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# Physico-chemical and biological characterization of an aquifer polluted with ETBE

Yves Benoit <sup>(1)</sup>, Laure Chancerelle <sup>(2)</sup>, Alain Dumestre <sup>(3)</sup>,  
Joanna Falcao Salles <sup>(4,5)</sup>, Françoise Fayolle-Guichard <sup>(1)</sup>,  
Geneviève Grundmann <sup>(4,5)</sup>, Martina Kyselková <sup>(4,5)</sup>, Jeanne Morinière <sup>(3)</sup>, Pascal  
Pandard <sup>(2)</sup>, Hans H. Richnow <sup>(6)</sup>, Mònica Rosell <sup>(6)</sup>

- (1) IFP, Département "Biotechnologie", F-92852 Reuil-Malmaison Cedex  
(2) INERIS, Parc Technologique Alata, BP2, 60550 Verneuil-en-Halatte  
(3) SERPOL SA, 2 Chemin du Génie BP 80, F- 69633 Vénissieux Cedex  
(4) Université Lyon 1, Villeurbanne-69622, Lyon, France;  
(5) CNRS, UMR5557, Écologie Microbienne; Villeurbanne; France  
(6) Department of Isotope Biogeochemistry, Helmholtz -Centre for Environmental Research-UFZ, Leipzig, Germany

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## Introduction

Petroleum compounds and among them, gasoline, is the most massively used chemicals worldwide and, as a consequence gasoline derived compounds are the most frequently found contaminants in groundwater. Apart from the petroleum derived components in gasoline, chemicals are added to in order to meet specific requirements. Oxygenates such as methyl *tert*-butyl ether (MTBE) or ethyl *tert*-butyl (ETBE) were chosen because of their high octane index. Such water soluble compounds are typically detected in groundwater after a contamination by gasoline.

The environmental impact of ether fuels on groundwater was not estimated prior to their utilization and the actual level of MTBE and ETBE contamination is not known in Europe even after several years of systematical use. Moreover, the knowledge on the ecology of MTBE- and ETBE-biodegradation is quite poor.

Two directives have been adopted in the EU to promote the use of biofuels through tax incentives, including the addition of bioethanol to gasoline. Some European MTBE producers have taken advantage of the subsidies by converting their plants to produce ethyl *tertiary*-butyl ether (ETBE) using bioethanol. ETBE is presently the main way of the bioethanol utilization as an alternative to straight ethanol. According to Lyondell, in 2008 there were 60 ETBE production units in Europe (installed production capacity 5.75 Mt). ETBE is produced mainly in France, Spain and Poland. In 2008, 4.5% of the whole gasoline sold in Europe was containing ETBE and half of the ETBE produced was synthesized using bioethanol raw material. In Europe, the maximal authorized ETBE concentration in gasoline has now reached 22% (v/v). More specifically, in France 1259 millions of liters of ETBE were incorporated in gasoline in 2008, i.e. 10.3% of gasoline (v/v).

One of the main properties of MTBE and ETBE is their high water solubility (40 and 10 g.L<sup>-1</sup>, respectively). In Europe, detectable levels of MTBE have been reported in samples from rivers in Germany (Achten & Puttmann, 2000, 2002a, 2002b) and Rosell i Linares (2006) reported several cases of MTBE-polluted freshwater in different European countries. No specific study was carried out concerning ETBE. When an MTBE-supplemented gasoline is spilled, the plumes generated by MTBE are much larger than those generated by benzene, the most soluble compound of gasoline (water solubility = 1.755 g.L<sup>-1</sup>). This persistence in groundwater of MTBE is the consequence of i) its high solubility ii) its low adsorption on organic matter and iii) its recalcitrance to biodegradation. No data are available on the environmental fate of ETBE but its physico-chemical properties, very similar to those of MTBE, should lead to a similar environmental behaviour. The European Commission has completed risk assessments on MTBE and ETBE and concluded that there were no health concerns with these products "in the case of non-repeated swallowing, contact with the skin and inhalation" but nothing is known about the possible risk following a long-lasting exposition to low concentrations of MTBE or ETBE when aquifers are polluted by such compounds. Moreover, these two compounds have very low odour and taste thresholds in water (ETBE: 13 and 47 µg.L<sup>-1</sup>, respectively and MTBE: 10 and 24 µg.L<sup>-1</sup>, respectively) rendering water undrinkable when present at very low concentrations. The EU legislation in preparation should propose a limit of 20 µg.L<sup>-1</sup> for MTBE and ETBE in water to ensure quality.

## Presentation of the project

The 3-year long project TISATIE is granted by the International Competitiveness Cluster Lyon & Rhône-Alpes (Thematic: Chemicals/Environment). It is focused on a French ETBE/MTBE polluted site (gas station) and aimed: (1) at improving our knowledge on the environmental fate of ETBE and (2) at defining a cleaning process of the site following its characterization.

An integrative strategy involving different biological tools was used to characterize and monitor the site. It included:

-The choice of the site and its physico-chemical and geological characterization. This required implementing analytical methods dedicated to the trace analysis of ETBE and MTBE and also of their common biodegradation product, *tert*-butyl alcohol (TBA).

-The evaluation of the environmental impact of the pollution was determined by two means. (i) Estimation of the ecotoxicity of water sampled on the site by using standardized methods measuring the effects of water on different biological systems. (ii) Characterization of the microbial diversity on different points of the site using a phylogenetic DNA microarray.

- The determination of the ETBE, MTBE and BTEXs biodegradation capacities of the indigenous microflora. This part was carried out by classical cultivation methods using as inoculums water sampled on the site. We also used the compound-specific stable isotope analysis (CSIA) to determine if ETBE biodegradation has occurred in the site and in microcosm studies to elucidate the predominant reaction mechanism. Moreover, efficiency of biodegradation has been evaluated by performing the same ecotoxicological tests after biological treatment.

The results obtained from these different studies allowed us determining which remediation process could be the most efficient on this site (biostimulation and/or bioaugmentation). Following pilot tests performed at laboratory scale, a process has been recently implemented on the site.

The different partners of the project are SERPOL (project coordinator), IFP (Biotechnology Department), Université Lyon1 (Microbial Ecology Laboratory, LEM). The ecotoxicological study was carried out by INERIS. The CSIA experiments were carried out at Helmholtz -Centre for Environmental Research-UFZ (Department of Isotope Biogeochemistry, Germany).

## Analytical methods.

### - Choice and characterization of the site

The choice of the pilot site, a working gas station, was initiated by a study to determine the vulnerability, the presence of pollution by gasoline, ETBE and MTBE being essential for the final choice.

To carry out this study, different analytical methods (GC and LC) were used to analyze both the aquifer and the soil and characterize the pollution levels on different part of the site. We determined:

- the total and solubilized organic carbon in the aquifer (TOC, DOC)
- the water soluble fuel ethers (MTBE, ETBE) and associated alcohol (TBA) concentrations
- the total soluble hydrocarbons (total HC and BTEXs)
- the electron acceptors
- the presence of metals, pesticides and chlorinated compounds.

### - Physico-chemical characterization of the aquifer:

The hydrogeology of the site was determined (piezometers) and physico-chemical parameters were scanned: pH, temperature, redox potential, dissolved oxygen, concentrations.

### - Determination of the ecotoxicity of the aquifer

In order to complete chemical analysis approach, an ecotoxicological survey of groundwater has been organized on several piezometers.

The following commonly used tests have been carried out, according to ISO standards

\*Determination of the inhibition of the mobility of *Daphnia magna Straus*.

\*Freshwater algal growth inhibition test with unicellular green algae (*Pseudokirchnerella subcapitata*)

\*Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test)

Due to samples specificity (*i.e.* low dissolved oxygen concentration, fine particles in suspension, volatile compounds like BTEX, ETBE, MTBE), the test conditions have been adapted to reoxygenate

samples and reduce volatilization (solution prepared in refrigerated vessels, tests performed in closed vessels).

#### - Determination of the biodegradation potential of the indigenous microorganisms.

The biodegradation capacity of ETBE, MTBE and BTEXs was estimated by using the indigenous microflora after filtration (0.22  $\mu$ ) as the inoculum (10%, v/v) in a mineral medium (MM). ETBE, MTBE and a mixture of BTEXs were added as the carbon and energy source (100 mg.L<sup>-1</sup>). Cultivation was carried out in closed flasks under aerobic conditions. The oxygen available in the flasks being non-limiting regarding the Theoretical Oxygen demand (Th.O.D.) calculated for the different substrates. The biodegradation is monitored by measuring the residual substrate concentrations in culture samples (triplicates)

When biodegradation of a substrate was observed, the culture was re-inoculated in fresh MM in the presence of the substrate to confirm the biodegradation capacities.

#### - Microbial diversity determination

\* DNA was extracted from 1 litre water sample with Ultraclean Water DNA kit (MoBio Laboratories, Carlsbad, CA, USA). The universal eubacterial primers 77-pA and pH (Sanguin *et al.*, 2008) were used to amplify *rrs* from DNA extracts obtained from environmental samples. The PCR reaction mixture, *in vitro* transcription, RNA purification and hybridization were as described in (Sanguin *et al.*, 2008). The slides were scanned at 532 nm with a 10  $\mu$ m resolution, using a GeneTac LS IV scanner (Genomic Solutions, Huntingdon, UK). Images were analyzed with the GenePix 4.01 software (Axon, Union City, CA). Finally, a given feature probe was considered positive when (i) hybridization signals were superior to the mean signal of the negative controls and (ii) at least 3 of 4 replicate spots were hybridized.

\* Purified PCR products were cloned into the plasmid vector pGEM-T (pGEMs-T Easy Vector System kit; Promega, Charbonnières, France) according to the manufacturer's protocol. Fifty clones were sequenced on both strands (CoGenics, Meylan, France).

#### - CSIA experiments

Groundwater samples were taken in November 2009 from 12 wells along the contamination plume in order to compare their ETBE carbon and hydrogen isotopic signatures to the ones close to the source. Depending on the ETBE concentration, the samples were preserved with approximately 70 g of NaCl in 240-mL serum bottles for manual headspace injection (HS) or 1-L bottles preserved with 1% (w/w) trisodium phosphate dodecahydrate (Na<sub>3</sub>PO<sub>4</sub>•12H<sub>2</sub>O or TSP) for purge and trap (P&T) pre-concentration step. Just prior injection into the gas chromatography-combustion-isotope ratio monitoring mass spectrometry systems (GC-C-IRM-MS, for details check Rosell *et al.* 2007), each sample for P&T was diluted in distilled water to a final concentration of around 100  $\mu$ g/L in three 120-mL serum bottles. These bottles were placed in a modified AquaTek 70 Autosampler coupled to a Velocity XPT Accelerated Purge and Trap System (both from Teledyne Tekmar, Mason, OH, USA). This P&T system was only connected to the instrument measuring carbon isotope composition.

In all cases, a DB-MTBE column (60 m length x 0.32 mm I.D. x 1.8  $\mu$ m film thickness from Agilent Technologies, Waldbronn, Germany) was used. Carbon delta values obtained by the two methodologies were standardized by aqueous standard injections in order to correct isotopic fractionations caused by the extraction techniques (Zwank *et al.* 2003).

In parallel, the isotope enrichment factors of an ETBE degrading enrichment culture obtained from well PZ1 were evaluated with resting cells (optical density at 700 nm was 0.1) under different oxygen concentrations (oxic versus hypoxic) and incubation temperatures (12°C vs. 30°C). All the cultures were incubated in a mineral salt medium containing vitamins and ETBE as sole source of carbon and energy. The monitoring of oxygen concentration was made through a non-invasive technique which requires oxygen sensitive optode spots (POF-PSt3) glued inside of the serum bottles and a fiber optic device (FIBOX 3 trace, both from PreSens, Germany) connected to a computer, see more details in Rosell *et al.* 2010.

## **Results and discussion**

The results presented here were obtained mainly during the first two years of the project.

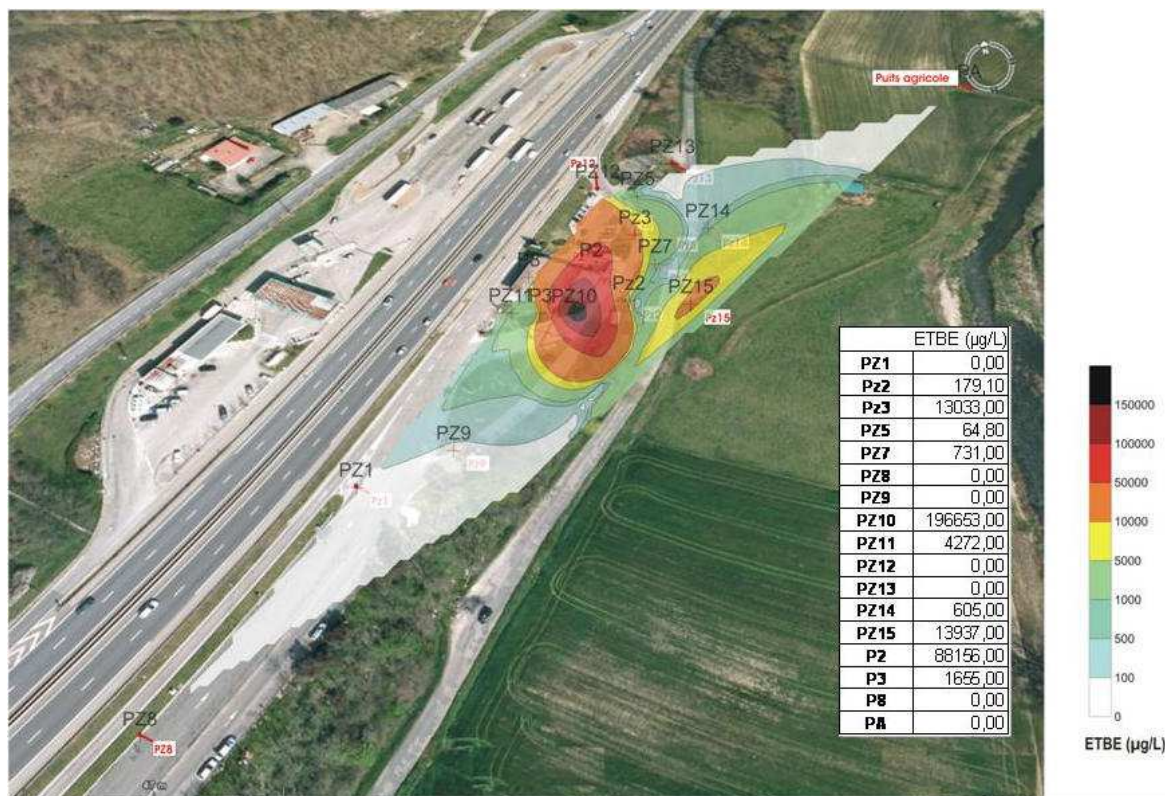
#### -Site description and characterization of the pollution

The site of the study was finally chosen among three possible sites located in the Rhône-Alpes district in France. The site is situated in the valley of a river which is a tributary of the river Rhône and located on alluvia composed of sandy gravel laying on a rocky substratum mainly composed of gneiss and mica. The water table has been detected at 5 m depth. The aquifer is flowing on top of this substratum

through slimy ground, developing high water permeability. As a hydraulic point of view, the aquifer detected right below the site is connected to the alluvium nappe of the river. This river has been considered as vulnerable due to its proximity to the surface and to the vulnerability of the hydraulic connection with the alluvium nappe.

Around the site, this potentially vulnerable aquifer is used for sensitive usage (such as agricultural irrigation) or for non sensitive applications (industrial wells). The pollutant characterization revealed a hydrocarbon pollution source mainly composed of light weathered gasoline with ether additives and trace of gasoil. Impacted waste fills and polluted interstitial air-ground were also detected in many other areas of the site (upstream and downstream the source area).

Concerning the groundwater impact, a floating organic phase composed of (i) weakly degraded gasoline (dissolved BTEXs and alkanes) and (ii) gasoil has been detected at different depth levels of the aquifer. This organic phase is mainly composed of organic pollutant such as ETBE and BTEXs. The contamination plume observed on the site before treatment regarding ETBE (Fig.1). Compared to fuel oxygenate ether's, the length of the BTEXs plume is smaller and the pollutions concentrations also.



**Figure 1. ETBE concentrations on the site**

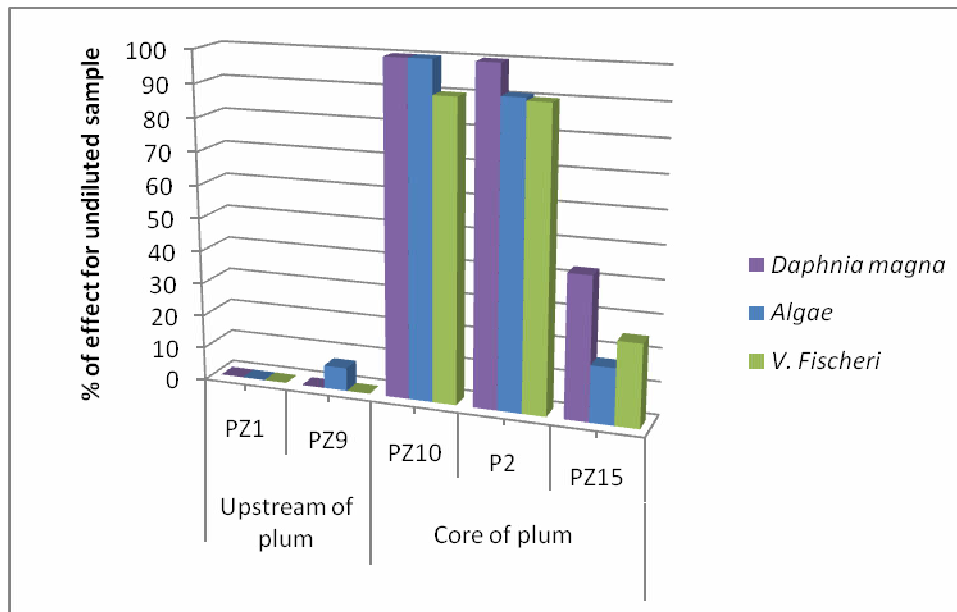
Downstream, the environmental impact is focused in the direction of identified targets (agricultural irrigation wells). The concentrations curves of dissolved pollutants in the aquifer revealed a greater mobility of ethers (Figure 1) compared to BTEXs. (results non presented).

The analysis performed on water samples pumped in the aquifer near the source (P2) revealed the following organic pollutants: Total dissolved organic content of  $200 \text{ mg.L}^{-1}$ , ETBE:  $197 \text{ mg.L}^{-1}$ , MTBE:  $0.9 \text{ mg.L}^{-1}$ , TBA:  $4.2 \text{ mg.L}^{-1}$ , BTEX:  $3.2 \text{ mg.L}^{-1}$  and Total soluble hydrocarbons:  $11 \text{ mg.L}^{-1}$

In complement, the water composition also revealed the presence of heavy soluble metals especially arsenic ( $50 \text{ µg/l}$  in a well). The highest permissible concentration for drinking water is set at max.  $10 \text{ µg.L}^{-1}$  (Directive 98/83/CE - November 1998). In P2, the aquifer is strictly anoxic with a total depletion of electron acceptor (oxygen, nitrates & sulphates). The anoxic conditions are closely linked to the biological activity of autochthonous aerobic and anaerobic microorganisms. The soluble fraction of BTEXs is easily biodegraded under aerobic conditions leading to the anoxic conditions observed near the area source. Despite MTBE traces, the major pollutant is ETBE with concentrations up to  $200 \text{ mg.L}^{-1}$  and TBA, a potential by-product of both fuel oxygenates biodegradation. ETBE biodegradation capacities of indigenous microflorae have been demonstrated for two different water samples upstream and in the middle of the site. ETBE accumulation in the aquifer is the consequence of (i) its high water solubility and (ii) the total oxygen depletion.

**-Toxicity of samples of the aquifer at different locations on the site**

Ecotoxicity test results show high toxicity level of water sampled in the pollution plum (PZ10, P2 and PZ15 piezometers), compared to PZ1 and PZ9 samples, located upward of the pollution plum (Fig. 2).



**Figure 2. Ecotoxicity of the core and upward pollution plum samples (undiluted)**

It can be highlighted that a same toxicity level is measured for P2 and PZ10 samples. These two piezometers are located in the core of pollution plum. The downstream sample PZ15 shows a lower toxicity (see EC<sub>50</sub> values in Table 1). These results can be linked with a lower ETBE concentration, but it should be noticed that other compounds like BTEXs and metals have been detected in the site that can also contribute to the observed toxicity.

**Table 1. EC<sub>50</sub> values on P2, PZ10 and PZ15 water samples**

Ecotoxicity tests	P2	PZ10	PZ15
Inhibition of light emission of <i>V. fischeri</i> (30 min)	EC <sub>50</sub> : 17.3%	EC <sub>50</sub> : 17.8%	EC <sub>50</sub> > 80%
Inhibition of <i>D.magna</i> mobility (48 h)	EC <sub>50</sub> : 16.0%	EC <sub>50</sub> : 12.2%	EC <sub>50</sub> > 100%
Inhibition of algal growth (72 h)	EC <sub>50</sub> : 54.7%	EC <sub>50</sub> : 70.8%	EC <sub>50</sub> > 98.7%

Globally, this combination of test allows to clearly discriminating between polluted and unpolluted samples. These results show also that *Daphnia magna* and *Vibrio fischeri* are the most sensitive organisms as monitoring tools for this type of pollution.

**-Biodegradation capacity of indigenous microflorae**

The biodegradation capacity of the indigenous microorganisms sampled at two locations of the site (see Fig. 1), PZ1 upward of the pollution plum and P2 located in the plum, under aerobic conditions was determined.

The biodegradation of BTEXs was estimated on a mixture of BTEXs (concentration of each target compound was 18 mg.L<sup>-1</sup>) and the biodegradation percentage was calculated on the residual concentration measured in the control (non-inoculated). The results are shown in Table 2 for both microflora (PZ1 and P2).

**Table 2. Biodegradation capacities of BTEXs by indigenous microflora from two locations on the site (PZ1 and P2)**

BTEX provided in the mixture *	Biodegradation of BTEXs (%)	
	Water sampled in well P2	Water sampled in well PZ1

<b>Benzene</b>	100%	100%
<b>Toluene</b>	99.4%	98.9%
<b>Ethylbenzene</b>	98.9%	97.2%
<b>m-Xylene</b>	98.3%	98.3%
<b>p-Xylene</b>	98.3%	95.5%
<b>o-Xylene</b>	98.4%	95.6%
<b>Total BTEXs</b>	98.9%	96.9%

\* No degradation was observed in the control

The biodegradation capacity of the microflora towards ETBE and MTBE ( $100 \text{ mg.L}^{-1}$ ) were determined after inoculating MM medium (triplicates). When degradation occurred, the culture was used as the inoculum for a secondary cultivation on MTBE or ETBE. The MTBE and ETBE biodegradation rates were calculated from a first addition of the substrates (Table 3).

The biodegradation of ETBE was observed on both cases at similar rates whereas no MTBE biodegradation by P2 microflorae was observed. The ETBE biodegradation rate was superior to that of MTBE using the microflora of PZ1 (x14).

The transient production of TBA was observed in the case of PZ1 after 3 successive additions. In the case of P2, the third addition of ETBE leads to the accumulation of TBA that was not further degraded. This shows that it is very important to monitor also the TBA concentration on sites polluted by ETBE or MTBE.

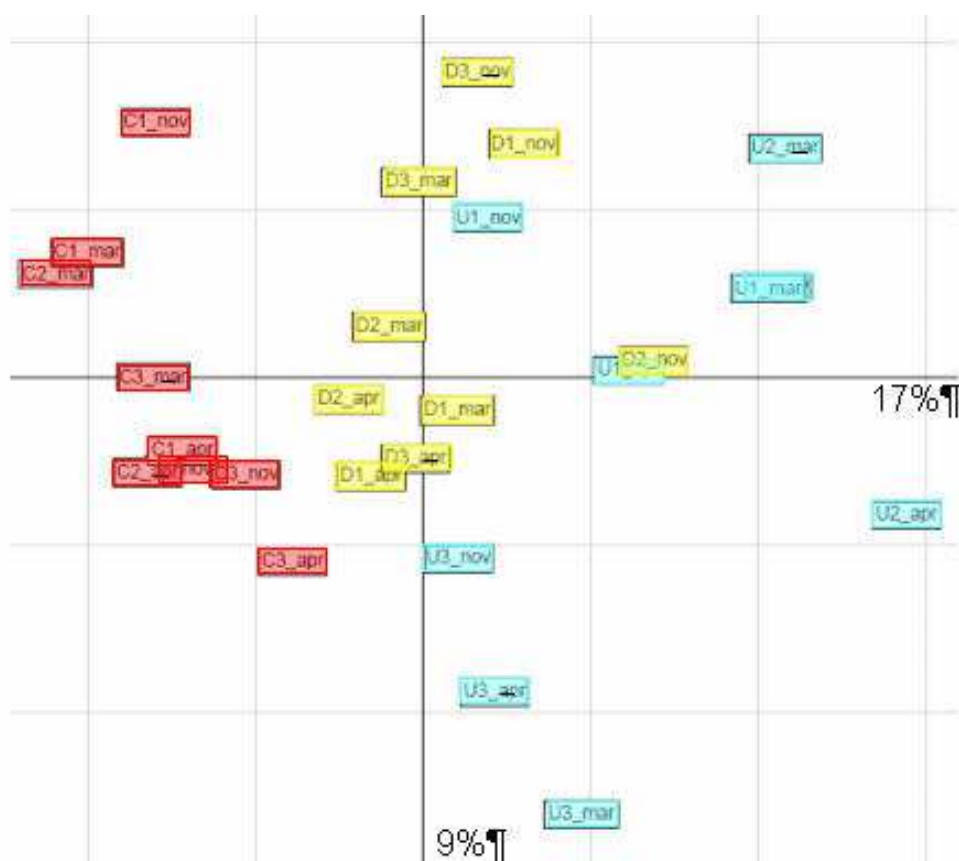
**Table 3. Biodegradation capacities of ETBE and MTBE by indigenous microflora from two locations on the site (PZ1 and P2)**

Origin of the indigenous microflorae	Biodegradation capacity of:	
	ETBE ( $\text{mg.L}^{-1}.\text{d}^{-1}$ )	MTBE ( $\text{mg.L}^{-1}.\text{d}^{-1}$ )
Well PZ1	19.6	1.4
Well P2	24.7	No

#### - Microbial diversity on the site and in the enrichments

Enrichment batches were set in the laboratory using water samples from a polluted well (P2). ETBE, TBA or BTEXs were added to the water sample. After 4 week incubation, communities were analysed using cloning and sequencing method. In the three cases, when degrading activity was detected, there was a shift toward Beta Proteobacteria although communities were specific of each pollutant. This indicates the large initial diversity in this water system. There was a prevalence of *Hydrogenophaga* in the case of ETBE and TBA and a prevalence of *Acidovorax* in the case of BTEXs. Among *Hydrogenophaga* genus *H. flava* is known for its capacity to degrade MTBE but it has never been mentioned for ETBE degradation. *Acidovorax* has never been mentioned to degrade BTEXs.

PCA analysis microarray data on water sampled in 9 wells at three dates (Fig. 3), separates three zones on the site corresponding to the center of the main pollution, upstream and down stream of the pollution. The bacterial community down stream of the main pollution zone was positioned in between the center and the upstream zone. This variability covered, including the time effect, 25% of the total variability. The complexity of a natural site with multiple pollutions might be responsible for the large unexplained variability (75%). Specific *ethb* gene PCR (targeting 4 sequences among 6 available) indicated the presence of this gene in water wells where ETBE was present.



**Figure 3. PCA analysis of microarray data of water samples from 9 wells, at three sampling dates**

November 08, March09, April 09. Center (C), Downstream (D), Upstream (U).

#### -CSIA experiments

Despite the wide range of ETBE concentrations (from  $64 \mu\text{g.L}^{-1}$  to  $120 \text{mg.L}^{-1}$ ) covered by the application of two different injection techniques (HS and P&T), no significant carbon ETBE isotopic fractionation was detected when comparing  $\delta^{13}\text{C}$  along the contamination plume (average  $-27.3 \pm 0.3\text{‰}$ ). The difference of  $\delta^{13}\text{C}$  respect the ones obtained closer to the source rarely exceeded the total analytical uncertainty  $\pm 0.5\text{‰}$  (Sherwood Lollar et al. 2007). The same was observed for hydrogen (mean  $\delta^2\text{H} = -200 \pm 7\text{‰}$ ), although the applied HS technique was only able to analysed samples with ETBE concentration higher than  $1 \text{mg.L}^{-1}$ . However, the fact that ETBE isotope fractionation in the field was not detected is not necessarily related to a lack of biodegradation. As observed in the microcosms studies performed with a concentrate of cells obtained originally from well PZ1, the related ETBE degrading community produces a very low carbon and almost non detectable hydrogen fractionation of ETBE during its complete biodegradation (see Table 4).

The ETBE carbon enrichment factors ( $\epsilon_c$ ) obtained under different oxygen and temperature conditions did not vary significantly. Therefore, the ETBE degraders from this enrichment seem to follow the same enzymatic reaction mechanism under the studied conditions. Comparing to previous studies, the obtained  $\epsilon_c$  are similar to the ones observed for *Aquicola tertiaricarbonis* L108 and *Rhodococcus ruber* IFP2001. In both strains, the initial monooxygenase reaction attacking the ethoxy group of ETBE is catalyzed by a cytochrome P450-type enzyme (CYP249) encoded by the *ethABCD* genes which has been found to be highly conserved in other strains able to grow on ETBE (Malandain et al. 2010). The low exhibited fractionation pattern discarded any implication of *Pseudonocardia tetrahydrofuranoydans* K1 ( $\epsilon_c = -1.7$  and  $\epsilon_H = -73\text{‰}$ ) attributed to the expression of a tetrahydrofuran monooxygenase. These results fitted perfectly with the detection of the *ethB* gene in the field site.

**Table 4. Comparison of carbon and hydrogen enrichment factors ( $\epsilon$ ) and  $\Lambda$  values ( $\Delta\delta^2\text{H}/\Delta\delta^{13}\text{C}$ ) for aerobic biodegradation of ETBE by enrichment culture from site PZ1 at different conditions and other strains reported in the literature. All the values have associated their  $\pm 95\%$  confidence intervals (CI)**



Culture	$\epsilon_C$ [‰]	B			$\epsilon_H$ [‰]	$\Delta$	Reference
		$R^2$	[%]	N			
<b>Pure cultures</b>							
<i>Aquicola tertiarycarbonis</i> L108	-0.8 ± 0.1	0.98	95	10	-11 ± 3	13 ± 1	Rosell et al. 2007
<i>Rhodococcus ruber</i> IFP2001	-0.8 ± 0.1	0.96	96	16	-11 ± 4	10 ± 1	Rosell et al. 2007
<i>Pseudonocardia tetrahydrofuranoxydans</i> K1	-1.7 ± 0.2	0.98			-73 ± 7	49 ± 4	McKelvie et al. 2009
<b>Enrichment from well PZ1</b>							
Oxic at 30°C	-0.4 ± 0.3	0.94	90	5	ns (-0.3)	na	This study
Hypoxic at 30°C	-0.7 ± 0.2	0.95	89	5	ns (+12)	na	This study
Oxic at 12°C	-0.5 ± 0.2	0.98	86	7	ns (+6)	na	This study
Hypoxic at 12°C	-0.6 ± 0.1	0.95	85	5	ns (+5)	na	This study

N: number of data points; na: not applicable; ns: not significant, B: maximum percentage of ETBE biodegradation analysed

## Conclusions

- Concerning the tools used to characterize the site, the results obtained since the beginning of the project revealed their complementarity: physico-chemical tools (pollutants quantification), microbial tools (evaluation of the biodegradation capacities), stable isotope tools (predominant enzymatic reaction mechanism), molecular biological tools (microbial ecology), and ecotoxicological tools (environmental impact evaluation).
- Concerning the site, its characterization revealed the high potential of water transfer upstream the site with the presence of BTEXs (37 mg.L<sup>-1</sup> at PZ10) and huge ETBE concentrations (196 mg.L<sup>-1</sup> at PZ10), demonstrating the great water mobility and the persistence of ethers in the aquifer. Aerobic biodegradation of pollutants near the hot spot creates local anoxic conditions, a depletion of the other electron acceptors and an acidification leading to a change of metals speciation and more specifically of arsenic (As<sup>3+</sup>) solubility (50 µg.L<sup>-1</sup>).
- Our studies have shown the aerobic biodegradation capacities of indigenous microflora. The microflora present in the water sampled upstream the site or in the site had both the potential to degrade a mixture of BTEXs and ETBE. MTBE was degraded only by the microflora present in PZ1 (upstream the site).
- The microbial approach delineated communities corresponding to meaningful situations on the site. This could serve as a reference to follow community modifications after remediation or to identify similar polluted situations. Furthermore, using an enrichment procedure, a natural ETBE degradation potential was shown. The various dominant taxa encountered at close distances suggest the existence of gene transfer.
- The results also revealed ecotoxicological effects near the hot spot (P2 and PZ10) where the different pollutants are present (BTEXs, ETBE, MTBE, TBA and also As<sup>3+</sup>) but also in the plume (at PZ15) where ETBE is the only organic compound detected emphasizing the possible ecotoxicological effect of this oxygenate.

## Perspectives

Experimental laboratory biodegradation tests (6 litres bioreactors) will be done on polluted water samples withdrawn from an impacted area of the site (PZ10). Biostimulation (O<sub>2</sub>, nitrate, and Fe) of intrinsic biodegradation capacities of ethers and BTEXs will be compared to bioaugmentation with enriched autochthonous microorganisms. The objective is to recommend the best available biodegradation strategy and extrapolate a bio treatment at site scale during the second half of 2010. After complete biodegradation at pilot scale, the effect of the treatment on pollutants concentrations (EtBE, MtBE and BTEXs) will be quantified as well as the water ecotoxicity in order to evaluate the environmental impact reduction. The evolution of the microbial consortia will be also tested with the DNA array described previously.

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## References.

- Achten C, Kolb A, and Puttmann W (2002a) Methyl *tert*-butyl ether (MTBE) in river and wastewater in Germany. *Environ. Sci. Technol.* **36**:3652-3661.
- Achten C, Kolb A, and Puttmann W (2002b) Occurrence of methyl *tert*-butyl ether (MTBE) in riverbank filtered water and drinking water produced by riverbank filtration. *Environ. Sci. Technol.* **36**:3662-3670.
- Achten C, and Puttmann W (2000) Determination of methyl *tert*-butyl ether in surface water by use of solid-phase microextraction. *Environ. Sci. Technol.* **34**:1359-1364.
- Malandain, C.; Fayolle-Guichard, F.; Vogel, T. M. (2010) Cytochromes P450-mediated degradation of fuel oxygenates by environmental isolates. *FEMS Microbiol. Ecol.*, **72**:289-296.
- McKelvie, J. R.; Hyman, M.; Elsner, M.; Smith, C. A.; Aslett, D.; Lacrampe-Couloume, G.; Sherwood Lollar, B. (2009) Isotopic fractionation of MTBE suggests different initial reaction mechanisms during aerobic biodegradation. *Environ. Sci. Technol.* **43**:2793–2799.
- Rosell i Linares M (2006) *Vigilància ambiental de metil tert-butil eter (MTBE), un additiu de la gasolina, en aigües i sols*. Thesis, Universitat de Barcelona, Spain.
- Rosell, M.; Barcelo, D.; Rohwerder, T.; Breuer, U.; Gehre, M.; Richnow, H. H. (2007) Variations in <sup>13</sup>C/<sup>12</sup>C and D/H enrichment factors of aerobic bacterial fuel oxygenate degradation. *Environ. Sci. Technol.* 2007, **41**:2036-2043.
- Rosell, M.; Finsterbusch, S.; Jechalke, S.; Hübschmann, T.; Vogt, C.; Richnow, H. H., (2010) Evaluation of the Effects of Low Oxygen Concentration on Stable Isotope Fractionation during Aerobic MTBE Biodegradation. *Environ. Sci. Technol.* **44**:309-315.
- Sanguin H, Kroneisen L, Gazengel K, (2008), Development of a 16S rRNA microarray approach for the monitoring of rhizosphere *Pseudomonas* populations associated with the decline of take-all disease of wheat. *Soil Biology & Biochemistry* **40**: 1028-1039.
- Sherwood Lollar, B.; Hirschorn, S. K.; Chartrand, M. M. G.; Lacrampe-Couloume, G. (2007) An approach for assessing total instrumental uncertainty in compound-specific carbon isotope analysis: Implications for environmental remediation studies. *Anal. Chem.* **79**:3469-3475.
- Zwank, L.; Berg, M.; Schmidt, T. C.; Haderlein, S. B. (2003) Compound-specific carbon isotope analysis of volatile organic compounds in the low-microgram per liter range. *Anal. Chem.* **75**:5575-5583.