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Effect of environmental conditions and microbial communities on ETBE biodegradation potential in groundwater

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Effect of environmental conditions and microbial communities on ETBE biodegradation potential in groundwater

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ABSTRACT

This report describes research carried out to determine: a) the environmental conditions which may support Ethyl *tert* butyl ether (ETBE) biodegradation in groundwater, b) the prevalence of ETBE biodegradation potential at ETBE-release sites (mainly) within Europe, and c) the organisms and mechanisms involved in aerobic ETBE biodegradation. The research included laboratory biodegradation (microcosm) studies, using samples from an ETBE-impacted site in France, and complementary microbiological studies of groundwater samples collected from several ETBE-impacted sites.

The results of this research suggest that biodegradation of ETBE is likely to occur in groundwater in the presence of dissolved oxygen and is not inhibited by the presence of other ether oxygenates, such as MTBE. Hydrochemical assessments, based on the analysis of dissolved ETBE, TBA and dissolved oxygen, can be used to demonstrate ETBE biodegradation in groundwater at an ETBE-release site. An additional line of evidence that may be relevant at some sites is microbiological assessment of ETBE biodegradation potential. This is site-specific and may be appropriate in cases where the hydrochemical assessment suggests that conditions are favourable for ETBE biodegradation but this is not observed. In this case, the presence of the *ethB* gene in the aquifer microbial community should be determined using aquifer material (e.g. core samples) or mixed groundwater-aquifer material (e.g. sampled from monitoring wells).

KEYWORDS

ETBE, Gasoline ether oxygenates, Groundwater, Aquifer, Biodegradation, *ethB*

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SUMMARY

Ethyl *tert* butyl ether (ETBE) is a gasoline additive that is predominantly used in European fuel blends to increase fuel octane number, improve combustion efficiency and reduce vehicle emissions. Accidental releases of ETBE to the subsurface environment can occur, which may lead to impacts on groundwater. This report describes research carried out to determine: a) the environmental conditions which may support ETBE biodegradation in groundwater, b) the prevalence of ETBE biodegradation potential at ETBE-release sites (mainly) within Europe, and c) the organisms and mechanisms involved in aerobic ETBE biodegradation. The research included laboratory biodegradation (microcosm) studies, using samples from an ETBE-impacted site in France, and complementary microbiological studies of groundwater samples collected from several ETBE-impacted sites.

Aerobic biodegradation of ETBE was assessed in laboratory microcosm studies using aquifer material and groundwater from ETBE-impacted and non-impacted locations. These experiments were amended with ETBE only, or ETBE and methyl *tert* butyl ether (MTBE) as co-substrates. ETBE biodegradation was initially slow for non-impacted locations but repeated additions of ETBE resulted in faster biodegradation, with similar rates between non-impacted and impacted locations. When MTBE was present as a co-contaminant with ETBE, such as may occur in groundwater impacted by both oxygenates, there was no significant effect on ETBE biodegradation and both compounds were biodegraded to *tert*-butyl alcohol (TBA). Microbiological analysis showed that ETBE-degrading organisms eventually dominate the aquifer microbial community and these persist during periods when ETBE is not present in groundwater. This implies that the potential for ETBE biodegradation is maintained in the aquifer microbial community. Members of the Comamonadaceae family increased in number within the microbial community after exposure to ETBE, suggesting the involvement of these organisms in ETBE biodegradation. This observation was confirmed by DNA Stable Isotope Probing (SIP), which showed that isotopically-labelled ETBE was biodegraded and incorporated in the DNA of bacteria of the Comamonadaceae family. The culturing of bacterial isolates suggested that there is a diverse group of organisms present with the capability to biodegrade ETBE at the site studied. In general, there was no significant difference in ETBE biodegradation potential between the unsaturated and saturated zone. However, aerobic biodegradation of ETBE was found to be slower at 20°C than 12°C. This implies that biodegradation rates may vary if experiments are carried out at temperatures which are different from *in situ* temperatures to which the microorganisms are adapted. This factor should be considered in the design of laboratory studies to assess ETBE biodegradation.

A survey of ETBE-impacted sites indicated that organisms containing the *ethB* gene may not be present at all sites, but when present ETBE biodegradation potential was confirmed. The absence of *ethB* gene-containing organisms at ETBE-release sites was correlated with groundwater conditions in which hydrochemical assessments showed that ETBE biodegradation was not observed, i.e. low ETBE concentration or dissolved oxygen. However, the absence of the *ethB* gene in organisms sampled at ETBE-impacted sites does not imply the absence of ETBE biodegradation potential, as many other organisms were identified in the European groundwater survey which could biodegrade ETBE but did not contain the *ethB* gene. This supports the importance of a hydrochemical assessment to demonstrate ETBE biodegradation. The identification of specific groups of ETBE-degrading organisms is not essential to demonstrate this potential.

Moreover, laboratory and field studies were conducted to determine the location of the *ethB* gene-containing organisms (ETBE-degraders) in samples from groundwater monitoring wells. The results show that most ETBE-degrading organisms are attached to the aquifer material. Therefore, aquifer material or a mixed groundwater-aquifer material 'slurry' sample should be collected to characterise the aquifer ETBE biodegradation potential, where this is based on the analysis of organisms or the detection of specific genes.

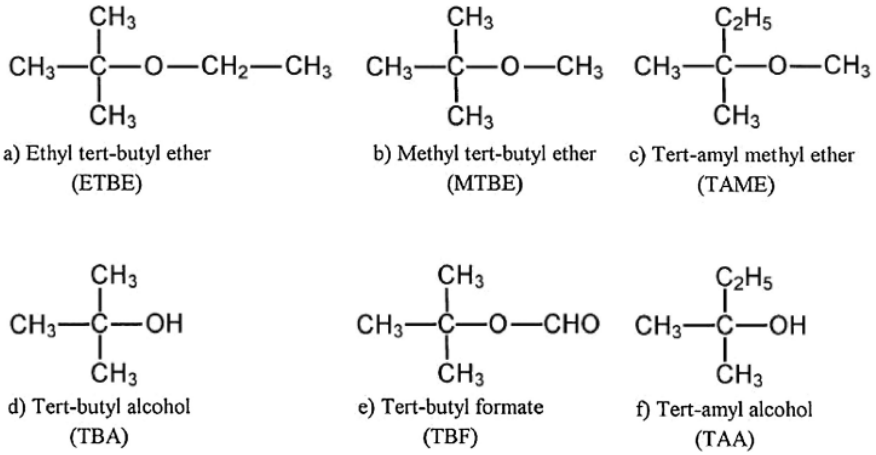
In summary, the results of this research suggest that biodegradation of ETBE is likely to occur in groundwater in the presence of dissolved oxygen and is not inhibited by the presence of other ether oxygenates, such as MTBE. Hydrochemical assessments, based on the analysis of dissolved ETBE, TBA and dissolved oxygen, can be used to demonstrate ETBE biodegradation in groundwater at an ETBE-release site. An additional line of evidence that may be relevant at some sites is microbiological assessment of ETBE biodegradation potential. This is site-specific and may be appropriate in cases where the hydrochemical assessment suggests that conditions are favourable for ETBE biodegradation but this is not observed. In this case, the presence of the *ethB* gene in the aquifer microbial community should be determined using aquifer material (e.g. core samples) or mixed groundwater-aquifer material (e.g. sampled from monitoring wells).

1. INTRODUCTION

1.1. BACKGROUND

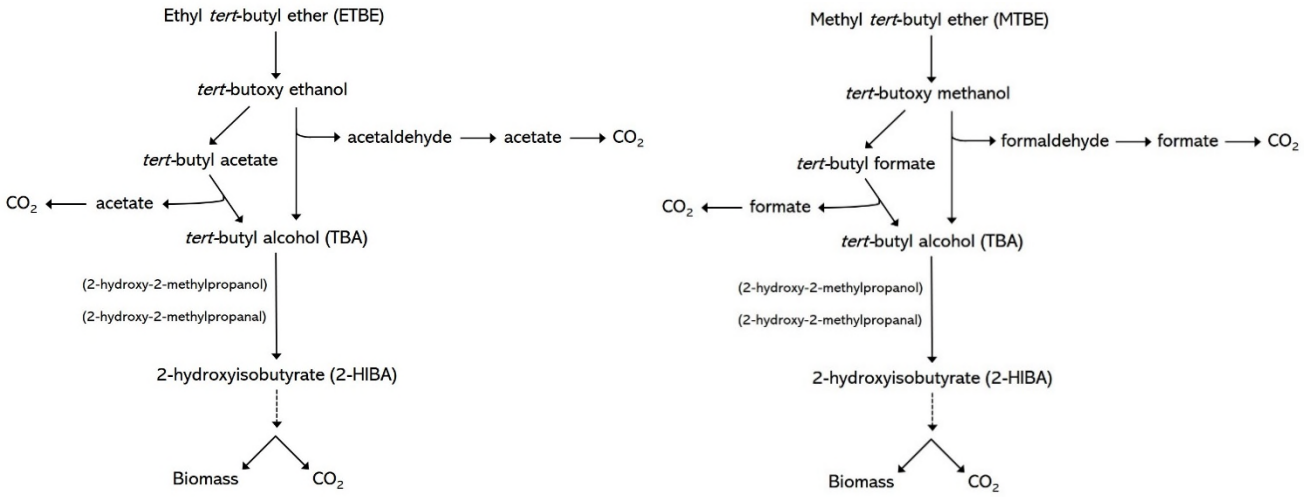
Ethyl *tert* butyl ether (ETBE) has now, in response to EU biofuels legislation (EU Renewable Energy Directives;2009/28/EC), replaced methyl *tert* butyl ether (MTBE) as the dominant gasoline ether oxygenate (GEO) used in European fuels to increase fuel octane number, improve combustion efficiency and reduce vehicle emissions. The molecular structure of the most common GEOs (ETBE, MTBE and *tert*-Amyl methyl ether (TAME)) and their major metabolic intermediates is shown in **Figure 1**.

Figure 1. **M**olecular structure of gasoline ether oxygenates used in gasoline formulations, a. ETBE, b. MTBE, c. TAME, and their major biodegradation organic metabolites, d. TBA, e. TBA, f. TAA. Image reproduced from Thornton et al. (2020).



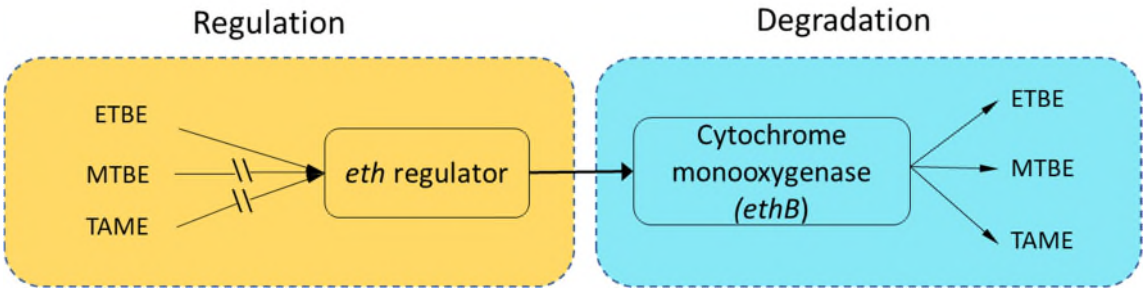
Accidental releases of ETBE to the subsurface environment can lead to groundwater impacts from ETBE in mixtures with other gasoline components or ETBE as a sole chemical. While ETBE has a similar environmental hazard profile to MTBE based on its properties, there is limited literature internationally (and less so for European contexts) on the biodegradation potential of this GEO in groundwater. The pathways proposed for aerobic biodegradation of ETBE and MTBE, respectively, are shown in **Figure 2**.

Figure 2. Proposed pathway for aerobic biodegradation of ETBE and MTBE (adapted from Thornton et al., 2020*)*



The microorganisms and genes that facilitate ETBE biodegradation in groundwater remain poorly characterised (as reviewed by Thornton et al., 2020). Moreover, interactions between microbial populations within aquifer microbial communities that are responsible for the degradation of specific compounds (e.g. ETBE or intermediate metabolites such as TBA), which ultimately lead to complete ETBE removal in groundwater, are unclear. One gene cluster (*eth*) has been reported in the literature to be important for the biodegradation of ETBE. This gene cluster encodes amongst others, a cytochrome monooxygenase, that is encoded by the *ethB gene*. This enzyme is responsible for initiating the aerobic biodegradation of ETBE, resulting in the production of the intermediate metabolite TBA. Given that the *ethB* gene encodes a monooxygenase enzyme, the presence of dissolved oxygen in groundwater is an essential prerequisite for biodegradation. There are limited reports that suggest other enzymes could be involved in ETBE biodegradation, although this is inconclusive. While GEOs are structurally related compounds (**Figure 1**), only ETBE is known to interact with the *ethB* gene regulator (which controls gene expression), ultimately resulting in the production of the *ethB* enzyme and ETBE biodegradation (**Figure 3**). However, the *ethB*-encoded enzyme has less specificity than the regulator, as this enzyme can initiate biodegradation of ETBE, MTBE and TAME.

Figure 3. Characterised genes and pathways involving the ethB gene for aerobic ETBE biodegradation. ETBE interacts with the regulator product (left box), activating the expression of the ethB gene (right box), resulting in the production of the cytochrome monooxygenase and biodegradation of ETBE, MTBE and TAME. A solid line indicates the process is known to happen, whereas // denotes this process does not occur.



Given the broad degradation capability of the *ethB*-encoded enzyme, it could by hypothesised that other characterised enzymes supporting GEO biodegradation would have activity towards all GEOs. For example the *mdpA* gene (which encodes a monooxygenase) is known to facilitate the biodegradation of MTBE, but has no known activity towards ETBE. Therefore, the presence of the *mdpA* gene at an ETBE-release site would imply a previous release of MTBE and that only MTBE would be biodegraded.

In addition, the environmental conditions which influence the potential for ETBE biodegradation in groundwater have not been adequately studied. In particular, the hydrogeochemical conditions in aquifers which support ETBE biodegradation and the effect of co-contaminants, such as other GEO, which may occur in mixtures with ETBE at gasoline release sites must be identified. Collectively, this information is needed to (i) identify which factors influence the potential for ETBE biodegradation in groundwater; (ii) improve the technical basis to predict conditions at sites where ETBE biodegradation may occur, and (iii) develop appropriate sampling and monitoring procedures for the assessment of this potential at ETBE-impacted and GEO-release sites, to support existing technical guidance.

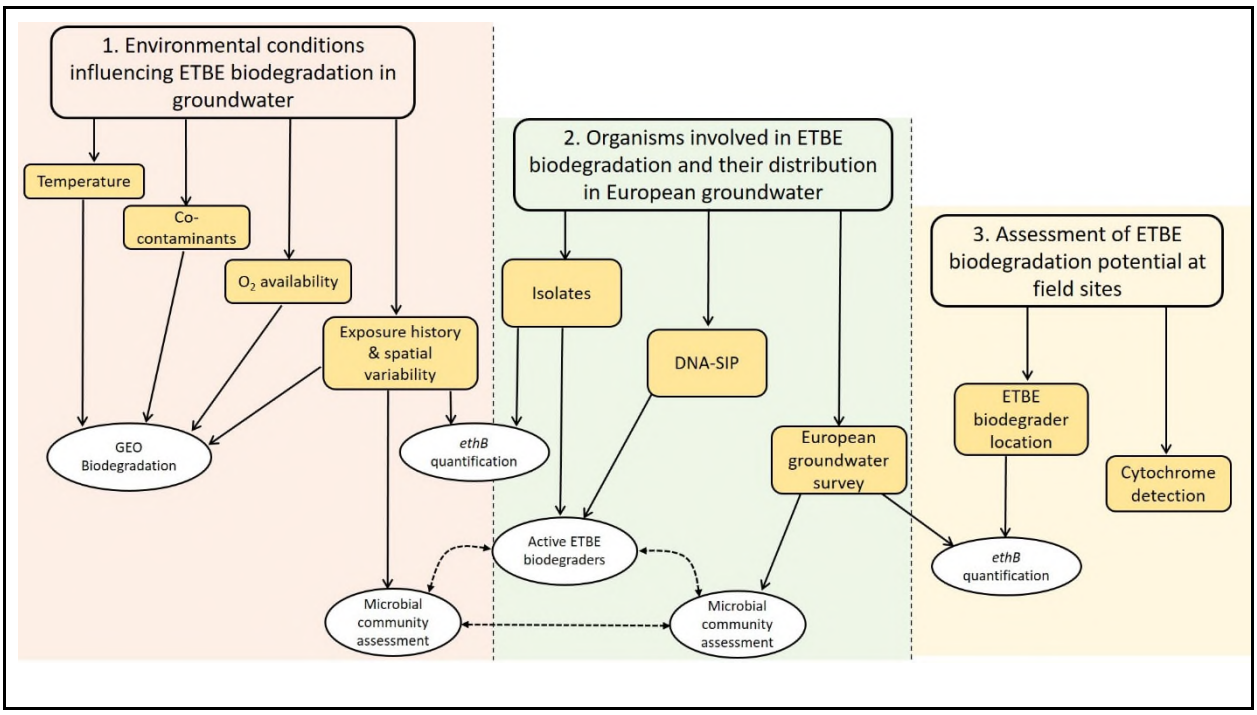
1.2. OBJECTIVES

To improve the knowledge of environmental conditions which influence the potential for ETBE biodegradation in groundwater, the objectives of this research were to:

1. Examine biodegradation of ETBE in groundwater, as a sole substrate and in mixtures with other GEOs
2. Characterise the diversity and function of the microbial community for ETBE biodegradation and identify relevant organisms and biodegradative genes responsible
3. Determine the presence of known ETBE-degrading organisms in European groundwater

The approach followed is shown in **Figure 4,** which illustrates the key activities completed under the objectives and their relationships to specific outputs. The research findings underpinning each objective are described in this report and the important observations highlighted.

Figure 4. Project overview highlighting key activities for each objective and how these link together. Solid arrows indicate each task with the resulting output and the dotted arrows show how the outputs can be interlinked for analysis.



2. METHODS

2.1. EXPERIMENTS EVALUATING ETBE BIODEGRADATION

An analysis of environmental conditions which may influence ETBE biodegradation in groundwater was undertaken using laboratory microcosm experiments, constructed with groundwater and aquifer material sampled from an ETBE-impacted site in France. The microcosm study considered:

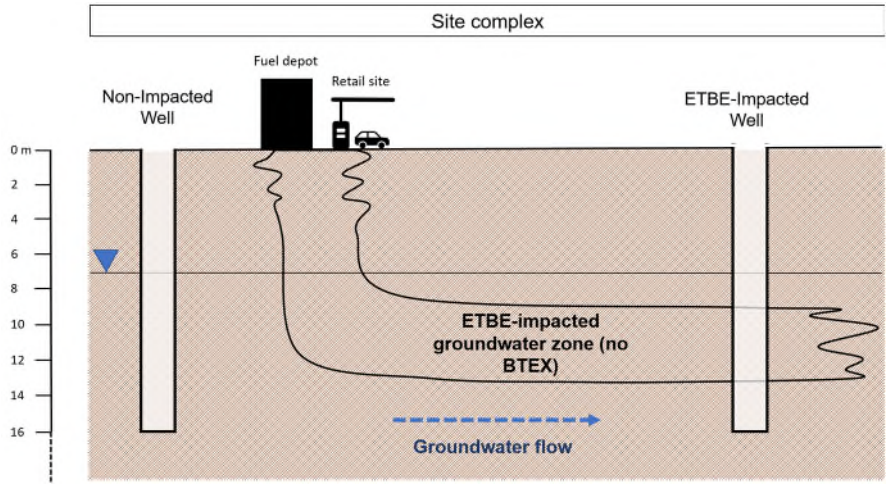
(i) exposure to ETBE;

(ii) variation in biodegradation potential between the unsaturated and saturated zone; and

(iii) the effect of co-contaminants (MTBE) on ETBE biodegradation and associated microbial community development.

Groundwater and aquifer material for these experiments was sampled from locations which represented current ETBE-impacted (I) and non-impacted (N) zones (i.e. no history of ETBE contamination) (**Figure 5**), as well as locations spanning the water table ('x', unsaturated zone to 'z', saturated zone) at the field site. The microcosms designated "unsaturated zone" comprised aquifer material sampled from the unsaturated zone at the site, which was then saturated with groundwater in the experiments (see Nicholls et al., 2020 for full details). The microcosms were amended with ETBE, MTBE and dissolved oxygen, as appropriate, at concentrations which were representative of a typical gasoline release into an aerobic aquifer. These microcosms were sampled for geochemical and microbiological analysis at intervals over 214 days. First-order biodegradation rates (day^{-1}) were estimated by regression of the natural log (ln) of GEO concentration versus time, for the linear section of the biodegradation profile.

Figure 5. Schematic highlighting typical sampling locations at sites used in this research. Non-impacted monitoring wells had no detectable organic compounds and ETBE-impacted locations had no detectable BTEX. These sites and monitoring wells were used in the microcosm study (section 3.1), DNA-SIP microcosms (section 3.2) and ETBE-degrader location study (section 3.3).



In a related previous study, the effect of groundwater temperature on ETBE biodegradation was examined in microcosm experiments constructed with groundwater and aquifer material from an MTBE/TAME-impacted site in the UK. These microcosms were amended with ETBE and ETBE/MTBE/TAME. Aerobic biodegradation of ETBE was evaluated at a groundwater temperature of 12°C (corresponding to the *in situ* temperature at the site) and 20°C, as representative of conditions which may be considered an upper temperature limit for the majority of European aquifers[1]. The results from this previous study are also reported in this report (section 3.1).

2.2. ETBE-DEGRADING ISOLATES

Individual ETBE-degrading bacterial isolates, originally obtained from a diverse community, were characterised, e.g. for their ability to degrade different GEOs and presence of known genes (*ethB*), etc. ETBE-degrading organisms were sampled at different stages in the microcosm study (section 3.1). To achieve this, groundwater-aquifer material samples were spread onto minimal media agar plates amended with ETBE. Minimal media is a culture medium that provides essential nutrients for bacterial growth, such as inorganic salts and is more representative of groundwater chemistry than most other defined, nutrient-rich media. The use of agar plates enables individual bacterial isolates to be obtained from a diverse bacterial community. Individual isolates were picked from these plates and tested for their metabolic activity on minimal media amended with either MTBE or TBA, to determine if the organisms could biodegrade other GEOs and the intermediate metabolite of ETBE biodegradation (i.e. capability to fully or partially biodegrade ETBE). Candidates of interest were selected based on the metabolic function, and a polymerase chain reaction (PCR) was performed to determine if the *ethB* gene was present (**Table 1**), as well as 16S rRNA gene sequencing to identify the organism.

2.3. DNA-SIP

DNA-SIP was used to identify active ETBE-degrading organisms in samples from ETBE-amended microcosms (section 3.1). ETBE labelled with ^{13}C on the ethyl group (heavy carbon) was added to a subset of microcosms in a separate experiment. As ETBE degraders utilise the ^{13}C carbon, it is incorporated into their DNA (isotopically heavy DNA) which can be separated from unlabelled DNA (isotopically light DNA) by centrifugation. Sequencing of the heavy and light DNA fractions allows primary ETBE-degraders to be identified.

2.4. CYTOCHROME DETECTION

To further support the molecular analysis of field samples for the assessment of ETBE biodegradation potential, the feasibility of cytochrome detection using a haem-staining method was investigated. To achieve this, the *ethB* gene-containing organism *Aquincola tertiaricarbonis* L108 was cultured and analysed for the expression of the cytochrome enzyme in total proteins.

[1] Tissen, C., Benz, S.A., Menberg, K., Bayer, P. and Blum, P. (2019). Groundwater temperature anomalies in central Europe, Environmental Research Letters, 14, 104012, https://doi.org/10.1088/1748-9326/ab4240

Table 1. Overview of molecular methods used in this research.

Method	Objectives
Polymerase chain reaction (PCR)	Detection of target genes in organisms, i.e. presence or absence of known biodegradative genes
Quantitative real time PCR (qRT-PCR)	Quantification of target genes; allows changes of the specific gene-containing organisms to be monitored
16S rRNA deep sequencing	Assessment of bacterial community diversity and taxonomic composition
DNA-SIP	*In situ* identification of primary ETBE-degraders in a microbial community, i.e. active ETBE-degrading organisms
Isolates	Identification of bacteria in a sample that can biodegrade ETBE (although not necessarily the dominant degrader) or other related co-substrates (e.g. MTBE) and metabolites (e.g. TBA), according to ability to grow on the specific organic compounds indicated

2.5. GROUNDWATER SURVEY AND LOCATION OF ETBE-DEGRADING ORGANISMS

A field study was undertaken to determine the distribution and location of *ethB* gene-containing organisms in pumped borehole samples containing aquifer material and groundwater which were collected from 5 ETBE-impacted sites (France (re-sample of same site in section 3.1), The Netherlands (2 sites), Turkey and the UK). Mixed samples of groundwater and aquifer material were collected after purging (pumping) monitoring wells at each site for the equivalent of 1, 3 and 6 borehole volumes. Groundwater well purging is often used prior to sampling groundwater for water quality analyses and purging often yields very turbid groundwater, rich in suspended solids if the well is pumped at a high rate. Aquifer material and groundwater in each sample was separated by filtration (using a 5 µm and 0.2 µm filter) and analysed for DNA yield and quantification of the *ethB* gene copy number.

As used for the microcosm studies, typically two monitoring well locations - ETBE-impacted (with no detectable benzene, toluene, ethylbenzene, xylene (BTEX) concentration) and non ETBE-impacted (with no detectable organic compounds) - were selected for sampling at the ETBE-release sites. These locations were identified from the available groundwater quality data at the respective sites. **Figure 5** shows a typical site layout and groundwater sampling locations used.

3. RESULTS AND DISCUSSION

3.1. ENVIRONMENTAL CONDITIONS INFLUENCING ETBE BIODEGRADATION IN GROUNDWATER

3.1.1. Biodegradation of ETBE and exposure history

In general biodegradation of ETBE occurred after a longer lag period (range of 33 to 59 days) in microcosms that contained aquifer sediment sampled at locations with no known previous exposure to ETBE, compared with locations impacted by ETBE, where biodegradation started immediately (**Figure 6**). The first-order biodegradation rate for ETBE generally increased from a mean value of 0.21 day^{-1} in locations with no previous exposure to ETBE to a mean value of 0.3 day^{-1} in locations impacted by ETBE, and was also higher following repeated exposure to ETBE in both non-impacted and impacted locations (**Table 2**).

These data suggest that a release of ETBE into an aerobic aquifer which has not previously been impacted by ETBE is likely to result in a lag period of a few weeks to a few months before biodegradation of ETBE may occur. Initial ETBE biodegradation rates may also be relatively slow. This lag period and relatively low initial biodegradation rate most likely reflect the adaptation of the aquifer microbial community to the initial ETBE exposure, specifically the time required for the induction of enzymes necessary for ETBE metabolism in ETBE-degrading organisms and the development of sufficient biomass of ETBE-degrading microorganisms (containing the *ethB* gene, although other routes could be possible) for detectable biodegradation. Aerobic biodegradation of ETBE may occur without a lag period and at a faster rate in aquifers exposed to ETBE from previous gasoline releases, due to a population of ETBE-degrading organisms established from the previous exposure. This potential is predicated on the presence of ETBE-degrading organisms within the ETBE-impacted zones, which are expected to increase in relative abundance in the aquifer microbial community after renewed exposure to ETBE. Repeated exposure to ETBE resulted in shorter lag periods and increased biodegradation rates, towards similar values in both non-impacted and ETBE-impacted locations (**Table 2**).

3.1.2. Indicators of ETBE biodegradation

Changes in several hydrochemical parameters measured in groundwater during the microcosm study confirmed the aerobic biodegradation of ETBE: (i) a reduction in ETBE concentration, (ii) the production of TBA, and (iii) a decrease in the dissolved oxygen concentration. These measurements provide three lines of evidence for ETBE biodegradation, as would typically be required using a monitored natural attenuation (MNA) approach for an ETBE-impacted site. Further to this, an increase in the proportion of *ethB* gene-containing organisms is an additional line of evidence for aerobic ETBE biodegradation that supported the hydrochemical results.

TBA was observed at varying concentration during ETBE biodegradation, from being absent (**Figure 6a**) to relatively high concentrations (e.g. 1200 µg L^{-1}) where ETBE biodegradation rates are high (**Figure 6e**). Aerobic biodegradation of ETBE is expected to result in the production of TBA as a metabolite, confirming an aerobic pathway for ETBE metabolism (**Figure 2**). Observed concentrations of TBA in groundwater will, however, depend on the relative rates of ETBE and TBA biodegradation. In specific cases TBA biodegradation may be overall rate-limiting for ETBE biodegradation, while in other cases no TBA accumulation occurred during

ETBE biodegradation. TBA is thus an indicator for ETBE (or MTBE) biodegradation, but the absence of TBA does not exclude biodegradation either.

Biodegradation of ETBE under aerobic conditions, with the production of TBA, results in a corresponding decrease in dissolved oxygen concentration (**Figure 6**). High rates of ETBE and TBA biodegradation may result in rapid consumption of dissolved oxygen.

The proportion of *ethB* gene-containing organisms increased significantly in the aquifer microbial community after exposure to ETBE, in locations with no previous exposure to ETBE, as well as in ETBE-impacted locations (**Figure 7**). The *ethB*-gene is thus an indicator for aerobic biodegradation of ETBE, but the absence of the *ethB*-gene does not exclude biodegradation either.

3.1.3. Resilience of the ETBE degrading microbiological community

After initial exposure to ETBE the proportion of *ethB* gene-containing organisms remained relatively stable in aquifer microbial communities from both non-impacted and ETBE-impacted locations. This included periods of up to three months of 'starvation' between episodes of repeated exposure, implying that these organisms have resilience during nutrient limitation (**Figure 7**). This indicates that the aerobic biodegradation potential for ETBE may be retained within the aquifer microbial community over an extended period and is quickly re-established with repeated exposure to ETBE, as evidenced by the corresponding biodegradation rates (**Table 2**).

Biodegradation of ETBE is expected to occur under aerobic conditions in groundwater where dissolved oxygen is not limiting and the indigenous aquifer microorganisms contain the ethB gene that supports ETBE biodegradation under these conditions. However, ETBE may also be biodegraded by organisms which do not possess the ethB gene, as identified through the isolation of ETBE-degrading organisms (section 3.2). The detection of the ethB gene in microorganisms responsible for ETBE biodegradation indicates that dissolved oxygen is needed to induce the enzymes necessary for ETBE biodegradation by these organisms. Therefore, dissolved oxygen should be monitored in groundwater as a basis to deduce locations where aerobic biodegradation of ETBE can be supported in principle.

Figure 6. ETBE, TBA and dissolved O_2 concentrations in microcosms constructed with groundwater and aquifer material from field site in France, at a) depth 'x' (8-9 m) and b) 'z' (13-14 m) located upgradient of the ETBE-impacted zone (N), and groundwater and aquifer material from depths c) 'x' (8-9 m), d) 'y' (9-10 m) and e) 'z' (13-14 m) located within the ETBE-impacted zone (I). ETBE was the sole carbon source added to the microcosms. Empty symbols are live microcosms and filled symbols are sterile controls. Key: N = Non-impacted monitoring well, I = ETBE-impacted monitoring well, X = 8-9 m depth, Y = 9-10 m depth, Z = 13-14 m depth, E = ETBE only addition, EM = ETBE and MTBE additions.

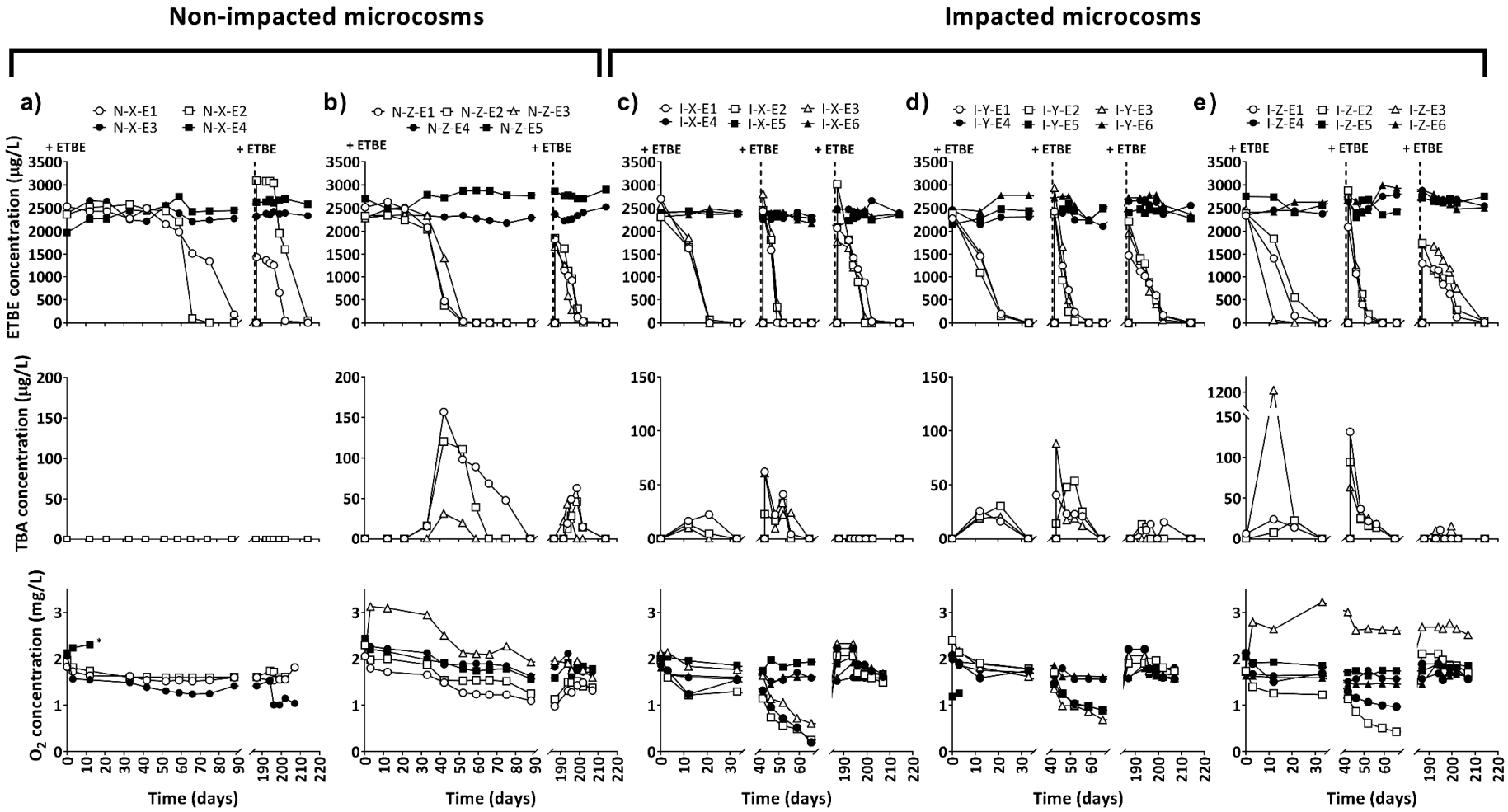
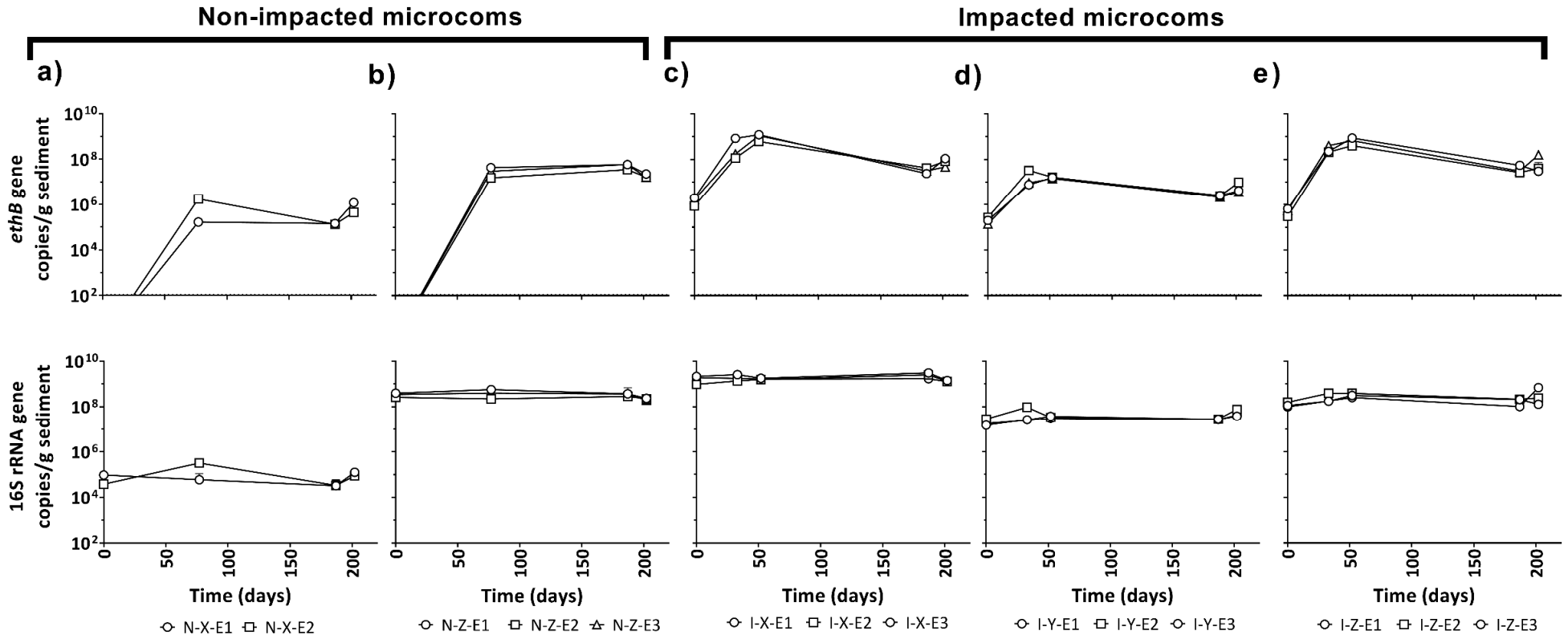


Table 2. First-order biodegradation rates (day^{-1}) estimated for microcosm experiments according to GEO amendment. Values indicated by "nd" could not be determined due to insufficient data. Key: N = Non-impacted monitoring well, I = ETBE-impacted monitoring well, X = 8-9 m depth, Y = 9-10 m depth, Z = 13-14 m depth, E = ETBE only addition, EM = ETBE and MTBE additions.

	ETBE (day^{-1})			MTBE (day^{-1})		
Microcosm	1	2	3	1	2	3
N-X-E1	0.06	0.32				
N-X-E2	0.22	0.22				
N-X mean	0.14	0.27				
N-Z-E1	0.24	0.26				
N-Z-E2	0.29	0.3				
N-Z-E3	0.32	0.38				
N-Z mean	0.28	0.31				
I-X-E1	0.34	0.4	0.36			
I-X-E2	0.35	0.53	0.18			
I-X-E3	0.33	0.54	0.36			
I-X mean	0.34	0.49	0.3			
I-Y-E1	0.22	0.33	0.2			
I-Y-E2	0.22	0.39	0.27			
I-Y-E3	0.23	0.34	0.21			
I-Y mean	0.22	0.35	0.23			
I-Z-E1	0.3	0.34	0.23			
I-Z-E2	0.37	0.41	0.18			
I-Z-E3	nd	0.31	0.29			
I-Z mean	0.34	0.35	0.23			
N-X-EM1	0.25	0.19		0.16	0.24	
N-X-EM2	0.21	0.25		0.13	0.16	
N-X-EM3	0.11	0.14		0.1	nd	
N-X mean	0.19	0.19		0.13	0.20	
I-X-EM1	nd	0.95	0.25	nd	0.36	0.25
I-X-EM2	nd	0.59	0.39	nd	0.22	0.29
I-X-EM3	nd	0.99	0.32	nd	0.32	0.29
I-X mean	nd	0.84	0.32	nd	0.30	0.28

Figure 7. *eth*B and 16S rRNA gene copy numbers for microcosms constructed with groundwater and aquifer material from field site in France, at a) depth 'x' (8-9 m) and b) 'z' (13-14 m) located upgradient of the ETBE-impacted zone (N), and groundwater and aquifer material from depths c) 'x' (8-9m), d) 'y' (9-10m) and e) 'z' (13-14m) located within the ETBE-impacted zone (I). ETBE was the sole carbon source added and individual data points represent sampling periods after repeated additions of ETBE to microcosms. Key: N = Non-impacted monitoring well, I = ETBE-impacted monitoring well, X = 8-9 m depth, Y = 9-10 m depth, Z = 13-14 m depth, E = ETBE only addition, EM = ETBE and MTBE additions.



3.1.4. Variation in ETBE biodegradation potential between the unsaturated and saturated zone

ETBE was biodegraded after a lag period which was generally longer (range of 59 to 66 days) in the unsaturated zone than the saturated zone (range of 33 to 42 days) at locations with no previous ETBE exposure (**Figure 6a, b**). Furthermore, the ETBE biodegradation rate was lower in the unsaturated zone (mean of 0.14 day^{-1} at depth "x" in **Table 2**) than the saturated zone (mean of 0.28 day^{-1} at depth "z" in **Table 2**) at locations with no previous exposure to ETBE. However, repeated exposure to ETBE generally resulted in a greater increase in ETBE biodegradation rates, especially in the unsaturated zone relative to the saturated zone in the non-impacted locations. There was no consistent difference in the lag period between the unsaturated and saturated zones at ETBE-impacted locations (**Figure 6c, d, e**).

In general, ETBE biodegradation rates were a factor of 2 higher in the saturated zone than in the unsaturated zone. Observed differences in ETBE biodegradation rate and lag period with depth most likely reflect local-scale variation in the distribution of ETBE-degrading organisms and environmental conditions (e.g. nutrient and oxygen availability), which is a characteristic feature of aquifers. Furthermore, there is a similar response of ETBE-degrading organisms in the unsaturated zone and saturated zone to ETBE exposure as the relative abundance of *ethB* gene-containing organisms increases after exposure to ETBE in both zones, and this potential subsequently persists.

3.1.5. Effect of co-contaminants

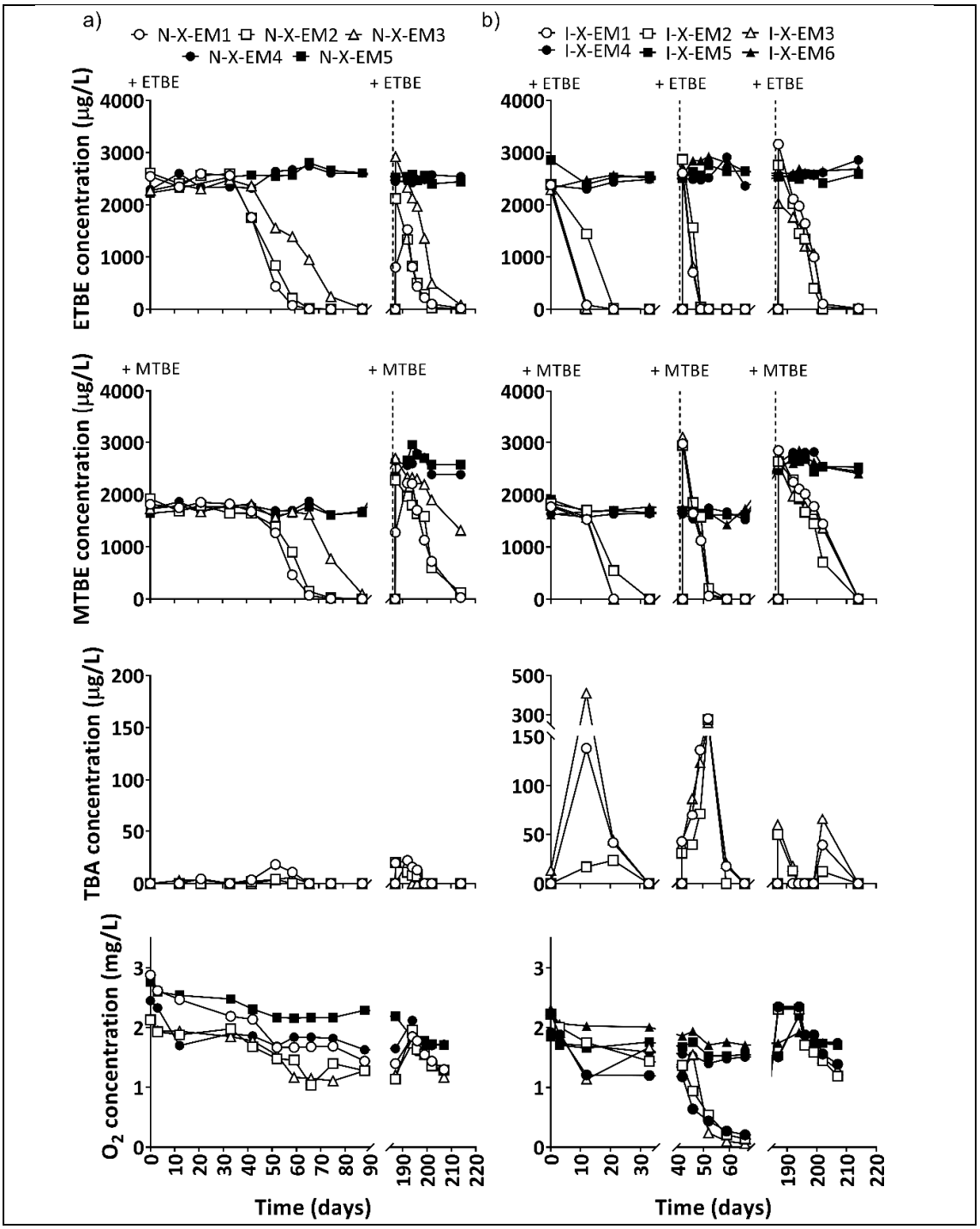
Both ETBE and MTBE were biodegraded in mixtures of these GEOs, but ETBE was biodegraded before, and in preference to, MTBE regardless of ETBE-exposure history (**Figure 8**). The corresponding biodegradation rates for ETBE-impacted locations were higher (respectively a maximum of 0.99 day^{-1} for ETBE and 0.36 day^{-1} for MTBE) than for locations with no previous exposure to ETBE (respectively a maximum of 0.25 day^{-1} for ETBE and 0.24 day^{-1} for MTBE) (**Table 2**). Biodegradation rates for MTBE increased after ETBE was almost fully biodegraded (**Table 2**) and TBA accumulated as a transient metabolite of both ETBE and MTBE biodegradation (**Figure 8**).

At GEO release sites mixtures of ETBE and MTBE in groundwater can occur from separate releases of gasoline containing these GEOs, or a release of gasoline containing both GEOs. Aerobic biodegradation of both ETBE and MTBE to TBA may occur concurrently in groundwater at locations with *and* without previous exposure to these GEO. There was no evidence of inhibition of ETBE biodegradation by MTBE, whereas ETBE was biodegraded preferentially before MTBE at the site studied. However, it should be noted that the predominant GEO present at this site was ETBE, so these observations may be site-specific.

The *ethB* gene was detected in GEO-degrading organisms sampled from both non-impacted and ETBE-impacted locations. As also demonstrated with the ETBE-only amended microcosms, additions of ETBE resulted in an increase in the *ethB* gene-containing organisms, which persisted during the starvation period. Therefore, the potential for aerobic biodegradation of ETBE and MTBE was retained within the aquifer microbial community and biodegradation activity was quickly re-established upon exposure to a mixture of ETBE and MTBE **(Figure 8**). However, the *mdpA* gene, which facilitates aerobic biodegradation of MTBE only (section 1.1), was not detected. This implies that the *mdpA* gene is not necessary for MTBE biodegradation or that MTBE was biodegraded by organisms using a different (unknown) gene. It is also plausible that both ETBE and MTBE can be biodegraded concurrently via their

respective pathways (**Figure 2**), by *ethB* gene-containing organisms in the microbial community. While this observation may be specific to the site studied, it suggests that mixtures of ETBE and MTBE can be biodegraded aerobically in groundwater when *ethB* gene-containing organisms are present in the aquifer microbial community. Moreover, these results suggest that ETBE may induce activity in organisms able to biodegrade both ETBE and MTBE. This has important implications for releases of ETBE to groundwater at former MTBE-release sites with no evidence of MTBE biodegradation, in that biodegradation of both GEOs may be stimulated.

Figure 8. ETBE, MTBE, TBA and dissolved O_2 concentrations for microcosms constructed with groundwater and aquifer material sampled at field site in France from depth 'x' (8-9 m) at a) location upgradient of the ETBE-impacted zone (N), and b) location within the ETBE-impacted zone (I). Empty symbols are live microcosms and filled symbols are sterile controls. Two additions of ETBE and MTBE were made to N microcosms and three additions of ETBE and MTBE were made to I microcosms. Key: N = Non-impacted monitoring well, I = ETBE- impacted monitoring well, X = 8-9 m depth, EM = ETBE and MTBE additions.

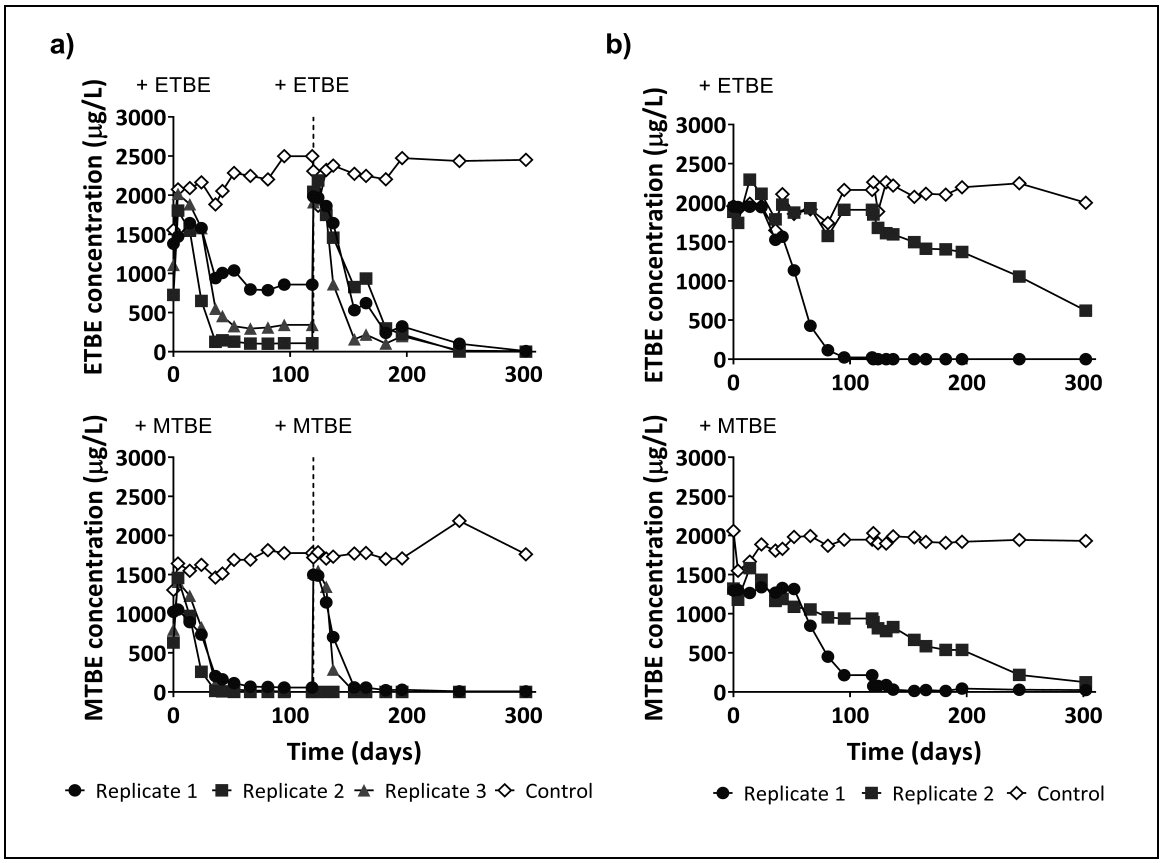


3.1.6. Effect of groundwater temperature

The biodegradation of ETBE and MTBE at different temperatures was determined experimentally in a microcosm study, established using aquifer material and groundwater from an MTBE- and TAME-release site (UK). Biodegradation rates of both GEOs were faster at 12°C than at 20°C over the same period, albeit with variability between replicates. This variability was predominantly noted in the 20°C replicates, where one replicate fully biodegraded ETBE in under 100 days, whereas ~75 % was biodegraded in the other replicate after 300 days (**Figure 9**). This result contrasts with the expected outcome that the biodegradation rate of organic compounds usually increases with temperature. It may be site-specific but suggests that GEO-degrading organisms are sensitive to temperature fluctuations, or that different populations of GEO-degrading organisms become dominant at different temperatures. The genes known to support ETBE (*ethB*) and MTBE (*mdpA*) biodegradation were present in all microcosms at 12°C, but were absent from replicate 2 microcosm at 20°C, corresponding with the observed biodegradation. It is evident that groundwater temperature has an important influence on GEO-degrading organisms and therefore biodegradation activity. In this study ETBE and MTBE biodegradation rates were significantly *reduced* at a higher groundwater temperature. This was also evident for the *ethB* and *mdpA* gene copy numbers, which indirectly indicate the relative abundance of the respective GEO-degrading organisms.

Therefore, geographical, daily or seasonal variation in groundwater temperature may result in significant differences in GEO biodegradation rates at GEO-release sites. Moreover, biodegradation rates may vary if experiments are carried out at temperatures which are different from *in situ* temperatures to which the microorganisms are adapted. This means that laboratory-based assessments (e.g. microcosms) of GEO biodegradation potential for sites should be conducted at *representative* groundwater temperatures.

Figure 9. Biodegradation of ETBE and MTBE in microcosms incubated at a) 12 °C and b) 20 °C. Filled symbols indicate live microcosms and empty symbols are sterile controls.



3.2. ORGANISMS INVOLVED IN ETBE BIODEGRADATION AND THEIR DISTRIBUTION IN EUROPEAN GROUNDWATER

A limited number of organisms have been identified with the metabolic capability to aerobically biodegrade ETBE in groundwater (see Thornton et al., 2020). While informative, it is likely that there are other organisms which can biodegrade ETBE aerobically, or potentially anaerobically. The literature has identified organisms containing the *eth* gene cluster as important ETBE degraders. However, it is possible that alternative *eth*-independent degradation routes exist but are yet uncharacterised. Results are presented below from experiments which were established to further characterise functional ETBE-degrading organisms and determine their distribution between ETBE-release sites. This analysis was undertaken by:

(i) (i) isolating ETBE-degrading organisms from microcosms (section 3.1),

(ii) (ii) analysing the DNA of organisms fed with isotopically labelled ETBE (13C-ETBE) in a DNA-SIP experiment, and

(iii) (iii) sampling organisms in groundwater from several sites impacted by ETBE (groundwater survey).

3.2.1. Identification of ETBE-degraders from microcosms

Microcosm studies (section 3.1) showed that the addition of ETBE to these systems did not significantly increase total microbial cell numbers, but did increase the relative abundance of *ethB* gene-containing organisms by many fold (**Figure 7**). The stability in total cell numbers (microbial biomass) in these systems indicates a nutrient limitation, other than carbon (e.g. nitrogen (N) or phosphorous (P)). Regardless of this nutrient limitation, ETBE-degrading organisms dominate these communities within these constraints, as determined through the ratio of *ethB*:16S rRNA gene copies (>1). This implies that natural microbial communities in aquifers (an oligotrophic environment) are typically nutrient limited and that the addition of P- or N-containing nutrients would be required to stimulate ETBE biodegradation rates further (within the constraints imposed by oxygen solubility). It should be noted that in the above microcosm studies, N and P concentrations were measured and P was below detection.

Deep sequencing of the microcosm microbial communities (section 3.1) identified 109 organisms that responded to the addition of ETBE. These were dominated by members of the Comamonadaceae, but numerous other bacterial strains also responded, particularly at previously non-impacted sites (see Nicholls et al., 2020). It should be noted that this dominance could be a site-specific observation. However, it demonstrated that certain community members come to dominate, regardless of the starting microbial population. This finding is also important as this group of organisms have not been commonly reported to be ETBE degraders, indicating that a variety of organisms possess the ability to biodegrade ETBE. However, the diversity of responding organisms indicates that the capacity to degrade ETBE (or its metabolites) is most likely the key determinant of microbial community composition, rather than specific taxonomic groups. This diversity could be useful for the implementation of biostimulation strategies, especially at sites which prove difficult to remediate. For example, certain organisms may biodegrade ETBE at a faster rate under specific conditions that could be achieved through biostimulation, when necessary.

A key challenge is to link correlation between microbial populations and observed degradation behaviour with function. These responding organisms could be ETBE degraders, degraders of ETBE metabolites (e.g. acetate or TBA), or simply responding to increased carbon flow through the system. Therefore, additional experiments described below were undertaken to provide functional information.

3.2.2. GEO-degrading bacterial isolates

The isolation of organisms and subsequent analysis allows for a greater understanding of each organism at an individual level, whereas microcosms assess behaviour and changes at a community-wide level. Organisms were isolated from ETBE-amended microcosms (section 3.1) and grown on media containing ETBE, MTBE and TBA. They were sequenced for identification and analysed for the presence of the known ETBE-degradative gene, *ethB*.

Over 900 isolates were obtained initially, with this number being reduced by grouping organisms based on growth behaviour (i.e. ability to grow on all substrates, etc.). The number of isolates was further reduced by the selection of organisms that possessed the *ethB* gene and those that lacked this gene.

Of the isolates obtained, 30 grew on ETBE and did not possess the *ethB* gene (*ethB* negative). Examples of organisms isolated include (at the genus level) *Ralstonia*, *Bradyrhizobium* and *Mesorhizobium*. The identification of non-*ethB* gene-

containing ETBE-degrading organisms from the microcosm study suggests that some observed ETBE biodegradation *could* be accounted for by these organisms and some by *ethB* gene-containing organisms. Over 50 bacterial isolates that grew on ETBE possessed the *ethB* gene (*ethB* positive) and a selected number of these organisms were sequenced. This included members of the Comamonadaceae family, identified as 'responders' in the initial microcosm study (section 3.1). *Methylibium* sp. (Comamonadaceae) were commonly identified, linking to the DNA-SIP observations.

3.2.3. DNA-SIP studies

Deep sequencing of the microcosm study identified organisms involved in the biodegradation of ETBE or intermediate metabolites – referred to as 'responders'. DNA-SIP was performed to identify organisms that were actively involved in the primary degradation of ETBE. The results showed that organisms belonging to the genera of *Methylibium*, *Leptothrix*, *Caulobacteria* and *Brevundimonas*, among others, were actively degrading ETBE in the SIP experiment. Of those, nearly half the ETBE-degrading organisms identified belong to the Comamonadaceae family, linking the observations made from section 3.1. The most abundant organism identified through DNA-SIP belonging to the Comamonadaceae family was a *Methylibium* sp.. While DNA-SIP is a technically complex laboratory method, it has utility for the identification of ETBE-degrading organisms, particularly at a site where the *ethB* gene is not present.

Organisms responsible for ETBE biodegradation have been identified in both the initial microcosm study (from ETBE-impacted locations) and by DNA-SIP analysis. This provides a basis to identify if these degraders are ubiquitous across all sites sampled in the groundwater survey (especially from ETBE-impacted zones).

3.2.4. Groundwater survey

Sites impacted by gasoline containing ETBE (mainly) within Europe were sampled for ETBE-degrading organisms, to identify the distribution of these in groundwater. This was achieved by the analysis of microbial communities using DNA extracted from pumped groundwater samples obtained from monitoring wells at the sites. Groundwater samples were obtained from sites in the following countries:

1. France
2. UK
3. Netherlands, FD (fuel depot)
4. Netherlands, RS (retail site); and
5. Turkey (non-European side of Turkey)

Two additional sites (in the UK and Spain) provided groundwater but no DNA could be obtained from these samples, probably because the sampled groundwater was free from suspended solids. Therefore, an analysis of the microbial communities at these sites was not possible.

DNA from samples collected from ETBE-impacted and non-impacted monitoring wells at the respective sites was sequenced. In addition, purge water samples obtained after pumping 1, 3 and 6 borehole volumes from these monitoring wells were analysed to assess the impact of the groundwater sampling protocol on the detection of ETBE-degrading organisms (section 3.3).

In summary, the microbial communities had an abundance of members of the gammaproteobacteria class (Appendix A). The major driver of differences was site-specific conditions rather than exposure to ETBE. Within a site, differences in

microbial community composition were evident between impacted and non-impacted locations. The presence of the *ethB* gene was determined for each sample, which revealed that this gene was present only in monitoring well F2 (France), and monitoring wells T1 and T2 (Turkey). Hydrochemical data collected from these sites offer an explanation regarding the occurrence of the *ethB* gene (**Table 3**). Where site conditions are favourable for *ethB* organisms, such as high ETBE concentration and dissolved oxygen, they are detected, but where conditions are unfavourable, they are not present. This is consistent with the lack of the *ethB* gene at sites N (RS and FD) and UK. In summary, the *ethB* gene was not detected at any site where either the ETBE concentration or dissolved oxygen was low.

Table 3. Hydrochemical analysis of groundwater overview of the five sites studied, including GEO and BTEX concentrations, dissolved oxygen and redox status, along with presence or absence of ethB gene.

Site	Monitoring well	GEO concentration (µg/L)			BTEX (µg/L)	Dissolved oxygen (mg/L)	Redox status (mV)	*ethB* gene detection
		ETBE	MTBE	TBA				
F	F1 F2	0 0 (previously 1400)	0 0	0 0	0 0	3.1 2.1	155 -43	× ✓
T	T1 T2 T3	7400 180 140	50 11 1	430 - -	1350 0 0	0.46 2.1 6.3	-62.1 109.7 330.1	✓ ✓ ×
N (FD)	N_FD1 N_FD2	0 81	<0.6 0.4	- -	0.61 0	No data but elevated Fe^{2+} and CH_4 imply both likely anoxic	- -	× ×
N (RS)	N_RS1 N_RS2 N_RS3 N_RS4	0.72 <0.2 0.84 <0.2	<0.3 <0.3 <0.3 <0.3	- - - -	0 0 0 0	No data but all likely oxic based on water chemistry	- - - -	× × × ×
UK	UK1 UK2	<1 129	<1 115	<10 14.4	<2 <2	0.73 0.06	332 141	× ×

The information from the DNA-SIP study and microcosm study was synthesised to examine the presence of the known ETBE-degrading organisms in the total microbial community at these sites. ETBE degraders and/or responsive organisms were found at the site in France, predominantly in samples from the ETBE-impacted monitoring well (F2). These organisms account for only a small proportion of the total microbial population, indicating that they are out-competed by other non-degraders within the microbial community. Establishing the reasons for the low relative abundance of the ETBE-degrading organisms and methods to increase the proportion of these would potentially allow biodegradation rates to be increased at contaminated sites if ETBE degraders were favoured. Known ETBE-degrading organisms were also detected at other sites, although there was no direct correlation between *ethB* gene detection and identification of the ETBE degraders. This suggests that the detection of specific organisms is not a reliable measure to determine ETBE biodegradation potential, whereas the presence of the *ethB* gene confirms this biodegradation potential.

3.3. ASSESSMENT OF ETBE BIODEGRADATION POTENTIAL AT FIELD SITES

3.3.1. Location of ETBE-degrading organisms in groundwater

The microcosm study (section 3.1) provided evidence that ETBE-degrading organisms were predominantly attached to the aquifer material rather than as planktonic cells in the groundwater. These initial data raised the hypothesis that *ethB* gene-containing ETBE-degrading organisms are found attached to the aquifer material at ETBE-release sites. Knowledge of ETBE-degrader location is important from a sampling perspective, i.e. to determine the most appropriate sample type for the detection of ETBE-degrading organisms. A combined laboratory and field study was therefore undertaken to investigate if the collection of groundwater samples alone or a mixed groundwater-aquifer material 'slurry' sample is the most effective basis to determine the presence of ETBE-degrading organisms at an ETBE-release site. Since groundwater sampling protocols typically involve the purging of monitoring wells prior to sample collection, to reduce sample turbidity, this study compared the distribution of ETBE-degrading organisms in groundwater and those attached to aquifer material, in samples collected after purging 1, 3 and 6 borehole volumes from monitoring wells at the field sites studied.

An initial experiment where aquifer material and groundwater were separated into different microcosms (constructed with inocula from the site in France) showed that ETBE biodegradation occurred in microcosms containing aquifer material only and not in microcosms containing groundwater only (**Figure 10**). This confirmed the predominant location of ETBE-degrading organisms (and therefore ETBE biodegradation potential) at this site. This was supported by:

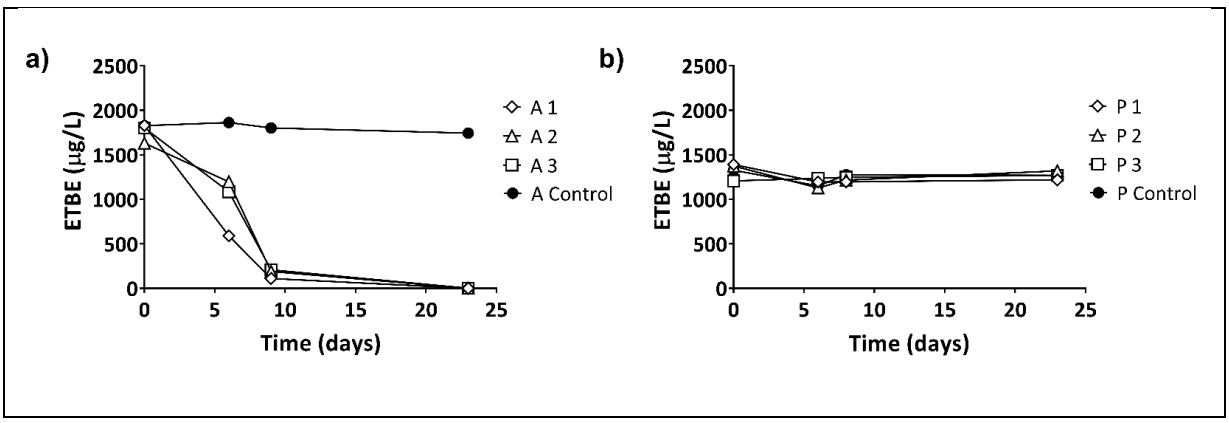
(i) evidence of ETBE biodegradation in microcosms containing aquifer material only (**Figure 10**);

(ii) a significant community of attached bacteria determined by fluorescence microscopy of grains;

(iii) recovery of >99 % of total DNA in mixed groundwater-aquifer material samples from organisms attached to grains, and

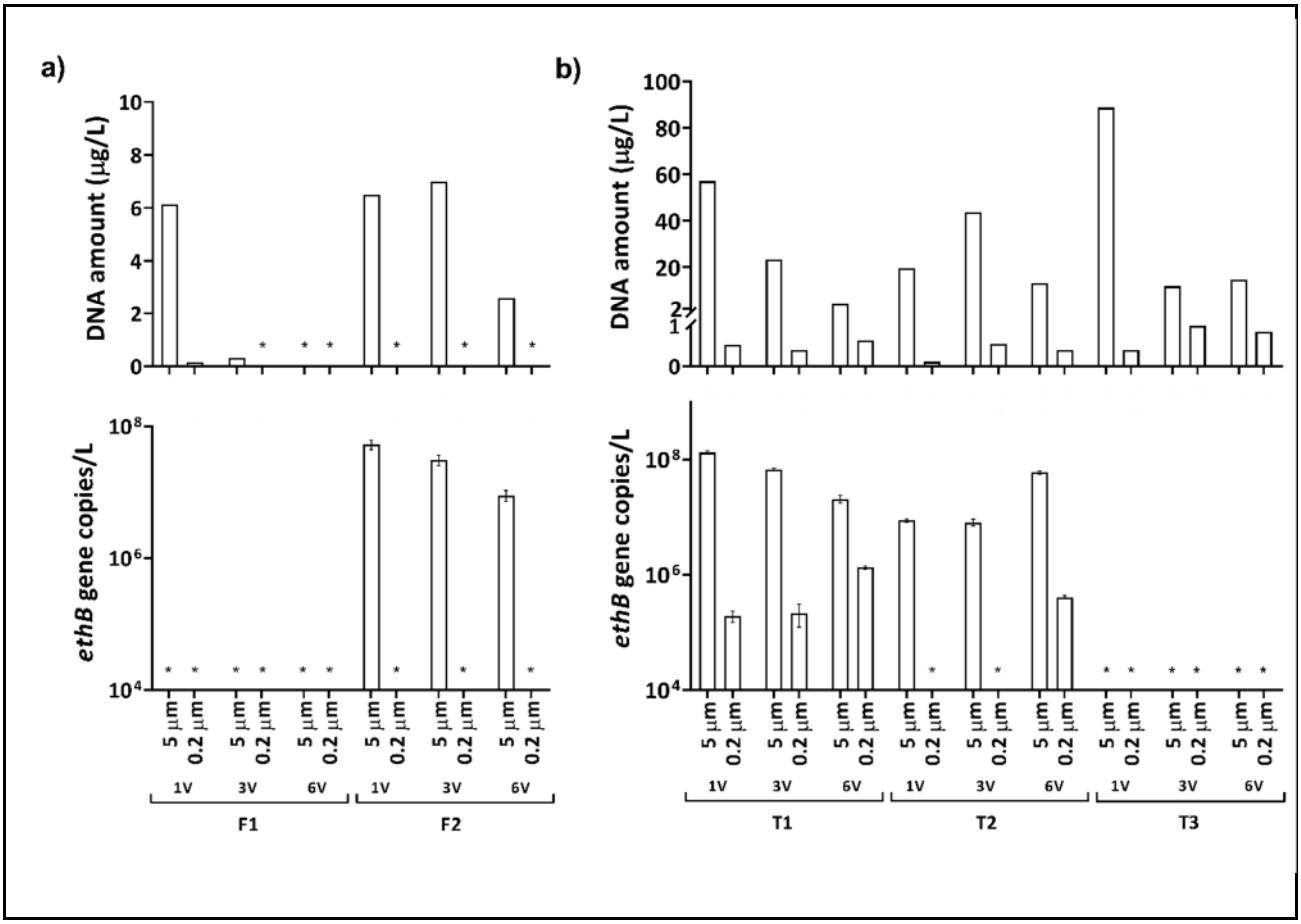
(iv) the detection of the ethB gene in samples of aquifer material only from these microcosms.

Figure 10. ETBE biodegradation in microcosms constructed with (a) aquifer material, and (b) absence of ETBE biodegradation in microcosms constructed with groundwater only. A1-3 and P1-3 are technical replicates. Sterile controls were run to ensure no ETBE loss (A control and P control).



Slurry samples were obtained from monitoring wells at the aforementioned five ETBE-impacted sites. DNA was recovered only from aquifer material and no DNA was recovered where 'clear' (sediment-free) groundwater was provided as a pumped sample. The samples from the field site in France confirmed the result of the former laboratory microcosm experiment, in that all the DNA was recovered from the aquifer material and the *ethB* gene was only detected in this fraction (**Figure 11**a). A similar result was also obtained for samples from the site in Turkey (**Figure 11b**).

Figure 11. Concentration of DNA and ethB gene copy number for pumped borehole samples from ETBE-impacted sites in (a) France, and (b) Turkey. Mixed (slurry) samples of aquifer material and groundwater were collected after purging 1, 3 and 6 borehole volumes (V) from two monitoring wells at the site in France (F1, non-impacted and F2, ETBE-impacted), and from three ETBE-impacted monitoring wells at the site in Turkey (T1, T2, and T3). The samples were filtered through a 5 µm filter to capture the attached microbial community on the aquifer material and the filtrate then filtered through a 0.2 µm filter to capture the planktonic microbial community in the groundwater. * indicates results are below detection limit.



The vast majority of DNA (and subsequent detection of *ethB* gene) was sampled from organisms attached to the aquifer material, such that sampling of 'clear' groundwater from pumped boreholes would not provide a reliable measure of the total aquifer microbial community. Moreover, where the *ethB* gene was detected in planktonic organisms, the ratio of *ethB*:total bacterial numbers was lower than that obtained when sampling attached organisms on the aquifer material. This indicates

that if only the planktonic community in the groundwater was sampled, the *ethB* gene-containing organisms would be underrepresented. These results imply that a 'clear' or sediment-free groundwater sample obtained from pumped monitoring wells, which may be collected within groundwater monitoring protocols for gasoline-release sites, will not provide a sensitive or representative sample of the aquifer microbial community, in terms of DNA recovery or *ethB* gene copy number. Therefore, it is recommended that a mixed aquifer material-groundwater (or 'slurry') sample is collected from pumped monitoring wells and filtered to provide a reliable and robust representative sample for the detection of *ethB* gene-containing organisms in ETBE-impacted aquifers. A minimal quantity of aquifer material (~1 g) is required to obtain sufficient DNA for this analysis, whereas a very large volume (>2 L) of groundwater would need to be filtered, with both technical and practical limitations.

3.3.2. Cytochrome detection

The detection of the *ethB* gene in organisms implies metabolic function, whereas the presence of the cytochrome enzyme confirms that the organisms have expressed the cytochrome enzyme and the environmental conditions are favourable for ETBE biodegradation. The haem staining method failed to give a positive result and subsequently coomassie staining confirmed the presence of total cell proteins. Therefore, a more sensitive method would be required for the detection of the cytochrome enzyme in an environmental sample to confirm ETBE biodegradation potential (where *ethB* gene-containing organisms are present).

4. CONCLUSIONS

The main conclusions from this research are summarised below.

This research has shown that ETBE can be biodegraded aerobically to TBA as an intermediate metabolite, and subsequently to CO_2, by a wide range of microorganisms in groundwater, both in isolation and in the presence of MTBE. Both GEOs appear to be biodegraded at comparable rates. There may be a period of adaptation of ETBE-degrading microorganisms in groundwater following an ETBE release, before biodegradation occurs, but this is short-lived (up to two months) and the potential for ETBE biodegradation appears to be retained within the aquifer microbial community for extended periods (minimum three months) in the absence of ETBE.

In the systems studied in this report, aerobic biodegradation of ETBE (and MTBE when present) in groundwater was facilitated by microorganisms which contain the *ethB* gene. The *ethB* gene is found in different classes of organisms, hence characterisation of *ethB* gene-mediated biodegradation should focus on functional genes rather specific taxonomic groups. However, data from these studies and the wider scientific literature have indicated that aquifer organisms which do not contain the *ethB* gene also have the capability to aerobically biodegrade ETBE. Therefore, this should be considered when undertaking microbiological studies of ETBE-degrading systems.

Biodegradation of ETBE is expected to occur in groundwater in the presence of dissolved oxygen and other ether oxygenates, such as MTBE. Groundwater monitoring at an ETBE-release site should therefore include measurement of ETBE (and other GEOs if present), TBA and dissolved oxygen to deduce ETBE biodegradation based on groundwater hydrochemistry. Aerobic biodegradation of ETBE can be interpreted from a relative decrease in the ETBE concentration, increase in TBA concentration and decrease in dissolved oxygen concentration in the groundwater. In some cases, TBA may be biodegraded rapidly during ETBE biodegradation and not accumulate in groundwater. Hence, while the accumulation of TBA may be evidence of ETBE biodegradation, the absence of TBA does not exclude biodegradation.

A microbiological assessment of ETBE biodegradation potential should focus on determining the presence of the *ethB* gene in the aquifer microbial community. This analysis may provide an additional line of evidence for ETBE biodegradation to support the primary lines of evidence obtained from the hydrochemical assessment. The absence of the *ethB* gene does not imply the absence of aerobic ETBE biodegradation potential, while the detection of this gene provides positive evidence of this potential.

ETBE-degrading organisms appear to be predominantly attached to aquifer materials and are unlikely to be detected in high numbers in groundwater. The wider significance of this result remains unknown, based on the relatively limited number of ETBE-release sites studied. However, this observation should be incorporated into the design of groundwater monitoring protocols, where an assessment of the aquifer ETBE biodegradation potential at a site is based on the detection of the *ethB* genes in the native microbial community. To fully capture the aquifer ETBE biodegradation potential, it is recommended that this analysis is undertaken on aquifer material (e.g. a core sample) or filtered samples of mixed groundwater-aquifer ('slurry') material which can be obtained from pumped monitoring wells. Sampling of clear groundwater for this assessment will provide a misleading result.

Considering these observations, biodegradation of ETBE in groundwater may in principle be enhanced by the addition of oxygen and nutrients (biostimulation), where these co-factors are found to be limiting. Molecular analysis (as described in this study) can be used to detect and quantify organisms which possess the known ETBE biodegradation gene (*ethB*), as a basis to assess the performance of such measures.

Three key areas of research are identified to further improve understanding of ETBE biodegradation in groundwater:

1. Further identification of non-*ethB* biodegradation routes. The bacterial isolates obtained from this study have enabled the identification of organisms that can degrade ETBE but lack the *ethB* gene. This data confirms that organisms lacking the *ethB* gene can degrade ETBE, although the metabolic pathway is unknown. As shown in this study, the *ethB* gene was not identified at all ETBE-release sites investigated. The isolates obtained therefore provide an opportunity to characterise non-*ethB* gene-containing organisms, to determine the alternative genes that are involved in ETBE biodegradation. This information would be novel, as a tool to improve the molecular-based characterisation of ETBE biodegradation potential at an ETBE-impacted site.

2. Determine if anaerobic ETBE biodegradation capability exists in the subsurface environment. In comparison with MTBE, very few studies have demonstrated the occurrence of anaerobic ETBE biodegradation in groundwater, or identified the organisms which may facilitate this process. This is an important knowledge gap since gasoline releases to groundwater typically result in the development of anaerobic conditions.

3. Understanding *ethB* gene transfer within the microbial community. The potential mobility of the *eth* genes within aquifer microbial communities can be exploited for bioremediation of GEO-impacted groundwater, through bioaugmentation. In this case, *ethB* gene-containing organisms can be introduced into the microbial community at a site where groundwater conditions are favourable for ETBE biodegradation, but the *ethB* genes are absent from the native microorganisms. The mobility of the *ethB* gene could potentially mean this gene is transferred between organisms that are suited to the specific aquifer environment to enable ETBE biodegradation. However, the fundamental understanding of the gene transfer mechanisms and organisms involved is lacking, which currently limits this management approach for GEO-release sites.

5. GLOSSARY

BTEX	Benzene, Toluene, Ethylbenzene, Xylene
DNA	Deoxyribonucleic acid
ETBE	Ethyl *tert* butyl ether
GEO	Gasoline ether oxygenate
Ln	Natural log
MNA	Monitored natural attenuation
MTBE	Methyl *tert* butyl ether
N	Nitrogen
O_2	Oxygen
P	Phosphorus
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SIP	Stable isotope profiling
TAA	*tert* amyl alcohol
TAME	*tert* amyl methyl ether
TBA	*tert* butyl alcohol
TBF	*tert* butyl formate
UK	United Kingdom

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7. REFERENCES

1. Thornton, S.F., Nicholls, H. C., Rolfe, S.A., Mallinson, H. E., Spence, M. J., 2020. Biodegradation and fate of ethyl tert-butyl ether (ETBE) in soil and groundwater: a review. *Journal of Hazardous Materials* **391**, 122046.

2. Nicholls, H. C., Mallinson, H. E., Rolfe, S. A., Spence, M. J., Thornton, S. F., 2020. Influence of contaminant exposure on the development of aerobic ETBE biodegradation potential in microbial communities from a gasoline-impacted aquifer. *Journal of Hazardous Materials* **388**, 122022

3. Nicholls, H. C., Rolfe, S. A., Mallinson, H. E., Spence, M. J., Thornton, S. F. (In preparation). Distribution of ETBE-degrading microorganisms in groundwater and implications for sampling of aquifer biodegradation potential: evidence from laboratory and field studies.

4. Nicholls, H. C., Mallinson. H. E., Thornton, S. F., Rolfe, S. A. (In preparation). Active ETBE-degrading organisms in a gasoline-impacted aquifer identified using DNA-SIP.

5. Nicholls, H. C., Mallinson, H. E., Rolfe, S. A, Thornton, S. F. (2018). Temperature and exposure history strongly influence GEO biodegradation in groundwater. Poster presented at Cefic-LRI and CONCAWE workshop on recent developments in science supportive to the persistence and biodegradation assessment, 27 Sept, Helsinki, Finland.

6. Nicholls, H. C., Mallinson, H. E., Rolfe, S. A, Thornton, S. F. (2019). Influence of exposure history and aquifer spatial variability on ETBE biodegradation potential and microbial community structure in groundwater. Platform presentation at GQ2019 Groundwater Quality Conference, 9-13 Sept, Liege, Belgium.

7. Mallinson, H. E., Nicholls, H. C., Spence, M. J., Rolfe, S. A., Thornton, S. F. (2019). Characterising microorganisms and genes responsible for aerobic biodegradation of ETBE in groundwater. Poster presented at GQ2019 Groundwater Quality Conference, 9-13 Sept, Liege, Belgium.

APPENDIX A: ORGANISMS IDENTIFIED IN GROUNDWATER SAMPLES FROM SITE SURVEYX`

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