al., 2003). These two genes code for monooxygenases which act on the initial attack of MTBE, ETBE and TAME (Lopes Ferreira et al., 2006). The *ethB* gene has been described to be found within an operon dedicated exclusively to ETBE degradation, however, its structure was also found to be held within two identical transposases, thus the elimination of the *eth* operon through homologous recombination seems to happen quickly. Thus probably sub strains exist within a single culture of the degrading strain *R. ruber* IFP 2001. The *alkB* gene on the other hand was stable, but is induced in the presence of alkanes, and not in the presence of the ethers, thus co-metabolism in this case would be best.

Though no *ethB* genes were found on biofilm samples, *alkB* amplification was positive in all biofilm samples and in strains M10 and E10. Bacterial isolates from the biofilms of the biofilters were cultivated in mineral media amended with MTBE, classified taxonomically and tested for the *alkB* gene. Seven of the 13 isolates were found to contain the gene, however, none of the isolates were found among the V3 bands from RNA extracts except for strain E10 and M10. And so we can only conclude that presence of the *alkB* gene in genomic DNA extracts does not entirely mean expression or function of this gene, nor biodegradation of MTBE. Also, we believe that the consortium composed of *R. ruber* E10 and *Acinetobacter calcoaceticus* M10 were the only taxonomically identified bacteria biodegrading MTBE in the biofilter E, and should therefore be used further, after seeking optimization of the environmental conditions for MTBE degradation and growth to improve its function. Certainly, the *R. ruber* E10 degrades MTBE better than strain M10 as shown in batch degradation tests in Chapter III.1, but the attachment “handicap” of E10 on Bioflow® 9 units does not make it suitable for the designed biofilter, thus the union of these strains in a biofilm have allowed for MTBE degradation. Entrapment of strain E10 in calcium alginate or water-in-oil-in-water could be used instead of the support material in this biofilter. Zhang et al. (2008) use aerobic granulation to self-immobilise strains in a bioreactor for MTBE removal, whereby removal efficiency is shown after 50 days of operational time.

Future work in oxygenate bioremediating biotechnologies should focus on ensuring minimalization of air stripping, as well as maintenance of active biomass within the systems,

without compromising the low-cost factor and dissolved oxygen concentrations. Further MTBE degrading gene markers should be searched and compared to literature to enable further understanding of the mechanisms which entail microbial ether degradation.

CAPÍTULO V. CONCLUSIONS - CONCLUSIONES

En base a los resultados obtenidos, así como a la revisión bibliográfica llevada a cabo, a continuación se presentan las siguientes conclusiones:

1. De las 9 cepas bacterianas aisladas e identificadas genéticamente, 3 de ellas: *Acinetobacter calcoaceticus* M10 (CECT 7739), *Rhodococcus ruber* E10 (CECT 7740) y *Gordonia amicalis* T3 (CECT 7741) mostraron capacidad biotransformar los oxigenantes MTBE, ETBE y TAME en medios minerales adicionados de éstos como única fuente de C y energía. La presencia de una fuente alternativa de carbono (etanol) mejoró marcadamente el crecimiento pero no la biotransformación de estos compuestos.

2. Los estudios de biodegradación llevados a cabo en ensayos en *batch* usando medios minerales adicionados de los oxigenantes MTBE, ETBE ó TAME como única fuente de C y energía, revelaron que el metabolito *tert*-butil-alcohol (TBA) fue biotransformado por las cepas M10 y E10, mientras que el metabolito *tert*-amil-alcohol (TAA) lo fue sólo por la cepa M10, por lo que esta última se mostró como cepa bacteriana biotransformadora de los oxigenantes MTBE y TAME, mientras que la cepa E10 lo fue sólo del MTBE.

3. La investigación llevada a cabo sobre la metodología para el análisis de oxigenantes, ha permitido determinar la tasa de hidrólisis del oxigenante TAME en muestras abióticas de agua almacenadas en oscuridad y temperatura controlada, siendo esta tasa mayor que en muestras inoculadas con la cepa bacteriana M10 y almacenadas bajo las mismas condiciones. Esta diferencia se acentuó a medida que se incrementaron los periodos de almacenamiento de las muestras. Estos resultados tienen especial importancia en los estudios sobre biodegradación de compuestos oxigenates.

4. El estudio sobre la comparación entre los distintos métodos disponibles para la extracción de ADN desde muestras de agua subterránea, reveló que el protocolo del *kit* comercial FastDNA® fue uno de los protocolos más reproducible y el que generó la aparición de bandas exclusivas.

5. Los estudios de citometría de flujo revelaron que las cepas fueron viables en presencia de los oxigenantes, siendo la actividad fisiológica bastante baja en la

mayoría de los casos, a excepción de la cepa *Rhodococcus ruber* E10 en presencia de MTBE, la cual mostró la mayor actividad fisiológica en ensayos en *batch.*

6. Las microfotografías generadas por la microscopia electrónica de barrido (*SEM*), los resultados de los ensayos de *FISH*, así como los estudios sobre producción de exopolisacárido *(EPS)* pusieron de manifiesto que la cepa *A. calcoaceticus* M10 y el consorcio M10-E10 se mostraron como óptimos inoculantes selectivos de un biofiltro diseñado para biorremediar aguas subterráneas contaminadas con oxigenantes de las gasolinas.

7. El biofiltro inoculado selectivamente con el consorcio M10-E10 fue el más efectivo en la eliminación del oxigenante MTBE. Esta eliminación estuvo unida a la reducción de la toxicidad del efluente, hecho comprobado mediante el ensayo Microtox® y a la reducción y estabilización de los cambios dinámicos en la población microbiana entrante.

8. Los resultados obtenidos de la correlación de Pearson realizada entre la biodiversidad del influente y la existente en las biopelículas establecidas, así como el estudio semanal de la dinámica microbiana del influente, revelaron que la biodiversidad observada en las biopelículas estuvo directamente relacionada con la biodiversidad presente en el influente (agua subterránea) a tratar.

9. La amplificación del gen *alkB* fue positiva en todas las muestras de biopelícula, así como en las cepas M10 y E10. Sin embargo, ninguna de las cepas aisladas e identificadas de las biopelículas con amplificación positiva al alkB, se encontraron entre las bandas de TGGE a partir de extracciones de ARN, exceptuando los extractos de la cepa E10 y M10, por lo que la presencia del gen *alkB* en los extractos de ADN genómico no significa la existencia de funcionalidad por parte del mismo.

10. Las cepas bacterianas *A. calcoaceticus* M10 y *R. ruber* E10, integrantes del consorcio M10-E10 fueron las únicas cepas identificadas con capacidad para biotransformar MTBE en el biofiltro diseñado y construido para la biorremediación de aguas subterráneas contaminadas con este oxigenante, bajo nuestras condiciones de estudio.

11. En el uso de biofiltros sumergidos aireados para el tratamiento de aguas subterráneas contaminadas con oxigenantes de las gasolinas, sugerimos que el control de aspectos tales como el fenómeno de *air stripping* y el mantenimiento de una biomasa activa dentro de los sistemas, sin comprometer el factor de bajo costo y las concentraciones de oxígeno disuelto, se considere imprescindible para mejorar su eficiencia.

Based on the results, as well as the review carried out, we present the following conclusions:

1. From the 9 strains isolated and genetically identified, 3 of them: *Acinetobacter calcoaceticus* M10 (CECT 7739), *Rhodococcus ruber* E10 (CECT 7740) and *Gordonia amicalis* T3 (CECT 7741) showed the ability to biotransform oxygenates MTBE, ETBE and TAME in mineral media as sole C and energy source. The presence of an alternative carbon source (ethanol) improved growth but not biotransformation of these compounds.

2. Biodegradation studies conducted in batch using mineral media amended with the oxygenates MTBE, ETBE or TAME as the sole C and energy source, showed that the metabolite tert-butyl alcohol (TBA) was biotransformed by strains M10 and E10, while the metabolite tert-amyl alcohol (TAA) was only biotransformed by the strain M10, therefore the the latter was found to be biotransform the oxygenates MTBE and TAME, whereas strain E10 only biotransformed MTBE .

3. Research conducted on the methodology for the analysis of oxygenates, demonstrated the rate of hydrolysis of TAME oxygenate water in abiotic samples stored in darkness and at a constant temperature. This rate was higher than their equivalent biotic samples with bacterial strain M10 when stored under the same conditions. This difference was accentuated as storage time increased. These results are particularly important for the study of biodegradation of oxygenates compounds.

4. The comparative study between methods for DNA extraction from a singular groundwater

sample revealed that the commercial kit protocol FastDNA® was one of the most reproducible protocols, and in addition, generated exclusive bands.

5. Flow cytometry analyses revealed that the strains were viable in the presence of oxygenates, however, their physiological activity was very low in most cases, with the exception of *Rhodococcus ruber* E10 in the presence of MTBE, which showed the highest physiological activity tests in batch.

6. From the micrographs generated by scanning electron microscopy (SEM), the results of FISH assays and studies on the production of exopolysaccharide (EPS), strain *A. calcoaceticus* M10 and consortium M10-E10 were selective as inoculants, optimal for the biofilter designed for bioremediation of oxygenate contaminated groundwater.

7. The biofilter inoculated with the consortium M10-E10 was the most effective in eliminating the oxygenate MTBE. This removal was linked to the reduction of effluent toxicity, as verified by the Microtox® analysis, and the reduction and stabilization of dynamic changes in the microbial population from the influent.

8. The results of the Pearson correlation conducted between the biodiversity of the influent and that established in the biofilms, as well as the weekly changes of the microbial community in the influent, revealed that the biodiversity observed in the biofilm was directly related to the biodiversity of the influent (groundwater).

9. The *alkB* gene amplification was positive in all biofilm samples as well as strains M10 and E10. However, none of the strains isolated and identified in the biofilms with positive amplification for *alkB* were found among TGGE bands from RNA extractions, except for strains E10 and M10, and thus the presence of the *alkB* gene in genomic DNA extracts does not necessarily infer MTBE degradation.

10. Bacterial strains *A. calcoaceticus* M10 and *R. ruber* E10, members of the consortium M10-E10, were the only strains identified capable of biotransforming MTBE in the biofilter designed for the bioremediation of groundwater contaminated with MTBE, under the conditions of our study.

11. We suggest that the control of aspects such as the phenomenon of air stripping and maintenance of an active biomass within the system (without compromising factor low cost and dissolved oxygen concentrations) is essential to improve efficiency for the use of submerged aerated biofilters for the treatment of groundwater contaminated with oxygenates in gasoline.

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