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Review

Biodegradation and fate of ethyl *tert*-butyl ether (ETBE) in soil and groundwater: A review

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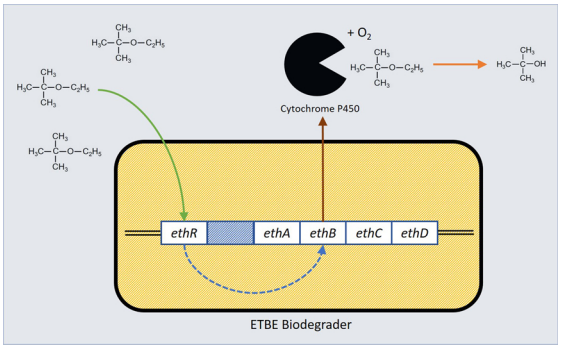
GRAPHICAL ABSTRACT



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ABSTRACT

This review summarises the current state of knowledge on the biodegradation and fate of the gasoline ether oxygenate ethyl *tert*-butyl ether (ETBE) in soil and groundwater. Microorganisms have been identified in soil and groundwater with the ability to degrade ETBE aerobically as a carbon and energy source, or via cometabolism using alkanes as growth substrates. Aerobic biodegradation of ETBE initially occurs via hydroxylation of the ethoxy carbon by a monooxygenase enzyme, with subsequent formation of intermediates which include acetaldehyde, *tert*-butyl acetate (TBAc), *tert*-butyl alcohol (TBA), 2-hydroxy-2-methyl-1-propanol (MHP) and 2-hydroxyisobutyric acid (2-HIBA). Slow cell growth and low biomass yields on ETBE are believed to result from the ether structure and slow degradation kinetics, with potential limitations on ETBE metabolism. Genes known to facilitate transformation of ETBE include *ethB* (within the *ethRABCD* cluster), encoding a cytochrome P450 monooxygenase, and *alkB*-encoding alkane hydroxylases. Other genes have been identified in microorganisms but their activity and specificity towards ETBE remains poorly characterised. Microorganisms and pathways supporting anaerobic biodegradation of ETBE have not been identified, although this potential has been demonstrated in limited field and laboratory studies. The presence of co-contaminants (other ether oxygenates, hydrocarbons and organic compounds) in soil and groundwater may limit aerobic biodegradation of ETBE by preferential metabolism and consumption of available dissolved oxygen or enhance ETBE biodegradation through cometabolism. Both ETBE-degrading microorganisms and alkane-oxidising bacteria have been characterised, with potential for use in bioaugmentation and biostimulation of ETBE degradation in groundwater.

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1. Introduction

Gasoline ether oxygenate (GEO) compounds, such as ethyl *tert*-butyl ether (ETBE), methyl *tert*-butyl ether (MTBE), *tert*-amyl methyl ether (TAME) and diisopropyl ether (DIPE) are oxygen-rich chemicals synthesised from feedstocks such as methanol, bioethanol and isobutylene (Ancillotti and Vittorio Fattore, 1998; Yuan, 2006; Umar et al., 2009; Concawe, 2012). These may be added to unleaded gasolines to increase fuel octane number, improve combustion efficiency and reduce vehicle emissions of carbon monoxide, ozone, nitrogen oxides and unburned hydrocarbons (EFOA (European Fuels Oxygenate Association), 2005; Babé et al., 2007; Westphal et al., 2010; Mikuš et al., 2013; Yee et al., 2013; Concawe, 2018a). Historically (pre-2000), MTBE was the ether oxygenate most commonly used in North America and much of Europe (Morales et al., 2009). However, other ether oxygenates are now favoured in several European markets, particularly ETBE ($(CH_3)_3C-O-CH_2CH_3$), which can be synthesized from renewable (bio)ethanol sources and fossil-based isobutylene (Stupp, 2007; Concawe, 2012; Le Digabel et al., 2013, 2014; van der Waals et al., 2019). ETBE has superior properties as an octane enhancer in promoting gasoline combustion and reducing vehicle emissions compared with MTBE (Yuan, 2006; Dietz, 2007; Umar et al., 2009). Relative to ethanol, ETBE has an advantage in producing less CO_2 per unit of energy and lower greenhouse gas emissions (Croezen and Kampman, 2009). Currently most ETBE consumption is in western Europe, where it is used at concentrations up to 10–15 % (Rosell et al., 2007; Babé et al., 2007; EFOA (European Fuels Oxygenate Association), 2009; Concawe, 2012; 2018a; Mikuš et al., 2013; Yee et al., 2013), and other international markets such as Japan (Rosell et al., 2007; Argus, 2018; van der Waals et al., 2019).

The market share of ETBE in European GEO production increased from 15 % in 2002 to around 60 % in 2010 (Concawe, 2012). Global ETBE production exceeds 3 Ma tonnes (Global Bioenergies, 2020) and is expected to grow by 4 % annually (Merchant Research and Consulting Ltd, 2020). The widespread use of ether oxygenate compounds has led to their increased detection in soil (Iturbe et al., 2003; Rong, 2008), surface water bodies and groundwater (McAuley, 2003; Shih et al., 2004; Ayotte et al., 2005; Moran et al., 2005; Spence et al., 2005; Rosell et al., 2005, 2007; Carter et al., 2008; van Wezel et al., 2009; Fayolle-Guichard et al., 2012; CONCAWE, 2012; Bombach et al., 2015; Quast et al., 2016; van der Waals et al., 2018). In the case of MTBE the relatively high concentrations that were used originally in reformulated fuels in North America has contributed to large MTBE plumes in some aquifers (Tong and Rong, 2002; Shih et al., 2004; Babé et al., 2007; Stupp, 2007; Kamath et al., 2012; Connor et al., 2014; McDade et al., 2015). This is not the case for ETBE plumes, which tend to be much smaller, due primarily to the lower solubility of ETBE in water and the proportion used in gasoline (Rosell et al., 2005; Fayolle-Guichard et al., 2012; Concawe, 2012; 2018b; Bombach et al., 2015; van der Waals et al., 2018). A recent survey of 50 gasoline-impacted European sites reported ETBE and MTBE median plume lengths in groundwater (38 m and 37 m, respectively), which were generally comparable to median plume lengths of associated BTEX compounds (Concawe, 2018b). Contamination of groundwater with ether oxygenates renders the water unfit to drink due to their low taste and odour thresholds, which are reported as 2 μg L^{-1} and 1 μg L^{-1}, respectively (Nihlén et al., 1998; McGregor, 2007; USEPA, 2013, 2017a; USEPA, 2017b). No official reference toxicological values for ETBE are available, except chronic values determined by ECHA (ECHA (European Chemicals Agency), 2020) as Derived No Effect Levels (DNELs). Available ecotoxicological values are highly variable according to studies and evaluation bodies, but all lead to Predicted No Effect Concentration (PNEC) or No Observed Effect Concentration (NOEC) of at least one order of magnitude greater than the reported odour and taste thresholds in water (Rosell et al., 2007; WHO (World Health Organization), 2005; Fawell, 2007; Suffet, 2007; van Wezel et al., 2009). Thus, organoleptic aspects are the primary concern of groundwater contamination by ETBE.

The ultimate fate of ETBE in contaminated soil and groundwater is determined by its susceptibility to microbial transformation under prevailing conditions and in the presence of other organic compounds (Somsamak et al., 2001; Rosell el al., 2005; Dietz, 2007). ETBE is most likely to be released to groundwater as the sole ether oxygenate in fuel formulations. However, historical fuel oxygenate blending and storage practices, releases of varying fuel formulations and field observations show that mixtures of fossil-based fuels (containing MTBE and/or TAME) and bio-based fuels containing ETBE can arise in plumes at sites (Rosell et al., 2005; Auffret et al., 2009; Fayolle-Guichard et al., 2012; Bombach et al., 2015; van der Waals et al., 2018, 2019). In this context different aromatic constituents and other fuel additives in such mixtures can have a beneficial or detrimental effect on ETBE biodegradation (Dietz, 2007; Lin et al., 2007; Auffret et al., 2009; Fayolle-Guichard et al., 2012; Gunasekaran et al., 2013; Bombach et al., 2015; van der Waals et al., 2018). The existing literature on the fate of MTBE in the environment has been extensively reviewed (Squillace et al., 1997; Deeb et al., 2000; Prince, 2000; Stocking et al., 2000; Fayolle et al., 2001; Seagren and Becker, 2002; Zanardini et al., 2002; Fiorenza and Rifai, 2003; Davis and Erikson, 2004; Schmidt et al., 2004; Rosell et al., 2006; Debor and Bastiaens, 2007; Concawe, 2012; Hyman, 2013). However, the current state of knowledge on the biodegradation and fate of ETBE in the subsurface has not been comprehensively examined. In particular, the potential for biodegradation of ETBE in soils and groundwater under conditions which are known to support biodegradation of MTBE, the effects of other gasoline components on ETBE biodegradation, and the microorganisms facilitating ETBE

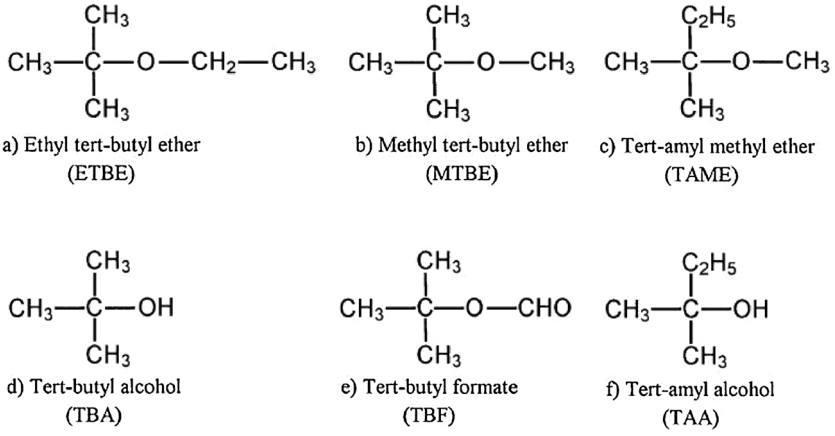

Fig. 1. Molecular structure of ETBE, MTBE and TAME (a–c) and their major metabolic intermediates (d–f), reproduced from Kharoune et al (2001b).

biodegradation have not been fully evaluated.

This paper reviews the literature from laboratory and field-based studies on the microorganisms, metabolic pathways and environmental conditions currently known to support ETBE biodegradation in soil and groundwater. This includes consideration of the effects of other oxygenate and hydrocarbon compounds present in gasoline on ETBE biodegradation potential.

2. Influence of ETBE properties on subsurface behaviour

Each oxygen atom on the ETBE molecule is linked to two hydrocarbon groups via carbon atoms (Fig. 1), forming a carbon-oxygen-carbon sequence in the branched structure. Whereas MTBE and TAME are composed of a *tert*-butyl group bonded to a methoxy group, the *tert*-butyl group in ETBE is bonded to an ethoxy group (François et al., 2002 Yuan, 2006; Fayolle-Guichard et al., 2012).

The chemical properties of ETBE are compared in Table 1 with those of other common fuel oxygenates, including tertiary-butyl alcohol (TBA), and benzene (as a proxy for BTEX with which ETBE may be associated in groundwater plumes). These properties vary markedly with temperature and influence the distribution of ETBE between phases (e.g. pure phase, gas phase, aqueous phase, solid phase), the relative importance of various processes (e.g. vaporisation, volatilisation, dissolution and sorption) that control the fate of ETBE in the subsurface (Moyer, 2003; Fischer et al., 2004; Rosell et al., 2007; Stupp, 2007) and biodegradation potential (Kharoune et al., 2002; Moyer, 2003; Rosell et al., 2005; Dietz, 2007; Purswani et al., 2008; Bartling et al., 2011). There have been limited studies of the distribution of ETBE in the subsurface environment but the available data suggest that ETBE is not a significant contaminant in European soils or groundwater (Concawe, 2012).

Considering these properties, ETBE has similar phase partitioning and transport behaviour to MTBE and TAME when released to the subsurface as a component of gasoline (Dietz, 2007). With a similar vapour pressure (*Vp*) to benzene (Table 1) ETBE will readily vaporise from gasoline and migrate with benzene in the vapour phase through the unsaturated zone and soil zone (Bartling et al., 2011). The aqueous solubility of ETBE is an order of magnitude greater than benzene but significantly less than that of MTBE. Hence, ETBE may be released preferentially to BTEX from gasoline into the dissolved phase (soil pore water, unsaturated zone pore water or groundwater), according to its effective solubility, as documented for MTBE releases (Moyer, 2003; Spence et al., 2005; Thornton et al., 2011, 2013). Moreover, this solubility may in principle result in ETBE plumes extending further than the corresponding hydrocarbon plume in a typical gasoline release to groundwater, as is often observed for MTBE releases (Chen et al., 2005; Rosell et al., 2005; Spence et al., 2005; Stupp, 2007; Fayolle-Guichard et al., 2012; Concawe, 2012; Connor et al., 2014; McDade et al., 2015). However, the lower aqueous solubility of ETBE relative to MTBE is likely to result in smaller ETBE plumes in groundwater than for comparable MTBE releases in the absence of other controlling factors (Rosell et al., 2005; Yuan, 2006; Yee et al., 2013). Ether oxygenate aqueous solubility generally increases by a factor of 2 as groundwater temperature decreases from 20 °C to 5 °C, with a consequent higher potential rate of spreading from unleaded releases in colder climates (Fischer et al., 2004). Temporal variation in ETBE aqueous solubility within plume sources should be considered when modelling the environmental risk, fate and transport of ether oxygenates in groundwater (Thornton et al., 2013). Models describing the temperature-dependency of ether oxygenate aqueous solubility are available for fate and transport assessments in surface water and groundwater, risk assessment and remediation design (Gonzalez-Olmos and Iglesias, 2008). The dimensionless Henry's Law constant (*H*) for ETBE which describes the temperature-dependent equilibrium partitioning of organic chemicals between aqueous and gaseous phases, is comparable to MTBE and lower than that of benzene, implying a lower potential for volatilisation of ETBE from the dissolved phase to the gaseous phase than for benzene. ETBE is therefore less likely be found in soil gas than benzene when the source is dissolved phase. Consequently at typical groundwater temperatures volatilisation has limited influence on the transport of ETBE in the subsurface.

The low reported organic carbon-water partition coefficient (log K_{oc}) and octanol-water partition coefficient (log K_{ow}) for ETBE indicates that sorption to aquifer materials will be negligible and unlikely to limit migration of ETBE plumes in groundwater (Fayolle et al., 2001; Moyer, 2003; Dietz, 2007). However, sorption of ETBE, as with other GEO, is expected to be higher in soils than aquifers due to the greater organic carbon content (Huttunen et al., 1997; Linnemann, 2003; Smith and Lerner, 2007; Kamalan et al., 2009; Zhu and Bi, 2011; Fayolle-Guichard et al., 2012). Reported values of K_{oc} between 68–136 L kg^{-1} for sorption of ETBE onto soils suggest that both the quantity and composition of soil organic matter (SOM) are important (Zhu and Bi, 2011). The same study documented a decrease in ETBE sorption coefficients with increasing temperature (from 15 − 40 °C) and negative calculated sorption enthalpy (DH\0), indicating that ETBE sorption onto soil is exothermic and appears to be controlled by short-range van der Waals forces and other specific interactions. Both linear and Freundlich isotherms describe ETBE sorption to soil components across 3 orders of concentration (Kamalan et al., 2009; Zhu and Bi, 2011). Balseiro-Romero et al. (2014) showed that ETBE mobility in soils can be enhanced by humic substances and plant root exudates (e.g. organic acids), although this phenomenon is confined primarily to the upper soil horizon and plant rhizosphere. In combination, the aqueous solubility, Henry's Law constant and organic carbon-water partition

Table 1
Selected properties of ether oxygenate compounds, TBA and benzene.

	ETBE	MTBE	TAME	TBA	Benzene
Molecular formula	$C_6H_{14}O$	$C_5H_{12}O$	$C_6H_{12}O$	$C_4H_{10}O$	C_6H_6
Molecular weight (g mol^{-1})	102.18	88.15	102.18	74.12	78.11
Boiling point (°C)	72.8	55.2	85 – 86	82.8	80.1
Density at 25 °C (kg L^{-1})	0.752	0.741	0.764	0.786	0.879
Solubility in water (g L^{-1})	12	43 – 51	20	fully miscible	1.8
Vapour pressure (Pa)	11965 – 16500	17600 – 60500	10112 – 13263	5413 – 5852	12692
Henry's Law constant[a] (-)	0.0564 – 0.0972	0.0216 – 0.0555	0.054 – 0.1096	0.0004 – 0.0006	0.221
Log K_{ow} (-)	1.74 – 1.92	0.94 – 1.6	1.55 – 1.95	0.35 – 0.73	2.13
Log K_{oc} (-)	0.94 – 1.57	0.02 – 1.1	1.62	0.4	1.56 – 2.15

Notes : Data compiled from Fayolle et al (2001); Moyer (2003); Babé et al (2007); Rosell et al (2007); Dietz (2007); Concawe (2012); Mikuš et al (2013) and USEPA (2017a).

Table 2
Microorganisms reported to degrade ETBE under aerobic conditions.

Strain	Inocula	Conditions and growth substrate(s)	Temp	Degradation behaviour and utilisation rate	Co contaminants	Reference [1]
Mycobacterium vaccae JOB5 ENV425	Strain from ATCC collection Native isolates in mixed culture from uncontaminated soil, USA	Enriched on propane with R2A or minimal salt medium	30 °C	ETBE degraded at variable rate according to growth substrate but with maximum utilisation rate of 30 mg L^{-1} day^{-1}	Not compared in mixture	Steffan et al (1997), **Cometabolism**
Gordonia terrae IFP 2001 *Rhodococcus equi* IFP 2002	Activated sludge from urban wastewater treatment plant, France	Isolated and enriched on ETBE with minimal media	30 °C	ETBE degraded as sole carbon and energy source at 67 mg $L^{-1}$$day^{-1}$	Not compared in mixture, but no degradation of MTBE or TAME in isolation	Fayolle et al (1998)
Rhodococcus ruber IFP 2001	Formerly identified as *Gordonia terrae* IFP 2001, France	Grown on minimal media with ETBE	30 °C	ETBE degraded as sole carbon and energy source with stoichiometric accumulation of TBA	Not compared in mixture	Chauvaux et al (2001)
Gordonia terrae IFP 2001 *Gordonia terrae* IFP 2007	Activated sludge from an urban wastewater treatment plant, France	Grown on minimal media with ETBE. *G. terrae* IFP 2007 derived from *G. terrae* IFP 2001	30 °C	ETBE degraded as sole carbon and energy source with stoichiometric accumulation of TBA Cometabolism of MTBE and TAME (with ethanol) to TBA and TAA, respectively	Not compared in mixture	Hernandez-Perez et al (2001)
Comamonas testosteroni E1 Unidentified strain – CDC group A-5	Isolated from gasoline-polluted soil, France	Consortium enriched with ETBE, strains isolated with ETBE on minimal media	28 °C	ETBE degraded completely as sole carbon and energy source with no accumulation of TBA or TBF	Not compared in mixtures but ETBE-degrading strains also degraded MTBE, TAME, TBA and TBF as sole carbon and energy source	Kharoune et al (2001a)
Mycobacterium austroafricanum IFP 2012	Activated sludge from an urban wastewater treatment plant, France	Strain grown on mineralal media with 300 mg L^{-1} MTBE, TAME or TBA; enriched and isolated on TBA	30 °C	Slow and incomplete degradation of ETBE, compared with MTBE, TAME and TBA, when fed separately; TBA produced from ETBE degradation mineralised	Not compared in mixture	François et al (2002)
Mycobacterium austroafricanum IFP 2012 *Mycobacterium austroafricanum* IFP 2015	Activated sludge from urban wastewater treatment plant, France Drain water at an MTBE-supplemented gasoline storage tank, France	Enriched on MTBE with mineral media	30 °C	ETBE degraded (3x faster for IFP 2015 than IFP 2012); TBA degraded	Not tested	Lopes Ferreira et al (2006a)
Aquincola tertiaricarbonis L108	MTBE-contaminated groundwater, Germany	Grown on ETBE in minimal salt solution supplemented with vitamins	30 °C	ETBE degraded as sole carbon and energy source, with max growth rate at pH 7 and 30 °C of 0.06 hr^{-1}, max growth yield of 0.53 g dm (g ETBE)$^{-1}$, max degradation rate of 1.11 mmol ETBE h^{-1} g^{-1}, and μ_{max} = 0.06 h^{-1}	Not compared in mixture	Müller et al (2008)
Acinetobacter calcoaceticus M10 *Arthrobacter* sp. MG *Gordonia amicalis* EA *Nocardioides* sp. E7 *Rhodococcus ruber* E10	Hydrocarbon-contaminated soil (surface 0 – 15 cm), Spain	Enriched with 200 mg L^{-1} ETBE, MTBE and TAME in minimal media; isolated and grown with 100 mg L^{-1} ETBE and mineral media (MM) or mineral-plus (MM+) medium (cometabolic media supplemented with yeast extract and ethanol)	30 °C	ETBE degraded as sole carbon and energy source; degradation rate not given	*Acinetobacter calcoaceticus* M10 – ETBE degraded in MM, inhibited in MM+. *Arthrobacter* sp. MG and *Gordonia amicalis* EA – ETBE degraded in MM + only *Nocardioides* sp. E7 and *Rhodococcus ruber* E10 – ETBE degraded greater in MM + than MM	Purswani et al (2008), **Cometabolism** Purswani et al (2011)
Rhodococcus wratislaviensis IFP 2016 *Rhodococcus aetherivorans* IFP 2017	Isolated from consortium obtained with microorganisms combined from wastewater treatment plant, pristine forest soil and gasoline-contaminated soil, France	Consortium grown on mixture of BTEX, octane, hexadecane, 2,2,4-trimethylpentane, cyclohexane, cyclohexanol, naphthalene, MTBE, ETBE, TBA, 2-ethyl hexyl nitrate with mineral media; Isolates grown on mixture with mineral media and vitamin solution	30 °C	Accumulation of TBA from ETBE and MTBE degradation by consortium and isolates (individually and in co-culture), with no further degradation IFP 2017 degraded ETBE at rate of 116 µmol ETBE hr^{-1} g^{-1} (dry weight)	IFP 2016: ETBE degraded by 25 %. BTEX inhibited MTBE. Order : BTEX > MTBE > ETBE IFP 2017: BTEX = 1.5-fold lower ETBE degradation (MTBE 24-fold lower). MTBE – no significant effect on ETBE, no MTBE degradation in presence of ETBE. Order : BTEX > ETBE > MTBE	Auffret et al (2009), **Cometabolism**

(*continued on next page*)

Table 2 (*continued*)

Strain	Inocula	Conditions and growth substrate(s)	Temp	Degradation behaviour and utilisation rate	Co contaminants	Reference [1]
Pseudomonads (*Pseudomonas aeruginosa* BM-B-450 and *Pseudomonas citronellolis* BM-B-447 isolated but not tested with ETBE)	Consortium from gasoline-polluted soil, Mexico	Pure strains isolated on nutrient agar and grown on 75 mg L^{-1} pentane and 35 mg L^{-1} MTBE in liquid media	30 °C	1.5 mg L^{-1} ETBE degraded to 92% with n-pentane and MTBE by consortium at max degradation rate of 78.8 $mg_{protein}{}^{-1}$ h^{-1}	Not compared in mixture	Morales et al (2009), **Cometabolism**
Pseudonocardia tetrahydrofuranoxydans K1.	Enriched from wastewater treatment plant sludge, Germany	Strain originally grown on tetrahydrofuran (THF) as sole carbon source and fed MTBE (1000 mg L^{-1}), ETBE (750 mg L^{-1}) and TAME (575 mg L^{-1}) in sodium phosphate buffer	Not given	ETBE degraded cometabolically to TBA with MTBE and TAME, using THF as carbon source	MTBE and TAME also biodegraded to TBA and TAA,. respectively, but no further biodegradation of TBA or TAA by THF-grown cells	McKelvie et al (2009), **Cometabolism**
Rhodococcus ruber IFP 2001 *Rhodococcus. zopfii* IFP 2005 *Gordonia* sp. IFP 2009	Activated sludge from wastewater treatment plant, France	Cultured on mineral media with 250 mg L^{-1} ETBE	30 °C	ETBE degraded as sole carbon and energy source with accumulation of TBA at rates from 1.6-3.5 mmol ETBE g^{-1} dry weight h^{-1}	MTBE and TAME only degraded to TBA after growth on ETBE but not compared in mixture. Presence of easily degradable substrate (e.g. incubation on ETBE in presence of Luria-Bertani medium) inhibited the induction process.	Malandain et al (2010)
Rhodococcus wratislaviensis IFP 2016 *Rhodococcus aetherivorans* IFP 2017 *Aquinola tertiaricarbonis IFP 2003*	Mixed culture IFP (MC-IFP) from gasoline-contaminated groundwater, France	Each strain grown on 300 mg L^{-1} ETBE in mineral media	30 °C	ETBE degraded at rate of 19.9 mg L^{-1} day^{-1} to CO_2 without accumulation of TBA when native groundwater innoculated with mixed culture of all 3 strains	BTEX present as co-contaminant and biodegraded concurrently with ETBE by native consortia	Fayolle-Guichard et al (2012)
Rhodococcus sp. IFP 2042	MTBE and TBA-contaminated groundwater, USA	Strain enriched from groundwater on mineral media using ETBE as growth substrate, then isolated	30 °C	ETBE degraded as sole carbon and energy source with accumulation of TBA	Not compared directly but complete degradation of ETBE to CO_2 and biomass when *Rhodococcus* sp. IFP 2042 mixed with TBA-degrader *Bradyrhizobium* sp. IFP 2049	Le Digabel et al (2013)
Aquinola tertiaricarbonis L108	MTBE-contaminated groundwater	Grown on 300 mg L^{-1} ETBE in mineral salt solution supplemented with vitamins	30 °C	ETBE degraded as sole carbon and energy source with accumulation of TBA	MTBE and TAME also degraded to TBA and TAA but not compared in mixtures	Schuster et al (2013)
Xanthomonas sp. *Methylibiumsp*. *Methylobacillus* sp. *Methylovorus* sp	Gasoline-contaminated groundwater, Spain	Grown on 50 mg L^{-1} ETBE in minimal media	30 °C	Degradation of 47 % ETBE as sole carbon and energy source in 5 days with continued degradation up to 51% in 9 days	Reduced rate of ETBE degradation (14 % in 9 days) in presence of 50 mgL^{-1} BTEX	Gunasekaran et al (2013)
Achromobacter xylosoxidans MCM2/2/1	Gasoline-contaminated soil, Spain	Grown on 100 mg L^{-1} MTBE in mineral medium with yeast extract	30 °C	100 mg L^{-1} ETBE degraded as sole carbon and energy source to 42 % in 6 days with no TBA accumulation	Not compared in mixture	Gunasekaran et al (2014)
Rhodococcus sp. IFP 2040 *Rhodococcus* sp. IFP 2041 *Rhodococcus* sp. IFP 2043 *Betaproteobacteria* IFP 2047 *Pseudonocardia* sp. IFP 2050	MTBE-contaminated soil, Belgium (BE1) MTBE, TBA, TBF-contaminated groundwater, Germany (GE1) MTBE-contaminated soil, Belgium (BE1) MTBE, TBA, TBF-contaminated groundwater, Germany (GE1) MTBE, TBA-contaminated groundwater, France (FR5)	Strain enriched from respective sample on mineral media using ETBE (200 mg L^{-1}) as growth substrate, then isolated	30 °C	ETBE degraded as sole carbon and energy source with accumulation or degradation of TBA Variable yield of 0.11-0.43 mg biomass mg^{-1} ETBE degraded and degradation time of 4-42 days	Not compared in mixture	Le Digabel et al (2014)

1. "**Cometabolism**" refers to studies demonstrating cometabolism of ETBE with the organism indicated.

coefficient of ETBE imply that it may migrate more rapidly and extend further than other gasoline components, such as BTEX compounds, in groundwater (Shih et al., 2004; Fayolle-Guichard et al., 2012).

3. Aerobic biodegradation of ETBE

3.1. Biodegradation pathways and intermediate compounds

Microorganisms reported to biodegrade ETBE under aerobic conditions are summarised in Table 2, with a phylogenetic tree of those organisms for which 16S rRNA gene sequences are available shown in Supplementary Figure Class. The majority of organisms identified as ETBE degraders to date are in the Actinobacteria (largely members of the Nocardiaceae), but organisms in the beta and gamma Proteobacteria have also been found. The aerobic biodegradation pathway for the *tert*-alkyl ethers is well established (White et al., 1996; Deeb et al., 2000; Fayolle et al., 2001, 2003; Schmidt et al., 2004; Hyman, 2013). This was first proposed for ETBE by Kharoune et al. (2001a), with later revision by Müller et al. (2007). Fig. 2 shows the pathway for ETBE catabolism (compared with MTBE) to the intermediate 2-hydroxyisobutyric acid (2-HIBA), with related intermediate compounds. The first step involves hydroxylation of carbon in the ethoxy group of ETBE adjacent to the ether bond by a monooxygenase enzyme, forming an unstable hemiacetal intermediate, *tert*-butoxy ethanol (denoted by square brackets in Fig. 2), which is subsequently converted to TBA (Fayolle et al., 2001; Kharoune et al., 2001a; François et al., 2002; Lopes Ferreira et al., 2006b; Rohwerder et al., 2006; McKelvie et al., 2009). This occurs either directly by dismutation, with accompanying production of TBA and acetaldehyde (White et al., 1996; Kharoune et al., 2001a; Rohwerder et al., 2006; Schuster et al., 2013), or by dehydrogenation to an ester intermediate, which is then hydrolysed to TBA (Piveteau et al., 2001; Lopes Ferreira et al., 2006a). The exact nature of this ester intermediate is uncertain. In the biodegradation pathway for MTBE this ester is *tert*-butyl formate (TBF) and Kharoune et al. (2001a) proposed that this was also the case for ETBE. However, Müller et al. (2007, 2008) suggested that the ester intermediate for ETBE is *tert*-butyl acetate (TBAc). The two-carbon acetate moiety of TBAc corresponds more closely to the original ethyl moiety of ETBE than the one-carbon formate moiety found in TBF and is more likely to be the relevant ester intermediate of ETBE (Lopes Ferreira et al., 2006a). The intermediate one- and two-carbon aldehydes and acids are expected to be oxidized by conventional dehydrogenase systems (Rohwerder et al., 2006; McKelvie et al., 2009). TBA is then hydroxylated by a monooxygenase to 2-hydroxy-2-methyl-1-propanol (MHP), with further enzymatic oxidation to 2-hydroxyisobutyric acid (2-HIBA) (Steffan et al., 1997; Fayolle et al., 2001; Lopes Ferreira et al., 2006a; Schuster et al., 2013). The *tert*-butyl moiety is maintained until 2-HIBA, after which this structure is destroyed with subsequent mineralisation to CO_2 and biomass (Kharoune et al., 2002). This occurs via reactions producing 2-propanol, acetone, hydroxyacetone and pyruvic acid (Steffan et al., 1997; Church et al., 2000; Rohwerder et al., 2006; Schuster et al., 2013).

MTBE and structurally related compounds, such as ETBE, have been reported to be resistant to microbial transformation in the environment (Suflita and Mormile, 1993; Yeh and Novak, 1994, 1995; Fayolle et al., 2001; Somsamak et al., 2001; François et al., 2002). It has been suggested that this is due to the presence of a stable ether linkage and branched (sterically hindered) tertiary carbon structure which may be

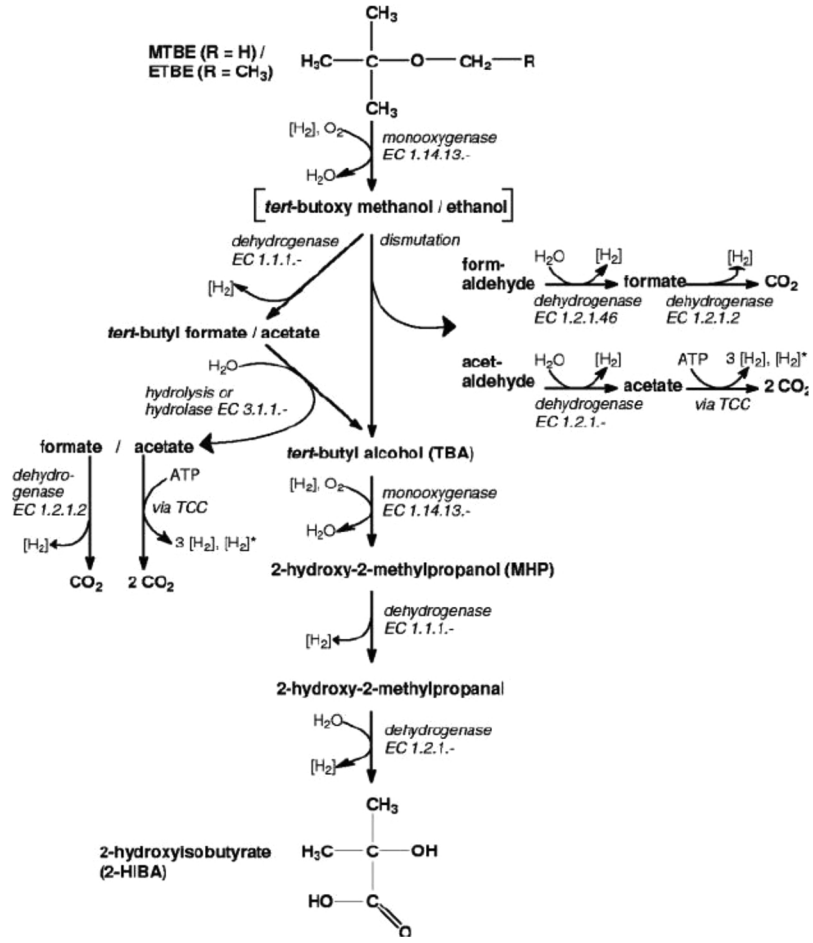

Fig. 2. Proposed aerobic pathway for biodegradation of MTBE and ETBE to 2-HIBA, reproduced from Müller et al (2007).

resistant to enzymatic attack (Kharoune et al., 2001b; Yuan, 2006; Müller et al., 2008; McKelvie et al., 2009; Morales et al., 2009; Gunasekaran et al., 2014; Bombach et al., 2015). Observed yields were generally too low to support microbial growth on these substrates (Fayolle et al., 2003; Zein et al., 2006; Müller et al., 2007; Le Digabel et al., 2013, 2014). Cleavage of the ether bond (o-demethylation) in oxygenates requires significant energy investment by microorganisms when this is the only carbon and energy source, and energy loss during metabolism is considered to be an important factor contributing to the typically low biomass yield efficiency (Suflita and Mormille, 1993; Yeh and Novak, 1994; Mormile et al., 1994; White et al., 1996; Steffan et al., 1997; Fayolle et al., 1998; Prince, 2000; Fortin et al., 2001; Kharoune et al., 2002; Fayolle et al., 2003; Müller et al., 2007; Morales et al., 2009; Le Digabel et al., 2014). Explanations of these restrictions on ether oxygenate biodegradation have focused on a fundamental analysis of substrate fluxes available for biomass production during metabolism by the relevant pathways (Fayolle et al., 2003; Müller et al., 2007; Le Digabel et al., 2013, 2014). The study by Müller et al. (2007) showed that MTBE and ETBE are theoretically effective heterotrophic growth substrates, with maximum biomass yields of 0.87 g g^{-1} and 0.75 g g^{-1}, respectively. This is capable of providing sufficient energy equivalents in the potential assimilatory routes to incorporate carbon into biomass without the need to dissimilate additional substrate. However, due to slow degradation kinetics, the energy needed to degrade these oxygenates may only be sufficient for cell maintenance, thus requiring relatively high threshold substrate concentrations, S_{min}, to support growth (Müller et al., 2007). Catalysing the cleavage of the ether bond in ETBE is therefore likely to control the subsequent substrate flux and energy cascade in different microorganisms for cell growth, such that actual growth yields may be very low and approach zero (Hernandez-Perez et al., 2001; Müller et al., 2007; Le Digabel et al., 2014). Low biomass yields on both MTBE and ETBE have indeed been reported when degradation rates of these ethers are slow, leading to the possibility that maintenance energy rates may exceed energy generation rates needed for growth (Le Digabel et al., 2014). In cases where substrate concentrations fall below S_{min} metabolism of ETBE as a carbon and energy source may be restricted (and only occur by cometabolism), potentially limiting ETBE biodegradation in groundwater (Zein et al., 2006; Babé et al., 2007; Debor and Bastiaens, 2007; Schirmer and Martienssen, 2007).

The intermediates TBA and 2-HIBA are often reported to be biodegraded faster than the parent compounds and thus not accumulate (e.g. Kharoune et al., 2001b, 2002; Zein et al., 2006; Babé et al., 2007; Müller et al., 2008; Fayolle-Guichard et al., 2012; Gunasekaran et al., 2013, 2014; Kyselková et al., 2019). This is consistent with accessibility and cleavage of the ether bond being the major limiting step in oxygenate biodegradation, rather than assimilation of the intermediates in later stages of metabolism (Yeh and Novak, 1994; Kharoune et al., 2001a, b; Müller et al., 2008). Structural differences between MTBE and ETBE, specifically the respective presence of a methyl and ethyl group adjacent to the ether bond, can influence the biodegradation potential of these oxygenates (White et al., 1996; Prince, 2000; François et al., 2002; Kharoune et al., 2002; Yuan, 2006; Müller et al., 2007). Concerning ETBE, the ethoxy carbon of ETBE is more susceptible to biodegradation than the corresponding methoxy carbon in MTBE, as the carbon atom is located further away from the ether bond and therefore less sterically hindered (Fayolle et al., 1998; Kharoune et al., 2001a, b; Yuan, 2006). The C2-carbon compounds (acetaldehyde and acetate) generated by the initial hydroxylation step are also considered to be more easily metabolised than the corresponding C1-carbon intermediates produced during MTBE biodegradation (Fayolle et al., 1998; 2001; Kharoune et al., 2001a), which requires specific organisms (methylotrophs) to utilise them (Piveteau et al., 2001; Lopes Ferreira et al., 2006a). Consequently, these C2-carbon metabolites are often used rapidly as carbon substrates in ETBE biodegradation and seldom accumulate (Hernandez-Perez et al., 2001). Conversely, the accumulation of TBA and other organic intermediates has also been reported in some studies (e.g. Yeh and Novak, 1995; Steffan et al., 1997; Kharoune et al., 2002; Babé et al., 2007; Auffret et al., 2009; Malandain et al., 2010; Fayolle-Guichard et al., 2012; Le Digabel et al., 2013, 2014; van der Waals et al., 2019). This may imply that ETBE is not a viable growth substrate in such cases and that the intermediates are not used as a carbon and energy source (Fayolle et al., 2001; Hernandez-Perez et al., 2001), such that their mineralisation is rate-limiting for overall biodegradation (Martienssen et al., 2006; Le Digabel et al., 2014). Differences in the relative transformation rate of ETBE and TBA by pure strains and mixed cultures from natural consortia are also reported to result from variation in the efficiency of separate enzymes used by one microorganism for each substrate, degradation by the same enzyme (e.g. monooxygenase) with different efficiencies for each substrate, and commensalistic metabolism of the parent and intermediate compounds by different microorganisms in consortia (Kharoune et al., 2001a; Béguin et al., 2003; Lopes Ferreira et al., 2006a; Babé et al., 2007; Lin et al., 2007; Morales et al., 2009; Le Digabel et al., 2014).

3.2. Genes and enzymes involved in aerobic ETBE biodegradation

3.2.1. Eth genes and cytochrome P450 monooxygenase

Genes known to be involved in GEO biodegradation are summarised in Fig. 3 with respect to the different substrates reportedly metabolised.

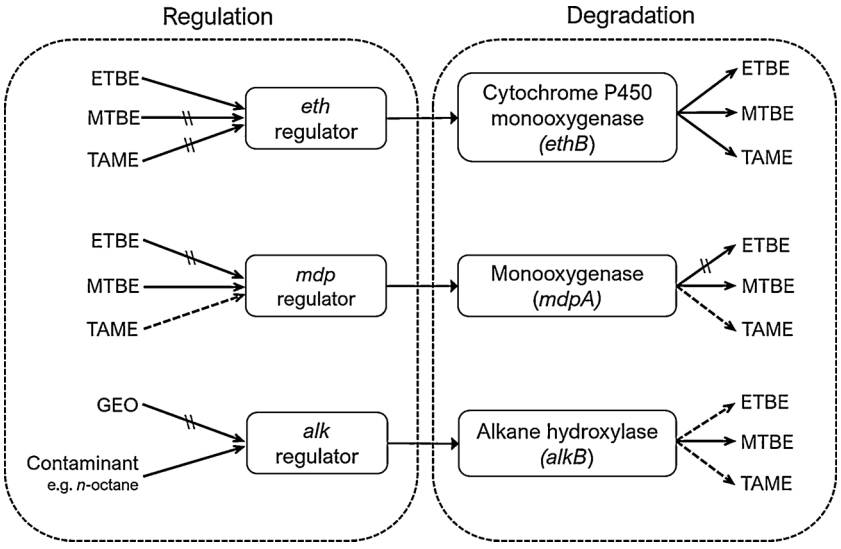

Fig. 3. Three characterised genes and pathways involved in GEO biodegradation, the *eth*, *mdp* and *alk* genes, based on the literature. Solid lines indicate evidence of this process, dotted lines denote insufficient evidence to confirm this process, and a solid line with \\ embedded denotes this process is known not to occur. The regulator gene products (left box) activate the expression of the degradative genes (right box).

The *eth* genes are the most extensively characterised gene cluster implicated in aerobic biodegradation of ETBE. *Rhodococcus ruber* IFP 2001 (formerly *Gordona terrae*) was identified for its ability to biodegrade ETBE (Fayolle et al., 1998) and subsequently a gene cluster responsible for catalysing the oxidation of ETBE was identified. This gene cluster, *ethRABCD*, encodes an AraC/XylS-type positive transcriptional regulator (*ethR*), a ferredoxin reductase (*ethA*), a cytochrome P450 monooxygenase (*ethB*), of the CYP 249 family, ferredoxin (*ethC*) and a protein of unknown function (*ethD*) (Chauvaux et al., 2001; Béguin et al., 2003; Hyman, 2013; Schuster et al., 2013). The 10 kDa *ethD* protein is essential for degradation activity (Chauvaux et al., 2001), while *ethA* and *ethC* are known to be involved in electron transport to *ethB*.

The *eth* genes are flanked by two identical IS3-type class II transposons. These can form a hairpin and undergo homologous recombination, resulting in *eth* deletion mutants (Chauvaux et al., 2001). The loss of *eth* genes occurs at a high frequency under non-selective conditions (Urios et al., 2002), as demonstrated through successive transfers on Luria Broth medium. High sequence similarities (99 %) of *ethB* genes have been reported (Malandain et al., 2010) between organisms belonging to the Phylum Actinobacteria. This suggests that horizontal transfer of the hairpin structure between these species is most likely a recent event (Schuster et al., 2013). Current NCBI databases show a minimum nucleotide identity of 97 % between *ethB* genes.

Monooxygenases (e.g. *ethB* and *mdpA*) have been widely implicated in catalysing the first step of oxidative catabolism (production of an hemiacetal leading to cleavage of the ether bond) in ETBE and other ether oxygenates (Chauvaux et al., 2001; Fayolle et al., 2001; Kharoune et al., 2001a; Hernandez-Perez et al., 2001; Béguin et al., 2003; Müller et al., 2008; Auffret et al., 2009; Malandain et al., 2010; Fayolle-Guichard et al., 2012; Schuster et al., 2013; Gunasekaran et al., 2014; van der Waals et al., 2019). However, these monooxygenases differ in their substrate specificity, for example, hydroxylation of only methoxy or methoxy and ethoxy groups of tert-alkylethers (Schuster et al., 2013). The degradation of ETBE by *Rhodococcus ruber* IFP 2001 is initiated when the cytochrome P450 monooxygenase encoded by *ethB* incorporates oxygen into ETBE, forming *tert*-butoxy ethanol. *Tert*-butyl acetate (TBAc) is then formed through the possible action of dehydrogenases (Lopes Ferreira et al., 2006a). Acetate and *tert*-butyl alcohol (TBA) are produced, with further metabolism of these to biomass and carbon dioxide (Malandain et al., 2010). Isolated strains of bacteria possessing the ability to grow on ETBE have different metabolic capabilities, resulting in either partial breakdown of ETBE or complete mineralisation. Examples include *Betaproteobacteria* IFP 2047 and *Actinobacteria* IFP 2050, isolated from contaminated groundwater in Germany and France, respectively, which can degrade both ETBE and TBA (Le Digabel et al., 2014). TBA produced from aerobic ETBE biodegradation may accumulate as a transient intermediate or be metabolised, according to the metabolic capability of the species responsible. The *mdpJ* gene, encoding a TBA hydroxylase, has been shown to facilitate TBA degradation in an ETBE degrader, *Aquincola tertiaricarbonis* L108 (Schuster et al., 2012), and MTBE degrader, *Methylibium petroleiphilum* PM1 (Joshi et al., 2015). *Rhodococcus ruber* IFP 2001 was isolated for the ability to degrade ETBE as the sole carbon and energy source, with accumulation of TBA (Hernandez-Perez et al., 2001). Some studies have shown the importance of syntrophic interactions for mineralisation of ETBE without the accumulation of TBA. *Rhodococcus* sp. IFP 2042 was reported to degrade ETBE to TBA and *Bradyrhizobium* sp. IFP 2049 degraded TBA to biomass and carbon dioxide (Le Digabel et al., 2013). These organisms were isolated from MTBE- and TBA-contaminated groundwater. To date, there is limited knowledge of the geographic or taxonomic distribution of aerobic ETBE-degrading organisms (Table 2).

3.2.2. The eth gene cluster regulator and cytochrome specificity

Malandain et al. (2010) studied three different strains able to grow on ETBE, *Rhodococcus* ruber IFP 2001, *Rhodococcus zopfii* IFP 2005 and *Gordonia* sp. IFP 2009, all possessing the *ethB* cytochrome P450 monooxygenase and able to degrade MTBE and TAME. ETBE was the favoured substrate as ETBE degradation rates were 10x that of MTBE or TAME. Each strain utilised ETBE at rates between 1.6–3.5 mmol ETBE g^{-1} dry weight h^{-1}. Transcriptional analysis showed that *ethB* transcription rates were greater in IFP 2005 than the other strains, possibly explaining the greater rate of ETBE degradation for this organism. MTBE and TAME did not induce *ethB* expression, suggesting that these compounds do not interact with the *eth* regulator (*ethR*). Thus the specificity of the *eth* cytochrome system is due to the regulator rather than the cytochrome itself and is an important consideration for the biodegradation of ETBE with other oxgenate co-substrates in ether-contaminated groundwater, since the presence and expression of the *eth* genes would have to be studied systematically to evaluate the possibility of natural attenuation (Malandain et al., 2010).

Aquincola tertiaricarbonis L108 contains the *eth* gene cluster (*ethABCD*) but lacks the regulator, *ethR*, resulting in constitutive expression of the *eth* gene cluster. This organism can degrade ether oxygenates at high rates and without a lag, due to the expression of the *eth* monooxygenase at high levels, even when grown on non-ether substrates. It can also degrade TBA, indicating that it has metabolic pathways for complete mineralisation. The authors, Müller et al. (2008) and Schuster et al. (2013), also report that this microbe degrades ETBE, MTBE and TAME at similar rates, but with ETBE somewhat favoured. This is supported by Pannier et al. (2010), who reported that *A. tertiaricarbonis* L108 degraded MTBE and ETBE at similar rates when fed these substrates as the sole carbon source, although ETBE was the preferred substrate in mixtures of these compounds, thus showing a higher affinity for ETBE. However, ethers with larger residues, n-hexyl methyl ether, tetrahydrofuran, and alkyl aryl ethers, were not degraded at significant rates, indicating the residue in the ether molecule which is not hydroxylated during degradation also contributes to the determination of substrate specificity (Schuster et al., 2013). The higher resistance of MTBE and TAME to biodegradation compared with ETBE is reported to reflect the greater difficulty of inserting an oxygen atom to yield the hemiacetal via hydroxylation of methoxycarbon in MTBE and TAME by the cytochrome P450, due to proximity of the ether bond (Kharoune et al., 2001a; Malandain et al., 2010).

3.2.3. Alternative genes and enzymes

While most molecular research into ETBE biodegradation has focused on the *eth* gene cluster, there is also limited evidence for the involvement of alternative enzymes. For example, *Achromobacter xylosoxidans* MCM2/2/1, isolated from ETBE- and MTBE-contaminated soil, was shown to degrade both compounds and possessed a cytochrome P450 gene (CYP) encoding a protein distinct from *ethB*. The cytochrome (*CYP*) gene was 88 % and 90 % similar at the nucleotide and amino acid level, respectively, to the *thcB* gene of *A.xylosoxidans* A8, with this gene often described in bacteria with broad substrate specificity for xenobiotic compounds. The *CYP* gene was only 23 % similar at the amino acid level to the *ethB* gene of *R. zopfii* (Gunasekaran et al., 2014). The authors (*op cit*) acknowledge that experimental confirmation is needed to establish the role of this protein in ETBE and MTBE biodegradation. The data is inconclusive as the organism expresses the *CYP* in the absence of MTBE and expression actually decreases upon MTBE exposure. Furthermore, detection of the *eth* genes was apparently not attempted in this study, which could account for the GEO biodegradation. Additional studies addressing this are needed to clarify the exact role of the reported cytochrome. However, it is plausible that alternative cytochromes could also be responsible for ETBE biodegradation.

In other studies strains that can degrade ETBE have been identified, but the *eth* genes could not be specifically detected, suggesting that alternative enzymes may be involved. The ETBE degrader *Rhodococcus* sp. IFP 2042 did not have detectable *ethB* or *ethR* genes but instead several *alkB* genes encoding alkane hydroxylases, a non-heme iron

containing oxygenase, were identified (Le Digabel et al., 2013). Similarly, *alkB* genes, rather than *eth* genes, were also found in strain *R. wratislaviensis* IFP 2016 which degraded ETBE in mixtures with various alkanes (e.g. BTEX, octane) but not on its own (Auffret et al., 2009). *alkB* genes have been implicated in MTBE biodegradation, where their expression is induced in the presence of alkane co-contaminants (Bravo et al., 2015) or TBA (Lopes Ferreira et al., 2007), but with a potentially broad range of activity against ether oxygenates (Steffan et al., 1997). Nonetheless, alkane hydroxylases induced in some Pseudomonad strains (*P. mendocina* KR1 and *P. aeruginosa*) grown on *n*-octane have been reported with restricted activity towards MTBE and TAME, rather than ETBE (Smith and Hyman, 2010), suggesting a clear preference for methyl ethers with no reactivity towards ETBE. It should be noted, however, that the detection of the *alkB* gene is limited due to nucleotide variation of this gene and so primer choice consequently determines the effectiveness of the detection methods (Jurelevicius et al., 2013).

While the cytochrome P450 monooxygenase (encoded by *ethB*) displays the ability to degrade different GEO, monooxygenases linked to MTBE degradation do not appear to possess this broad GEO degradation activity. The MTBE-degrading organism *Methylibium petroleiphilum* PM1 contains a gene induced in the presence of MTBE, *mdpA*, that encodes a monooxygenase (Schmidt et al., 2008). However, *M.petroleiphilum* PM1 has no known activity towards ETBE (Kane et al., 2007). Another microorganism, *Methylibium* sp. strain T29, isolated from a gasoline-contaminated site, can degrade MTBE and TAME, but not ETBE. The organism possesses a monooxygenase with 84 % amino acid identity to the *mdpA* gene (Szabó et al., 2015) in *Methylibium petroleiphilum* PM1. This suggests that the characterised MTBE monooxygenase does not degrade ETBE but is specific to MTBE. Interestingly, van der Waals et al. (2019) recently reported degradation of ETBE by an algal-bacteria culture enriched from contaminated groundwater in which the *eth* gene encoding the cytochrome P450 subunit was not found in the microbial community, but instead a relatively high amount of *hcmA* genes were detected, leading the authors to conclude that ETBE was degraded via TBA and 2-HIBA according to the metabolic pathway involving 2-hydroxyisobutyryl-CoA mutase, suggested by Rohwerder et al. (2006). Further work is required to deduce if the *hcmA* gene is expressed during growth on ETBE.

4. Aerobic biodegradation of ETBE in environmental samples

Pure and mixed cultures able to partially transform ETBE to TBA or completely degrade ETBE aerobically in the natural environment have been characterised from soil, activated sludge and wastewater from treatment plants, and groundwater contaminated with gasoline and/or MTBE. Microorganisms isolated from these studies are summarised in Table 2.

4.1. Soil

Although soils potentially harbour a diverse range of methylotrophic bacteria capable of metabolising ether oxygenates (De Marco et al., 2004), the biodegradation of ETBE in soils has received little attention so far. Nonetheless several microorganisms have been isolated from uncontaminated and gasoline- or MTBE-contaminated soils for their ability to grow on ETBE as a sole carbon and energy source under aerobic conditions (Table 2 and references therein). Aerobic biodegradation of ETBE has also been demonstrated in mixed cultures and consortia sampled from soils exposed previously (Kharoune et al., 2001b, 2002; Gunasekaran et al., 2013) and not exposed (Steffan et al., 1997; Yuan, 2006; Bartling et al., 2011) to gasoline or ETBE. Phylogenetic analysis shows that many strains of aerobic ETBE-degrading microorganisms in soils belong to the Phylum Actinobacteria, with Rhodococcual species of particular importance. The isolation of these bacterial strains from aerobic soil environments with contrasting contamination history implies a diverse range of microorganisms able to biodegrade ETBE in isolation or simultaneously in mixtures with other GEO (e.g. MTBE and TAME) (Steffan et al., 1997; Kharoune et al., 2001b, 2002). Biodegradation of ETBE in soils by pure strains or consortia has been documented without a lag (Hernandez-Perez et al., 2001; Kharoune et al., 2001a, b) and with partial transformation to TBA (Steffan et al., 1997; Auffret et al., 2009; Purswani et al., 2011; Le Digabel et al., 2014) or complete mineralisation to CO_2 (Hernandez-Perez et al., 2001; Kharoune et al., 2001a, b, 2002; Morales et al., 2009; Gunasekaran et al., 2014; Le Digabel et al., 2014).

Relatively rapid aerobic ETBE metabolism without a lag is possible in soils which have not been exposed to this compound (Bartling et al., 2011). Degradation rates vary according to measurement method, ranging from 0.0091 day^{-1} to as high as 0.024 mmol L^{-1} ETBE day^{-1} g^{-1} soil (Yeh and Novak, 1995; Yuan, 2006). In consortia from gasoline-contaminated soils, ETBE biodegradation rates of between 0.57-0.78 mmol g^{-1} of cell protein day^{-1} and up to 0.93 mmol ETBE g^{-1} cell protein h^{-1} with biomass yields of 0.51 g protein g^{-1} ETBE have been reported (Kharoune et al., 2001a, 2002). Similarly, Kharoune et al. (2001b) recorded complete biodegradation of ETBE at a rate of 0.28 mmol L^{-1} day^{-1}, simultaneously with MTBE and TAME, by a consortium enriched from a gasoline-contaminated soil. Microorganisms (both pure strains and natural isolates) have also been characterised with the capability to metabolise ETBE after growth on other GEO (Purswani et al., 2008, 2011; Gunasekaran et al., 2014) and hydrocarbons such as propane and n-pentane (Steffan et al., 1997; Morales et al., 2009). Degradation rates as high as 0.29 mmol ETBE L^{-1} day^{-1} have been reported for *Mycobacterium vaccae* JOB5, isolated from soil and grown on propane, for an initial inoculum size of 0.08 g protein L^{-1} (Steffan et al., 1997). This offers the potential of using propanotrophs for bioaugmentation to overcome slow growth kinetics of organisms on ETBE in the development of groundwater treatment systems or bioremediation approaches for ETBE-contaminated aquifers, as demonstrated for MTBE (Debor and Bastiaens, 2007; Schirmer and Martienssen, 2007;).

Other studies have focused on the impact of ETBE on soil microbial processes and microbial community structure and function. Using a eukaryotic growth inhibitor (cyclohexamide), Kharoune et al. (2002) showed that bacteria, rather than fungi, are responsible for ETBE degradation in soils and that the accumulation or degradation of TBA is related to the balance and evolution of specific ETBE- and TBA-degraders in consortia, as reported for groundwater (see below). They found TBA accumulated at inoculum concentrations of <120 mg L^{-1} protein, with reduced ETBE biodegradation rate, but with larger inocula ETBE was degraded faster with no TBA accumulation. This was attributed to the time required for growth of active microorganisms at the expense of the ETBE-ethyl group prior to metabolism of the TBA, or an initially low number of TBA degraders relative to ETBE-degraders in the small inoculum. Clearly, this temporal change in total cell numbers and community of ETBE- and TBA-degraders can explain lags for ETBE biodegradation and transient accumulation of metabolites in natural consortia.

ETBE biodegradation may be inhibited at soil pore water concentrations greater than 15.7 mmol L^{-1} (Kharoune et al., 2002). Bartling et al. (2010) reported increased N-compounds (NH_4^+ and NO_2^-) in soil contaminated with ETBE and TAME, attributing this to toxicity towards nitrifying microflora and the release of proteins and amino acids from lysed bacteria mineralised by GEO-tolerant microorganisms. Bartling et al. (2011) used phospholipid fatty acid (PLFA) analysis (an indication of microbial biomass and community composition) to show that Gram-positive bacteria became more dominant than Gram-negative bacteria in the microbial community of a soil not previously exposed to contamination, during aerobic biodegradation of ETBE and TAME. The PLFA concentrations and proportion of Gram-positive bacteria were increased significantly after biodegradation of these GEO over 42 days, which was attributed to the Gram-positive bacteria having a more robust cell wall structure than Gram-negative bacteria,

or ability to persist in toxic conditions following soil contamination. This suggests Gram positive bacteria in soils may utilise ETBE as a carbon source, consistent with other studies in which similar microorganisms (e.g. *Acinetobacter calcoaceticus* M10, *Gordon terrae* IFP 2001, *Mycobacterium austroafricanum* IFP 2012) have been implicated in GEO biodegradation (Fayolle et al., 1998; Hernandez-Perez et al., 2001; François et al., 2002; Purswani et al., 2008).

4.2. Groundwater

The literature on aerobic biodegradation of ETBE in groundwater is sparse and in general restricted to studies assessing the development of treatment systems for ETBE-contaminated groundwater using pure and mixed cultures grown on various substrates (Fayolle et al., 1998; Chauvaux et al., 2001; Hernandez-Perez et al., 2001; François et al., 2002; Lopes Ferreira et al., 2006a; Zein et al., 2006; Auffret et al., 2009; McKelvie et al., 2009; Malandain et al., 2010; van der Waals et al., 2019), and a limited number of field studies examining the fate of ETBE in aquifers at gasoline-release sites (Müller et al., 2008; Fayolle-Guichard et al., 2012; Gunasekaran et al., 2013; Le Digabel et al., 2013, 2014; Schuster et al., 2013; Kyselková et al., 2019; Nicholls et al., 2020). In general, there is a lack of data on the geographical distribution of ETBE in groundwater within Europe compared with other GEO such as MTBE, and relatively few documented releases of ETBE to groundwater with concentrations above detection limits (<2 % of European sites sampled) (Concawe, 2012).

To date very few pure strains have been isolated in groundwater that can completely degrade ETBE and related organic metabolites (Table 2). However, *Aquincola tertiaricarbonis* L108 was identified by Müller et al. (2008) for its capacity to grow on MTBE, ETBE and TAME, in addition to TBA, TAA and 2-HIBA, with an effective growth rate (μ_{max}), growth yield and maximum degradation rate of respectively 0.06 h^{-1}, 0.53 g dry mass (g ETBE)$^{-1}$ and 1.11 mmol ETBE h^{-1} g dry mass^{-1} for ETBE, 0.1 h^{-1}, 0.48 g dry mass (g TBA)$^{-1}$ and 2.82 mmol TBA h^{-1} g dry mass^{-1} for TBA, and 0.17 h^{-1}, 0.54 g dry mass (g 2-HIBA)$^{-1}$, 3.27 mmol 2-HIBA h^{-1} g dry mass^{-1} for 2-HIBA at pH 7 and 30 °C. The relatively high rates for TBA and 2-HIBA indicate that their transformation did not limit ETBE metabolism (despite these metabolites possessing a tertiary carbon atom), and based on specific affinity ETBE (μ_{max}/K_s = 0.73 h^{-1} $mmol^{-1}$) was a significantly better substrate for strain L108 than MTBE (μ_{max}/K_s = 0.052 h^{-1} $mmol^{-1}$) (Müller et al., 2008). This exceptional metabolic capability and elevated substrate specificity for compounds with tertiary butyl groups was attributed to attack of the ethyl group on ETBE by a monooxygenase reaction catalysed by a cytochrome P450-type enzyme encoded by the *ethABCD* genes, with a different oxygenase system likely responsible for hydroxylating TBA in strain L108. Schuster et al. (2013) showed that L108 lacks the regulator *ethR* gene but constitutively expresses the P450 monooxygenase system at high levels, even when grown on non-ether substrates, suggesting a plausible mechanism for this behaviour. The high growth yield (≥0.5 g g dry mass^{-1}) on ETBE also suggests low maintenance requirements (shown to be theoretically possible by Müller et al. (2007)) may be an intrinsic property of this strain. However, metabolism of one GEO (e.g. ETBE) in aquifers may not automatically confer this capability for others (e.g. MTBE), where substrate-specific enzymes exist or are slow to develop (Yeh and Novak, 1995).

Gunasekaran et al. (2013) reported the biodegradation of ETBE as a sole carbon source by a consortium containing *Xanthomonas* sp., *Methylibium* sp., *Methylobacillus* sp. and *Methylovorus* sp. enriched from gasoline-contaminated groundwater, in which the dominant microorganism, *Xanthomonas* sp., is a genus from the γ-proteobacteria phylum and known to degrade petroleum hydrocarbons. The presence of *Methylophilus* sp. suggests this methylotroph plays an important role in ETBE degradation, given its ability to metabolise other GEO (De Marco et al., 2004). Other studies (Le Digabel et al., 2013, 2014) highlight the importance of cooperation between different microorganisms in the syntrophic degradation of ETBE by natural consortia in groundwater. Le Digabel et al. (2013) isolated two bacterial strains, IFP 2042 and IFP 2049, from MTBE/TBA-contaminated groundwater, with 16S rRNA genes similar to *Rhodococcus* sp. (99 % similarity to *Rhodococcus erythropolis*) and *Bradyrhizobium* sp. (99 % similarity to *Bradyrhizobium japonicum*), respectively, which completely degraded ETBE as sole carbon and energy source to CO_2. *Rhodococcus* sp. IFP 2042 degraded ETBE to TBA, which accumulated, and *Bradyrhizobium* sp. IFP 2049 degraded TBA to biomass and CO_2. A mixed culture of IFP 2042 and IFP 2049 degraded ETBE to CO_2 with a biomass yield (0.31 ± 0.02 mg biomass mg^{-1} ETBE) similar to the original ETBE enrichment (0.37 ± 0.08 mg biomass (dry weight) mg^{-1} ETBE). This provides a plausible explanation for the accumulation of TBA as a dead-end metabolite of ETBE, where TBA-degraders are absent from consortia (Somsamak et al., 2001; Schmidt et al., 2004), and suggests incomplete degradation of ETBE could occur in natural consortia dominated by ETBE-degraders similar to *Rhodococcus* sp. IFP 2042. Subsequently, Le Digabel et al. (2014) confirmed the role of microorganisms from the phyla *Actinobacteria* (*Rhodococcus* sp. IFP 2040, IFP 2041 and IFP 2043, and *Pseudonocardia* sp. IFP 2050) and *Proteobacteria* (*Betaproteobacteria* IFP 2047), isolated from MTBE-contaminated soil and groundwater from different geographical locations, in ETBE and/or TBA degradation (Table 2). IFP 2040, 2041 and 2043 degraded ETBE to TBA, whereas IFP 2047 and 2050 degraded ETBE and TBA to CO_2 and biomass. The study also showed that when ETBE degradation led to TBA accumulation other microorganisms, such as *Bradyrhizobium* (Le Digabel et al., 2013) and *Rubrivivax* sp. IFP 2047 (from Proteobacteria) may degrade TBA. Other *Betaproteobacteria* (order *Burkolderiales* with representatives from the genera *Polaromonas*, *Cupriavidus*, *Rhodoferax* and *Methylibium*) capable of utilising tertiary alcohols, including TBA (and TAA), as a sole carbon and energy source have also been characterised in activated sludge (Piveteau et al., 2001) and TBA-contaminated groundwater (Aslett et al., 2011). Strain CIP I-2052 (belonging to *Burkholderia cepacia* species) isolated by Piveteau et al. (2001) exhibited a substrate specificity for tertiary alcohols, with a maximum growth rate, degradation rate and growth yield on TBA of 0.032 h^{-1}, 0.48 mmol TBA·g^{-1} (cell dry mass) h^{-1} and 0.54 g·g^{-1}, respectively. CIP I-2052 also degraded formate and methanol (proposed metabolites of ETBE and MTBE) as sole carbon and energy sources, indicating the presence of a complete pathway for C1 compounds, and emphasising the importance of commensalistic action of these different facultative methylotrophic bacteria in complete ETBE degradation. More recently Kyselková et al. (2019) characterised the taxonomical composition of microbial communities and diversity of the *ethB* gene in ETBE-enrichment cultures established from a gasoline-contaminated aquifer. The composition of the enrichment cultures was distinct from their initial water samples, suggesting the importance of the rare biosphere as a reservoir of potential ETBE-degraders. Cultures originating from uncontaminated and contaminated locations were respectively dominated by *Mesorhizobium* and *Hydrogenophaga*, implying that distinct consortia with the same functional properties were present at the site. Moreover the *ethB* gene was conserved in these highly distinct microbial communities, which was attributed to dissemination of the *eth* genes by horizontal gene transfer amongst the various hosts. This mechanism is also reported to account for the presence of extremely conserved *eth* loci present amongst otherwise unrelated sequences in pure strains *R. zopfii* IFP 2005 and *Mycobacteriumsp*. IFP 2009 (Lopes Ferreira et al., 2006b).

The importance of environmental limitations on ETBE degradation by natural consortia in groundwater has received little attention. Fayolle-Guichard et al. (2012) compared biostimulation and bioaugmentation of ETBE degradation by indigenous microflora enriched from ETBE-contaminated groundwater with a mixed culture comprising known ETBE-degraders, *Rhodococcus wratislaviensis* IFP 2016, *Rhodococcus aetherivorans* IFP 2017 and *Aquincola tertiaricarbonis* IFP 2003. Biostimulation by amendment of native groundwater with either O_2 or

O_2 and nutrients did not promote ETBE degradation. However, after biostimulation and bioaugmentation by the enriched indigenous microflora the native consortium completely degraded ETBE to CO_2 and biomass at a rate (0.91 mg L^{-1} h^{-1}) with only transient accumulation of TBA. This was comparable to that for the exogenous mixed culture (0.83 mg L^{-1} h^{-1}) and similar to BTEX in each system (0.64 and 0.82 mg L^{-1} h^{-1}, respectively). Hence, the absence of ETBE degradation under biostimulated conditions most likely reflected a limited population of ETBE-degrading microorganisms in the native consortia, which increased after enrichment (Schirmer and Martienssen, 2007; Debor and Bastiaens, 2007). Surprisingly, the exogenous mixed culture was able to metabolise ETBE without accumulation of TBA at rates comparable to the native enriched consortia and concurrently with BTEX. These results are encouraging in demonstrating the ability of the mixed culture to compete successfully with the indigenous microorganisms and potential to develop biologically-based *in-situ* and *ex-situ* treatment options for ETBE-contaminated groundwater using either enriched native or exotic ETBE-degrading microorganisms, which overcomes common technical barriers to bioremediation of GEO in groundwater (Debor and Bastiaens, 2007; Schirmer and Martienssen, 2007; Thornton et al., 2016). However, further studies are needed to verify this capability and practical application in different contexts. More recently, van der Waals et al. (2019) have described aerobic biodegradation of ETBE to TBA at a rate of 34 mM day^{-1} (first order rate 0.4 day^{-1}) by a mixed algal-bacterial culture enriched from ETBE-contaminated groundwater, in which oxygen produced by the micro algae, *Chlorella* and *Scenedesmus*, was used by the native bacteria for ETBE metabolism, supporting the development of novel *ex situ* groundwater treatment systems without the need for external O_2 supplementation or aeration.

4.3. Co-metabolism of ETBE and aerobic biodegradation in mixtures with other fuel constituents

In cases where gasoline containing ETBE is released to soil or groundwater the resulting plume of dissolved constituents (in groundwater) will potentially comprise a mixture of hydrocarbon compounds and other fuel additives, which may have a synergistic or antagonist effect on ETBE biodegradation potential (Auffret et al., 2009; Fayolle-Guichard et al., 2012; Concawe, 2012; Bombach et al., 2015). There are, however, very few reports documenting the effect of co-contaminants on the fate of ETBE in soil or groundwater.

Preferential utilisation of BTEX or ethanol as co-contaminants is reported to suppress or completely inhibit aerobic biodegradation of ETBE by *Acinetobacter calcoaceticus* M10 cultured from hydrocarbon-contaminated soil (Purswani et al., 2008), *Rhodococcus wratislaviensis* IFP 2016 and *Rhodococcus aetherivorans* IFP 2017 cultured from gasoline-contaminated soil (Auffret et al., 2009) and a mixed consortium consisting of *Xanthomonas* sp., *Methylibium* sp., *Methylobacillus* sp. and *Methylovorus* sp. cultured from gasoline-contaminated groundwater (Gunasekaran et al., 2013), relative to metabolism in the absence of any substrate competition. This occurs by depleting the dissolved oxygen supporting ETBE respiration or by direct inhibition. In this respect enzymes that mediate the degradation pathway of aromatic ring structures in gasoline hydrocarbons are likely to be more easily inducible, available and with broader substrate degradation capacities within a consortia exposed to chronic contamination than an ether-degrading group of enzymes, which may have a different affinity for specific ether oxygenates (Auffret et al., 2009). Catabolic repression of enzyme induction in ETBE-degrading microorganisms (e.g. *Rhodococcus* strains) by the presence of such alternative substrates has been proposed to explain this behaviour (Yeh and Novak, 1995; Malandain et al., 2010). Other mechanisms which may limit ETBE biodegradation in gasoline-contaminated soil and groundwater include toxicity towards ETBE degraders from other GEO (Kharoune et al., 2001b, 2002; Lin et al., 2007) and metabolites of BTEX degradation (Dietz, 2007; Gunasekaran et al., 2013). More generally, inhibition of aerobic ETBE biodegradation in uncontaminated organic-rich soils can arise from preferential utilisation of more easily degradable, naturally occurring organic compounds (Yuan, 2006).

The biodegradation of ETBE in mixtures with other GEO is of interest in order to understand the effect of these structurally-related compounds on metabolic rates in multi-substrate systems and the removal pattern (e.g. preferential, sequential or simultaneous utilisation) which may occur for ETBE (Kharoune et al., 2001b). However, there are very few studies to date which have directly compared the biodegradation of ETBE in mixtures with other GEO (e.g. Kharoune et al., 2001b, 2002; Yuan, 2006; Zein et al., 2006; Lin et al., 2007). Most attention has focused on comparisons between ETBE, MTBE and TAME in isolation or biodegradation of MTBE and TAME by cells grown on ETBE (Table 2). Nethertheless, these studies provide insight on potential mechanisms which may affect ETBE biodegradation in mixtures with MTBE and TAME.

Biodegradation of ETBE as a sole carbon and energy source in preference to MTBE and TAME has been reported in pure cultures of *Gordonia terrae* IFP 2001, *Gordonia* sp. IFP 2009, *Rhodococcus ruber* IFP 2001, *Rhodococcus equi* IFP 2002 *and Rhodococcus zopfii* IFP 2005 (Fayolle et al., 1998; Hernandez-Perez et al., 2001; Malandain et al., 2010), *Comamonas testosteroni* E1 (Kharoune et al., 2001a) and *Rhodococcus wratislaviensis* IFP 2016 and *Rhodococcus aetherivorans* IFP 2017 (Auffret et al., 2009). *Methylibium petroleiphilum* PM1 is also reported to biodegrade ETBE by zero-order kinetics (0.0287 mmol h^{-1}), concurrently with MTBE, TAME and DIPE (Church et al., 2000). Similarly, strain *Aquincola tertiaricarbonis* L108 can mineralise ETBE, MTBE and TAME at comparable rates (Schuster et al., 2013), and *Achromobacter xylosoxidans* MCM2/2/1 is reported to degraded ETBE and MTBE without accumulation of TBA (Gunasekaran et al., 2014). The ability of these strains to metabolise the three GEO has been attributed to the induction of a cytochrome P450 enzyme with a broad substrate range but different affinity for each oxygenate, the preferred substrate being ETBE. While the *ethB* gene is induced by ETBE in many strains to support biodegradation of MTBE and TAME, the absence of the *ethR* regulator in *Aquincola tertiaricarbonis* L108 results in constitutive expression of the *eth* monooxygenase, providing this organism with the metabolic pathways for complete mineralisation of ETBE, MTBE and TAME (Schuster et al., 2013). This may confer the ability to biodegrade all three GEO in mixtures. However, MTBE and TAME may not support growth of ETBE-induced cells if a microorganism cannot grow on the C1 carbon sources resulting from metabolism of these two oxygenates (Fayolle et al., 1998; Purswani et al., 2008). Conversely, as reported for the methylotrophs *Mycobacterium austroafricanum* IFP 2012 and *Mycobacterium austroafricanum* IFP 2015, slow growth on ETBE relative to MTBE or TAME may imply an inability to metabolise the C2 compounds (or later intermediates) released from cleavage of the ethyl group on ETBE (François et al., 2002 Lopes Ferreira et al., 2006a).

ETBE has been reported to biodegrade as a sole carbon and energy source simultaneously with MTBE and TAME in mixed cultures from a continuous upflow bioreactor (Kharoune et al., 2001b), gasoline-contaminated soil (Kharoune et al., 2002), vadose zone soil (Yuan, 2006), aerobic bioreactor treating gasoline-contaminated groundwater (Zein et al., 2006) and wastewater treatment plant (Lin et al., 2007). In some cases, ETBE was degraded faster than MTBE and TAME in mixtures (respectively 73, 29 and 28 mg L^{-1} d^{-1}), although at a slower rate than that for the individual oxygenates (respectively 111, 23 and 28 mg L^{-1} d^{-1}), suggesting that mixtures of these compounds were more toxic for ETBE-degrading microorganisms in the consortia (Kharoune et al., 2001b; 2002). Similar inhibition of GEO degradation due to toxicity effects from parent compounds or intermediates in mixtures of ETBE and MTBE has been observed by Yuan (2006) and Lin et al. (2007). Other studies have reported limited biodegradation of ETBE relative to MTBE by consortia from MTBE-contaminated sites (Babé et al., 2007). This was attributed to the inability of the monooxygenase responsible

for initial attack on the methyl group of MTBE to attack the ethyl group of ETBE, or failure of ETBE to induce or derepress synthesis of the monooxygenase responsible for MTBE metabolism. This is consistent with proposed limitations on GEO biodegradation related to the oxygenate structure, and in particular the relative accessibility of enzymes to the methoxy carbon in MTBE or ethoxy carbon in ETBE due to the proximity of the ether bond (Kharoune et al., 2001b; Yuan, 2006; Malandain et al., 2010).

Cometabolism is a potentially important mechanism for ETBE biodegradation in gasoline-contaminated soil and groundwater, since it decouples bacterial cell growth and biomass maintenance from biodegradation of ETBE as a primary substrate (Müller et al., 2007). It enables biodegradation of the non-growth substrate (e.g. ETBE) to trace levels in the presence of an alternative carbon and energy source, using non-specific enzymes (e.g. oxygenases) that support transformation of both the growth substrate and ETBE (Dalton and Stirling, 1982; Janke and Fritsche, 1985; Morales et al., 2009; Hazen, 2010). To date aerobic cometabolism of ETBE has only been demonstrated using ethanol (Purswani et al., 2008), propane (Steffan et al., 1997), cyclohexane (Corcho et al., 2000), pentane (Morales et al., 2009), mixed hydrocarbon compounds (Auffret et al., 2009), and heterocyclic ether compounds (McKelvie et al., 2009) as growth substrates. Purswani et al. (2008) observed greater degradation of ETBE by *Arthrobacter* sp. MG, *Gordonia amicalis* EA, *Nocardioides* sp. E7 and *Rhodococcus ruber* E10 isolated from hydrocarbon-contaminated soil in mineral media supplemented with ethanol and yeast extract, than in mineral media with ETBE alone, whereas Corcho et al. (2000) reported cometabolism of ETBE, MTBE, TAME and TBA by a mixed culture able to grow on cyclohexane as a sole carbon and energy source. *Pseudonocardia tetrahydrofuranoxydans* strain K1 grown on tetrahydrofuran (THF) was shown by McKelvie et al. (2009) to cometabolise MTBE and ETBE to TBA and TAME to TAA. The ETBE-oxidizing activity in strain K1 was attributed to the THF-dependent expression of THF-monooxygenase, which initiates THF oxidation by generating 2-hydroxytetrahydrofuran from initial oxidation of a C–H bond in the methoxy group on MTBE and TAME, and in the ethoxy group on ETBE. In similar studies Steffan et al. (1997) and Morales et al. (2009) have shown the importance of alkane-oxidising bacteria as mediators of ETBE cometabolism in gasoline-contaminated soils. In the former (Steffan et al., 1997), strain *Mycobacterium vaccae* JOB5 and natural isolates of nocardioform bacteria ENV420, ENV421, and ENV425 obtained in mixed cultures from soil were able to degrade ETBE, MTBE, TAME and TBA after growth on propane. A cytochrome P450 enzyme (propane mono-oxygenase) was detected in propane-grown cells of strain ENV425. In the latter study a consortia dominated by Pseudomonad bacteria, in which *P. aeruginosa* and *P. citronellolis* were isolated as specific strains, cometabolised ETBE, MTBE, TAME, TBA and TAA after growth on *n*-pentane, presumable using enzyme systems with broad affinity for the methoxy, ethoxy and tertiary methyl groups in these respective ether oxygenates (Morales et al., 2009).

These studies are significant in demonstrating the occurrence of environmental isolates and pure strains which can grow on propane and also oxidise ETBE with other GEO by the induction of relevant enzyme systems. Alkane-oxidising bacteria are widespread in the environment and these hydrocarbons (e.g. propane, pentane) are typically present in gasoline-contaminated soils and groundwater containing GEO. Cometabolism thus opens up opportunities to overcome potential limitations on ETBE biodegradation, such as low cell yields and slow growth of organisms, in the development of biologically-based *in situ* treatment concepts (Schirmer and Martienssen, 2007; Morales et al., 2009). However a major concern of ETBE cometabolism is the accumulation of TBA in treated groundwater, noted in field studies of GEO-contaminated aquifers (Wilson et al., 2004; Martienssen et al., 2006; Babé et al., 2007; Aslett et al., 2011; Rasa et al., 2011; Fayolle-Guichard et al., 2012; Le Digabel et al., 2014). This therefore requires the identification of organisms with the capability for complete mineralisation of this ether oxygenate (e.g. *Aquinola tertiaricarbonis* L108) for effective bioremediation (Müller et al., 2008; Schuster et al., 2013).

In general, the effect of co-substrates on ETBE biodegradation potential in gasoline-contaminated soil and groundwater appears to be site-specific and in particular related to the presence of appropriate primary substrates in contaminant mixtures and microorganisms which have evolved the requisite enzymes to exploit these for growth. Common hydrocarbon co-substrates, such as BTEX, may limit or enhance ETBE biodegradation by different mechanisms, whereas cometabolism by alkane-oxidising bacteria offers some promise to enhance *in situ* bioremediation of ETBE contaminated groundwater using hydrocarbon compounds typically found in gasoline plumes. Most studies suggest that MTBE and TAME do not significantly affect the biodegradation of ETBE in mixtures of these GEO, which may be biodegraded simultaneously at comparable rates by monooxygenase enzymes with a broad substrate specificity. Differences in ETBE biodegradation in mixtures with MTBE and TAME appear to reflect the respective oxygenate structure and metabolic capability of the specific microorganisms involved. An important observation is that single substrate studies on ETBE biodegradation or fate in soil and groundwater should be interpreted with care as results may differ significantly when mixtures are likely to occur (Yeh and Novak, 1994).

5. Anaerobic biodegradation of ETBE in soil and groundwater

Anaerobic biodegradation of ETBE is of particular interest because gasoline plumes in groundwater rapidly become anaerobic due to consumption of available dissolved oxygen by biodegradation of hydrocarbon compounds (Wiedemeier et al., 1999; Hyman, 2013; van der Waals et al., 2019). Moreover, the demonstration of this potential offers the possibility of using anaerobic biodegradation processes for the management and remediation of ETBE-contaminated sites (Fayolle-Guichard et al., 2012; Bombach et al., 2015). Anaerobic biodegradation of ether oxygenates such as MTBE and TAME has been increasingly documented in mixed enrichment cultures and GEO-contaminated groundwater under various redox conditions (Somsamak et al., 2001, 2005; Somsamak et al., 2006; Kuder et al., 2005; Pruden et al., 2005; Wilson et al., 2000; Wilson, 2003; Wilson et al., 2005; Martienssen et al., 2006; Häggblom et al., 2007; Wei and Finneran, 2009; Youngster et al., 2008, 2010a,b; Ghasemian et al., 2012; Sun et al., 2012; Key et al., 2013; Liu, 2015; van der Waals et al., 2018). However, this potential is currently not well established for ETBE in the subsurface environment and detailed knowledge of the relevant pathways and microorganisms responsible is lacking (Hyman, 2013; van der Waals et al., 2019).

Many studies have found no evidence of anaerobic ETBE biodegradation under conditions characterised by denitrification (Somsamak et al., 2001; Mormile et al., 1994; Waul et al., 2009; Bartling et al., 2010; van der Waals et al., 2019), Fe-reduction (Somsamak et al., 2001; Waul et al., 2009; van der Waals et al., 2019), SO_4-reduction (Mormile et al., 1994; Somsamak et al., 2001; van der Waals et al., 2019), methanogenesis (Suflita and Mormile, 1993; Mormile et al., 1994) or anoxic (Hernandez-Perez et al., 2001; Rosell et al., 2005), even after extensive experimental periods, for example 870 days in the study of van der Waals et al. (2019). The lack of anaerobic ETBE biodegradation in these studies is speculated to reflect the resistance of the branched chemical structure of ethers to enzyme attack under anaerobic conditions (specifically the presence of the tertiary substituted carbon), absence of metabolic pathways in microorganisms able to catalyse this transformation under the provided redox state and electron acceptors (Mormille et al., 1994; Fayolle-Guichard et al., 2012), or preferential metabolism of more biodegradable organic compounds in the inocula used in specific cases (sediment and groundwater from landfill leachate-impacted aquifer, Mormile et al. (1994); peaty soil, Yeh and Novak (1994, 1995); gasoline-impacted estuarine sediment, Somsamak et al. (2001); sludge and soil from petroleum refinery sites and

manures, Waul et al. (2009)). Interestingly, Somsamak et al. (2001) demonstrated anaerobic biodegradation of MTBE and TAME but not ETBE in isolation under SO_4-reducing conditions, confirming that the microorganisms involved were able to cleave the methoxy bond on MTBE and TAME, but not the corresponding ethoxy group on ETBE. Biodegradation of MTBE and TAME under SO_4-reducing and methanogenic conditions is believed to initially occur by demethylation of the methyl group (O-demethylation) of these compounds to respectively TBA and TAA, and to be mediated by acetogenic bacteria which produce a C-1 compound or acetate via acetogenic pathways, with these compounds then serving as a carbon source for the methanogenic or SO_4-reducing communities (Somsamak et al., 2001, 2005; Somsamak et al., 2006; Häggblom et al., 2007; Youngster et al., 2008, 2010b). Anaerobic biodegradation of ETBE is likely to be similarly mediated, with the production of TBA indicating that cleavage of the ether bond in ETBE is the presumed first step in the pathway (Yeh and Novak, 1994). Hence, biodegradation of ETBE under these strictly anaerobic conditions may require specific microorganisms able to cleave the ethyl group on ETBE or involve cleavage of the ethyl and methyl groups on ethers by different reactions (Somsamak et al., 2001).

However, in a few cases anaerobic biodegradation of ETBE to TBA has been demonstrated in soils after a long lag (120 days) using NO_3^- as an electron acceptor and by methanogenic pathways (Yeh and Novak, 1994). This has also been observed under mixed redox conditions in gasoline-contaminated groundwater (Bombach et al., 2015). Relatively rapid anaerobic biodegradation (87 % in 83 days) of ETBE in aquifer microcosms containing inocula from an industrially contaminated site supplemented with ferulate, syringate, isopropanol and diethyl ether has recently been reported (van der Waals et al., 2018). No ETBE biodegradation occurred in unamended natural systems after 1140 days. This study is significant in that it offers the possibility of stimulating *in situ* anaerobic biodegradation of ETBE in groundwater by the addition of these substrates. TBA may not accumulate under such conditions, suggesting that metabolism of this intermediate is not the rate-limiting step for complete anaerobic ETBE biodegradation or that different microorganisms may facilitate TBA metabolism (Yeh and Novak, 1994). However, additional studies are needed to elucidate the potential for cometabolism of ETBE under anaerobic conditions.

In summary, a relatively limited number of studies have been conducted to evaluate anaerobic biodegradation of ETBE in soil and groundwater at field-sale. Most have shown that this potential is not widespread in the subsurface environment. This may imply that the organisms or catabolic enzyme systems responsible for anaerobic ETBE biodegradation in these environments are sparsely distributed (Mormile et al., 1994) or that the chemical and microbiological conditions necessary for biodegradation are sporadically developed and that this potential is site-specific (Yeh and Novak, 1994; Somsamak et al., 2005; Waul et al., 2009; Wei and Finneran, 2009; Youngster et al., 2010b; Fayolle-Guichard et al., 2012; Bombach et al., 2015; van der Waals et al., 2018). Even when microorganisms facilitating anaerobic biodegradation of ETBE exist, this potential may be limited in specific circumstances by preferential metabolism of co-contaminants in gasoline-contaminated groundwater (Yeh and Novak, 1994).

6. Conclusions and research needs

Compared with MTBE, the scientific literature on ETBE biodegradation in soils and groundwater is relatively limited and based mainly on laboratory studies with few field studies. However, it appears that the behaviour of ETBE in groundwater is broadly similar to that of MTBE, taking into account the differences in their physical and chemical properties. To date, a relatively restricted range of microorganisms have been identified with the ability to degrade ETBE aerobically as a carbon and energy source, or via cometabolism using alkanes as growth substrates. These microorganisms can degrade ETBE aerobically via a common pathway with MTBE and TAME, using similar genes and enzyme systems but leading to different intermediate compounds, according to their specific structure. Microorganisms and pathways facilitating anaerobic biodegradation of ETBE have not yet been elucidated, although very limited studies suggest anaerobic biodegradation may occur under mixed redox conditions. The presence of co-contaminants (other ether oxygenates, hydrocarbons and organic compounds) in soil and groundwater may limit aerobic biodegradation of ETBE by preferential metabolism and consumption of available dissolved oxygen or enhance ETBE biodegradation through cometabolism. However, this behaviour is site-specific and appears to depend on the respective microorganisms involved, substrate specificity of enzymes catalysing the metabolism and availability of specific substrates. These biodegradation mechanisms have particular relevance for gasoline plumes in groundwater, since the potential for ETBE biodegradation may evolve according to the biodegradation of other constituents in the plume and oxidant availability. Nonetheless, ETBE-degrading microorganisms and alkane-oxidising bacteria have been characterised which offer the potential to enhance *in situ* biodegradation of ETBE in groundwater, if required, through bioaugmentation and biostimulation.

Considering the current knowledge base on the fate of ETBE in soil and groundwater, gaps in understanding exist in the following areas:

1 The pathways, genes, enzymes, regulators and microorganisms facilitating ETBE biodegradation under aerobic, anaerobic and co-metabolic conditions, and their occurrence in soil and groundwater and across different geographical regions;
2 Understanding of the reasons for low microbial growth rates and cell yields on ETBE and the selectivity of enzymes supporting catabolism of ETBE and its intermediate compounds in mixtures with other ether oxygenates;
3 The effect of co-substrates, such as other ether oxygenates and gasoline hydrocarbons in representative compositions, on ETBE biodegradation potential in typical gasoline-contaminated settings, considering (i) substrate interactions which may suppress or inhibit biodegradation and cometabolism which may enhance biodegradation at field sites, and (ii) interactions between microorganisms in consortia facilitating biodegradation within such mixtures and the genes responsible for this behaviour;
4 The fate and potential for biodegradation of ETBE in the unsaturated zone, considering the different conditions which characterise this domain compared with soils and the saturated zone. The unsaturated zone may be an important location for natural attenuation and biodegradation of ETBE, as has been shown for gasoline hydrocarbons and MTBE.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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