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GROUNDWATER BIOREMEDIATION: Microbial populations involved in chloroethenes and BTEX contaminated aquifer processing

[AGR/16]

Martina Bertolini

(R12013)

Tutor: Prof. Lucia Cavalca

Ph.D. Dean: Prof. Ella Pagliarini

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ABSTRACT

Groundwater plays an important role in water supply around the world. 2 billion of people use aquifers as drinking water. Consequently, contamination of groundwater has a great social and economic impacts.

The use of organisms (microorganisms and plants) to remediate contaminated matrices, called bioremediation, is becoming more and more frequent. These techniques are cheaper than chemical and physical remediation techniques.

Chloroethenes, aromatic and aliphatic hydrocarbons are widely contaminant compounds because of their intensive use in industrial activity. It is possible to lower their concentration in the environment by means of microbial biodegradation in anaerobic and aerobic conditions.

In this study, an aquifer (located near Porto Marghera, Venice, Italy) contaminated by a leaching from a former landfill was analyzed. The contamination comprised chlorinated solvents, benzene, toluene, ethylbenzene and xylenes (BTEX) and aliphatic hydrocarbons. In 1995, an intervention with a pump and treat reactor was installed. Due to low efficiency and high maintenance costs of the physic-chemical treatment, the installation of a biological treatment, based on two permeable reactive biobarriers, was planned. After preliminary characterization of the microbial community at the site in order to evidence the presence of natural microbial populations involved in decontamination processes, in February 2016 a first biobarrier was installed to stimulate bacterial anaerobic organohalide respiration to dechlorinate chloroethenes. The injection of a reducing substrate was set up to create strong reducing conditions to improve the activity of anaerobic bacteria. A second biobarrier was meant to stimulate bacterial aerobic biodegradation of BTEX and aliphatic hydrocarbons. Urea, ammonium phosphate and O_2 were planned to be injected in the aquifer. Moreover, this treatment was also forecasted to be used for complete vinyl chloride aerobic biodegradation.

In order to define the presence of organo-halide respiring bacteria at the aquifer, laboratory-based anaerobic microcosm study was set up. The effect of the biostimulation intervention (i.e., the addition of a reducing substrate) was also monitored in comparison with natural attenuation processes.

Chlorinated ethenes were analyzed through gas-chromatography coupled to mass spectrophotometry (GC-MS). At microcosms scale, the natural organohalide respiration activity was influenced by the presence of reducing substrate, showing an increase of dechlorination of highly chlorinated ethenes, with a concomitant accumulation of vinyl chloride. Landfill active microbial community composition was determined through Illumina 16S rRNA sequencing of cDNA from RNA extracted from groundwater samples. Active organo-halide bacteria were quantified by quantitative Real Time PCR (q-PCR). Phylogenetic bacterial biomarkers for *Dehalococcoides*, *Geobacteriaceae*, and functional biomarkers *tceA* and *vcrA,* coding for chlorinated ethenes reductases*,* were applied. The ability of aerobic biodegradation of vinyl chloride, BTEX, aliphatic hydrocarbons and chlorobenzene was studied by the Most Probable Number (MPN) technique and q-PCR of *etnC* and *tbmD* genes, coding for alkene and toluene-benzene monooxygenases, respectively.

Once established the presence of bacterial natural attenuation activities for all the compounds, chemical and microbiological analyses were performed at field scale in order to monitor the efficacy of the bioremediation treatments. Moreover, the microbial community composition of anaerobic biobarrier was analyzed before and after 22 months of treatment, by 16S rRNA Illumina sequencing. Reducing substrate addition affected the microbial community composition at the site, causing an increase of fermentative bacteria, mainly belonging to Archaea domain, whereas typically recognized bacteria involved in organohalide respiration were not displayed. These data, along with a decrease in chlorinated solvents measured at the site, suggest a possible presence of a still unexplored biodiversity of OHR bacteria and further culturomics efforts will help to elucidate this. At the plume fringe in the aerobic part of aquifer, BTEX, chlorobenzene and aliphatic hydrocarbon degrading bacteria were characterized. Moreover, microbial consortia able to use vinyl chloride as sole carbon and energy form were selected, demonstrating the feasibility to remediate the site from the carcinogenic intermediate of organohalide respiration.

The microbiological work carried out during this Doctorate, along with hydrogeochemical data, demonstrated that a bioremediation intervention could successfully decontaminate this historical and naturalistically important site. Since the beginning of 2020, a full-scale biobarrier plant has been established and it is expected to run for 30 years in order to completely remediate the aquifer.

RIASSUNTO

L'acqua di falda ha un ruolo molto importante nell'approvvigionamento di acqua nel mondo: 2 miliardi di persone la usano come acqua da bere, rendendo la contaminazione degli acquiferi un grave problema sociale e economico.

Negli anni si sono diffuse nuove tecniche di risanamento delle matrici contaminate basate sull'uso di organismi come microorganismi o piante, che hanno preso il nome di biorisanamento. Oltre ad un'efficacia superiore e ad un minore impatto ambientale, il biorisanamento offre spesso un'alternativa più economica rispetto alle classiche tecniche di risanamento chimico-fisiche. I cloroeteni, benzene, toluene, etilbenzene e xileni (BTEX) e il clorobenzene sono composti contaminanti molto diffusi a causa del loro uso intensivo nelle attività industriali, che possono essere trasformati o degradati nell'ambiente grazie a diversi metabolismi microbici in condizioni aerobiche e anaerobiche. In questo lavoro, è stato analizzato un acquifero nei pressi di Porto Marghera (Venezia) contaminato da una percolazione di solventi clorurati, BTEX e idrocarburi alifatici e aromatici da una discarica limitrofa.

Nel 1995, è stato installato un intervento con un reattore *pump and treat*. A causa del basso tasso di degradazione e gli alti costi di questo intervento, è stato pianificato un intervento basato sulla stimolazione delle attività microbiche mediante l'installazione di due biobarriere reattive permeabili. Dopo una caratterizzazione preliminare della comunità microbica presente nel sito per evidenziare la presenza di una popolazione microbica autoctona coinvolta nel processo di decontaminazione, nel Febbraio 2016 una prima biobarriera è stata installata per stimolare la respirazione anaerobica dei solventi clorurati da parte di batteri anaerobici. Per creare un ambiente fortemente riducente è stato iniettato nell'acquifero un substrato riducente. Una seconda bio-barriera è stata installata per stimolare la degradazione batterica aerobica di BTEX e idrocarburi alifatici grazie all'iniezione di urea, ammonio fosfato e ossigeno. Con questo trattamento, inoltre, sarebbe stato possibile potenziare la biodegradazione aerobica del cloruro di vinile in presenza di microrganismi in grado di mineralizzarlo.

Per definire la presenza dei batteri che effettuano la respirazione degli alogenuri alchilici nell'acquifero, è stato predisposto uno studio in microcosmi in condizioni anaerobiche in scala di laboratorio. L'effetto dell'intervento di biostimolazione (l'aggiunta di substrato riducente) è stato monitorato comparandolo ai processi di naturale attenuazione. I cloroeteni sono stati analizzati attraverso il gas cromatografia accoppiata alla spettrometria di massa (GC-MS). È stata dimostrata la presenza nell'acquifero di attività microbiche in grado di trasformare i contaminati. La naturale attività della respirazione degli alogenuri alchilici veniva influenzata a scala di microcosmo dalla presenza del substrato riducente inducendo un aumento della declorurazione, sebbene portasse ad un accumulo di cloruro di vinile quale intermedio.

La struttura della comunità microbica attiva è stata determinata attraverso il sequenziamento Illumina del 16S rRNA del cDNA estratto dall'RNA dei campioni della discarica. I batteri attivi che respirano gli alogenuri alchilici sono stati quantificati mediante PCR quantitative Real Time (qPCR). Sono stati utilizzati marcatori filogenetici, *Dehalococcoides e Geobacteriaceae,* e funzionali, *tceA* and *vcrA,* codificanti per le reduttasi dei cloroeteni. La degradazione del cloruro di vinile, BTEX, idrocarburi alifatici e clorobenzene in condizioni aerobiche è stata determinata attraverso la tecnica del Most Probable Number (MPN) e di qPCR dei geni *etnC* e *tbmD* codificanti rispettivamente per le monoosigenasi degli alcheni e del toluene e del benzene.

Stabilita la presenza di un'attività batterica autoctona di attenuazione naturale, sono stati monitorati gli effetti dei trattamenti attraverso analisi chimiche e microbiologiche in scala di campo. È stata, inoltre, analizzata la comunità microbica della biobarriera anaerobica prima del trattamento e dopo 22 mesi, mediante sequenziamento Illumina dei16S rRNA.

È stato dimostrato che il trattamento anaerobico ha modificato la composizione della comunità microbica, favorendo i microorganismi fermentanti, in particolare quelli appartenenti al dominio degli Archea, ma senza determinare aumenti significativi dei tipi generi batterici in grado di

effettuare respirazione dei composti organoclorurati. I dati raccolti, insieme ad una diminuzione dei solventi clorurarti misurata nel sito, suggeriscono la possibile presenza di nuovi batteri in grado di declorurare i cloroeteni in condizioni anaerobiche e ulteriori sforzi per coltivarli verranno intrapresi.
Nel plume di contaminazione che raggiunge la parte aerobica dell'acquifero, sono stati isolati batteri degradanti BTEX, clorobenzene e idrocarburi alifatici. Inoltre sono stati selezionati consorzi microbici in grado di utilizzare il cloruro di vinile come unica fonte di carbonio ed energia, dimostrando la possibilità di risanare l'acquifero dall'intermedio carcinogeno che si forma dai processi di respirazione anaerobica dei solventi clorurati.
Il lavoro microbiologico condotto nel corso di questo Dottorato, unitamente a dati idro-geochimici, hanno dimostrato che è possibile risanare questo sito storico e di interesse naturalistico mediante un intervento di biostimolazione della flora microbica autoctona. Dall'inizio del 2020 è stato allestito un impianto *full-scale* e si prevede che il trattamento dovrà durare circa 30 anni per vedere decontaminato l'acquifero

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INTRODUCTION

Pollution of soil, water and air has been an issue since the last century. Industrial, agriculture and domestic sectors through contaminant discharges or incorrect disposals are the responsible of widespread contamination.

High water quality is one of 17 sustainable development goals of FAO for 2030 (6. Clean water and sanitation). Groundwater is very important reserve of water around the world. Around 30% of water used in the world derives from groundwater: 70% are used for irrigation, indeed about 39% of total irrigated area that produce 40% of the world's food production are irrigated with groundwater (Thiruvenkatachari et al., 2008; Siebert et al. 2010). Domestic and industrial uses are lower, 21% and 9% respectively (Margat and van der Gun, 2013), but 2 billion of people use aquifers as drinking water. The impact of each sector is different according to the characteristics of the considered country (population, climate and economic development) (Unesco, 2015).

Groundwater is affected by different contamination of inorganic and organic compounds. In 2011, in Europe, 10% of groundwater resulted to be contaminated by chlorinated hydrocarbons, the fourth contaminants after heavy metals, mineral oil and aromatic hydrocarbons (EEA 2011). In particular, in 2016, groundwater contaminated by tetrachloroethene (PCE) was present in 10 European countries with an area of contamination of 51,400 km^2, more than Swiss extension (WISE-SoW database, 2019).

Although initial environmental remediation strategies were developed through chemical and physical treatments of the contaminated matrices, since few years with increase of environment protection interest, also plants and microorganisms have taken part in remediation actions leading to the bioremediation approach.

BIOREMEDIATION

Contaminated sites are mostly remediated by conventional techniques (Figure 1). Biological treatments are applied on less than 50% of the total contaminated soil. Only Estonia has a massive (more than 95%) use of *ex situ* biological treatments. In Italy, around 22% of contaminated sites are treated by *in situ* or *ex situ* bioremediation treatments. Conventional physic-chemical treatments are usually very expensive, and they have a high impact of environment because in the majority of the cases they are not decisive in the degradation of contaminants compounds producing huge amounts of contaminated waste.

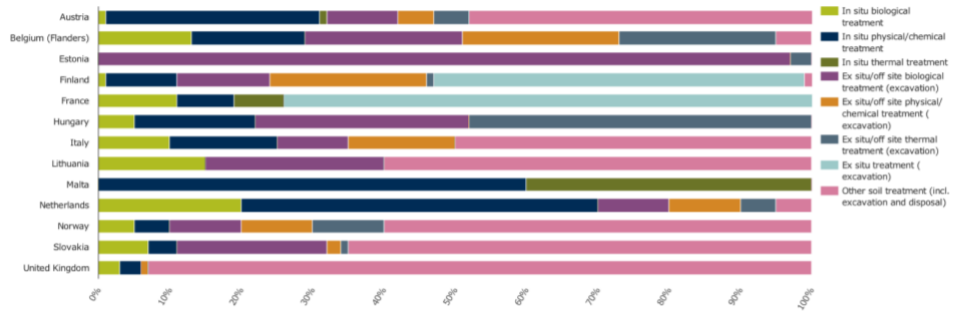

Figure 1 Most frequently applied remediation techniques for contaminated soil (European environment agency, 2014)

In the last decades, use of biological remediation techniques increased. These techniques have minor environment impacts and they are cheaper. In addition, some of them have a less impact on workers health (Majone et al., 2014).

Bioremediation is based on the use of organisms (in particular, plants and microorganisms) to remediate contaminated area through biological mechanisms such as degradation, detoxification, mineralization and transformation (Azubuike et al., 2016).

Bioremediation techniques can be divided in two main groups based on the place where remediation takes place: *ex situ* and *in situ* (Table1). Techniques belonging to the first group are based on the transportation of contaminated

matrices in a different place to be treated. On the other hand, *in situ* techniques treat contamination directly on site. This difference makes *in situ* techniques cheaper but also safer because there is no transport of contaminated matrix with the risk of leakage and workers contact with contaminant compounds (Boopathy et al., 2000).

Table 1 Bioremediation techniques that employ microorganisms

ex situ	**biopile**	
	windrow	
	bioreactor	
	land farming	
in situ	natural attenuation	
	enhanced	bioslurping
		bioventing
		biosparging
		permeable reactive barrier

Among bioremediation techniques that use microorganisms (Table 1), **biopile** is a *ex situ* bioremediation technique. Contaminated soil is removed and pilled to be treated on site. This procedure permits the aeration of soil, nutrient addition and creation of best conditions to improve biodegradation activity.
In addition to biopile, periodic turning of contaminated soil on windrows can improve biodegradation activity because increase aeration and uniform distribution of pollutants. Similar technique is the land farming. Soil is not pilled but it is tilled to increase aeration and improve microbial degradation.
Another *ex situ* bioremediation techniques is the use of **bioreactors**. Contaminated matrix is placed in bioreactor where it is treated in monitored conditions. This technique allows to treat different types of contaminant compounds in different conditions and in successive treatments (sequencing batch). Nevertheless, it is possible to treat only low amounts of contaminated matrix and it is expensive.

In *in situ* techniques, contaminated matrices are not removed from origin place. In the **natural attenuation** approach, no interventions are taken, being the natural environmental microbial community activities to carry on attenuation processes. For this reason, it is very cheap, but it takes longer time to remediate the site than other techniques. In this case, monitoring of efficacy of the process is essential.

Biostimulation is the used to stimulate microbial degradation thorough injection of nutrients or substrates in order to create favorable conditions for microbial organisms activity. **Air injection** is the stimulation of aerobic microbial degradation of contaminant compounds through increment of oxygen. It is injected in the vadose zone (**bioventing**) or in saturated zone (**biosparging**). Injection can be coupled with vapor extraction of contaminant and vacuum enhanced pumping to extract volatile compounds (**Bioslurping**) (Azubuike et al., 2016).

During the decision process of the best bioremediation technique, it is important to consider contaminant nature, contamination characteristics (depth and amount) and site features (position, social and economic importance, geologic and hydrologic analysis).

A common issue *in situ* bioremediation techniques is the formation of aquifer clogging due to a huge increase of biomass. Furthermore, over time an accumulation of the added unused growth substrate as well as intermediate compounds is often observed. Moreover, the addition of O_2 as treatment to improve aerobic biodegradation can lead the oxidation of other reduced species present in the aquifer (Frascari et al., 2015).

PERMEABLE REACTIVE BIO-BARRIER

The definition of Permeable Reactive Barriers (PRB) from USEPA is: “an emplacement of reactive materials in the subsurface designed to intercept a contaminant plume, provide a flow path through the reactive media, and transform the contaminant(s) into environmentally acceptable forms to attain

remediation concentration goals down gradient of the barrier" (Faisal et al., 2018).

PRB are a passive treatment technology for contaminated aquifers, where contaminant flow is directed by natural movement of plume and no energy is required. PRBs have less impact on environment (Higgins et al. 2009) and they are less dangerous for workers and cheaper. In fact, the main costs and environment impact are due to the construction of PRB and after installation they require low maintenance. PRB can be used to treat different type of contaminants (organic and inorganic compounds) because it is possible to choose different reactive material and removal mechanisms based on the characteristics of contaminated area and of the contaminants. Suitable decontamination mechanisms are degradation, precipitation and sorption. They can be reached through physical, chemical and biological treatments. In order to remediate multi-contaminated area, multi-barrier systems can be set up to exploit different decontamination mechanisms (Obiri-Nyarko et al. 2014). PRB can have two conformations: funnel-and-gate or continuous gate designs. In the first case the treatment zone is a delimited area where the contamination plume is converged thorough wall insert in the soil. The second conformation does not change natural flow direction of contamination plume because reactive zone is along all plume path (Obiri-Nyarko et al. 2014).

If decontamination is carried out by microorganisms, barriers are called bio-barrier (Careghini et al. 2013).

Permeable reactive bio-barrier (PRBB) technique is cheaper than classical remediation techniques such as pump and treat (P&T) method but also than permeable reactive barrier (PRB). Battelle, in report of 2012, compared construction and maintenance cost of PRB and PRBB and their environment impacts. In addition, in the same site, PRBB and P&T were compared. Two sites were analyzed. In one site PRB was used as remediation techniques with granular zero valent iron addition. In the other site, both PRBB and P&T methods were used. In PRBB, mulch (from recycled organic material) and

vegetal oil was injected in the aquifer. Both the sites were contaminated by TCE and their remediation rate could be considered similar. The final costs for 1'000L of treated groundwater were: 0.3$ for PRBB, 1.1$ for PRB e and 2.06$ for P&T.

The environmental impacts were analyzed. PRBB showed a less impact in GHGs (carbon dioxide, nitrous oxide and methane), NO_x, PM_{10} and SO_x emissions. Total energy used for the installation and maintenance was lower for PRBB. In addition, PRBB technique decreased risks of injury and fatality. On the other hand, PRB showed a minor impact on water consumption because it did not need to use of water source during its maintenance.

CHLORINATED ETHENES

Chloroethenes (CE) are tetrachloroethene (PCE), trichloroethene (TCE), *cis*-dichloroethene (*cis*-DCE), 1,1-*trans*-dichloroethene (1,1-DCE), 1,2-*trans*-dichloroethene (1,2-DCE) and vinyl chloride (VC). They are one of most frequently detected compounds in contaminated area around the world (McCarty, 2010). They are widely used in industrial sector. In particular, PCE and TCE are used as solvent because of high solvent properties and low fire and explosion potential, for waxes, resins, fats, rubbers, oils and in metal degreasing; but they are common also in some household products like dry cleaning solvents and paint products. Their presence in the environment is also due to inadequate disposal methods in the past (Moran et al., 2007; Beamer et al., 2012).

CE are colorless liquids or gases (VC above 7°C) and belong to the group of chlorinated volatile organic compounds (Cl-VOCs) together with other polychloromethanes and polychloroethanes. TCE and PCE are non-flammable and they are hardly soluble in water. DCE is highly flammable and it has modest solubility in water (Table 2). They have a typical chloroform-like odor. Due to their density, higher than water density, PCE and TCE form a dense non-aqueous phase liquid (D-NAPL) that penetrates through permeable groundwater aquifers, giving rise to plume.

Because of their wide presence in the environment, human exposure to chloroethenes occurs via different routes, such as dermal absorption, ingestion and inhalation (Huang et al., 2014). Most of the cases of intoxication are the result of repeated exposures to small doses (chronic exposure), instead of acute narcosis. CE cause injury to the central nervous, immune and endocrine systems (USA EPA, 2007). The exposure of these compounds shows a correlation with the formation of cancer in human being. In particular, PCE and TCE are associated with esophageal and cervical cancer and non-Hodgkin's lymphoma. VC is even more toxic. TCE and VC has been included in the group 1 by International Agency for Research on Cancer (IARC) (IARC, 1987 and 2020; Lynge et al, 1992) (Table 2).

They are present in the environment not only because of human activities, but also due to natural sources. Their concentrations in uncontaminated soils are about 0,001-0,1 mg organic chlorine g^{-1} of soil weight) (Öberg et al., 2004). CE are also produced naturally from volcanic activity, marine algae and halogenation processes between humic acid and soil microorganisms (Gribble, 1992; Gribble, 1994). The "natural" production of chlorinated hydrocarbons suggests the presence of microorganisms that are able to transform or degrade these compounds.

Table 2 Selected characteristics of chlorinated ethene[a]

Compound		Molecular Formula	Appearance	Water Solubility at 25°C (g L^{-1})[a]	Density (g cm^{-3})[a]	Vapor pressure at 20°C (kPa)[a]	Autoignition Temperature (°C)[a]	Carcinogenicity[b]	Law limits (µg L^{-1}) D.Lgs. 152/06
Vinyl chloride (VC)		C_2H_3Cl	Colorless gas	Slightly soluble	0.969 (-13°C)	516.95	472°	Group 1 (2012)	0.5
***cis*-dichloroethene (*cis*-DCE)**		$C_2H_2Cl_2$	Colorless liquid	1-5	1.28 (20°C)	26.66	460°	N	-
***trans*-dichloroethene (*trans*-DCE)**	1,1-*trans*-DCE		Colorless liquid	2.5	1.213 (20°C)	66.5	460°	Group 3 (1999)	0.05
	1,2-*trans*-DCE		Colorless liquid	<1.0	1.26 (25°C)	53.33	460°	N	60
Trichloroethene (TCE)		C_2HCl_3	Colorless liquid	1.280	1.46 (20°C)	7.8	> 410°	Group 1 (2014)	1.5
Tetrachloroethene (PCE)		C_2Cl_4	Colorless liquid	0.15	1.63 (20°C)	1.9	>650°	Group 2A (2014)	1.1

[a] CAMEO Chemicals

[b] IARC (International Agency for Research on Cancer

MICROBIAL DECHLORINATION OF CHLORINATED ETHENES

In the past, CE were considered recalcitrant to biodegradation, but to date, different microbial mechanisms have been elucidated. While anaerobic biodegradation is widely studied, little is known about pathways involved in aerobic biodegradation of these compounds.

Four CE degradation pathways are known: one anaerobic pathway (reductive dechlorination) and two aerobic pathways (aerobic oxidation and aerobic cometabolic degradation).

CE with a higher numbers of chlorine substituents have a higher tendency to undergo microbial reductive dechlorination. CE with a lower numbers of chlorine substituents are more easily degraded by microorganisms through the aerobic oxidative degradation because of their high grade of reduction (Figure 2) (Mattes et al., 2010).

Different studies showed an improvement of the efficiency of bioremediation applying a sequential anaerobic-aerobic biodegradation exploiting all the different degradation pathways in different conditions through indigenous microbial community (Tiehm et al., 2011).

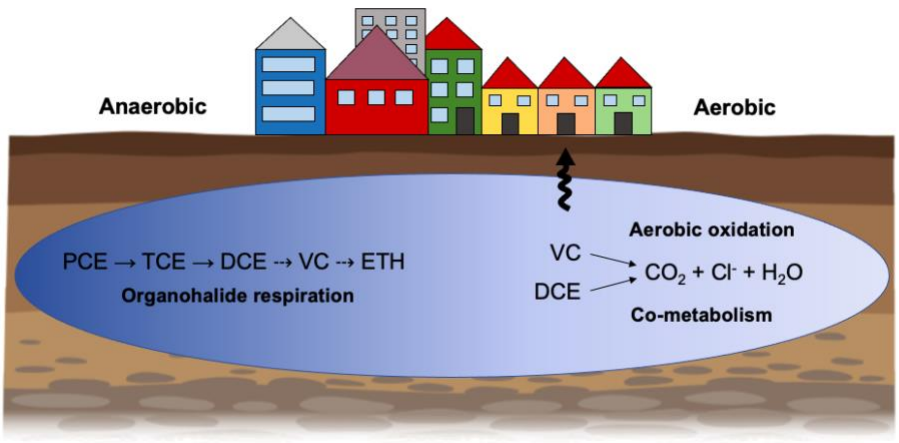

Figure 2 Chloroethenes degradation pathways

ORGANOHALIDE RESPIRATION

Organohalide respiration (OHR) is an anaerobic respiration process based on the use of CE as electron acceptors and hydrogen as electron donor. Alternative electron donors can be lactic or butyric acid produced during the fermentation of organic compounds. Dechlorinating bacteria compete with methanogens, sulfate reducing bacteria and acetogens for the use of hydrogen (Fennell et al., 1997; Yang et al., 2002; Matteucci et al., 2015). Additionally, they share the same range of redox potential in which their activity is efficient. Organohalide respiration occurs when redox potential is lower than -200 mV (from -210 to -470 mV) (Dolfing, 2016), methanogens act from around -175 to -400 mV and sulfate reducing bacteria optimal conditions are in presence of redox potential from -50 to -250 mV. However, dechlorinating bacteria are favored when hydrogen is present at low concentrations (Yang et al., 1998).

During dechlorination, chlorine atoms are sequentially replaced with hydrogen atoms, allowing the degradation of PCE to TCE, *cis*-DCE and VC down to ethene (Figure 3).

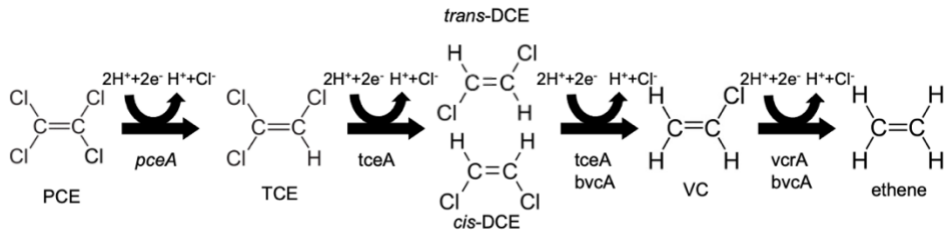

Figure 3 Anaerobic organohalide respiration of PCE to ethene. Genes involved in respiration were reported. Modified from: Parsons et al., 2004

The steps of the dechlorination from *cis*-DCE to ethene hardly happens bringing an accumulation of DCE and VC in the contaminated site thus impairing environmental quality (Bradley, 2003; Smidt and de Vos, 2004; Futagami et al., 2008; Abe et al., 2009). Greater difficulty of DCE and VC

dechlorination could be due a thermodynamical factors, based on prdocution of minor free energy (ΔG^0) during dechlorination reactions (Futagami et al., 2008).

OHR activity decreases with the decrease of the pH (Robinson et al., 2009).

The genes involved in organohalide respiration fall in the class of reductive dehalogenase homologous genes (*rdh* or RDases). They are: tetrachloroethene reductive dehalogenase (*pceA*), trichloroethene reductive dehalogenase (*tceA*), vinyl chloride reductase (*bvcA* and *vcrA*). Reductase coded by *pceA* are involved in degradation of PCE to TCE, that coded by *tceA* catalyzes TCE degradation to DCE or DCE to VC, that coded by *bvcA* degrades DCE to VC and VC to ethene, that coded by *vcrA* catalyzes the degradation of VC to ethene (Figure 3). Most of *rdh* use a corrinoid (coenzyme B12) as a cofactor (Futagami et al., 2008; Hug et al., 2013).

RDases can be subjected to horizontal gene transfer. According to genomic sequence analyses, they are clustered in genomic islands in high plasticity region with transposable elements (Richardson et al., 2013). Horizontal gene transfer occurs in *Dehalococcoides* and *Dehalogenimonas* (McMurdie et al., 2009) but it is also present in Firmicutes phylum (*Desulfitobacterium* and *Dehalobacter*) (Mailard et al., 2005; Kim et al., 2012).

Only few bacterial genera are known to perform anaerobic reductive dechlorination (Table 3). Keystone bacteria are *Dehalococcoides* (*Dhc*), obligate organohalide respiring bacteria, and *Dehalobium* belong to the Chloroflexi; *Desulfitobacterium*, *Dehalobacter* and *Clostridium* belong to the Firmicutes; *Comamonas*, *Geobacter*, *Desulfomonile*, *Desulfuromonas, Sulfurospirillum, Enterobacter* and *Shewanella* belong to the class Proteobacteria (Table 3).

Only few species (*Dehalococcoides mccartyi* strain BTF08 and *Dehalococcoides mccartyi* strain 195, previously *D. ethenogenes* by Maymó-Gatell et al., 1997b) of genus *Dehalococcoides* are able to degrade PCE to ethene and carry out a complete reductive dechlorination (Cichocka et al., 2010). Indeed, it is more common, CE are totally biodegraded by the activity

of different OHR bacterial consortia involved in different steps of degradation pathway (Di Stefano et al., 1991). Furthermore, in OHR microbial communities not only OHRB are present, but also other microorganisms with different roles (H_2 and corrinoid synthesis, protection of OHRB from oxygen). OHR microbial community can change according to electron donor fed, organohalide substrate and incubation conditions (Richardson, 2016). In some cases, in isolated OHRB, OHR activity rate decreases of more than four orders of magnitude than the same OHRB in mixed enrichment culture. Fermentative bacteria produce H_2 for OHRB activity, but sometimes this process can be syntrophic, because both bacterial populations reach beneficials. Methanogens can be not only a competitor for the use of H_2 but in some cases they can couple methanogenesis with the fermentation of fermentable products, e.g., *Methanosarcina* can produce H_2 from acetate (Heimann et al., 2006). Other bacteria have of corrinoid biosynthesis role. Corrinoid is a required cofactor for RDase enzymes but some OHRB are not able to produce it. Different bacteria showed a complete corrinoid biosynthetic pathway: *Spirochaetes, Sedimentibacter* and *Acetobacterium* (Ziv-El et al, 2011; Maphosa et al., 2012; Ziv-El et al., 2012). Another class of bacteria present in OHR microbial community are those that can use O_2 or that have an oxygen detoxification pathway in order to protect those OHRB that are strictly anaerobic (Richardson, 2016). On the other hand, OHRB can have detoxification function because high concentration of organohalide can inhibit fermentation activities.

Table 3 Known organohalide respiring bacteria that can dechlorinate chloroethenes (Atashgahi et al., 2016)

Phylum	Genus	Species	References
Chloroflexi	*Dehalococcoides*		Scholz-Muramatsu et al., 1995
		mccartyi 195	Maymo-Gatell, 1997, Löffler et al., 2013
		FL2	He, 2005
		mccartyi BAV1	Krajmalnik-Brown et al., 2004
		VS	Maymo-Gatell et al., 1997b
	Dehalobium		
		chlorocoercia	Miller et al., 2005
Firmicutes	*Desulfitobacterium*		Atashgahi, et al., 2016
		Viet1	Krumholz et al., 1996
		PCE1	Gerritse et al., 1996
		frappieri TCE1	Gerritse et al., 1999
		PCE-S	Miller et al., 1997
		hafniense Y51	Suyama et al., 2001
		hafniense DCB-2	Madsen et al., 1992
		dehalogenans JW/IU-DC1	Utkin qt al., 1994
		hafniense PCP-1	Dennie et al., 1998
		hafniense TCE1	Gerritse et al., 1999
		hafniense TCP-A	Breitenstein et al., 2001
		metallireducens 853-15A	Finneran et al., 2002
		hafniense G2	Shelobolina et a., 2003
		KBC1	Tsukagoshi et al., 2006
		B31e3	Yoshida et al., 2007

		hafniense JH1	Fletcher, 2008
		PR	Zhao et al., 2015
	Dehalobacter		Loffler et al., 1997
		restrictus PER-K23	Holliger et al., 1998
		TEA	Wild et al., 1996
	Clostridium	*bifermentans* DPH-1	He et al., 2003
Proteobacteria	*Comamonas*		Chen et al., 2013
	Geobacter		
		lovleyi	Sung et al., 2006
	Desulfuromonas		Maymo-Gallett et al., 1997b
		chloroethenica TT4B	Krumholz, 1997
		Michiganensis BB1 and BRS1	Sung et al., 2003
	Desulfomonile		
		tiedjei	DeWeerd et al., 1990
	Sulfurospirillum		
		multivorans	Luijten et al., 2003
		halorespirans PCE-M2	Holliger et al., 1993
		carboxydovorans	Jensen and Finster, 2005
	Enterobacter	*agglomerans* MS-1	Cupples et al., 2003
	Shewanella	*sediminis strain* HAW-EB3	Lohner and Spormann, 2013

The presence of other compounds, for example chloroform (CF) and 1,1,1-trichloroethane (1,1,1-TCA) can inhibit the biodegradation activity of *Dehalococcoides* (Maymó-Gatell et al., 2001; Yu et al., 2005).
CE can be reduced also through abiotic processes. These transformations are usually slower than organohalide respiration, but they can be relevant in some specific conditions, for example in presence of high concentration of reactive minerals as sulphide minerals (Tobiszewski et al., 2012). Chloride green rust and pyrite can slowly transform PCE and TCE to ethene and acetylene (Liang et al., 2009).

Reducing substrates as biostimulation factors

In order to improve the degradation by means of anaerobic OHR at field sites, biostimulation is widely used. A reductive fermentable substrate is added and through a fermentation it is transformed in reducing equivalents by fermentative bacteria (Conrad et al., 2010) decreasing redox potential conditions. Substrate has to provide to OHRB electron donor (H_2) but also carbon source. H_2 is then used by OHRB for OHR activity.
The reducing substrate is injected into the contaminant plume trough injection wells, thus creating a permeable reactive bio-barrier (PRBB) system. Different substrates can be used to enhance OHR: alcohols, organic acids, emulsified vegetable oil, complex organic materials (e.g., molasses) and plant-based materials (wood chip, corn cob) (Löffler et al., 2006). Several substrates are engineered in order to address some characteristics for their use at field scale (e.g., less thick for a better distribution of substrate, grade of fermentation and pH stability) (Steffan and Schaefer, 2016).
Selection of appropriate substrate is very important for the remediation success of the site. Determination of geologic and hydrogeologic characteristics of the site, concentration and nature of contaminants but also timeline and available budget are all important aspects to take into account during the selection of substrate. Characteristic of the site can affect distribution of electron donors in the treated contaminated zone. Substrates

are divided in two main groups: soluble and insoluble. The first one is quickly degraded therefore it needs frequently injections. Insoluble substrates are usually slowly fermented allowing a single injection or at periodic intervals of 1-5 years (Steffan and Schaefer, 2016). Soluble substrates can be easily distributed in the aquifer, but it is also quickly degraded, thus not reaching areas far from injection wells. Hydrogeological analysis of the site allows to determine if substrate achieves uniformly all contaminated area. Monitoring of substrate dispersion can help in determination of efficacy of the selected substrate. Depending on selected reductive substrate, it is possible to advantage OHRB over methanogens. Slowly fermented substrates (e.g., butyrate and propionate) release lower levels of H_2 favoring OHRB (Richardson, 2016). Mixture of substrates can be used to create best conditions for OHRB activity (Schöllhorn et al., 1994).

AEROBIC OXIDATION

Aerobic degradation of CE is less understood than OHR. In aerobic conditions, low CE can be degraded by two different mechanisms: oxidation and co-metabolism.

In aerobic conditions, CE can be used as carbon source by different bacteria. PCE degradation in aerobic conditions has not yet been found, TCE and DCE oxidation is rarely described. VC oxidation is most frequently reported (Dolinová et al., 2017).

Jennings et al. 2009 hypothesized two pathways for *cis*-DCE degradation based on an integrated omics approach, enzyme assays and compound-specific isotope analysis (CSIA). The two pathways are: glutathione S-transferases (GST)-catalyzed dehalogenation and monooxygenase-catalyzed epoxidation. In the first pathway, the two chloride atoms of *cis*-DCE are replaced with glutathione and hydroxyl group, respectively. Final product of pathway is glycolate. In the second pathway, *cis*-DCE is oxidized with formation of glyoxylate as final product. Glycolate can be transformed in

glyoxylate. Through glyoxylate cycle, glyoxylate became succinate and enters in the TCA cycle (Figure 4).

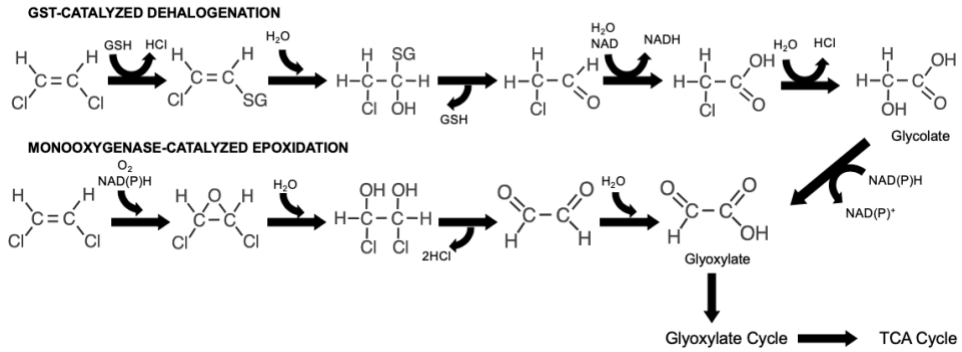

Figure 4 cis-DCE degradation pathway (Coleman et al., 2002)

Polaromonas sp. strain JS666 can use *cis*-DCE as sole carbon and energy source (Coleman et al., 2002b). Genome analysis of strain JS666 (Mattes et al., 2008) revealed the presence of genes for degradative enzymes of different compounds like CE and aromatic hydrocarbons and those for metal resistance.

TCE degradation as a sole carbon source was detected at microcosm scale, although bacteria involved in TCE degradation were not identified (Schmidt et al., 2014).

Pathway involved in VC oxidation is the same involved in ethene oxidation. Little is known about this pathway. Indeed, only the first two enzymes involved in VC-ethene oxidation were identified. All the other pathway steps were only assumed (Figure 5). The first enzyme involved in degradation is alkene monooxygenase (AkMO). With the addition of one oxygen atom, starting compounds are transformed in aliphatic epoxide (epoxyethane from ethene and chlorooxirane from VC). AkMO is composed by 4 subunits that are encoded by 4 genes, *etnA, B, C, D.* The second enzyme is epoxyalkane:coenzyme M transferase (EaCoMT) and it is encoded by *etnE*. This enzyme mediates the conjugation of epoxide, that is toxic for the cell,

with coenzyme M (Mattes et al., 2005) decreasing its toxicity. It was hypothesized that the last compound of degradative pathway enters in TCA cycle.

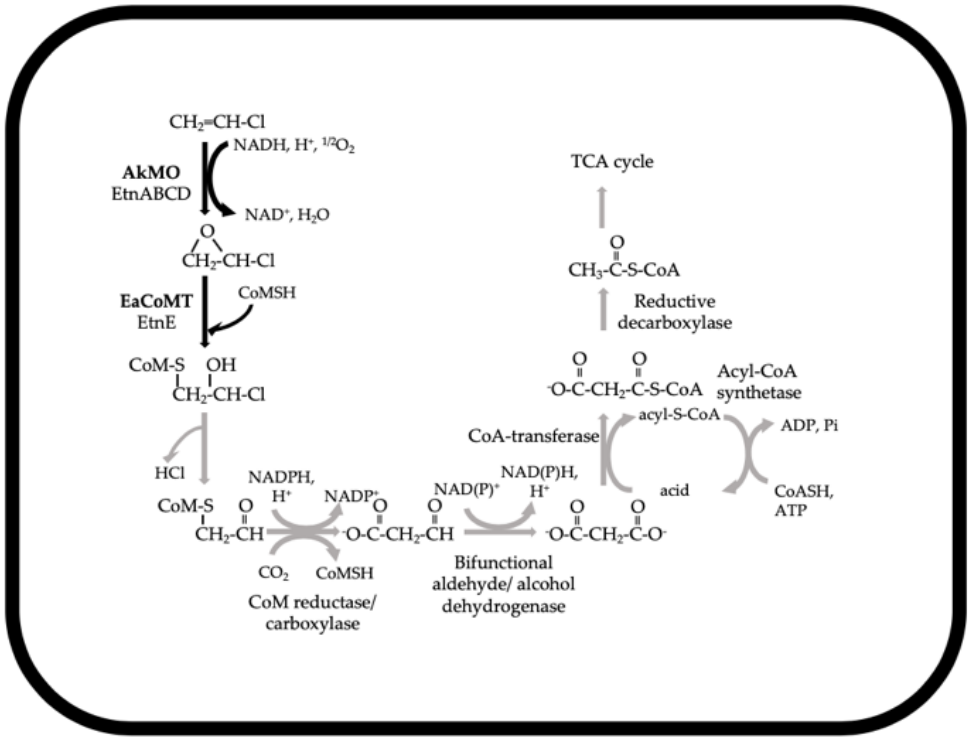

Figure 5 Aerobic biodegradation pathway of VC and ethene. Grey arrows: hypothetical reactions.

etnA, B, C, D, E genes are located on the same large linear plasmid as operon. Plasmid can be lost by degrading bacteria in VC starvation conditions also after 1 day (Figure 6) (Coleman et al., 2002). Furthermore, it was hypothesized a possible lateral transfer of this plasmid (Coleman et al., 2003). These genes show some difference in their sequence based on bacteria that degrade ethene or VC (Jin et al., 2010).

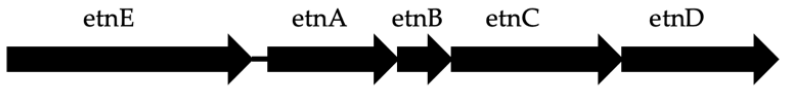

Figure 6 Gene cluster of the aerobic oxidative pathway for VC degradation (Coleman et al., 2002)

Only few VC-degrading bacteria genera are known: *Mycobacterium*, *Pseudomonas, Rhodococcus* and *Nocardioides* (Table 4). Oxidation of VC needs very low amount of oxygen (0.5-1.8 mg/L), for this reason VC oxidation was observed also in anaerobic conditions (Singh et al., 2004; Fullerton et al., 2014)

Table 4 Characterized bacteria that can degrade VC in aerobic conditions

Genus	Species/strains	References
Mycobacterium	*aurum* strain L1	Hartmans et al., 1985
	strains JS60, JS61, JS616, JS617	Coleman et al., 2002a
Nocardiodes	sp. strain JS614	Coleman et al., 2002a
Rhodococcus	*rhodochrous*	Malachowsky et al., 1994
Pseudomonas	*aeruginosa* strain DL1	Verce et al., 2000
	aeruginosa strain MF1	Verce et al., 2001
	putida strain AJ	Danko et al., 2004
Ochrobactrum	sp. strain TD	Danko et al., 2004
Ralstonia	sp. strain TRW-1	Elango et al., 2006
Brevundimonas	sp.	Paes et al., 2015
Rhodoferax	sp.	Paes et al., 2015

EtnC and EtnE phylogenetic trees were constructed (Figure 7) to show the variability of these enzymes. Protein sequences found on NCBI (National Center for Biotechnology Information) database through BLASTp (Basic Local Alignment Search Tool protein in protein) program based on the protein sequences of the enzymes EtnC and EtnE of *Nocardioides* sp. JS614 (accession number AAV52081.1 e AAV52084.1 on GenBank) were used. Only monooxygenase sequences retrieved from cultivable bacteria were analyzed. Sequences were analyzed with MEGA X software (Kumar et al., 2018). Sequence alignment was based on Muscle algorithm (Edgar, 2004) and phylogenetic trees were constructed using *Maximum Likelihood* method with matrix model of JJT (Jones et al., 1992). Methane monooxygenase was chosen as out-group.

All sequences of the two enzymes of interest belonged to only two genera: *Mycobacterium* (renamed *Mycolicibacterium* by Gupta et al. (2018) and *Nocardioides*. In *Mycolicibacterium* genus, and several strains were isolated and sequenced (Figure 7). EtnE deduced aminoacidic sequences were more conserved than EtnC sequences, which showed higher sequence variability.

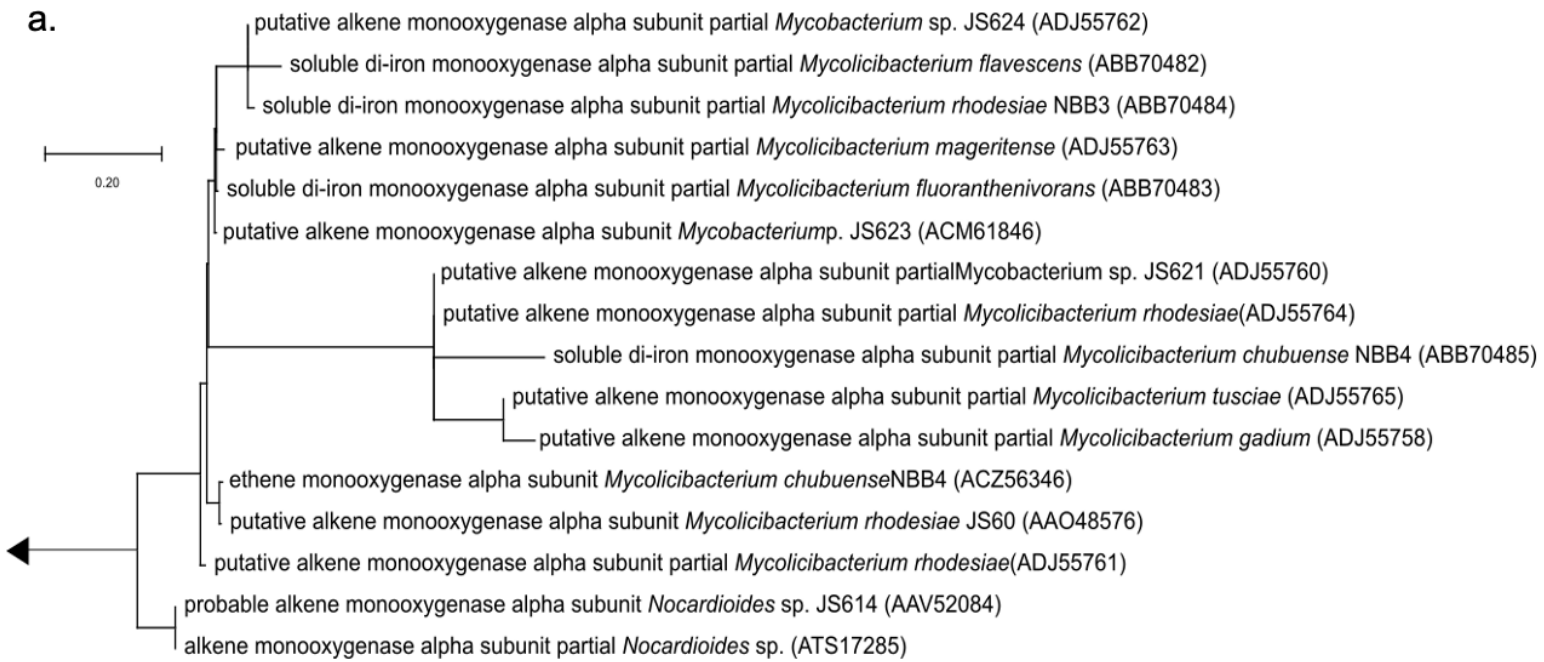
a.
0.20
putative alkene monooxygenase alpha subunit partial Mycobacterium sp. JS624 (ADJ55762)
soluble di-iron monooxygenase alpha subunit partial Mycolicibacterium flavescens (ABB70482)
soluble di-iron monooxygenase alpha subunit partial Mycolicibacterium rhodesiae NBB3 (ABB70484)
putative alkene monooxygenase alpha subunit partial Mycolicibacterium mageritense (ADJ55763)
soluble di-iron monooxygenase alpha subunit partial Mycolicibacterium fluoranthenivorans (ABB70483)
putative alkene monooxygenase alpha subunit Mycobacteriump. JS623 (ACM61846)
putative alkene monooxygenase alpha subunit partialMycobacterium sp. JS621 (ADJ55760)
putative alkene monooxygenase alpha subunit partial Mycolicibacterium rhodesiae(ADJ55764)
soluble di-iron monooxygenase alpha subunit partial Mycolicibacterium chubuense NBB4 (ABB70485)
putative alkene monooxygenase alpha subunit partial Mycolicibacterium tusciae (ADJ55765)
putative alkene monooxygenase alpha subunit partial Mycolicibacterium gadium (ADJ55758)
ethene monooxygenase alpha subunit Mycolicibacterium chubuenseNBB4 (ACZ56346)
putative alkene monooxygenase alpha subunit Mycolicibacterium rhodesiae JS60 (AAO48576)
putative alkene monooxygenase alpha subunit partial Mycolicibacterium rhodesiae(ADJ55761)
probable alkene monooxygenase alpha subunit Nocardioides sp. JS614 (AAV52084)
alkene monooxygenase alpha subunit partial Nocardioides sp. (ATS17285)

Figure 2 Phylogenetic trees of EtnC (a) and EtnE (b) protein sequences. Branches length corresponds to the number of substitutions and under each phylogenetic tree legend of scale was reported. Sequences of methane monooxygenase were used as outgroup (WP_040789699, WP_005572960, MSQ68733, WP_134211723, WP_017727839, PSR31298, WP_083042312, WP_059039071 e WP_066161679).

In contaminated soils and groundwaters, the presence of pollutant mixtures is more common than the occurrence of single compounds. For this reason, the transformations actually observed *in situ* often deviate from what theoretically expected. In fact, the biodegradation activity of *cis*-DCE decreases in presence of the other CE. In particular, the presence of 1,1DCE and TCE inhibits biodegradation of *cis*-DCE. Faster degradation of *cis*-DCE happens in presence of PCE than in presence of VC and *trans*-DCE. Higher concentrations of VC increase the period to degrade the same amount of *cis*-DCE (Zhao et al., 2010).

CO-METABOLIC DEGRADATION

CE can be degraded in co-metabolism. Bacteria does not use CE as carbon or energy source and their degradation is carried out by enzymes used by bacteria for other compounds. Usually, these enzymes are monooxygenases (Dolinová et al., 2017). Aerobic co-metabolic degradation was reported for all CE (Lange et al., 1997; Doughty et al., 2005). Different co-substrates that are used for bacteria growth are known: ammonium, cumene, ethane, ethene, isoprene, phenol, propane, methane and toluene. Their absence results in impairing co-metabolic degradation of CE. *Rhodococcus* sp. PB1 can degrade chlorinated ethenes while growing on propane (Frascari et al., 2008). TCE can be oxidized by *Pseudomonas putida* strain F1 in presence of toluene through toluene dioxygenase (TDO) (Lange et al., 1997). Butane monooxygenase (BMO) of *Pseudomonas butonovora* can degrade DCE in presence of butane (Doughty et al., 2005).

VC and TCE are oxidized by methanotrophic bacteria (Bowman et al., 1993; Yoon et al., 2011). Monooxygenases involved in oxidative degradation are methanol monooxygenases (MMO) that mediate conversion from methane to methanol. Two MMO are known: one in the membrane, particulate MMO (pMMO), and the other one in the cytosol, soluble MMO (sMMO). These two enzymes are encoded by *pmoA* and *mmoX* genes, respectively. pMMO is most frequently expressed than sMMO by methanotroph. Both MMO can

degrade CE but sMMO shows less specificity for methane, so it can degrade a wider range of substrates including CE and with higher rate (Lee et al., 2006). However, it was demonstrated that sMMO can be inhibited by high concentrations of CE (>50 μM) (Lee et al., 2006; Yoon et al., 2011). Facultative methanotroph (*Methylocystis, Methylocapsa, Methylocella*) were discovered suggesting a possible co-metabolic degradation of CE with co-substrates different from methane like acetate, pyruvate and ethanol (Dedysh et al., 2005; Dunfield et al., 2010; Im et al., 2011).

In two different studies, influence of methane and ethene on VC degradation was analyzed. In both studied, contemporary presence of ethene and methane increased VC oxidation. In Freedman et al., (2001), ethene presence improved VC degradation better than methane, also after a prolonged incubation. On the other hands, in Findlay et al. (2016) methane resulted as the best compound for VC degradation. In both studies, inoculums were from two different contaminated sites; so, these different results can be explained because of different composition of microbial community or hydrogeochemical characteristics of the site. Mechanisms that influence VC degradation rate are still little known.

BTEX

BTEX (benzene, toluene, ethyl benzene and xylenes) are VOCs (volatile organic compounds) (Table 5). They are monoaromatic hydrocarbons. They are one of the most widespread contaminants especially in air. Indeed, these compounds are not only used as industrial solvents in synthesis of different materials, but their emission in the environment is due to combustion processes and vehicle exhausts (El-Naas et al., 2014). As result of their relatively high vapor pressure, they can contaminate large regions.

Table 5 Chemical characteristic of benzene, toluene and chlorobenzene

Compound	Molecular Formula	Appearance	Water Solubility (mg L^{-1})[a]	Density (g cm^{-3})[a]	Vapor pressure (mm HG)[a]	Autoignition Temperature (°C)	Carcinogenicity[b]	Law limits (µg L^{-1}) D.Lgs. 152/06
Benzene	C_6H_6	Colorless volatile liquid	< 1	0.87	20	480°	Group 1	1
Toluene	C_7H_8	Colorless volatile liquid	1 to 5	0.87	76	592°	Group 3	15
Chlorobenzene	C_6H_5Cl	Colorless liquid	< 1	1.11	8.8	593°	-	40

[a] CAMEO chemicals

[b] IARC

They are responsible of stratospheric ozone depletion, greenhouse effect and global warming (Durmusoglu et al., 2010; Mohammad et al., 2007). Human daily exposure of these compounds is due to air breathing (vehicle exhausts but also cigarettes smoke) and food (Table 6). Indeed, exposure of BTEX through drinking water is low (Leusch and Bartkow, 2010).

Table 6 Estimated daily BTEX exposure (µg d^{-1}) (Leusch and Bartkow, 2010)

	Benzene	**Toluene**
Air breathing	90-1300	2-12000
Cigarette smoking	1800	2000
Food	Up to 250	Up to 64
Drinking water	Up to 10	Up to 43

Health effects of these compounds on human depend on the time of exposure and the amount of BTEX adsorbed by the body. Benzene is carcinogenic for human, indeed it is in Group 1 of International Agency for Research on Cancer (IARC). It can also enter in the body across the skin. Benzene affects mainly nervous system and blood forming organs as bone marrow bringing immune system diseases and bleeding disorders. Toluene affects nervous system with permanent brain damage due to repeatedly breathing. Chlorobenzene can create disturbances of central nervous system with muscle spasms. Toluene and chlorobenzene are accumulated in fat tissues (Leusch and Bartkow, 2010).

AEROBIC DEGRADATION

BTEX are efficiency degraded in aerobic conditions (An et al., 2001). Aromatic compounds can be consider degraded only if the ring is cleavaged (Smith et al., 1991). BTEX biodegradation pathways are based on two enzymes, monooxygenase and dioxygenase. Pathway of monooxygenase is called "*tol*".

Methyl and ethyl substituents of aromatic ring are transformed through subsequent oxidation in pyrocatechols and phenyl glyoxal substitute (Tsao et al., 1998; Khan et al., 2001). Dioxygenase pathway, also called "*tod*", attacks aromatic rings forming 2-hydroxy-substituted (Khan et al., 2001; Jindrová et al., 2002).

Aromatic degradation pathways are divided in two main steps: upper pathway and lower pathway. The first one is based on the transformation of aromatic compounds in dihydroxyl intermediate (Figure 8). In the second step, lower pathway, aromatic ring is cleavage. Then, resulted aliphatic compounds are oxidated to acetyl-CoA, pyruvate and acetaldehyde, that enter in TCA cycle (Figure 9) (Andreoni and Gianfreda, 2007; Jindrová et al., 2002). Some of the genes involved in aromatic degradation are toluene-dioxygenase (*tod* C1), toluene/benzene monooxygenase (*tbmD*), catechol 2,3 oxygenase (*c23o*), 1,2 dioxygenases (*cat1*/3) and catechol 2,3 oxygenase (*cat* 2.3.) (Hendrickx et al., 2006; Lillis et al., 2010; Garcia et al., 2006). The first two enzymes add two and one oxygen atom, respectively, to aromatic ring. The three catechol oxygenizes perform aromatic cleavage f aromatic compounds in the second phase of degradation pathway.

Chlorobenzene degradation follows the same pathways of the other aromatic compounds with an intermediated step of dehalogenation (Figure 10) (Mars et al., 1997).

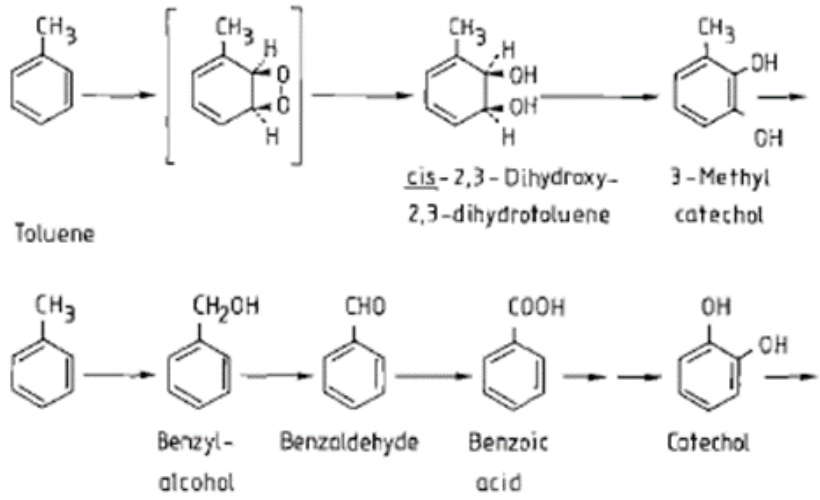

Figure 8 Initial steps of toluene aerobic oxidation (Smith et al., 1990)

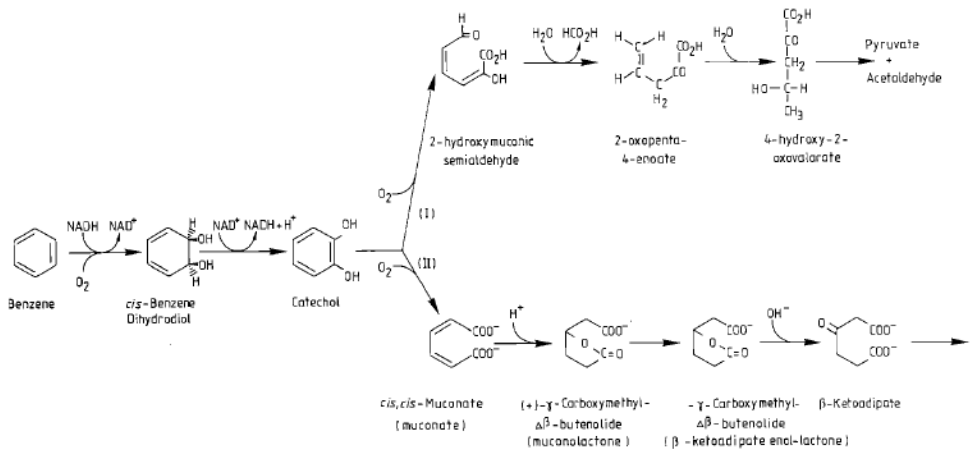

Figure 9 Aerobic Biodegradation of benzene. Upper and lower steps are reported (Smith et al., 1990)

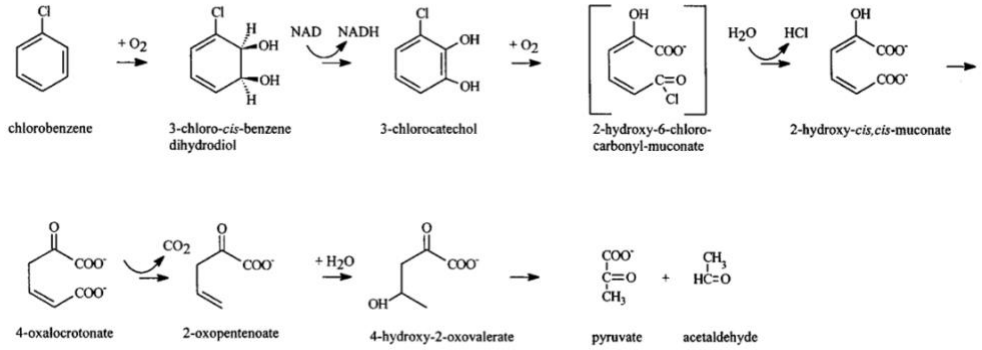

Figure 10 Complete aerobic biodegradation of chlorobenzene (Mars et al., 1997)

A lot of BTEX degrading bacteria are known. *Rhodococcus*, *Marinobacter*, *Acinetobacter* and *Pseudomonas* (Kim et al., 2002; Nicholson et al., 2004; Wang et al., 2006; Martina et al., 2012) were isolated from different environments. From sewage and fresh water, *Ralstonia*, *Microbacterium, Mycobacterium* and *Burkholderia* were isolated (Ryan et al., 2007; Cavalca et al., 2004; Johnson et al., 1997).

Oxidation of aromatic hydrocarbons was also met in hypoxic conditions. Kukor et al., 1996 demonstrated BTEX degradation presence in a petroleum contaminated aquifer with low oxygen concentrations (2 mg L^{-1} or less).

BTEX can be also degrade in anaerobic conditions. Bacteria can used nitrate as alternative electron acceptor instead of oxygens. Benzene is oxidated to CO_2, and this the oxidation is coupled with nitrate reduction (Coates et al., 2001), as well as toluene that can be degraded in nitrate reduction conditions (Chakraborty and Coates, 2004)

SITE OF INTEREST

The site analyzed in the present work is located in Northern Italy, near Venice, in the municipality of Mira-Dogaletto. In the site is present a former landfill into disuse that, due to the break of below clay lent, created a groundwater

contamination. The clay lent is natural layer of clay that delimits the different sections of the aquifer. Landfill was used during 1960s and 1970s as deposit of industrial wastes from the near petrochemical industrial site of Porto Marghera. The extension of landfill is of 16 hectares with an elevation above ground level of 11 m a.s.l. Contamination is comprised by different compounds: chlorinated solvents and petroleum derived hydrocarbons as BTEX. Total contaminant mass is calculated in 1'700'000 t. Contaminant compounds were found between 2.5 m and 10 m under a.s.l.

The area is surrounded by the Venice lagoon to the South and by an irrigation/ drainage groundwater channel to the North-side (Figure 11). It is below sea level, for this reason a drainage pump is used to maintain an adequate water level inside the channel affecting groundwater flow.

Although the aquifer is near the Venice lagoon, the electron conductivity of the aquifer is around 20 mS/cm. This value of electron conductivity is between potable water (2.5 mS/cm) and brackish water (30 mS/cm)**.** Further characteristics can be found at Chapter one of the present work.

In 2001, leaching from landfill was detected and double pump and treat (P&T) method was chosen in order to remediate the aquifer. Hydraulic barriers consisted of 14 wells: 7 proximal near the landfill and 7 distal wells near the channel. It could treat 18 m^3 h^{-1}. This type of remediation technique showed high operation and maintenance costs and a low efficiency in the degradation of multiple contaminant present in the aquifer.

Therefore, in 2016, microbial analysis were started to determine the presence of natural microbial community able to perform biodegradation activity at the site. Once the presence of bacterial activities useful for bioremediation were confirmed two sequenced multi permeable reactive bio barrier (PRBB) were set up to support P&T activity and in the future replace it (Figure 12). The purpose of these two PRBB was to stimulate the native microbial activity to perform: i) reductive dehalogenation of CE in the first anaerobic track of the groundwater plume, and ii) CE (mainly VC) and hydrocarbons oxidation in the last aerobic track of the plume in the proximity of the channel.

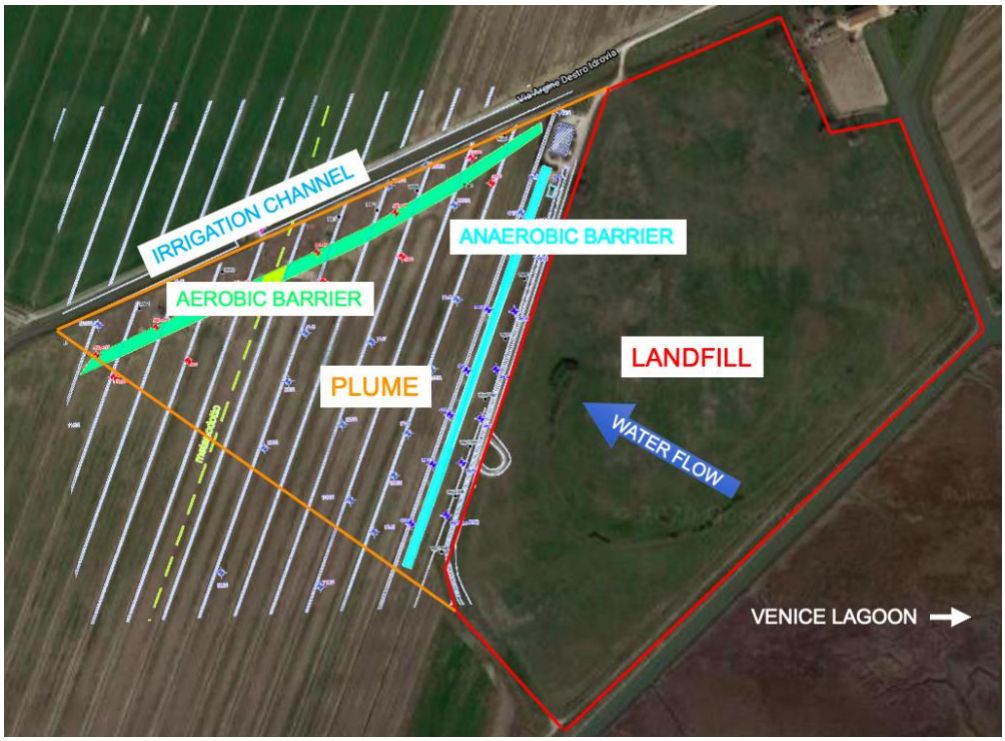

Figure 11 Setup map of two sequenced multi permeable reactive bio barriers in the site for the bioremediation of the aquifer

The first PRBB is located near the landfill (Figure 11) and it is characterized by anaerobic conditions. It is about 390 m long and consists of 39 wells 10 m deep. In the anaerobic PRBB, a reducing substrate is injected in the aquifer to create strong reductive conditions to promote biodegradation activity of anaerobic degrading bacteria and OHR bacteria (Figure 12). The substrate is injected with range dosage from 1.8 to 2.8 L day^{-1}. It is a liquid fermented vegetable-derived product composed of *Saccharum officinarum, Gramineae, Beta vulgaris* and *Chenopodiaceae*. Its composition is 64% water, 36% solids and 3.24% ash. It has a pH of 5, density of 1300 kg m^{-3} and high dynamic viscosity of 1500 mPa s. Its nitrogen content is of 3.8 g per L and its element composition is characterized by sodium (43%), potassium (21%) and magnesium (13%) that represent 77% of total residue (Table 7).

Table 5 Element compositions of reducing substrate. a. elemental composition by XRF analysis on the residue after glowing at 1025°C. b. trace elements in fresh undiluted reducing substrate (calculated from XRF analysis)

a.

Element	%
Na	43
K	21
Mg	13
S	6.9
Ca	5.5
P	4.8
Si	4.4
rest	1.3

b.

Element	mg kg^{-1}
Fe	120
Al	94
Cu	68
Zr	39
Mo	22
Mn	21
Th	16
Pb	14
Ba	10
Hf	8
Ni	2

Contamination plume flows with aquifer natural movement towards the irrigation channel, -10 m underneath field surface.

The second RPBB is located 25 m before the irrigation channel (Figure 11) and its purpose is the stimulation of aerobic degradation of VC produced during OHR carried out in the anaerobic path of the aquifer, and of aliphatic hydrocarbons and BTEX. It is about 500 m long and 10 m deep, with 53 recirculation wells and 43 injection wells. At the aerobic biobarrier level, urea, ammonium phosphate and O_2 are injected in the aquifer (Figure 12). For urea and phosphate supply a mixture of P and N with a ratio of 1:3 is used with a dosage of 3400 kg $year^{-1}$, O_2 was supplied through an injection of 1.0-1.8 m^3/h of air.

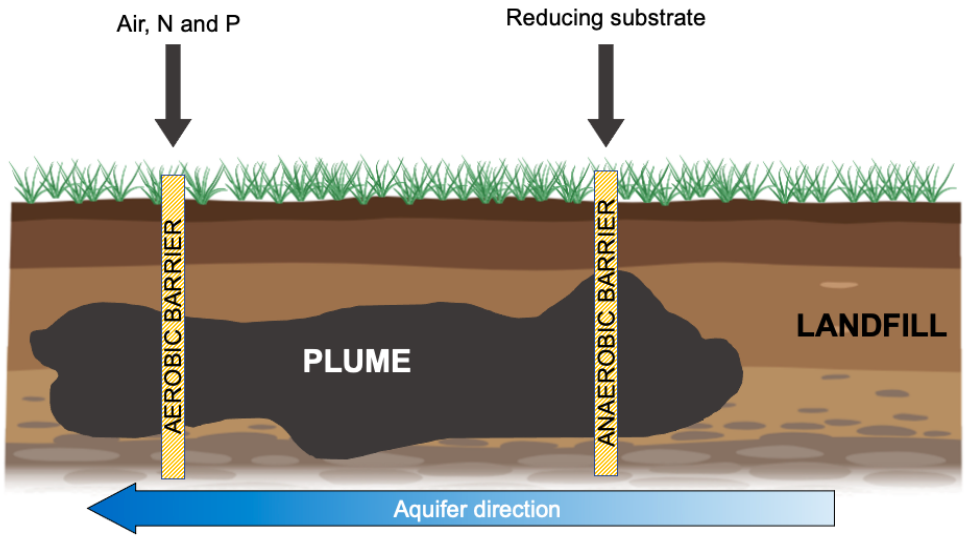

Figure 12 Biostimulation plan to improve degradation of contaminants by anaerobic and aerobic microbial community present in the aquifer

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AIMS

The aim of this PhD project was to analyze the microbial community and its remediation activity in a contaminated aquifer by landfill leaching under bioremediation through anaerobic and aerobic permeable reactive barriers. In particular, the actions undergone were:

- Characterization of natural microbial community present at the landfill and determination of its natural organo-halide respiration activity (Chapter 1).
- Evaluation of a biostimulation treatment on microbial community and on its reductive dehalogenation activity (Chapter 1)
- Determination of vinyl chloride biodegradation at aerobic permeable barrier in the aquifer, and effects of nutrients/air injection on degradation activity (Chapter 2)
- Ascertainment of presence of the native bacteria able to degrade aromatic compounds (toluene, benzene and chlorobenzene) in the aquifer. Monitoring of air/nutrients treatment impacts on degradation of these compounds, through quantification of functional biomarkers and isolation of bacteria that were able degrade those contaminants (Chapter 3).

RESULTS

CHAPTER 1

In situ CHARACTERIZATION OF THE MICROBIAL POPULATIONS INVOLVED IN ORGANOHALIDE RESPIRATION IN A CONTAMINATED AQUIFER FOR BIOREMEDIATION TREATMENT

Authors: Martina Bertolini[1], Sarah Zecchin[1], Giovanni P. Beretta[2], Laura Ferrari[3] and Lucia Cavalca[1]

[1] Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente (DeFENS), Università degli Studi di Milano Via Celoria 2, I-20133 Milano, Italy
[2] Dipartimento di Scienze della Terra Ardito Desio, Università degli Studi di Milano Via Mangiagalli 34, I-20133 Milano, Italy
[3]TAUW S.r.l. Italia

ABSTRACT

Chlorinated ethenes are excellent solvents in industrial activities due to their intrinsic characteristic. They are one of the main contaminants around the world because of inappropriate disposal in the past. Chlorinated ethenes are toxic for humans, for this reason they are strictly normed and their concentrations in the environment are monitored from all environmental protection agencies. For years, these compounds were considered recalcitrant to microbial remediation. Discovery of natural production of these compounds by algae and volcanos suggested a possible their bioremediation. Different bacteria were identified that are able to dechlorinate chloroethenes in anaerobic conditions (e.g., *Dehalococcoides*). In this study, aquifer contaminated by landfill leaching was analyzed to determine bioremediation potential of microbial community present in the groundwater. Further monitoring of microbial community, through molecular analyses, and monitoring of remediation rate, through chemical analyses, permitted to define

efficacy of implemented treatment (addition of reducing substrate in the aquifer).

INTRODUCTION

Tetrachloroethene (PCE), trichloroethene (TCE) and chloroethenes (CE) are widely used in industrial activities as solvents for waxes, resins, fats, rubbers, oils and in metal degreasing. They are also present in different house products as paint and dry-cleaning products. Because of their intensive use, they are among the prevalent contaminant compounds all over the world. Their presence in the environment is mainly due to their inadequate disposal methods (Moran et al., 2007; Beamer et al., 2012). PCE is toxic for humans, but degradation products, TCE, 1,1- and 1,2(*cis-trans*)-dichloroethene (DCE) and vinyl chloride (VC), are evenly more toxic (Linge et al., 1992; USA EPA, 2007). In particular, VC and TCE are carcinogen and they have been included in group 1 by International Agency for Research on Cancer (IARC).

PCE and TCE form a dense non-aqueous phase liquid (D-NAPL) with higher density with respect to water. Penetrating through permeable groundwater aquifers, D-NAPL gives rise to plume.

In order to remediate site affected to CE, bioremediation, is an efficient technique.

CE can be degraded under both anaerobic (organohalide respiration) and aerobic (metabolic and co-metabolic oxidation) conditions. High chlorinated ethenes are easier dechlorinated in anaerobic conditions with a consequent accumulation of DCE and VC. Conversely, these two compounds are oxidized more efficiently under aerobic conditions (Mattes et al., 2010). For this reason, in contaminated aquifers sequential anaerobic-aerobic biodegradation interventions can improve the efficiency of bioremediation as shown in previous studies (Tiehm et al., 2011; Yoshikawa et a., 2017; Weatherill et al., 2018).

During organohalide respiration (OHR), CE are used as electron acceptors and hydrogen as electron donors. Lactic and butyric acid can be used as

electron donors as well (Parsons, 2004), which derive from the fermentation of organic compounds (e.g., molasses, acetate, butyrate, propionate and lactate) (Liang et al., 2013). During bioremediation treatments, these organic compounds are added to the aquifer to improve OHR. During OHR, each chlorine atom is replaced by one hydrogen atom (Yang et al., 1998).

In the anaerobic environment of aquifers, organohalide-respiring bacteria (OHRB) compete with methanogens, sulfate reducing bacteria and acetogens for the use of hydrogen (Fennell et al., 1997; Yang and McCarty, 2002; Matteucci et al., 2015). However, OHRB are favored with a low concentration of hydrogen.

Only few bacterial genera are known to perform anaerobic organohalide respiration: *Geobacter*, *Desulfomonile* and *Desulfuromonas* belonging to the class Deltaproteobacteria, *Desulfitobacterium* and *Dehalobacter* belonging to the Firmicutes, *Sulfurospirillum* belonging to the Epsilonproteobacteria, *Dehalococcoides* (*Dhc*), an obligate organohalide-reducers, and *Dehalogenimonas* belonging to the Chloroflexi (Maymo-Gallett et al., 1997; Cichocka et al., 2010; Atashgahi and Smidt, 2016). Normally, for the complete biodegradation of CE, different bacterial consortia are involved in different steps of the degradation pathways (Di Stefano et al., 1991).

Genes involved in OHR are reductive dehalogenase homologous genes (*rdh*). Among these, tetrachloroethene reductive dehalogenase (*pceA)* is involved in reduction of PCE to TCE (Maillard et al., 2003), trichloroethene reductive dehalogenase (*tceA)* catalyzes TCE reduction to DCE or DCE to VC (Pöritz et al., 2013), vinyl chloride reductase (*bvcA*) reduces DCE to VC and VC to ethene (Krajmalnik-Brown et al., 2004), and vinyl chloride reductase (*vcrA*) catalyzes degradation of VC to ethene (Müller et al., 2004).

Although OHR is very well studied at laboratory scale, in field analyses aimed at demonstrating the role of OHRB in reductive dehalogenation and the effect of bio-stimulation intervention at contaminated sites are lacking.

In this work, an Italian aquifer affected by CE contamination due to petroleum hydrocarbons and CE leaching was considered. In field and laboratory based-

studies were performed in order to determine the feasibility of a bioremediation intervention. For this reason, molecular and chemical analyses were performed, to evidence the presence of OHRB in the indigenous microbial community, to monitor their response and to determine their efficiency in lowering CE concentration at the site, over a two-year bio-stimulation treatment.

MATERIAL AND METHODS

Site description (Geochemical characterization)

The study area is located in Dogaletto (Venice, Italy), next to a former landfill used for the collection of industrial wastes deriving from the near petrochemical plant of Porto Marghera. The landfill surface covers about 16 hectares with a total waste mass of 1'700'000 tons. The area is 3 m below the sea level and is surrounded by the Venice lagoon to the South- and by an irrigation/ drainage groundwater channel to the North-side. In order to drain waters from the fields and to maintain an adequate water level inside the channel, a drainage pump is used. This affects groundwater and surface water flow.

Due to a leaching on the bottom of the landfill, the underlying groundwater has been contaminated mainly by chlorinated compounds, petroleum hydrocarbons and BTEX.

Since 1995, a pump and treat (P&T) method has been applied. A hydraulic barrier was constructed and groundwaters were pumped from the aquifer to the treatment plant. Here, chemical reducing agents are added and hydrocarbons and solvents are adsorbed onto active carbon filters. Exhausted filters are then disposed to a controlled landfill site.

Due to high operation costs and low efficiency of this physic-chemical treatment, the feasibility of using a biological treatment intervention based on microbial reductive dehalogenation process has been evaluated at laboratory

and at field scales. In February 2016, permeable reactive bio-barriers were set up in order to exploit microbial activity for the biodegradation of the contaminants *in situ* (Figure S1). Bacterial anaerobic activity was stimulated by the addition of a reducing substrate (engineered molasses) in the aquifer (Figure 1). The bio-barrier was about 390 m long and consisted of 39 wells 10 m deep where water was recirculated and added of the substrate.

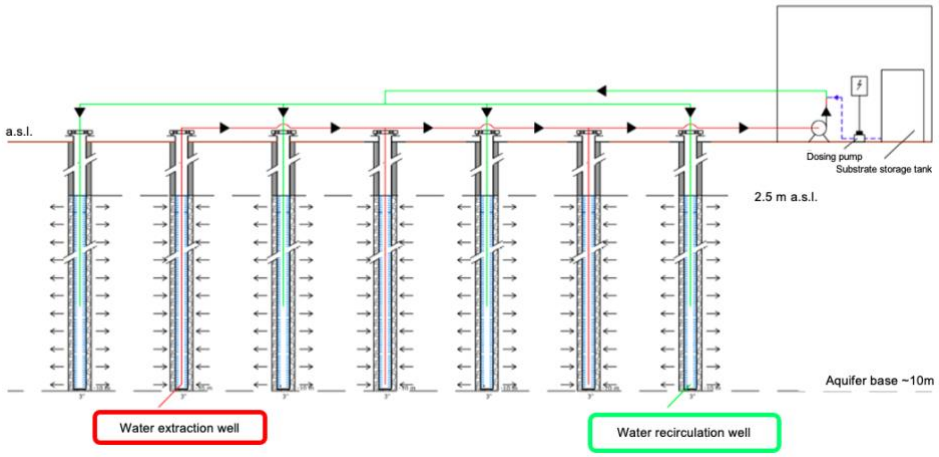


Figure 3 extraction and injection system located in anaerobic biobarrier for the addition of reductive substrate (TAUW Italia)

Analytical methods of chemical monitoring of the aquifer

Chlorinated ethenes were quantified every two months through gas chromatography – mass spectrometry system. Groundwater was sampled following equilibrium based static headspace preparation (EPA5021A 2014 method). Analysis were performed by AGROLAB Group. EPA8260D 2018 method for volatile organic compounds were selected for GC-MS analysis.

Groundwater sampling for microbiological analyses

Groundwater was sampled from two piezometers within the landfill (Pz22 and Pz25) and from four monitoring piezometers upstream (Pz13 and Pz16) and downstream (Pz10, and Pz3) the injection and recirculation plant (Figure 2).

For microcosm experiments, samples were withdrawn in February 2016 in 1L bottles fulfilled with waters to ensure anaerobic conditions. In February 2016, 60 L of groundwater were collected for RNA analyses.

For molecular analyses, groundwaters (20 L) were sampled every four months from May 2016 to January 2018, in fulfilled tanks to ensure anaerobic conditions.

Tanks and bottles were brought in cooler bag in the dark and stored in laboratory at 4°C until use for nucleic acid extraction and for the set-up of microcosm experiments.

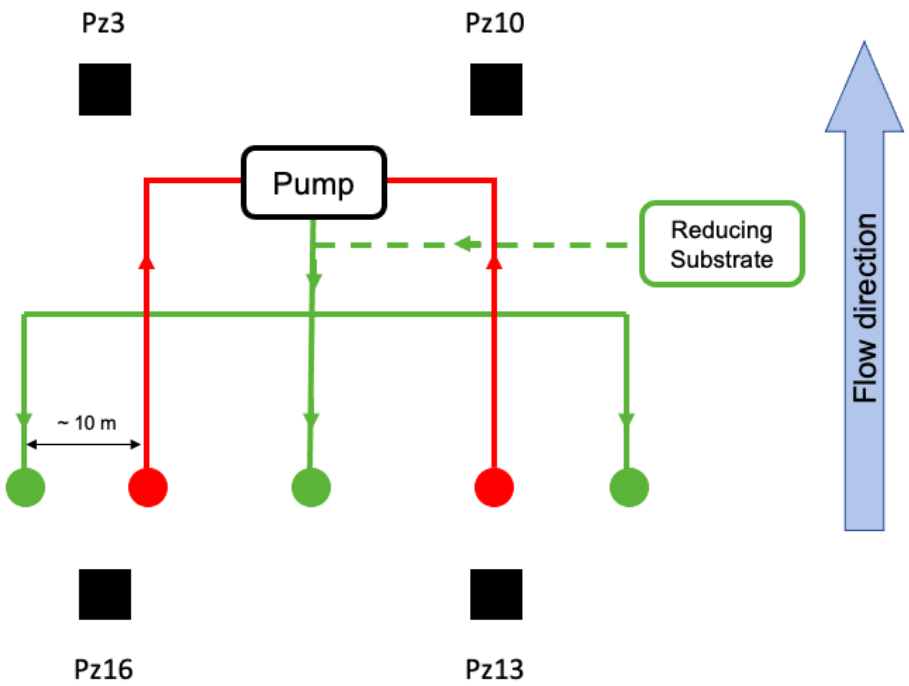


Figure 4 Monitoring piezometers position near anaerobic bio-barrier. Red lines are extraction wells, green lines are the injection wells

Microcosms experiments

In order to evaluate the ability of biodegradation of the native microbial populations towards chloroethenes mixture present at the site, microcosms experiments were set up with groundwater samples of the four piezometers withdrawn at the beginning of the campaign (May 2016 (T0)). Three conditions were considered: abiotic control (AB), groundwater (GW) and groundwater added of a reducing substrate (GWRS). For each condition, 100 mL serum bottles were added of 50 mL groundwater, 350 µL of 1:10 reducing substrate when appropriate and resazurin 0.1% w/v (redox indicator). Reducing substrate was liquid vegetable product based on molasses. Its nitrogen content was of 3.8 g L^{-1} with presence of different element, in particular, the main elements are sodium, potassium and magnesium that represent 77% of total residue. Reducing substrate pH was 5, density was 1300 kg m^{-3} and high dynamic viscosity of 1500 mPa s. Abiotic controls consisted of groundwater was autoclaved for 3 times, to be used in abiotic controls. Serum bottles were sealed with gray butyl rubber septa with crimped caps in anaerobic conditions in anaerobic cabinet (Forma Scientific, USA) under an atmosphere of N. Microcosms were incubated at 20°C in static conditions All the conditions were set up in triplicate.

Chloroethenes were quantified through analysis of the headspace in triplicate after 6 and 12 months of incubation. For the headspace sampling were used static headspace sampling (HS) method designed for the analysis of VOCs by GC (APATIRSA-CNR,2003). In HS, the sample was heated in a sealed vial in order that VOCs reach the equilibrium with the gas phase above the liquid. Then, gas phase was collected and introduced into the column of the gas-chromatograph for final determination by Gas Chromatography–Mass Spectrometry (GC-MS), using a 5975B Gas Chromatograph-Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with D3792 PoraBOND Q column (25m x 0.32 mm, 5.00 µm) (Agilent Technologies, Santa Clara, CA, USA). Helium at 250°C was used as carrier gas, injected manually with a split ratio of 3:1 and a split flow of 7.5 mL. Oven

temperature conditions were 40°C for 2 minutes and then 10°C/min to 260° for 7 minutes.

Ethenes relative concentration (%) was calculated referring the chromatographic area of the specific compound to the total chromatographic area obtained for all the compounds detected in sample.

Nucleic acid extraction methods

Groundwater biomass was filtered onto mixed cellulose/ester filters (MediaKap™ ME2M-050-18S ∅ 0.2 μm) (Cole-Parmer, USA) by using a peristaltic pump apparatus (Masterflex L/S Economy Variable-Speed Drive, 20 to 600 rpm with Masterflex L/S Easy-Load Head for High-Performance Tubing, PSF/CRS) (Cole-Parmer, USA). Filters were stored at -20°C until processed. RNA was extracted using RNA Power Soil ® Total RNA Isolation kit (Qiagen, Germany) in triplicate. Residual genomic DNA was removed using 1 μL of DNaseI for 1 μg of RNA (Thermo Fisher Scientific) according following protocol: 37°C for 30 min, addition of 4 μL of EDTA and incubation for 10 min at 65°C. RNA was reverse transcribed with iScript ™ cDNA Synthesis Kit (BIO-RAD) according given protocol. Gel agarose electrophoresis and 16S rRNA genes PCR amplification (protocol in SM) was used to determine purity of the RNA. Total DNA was isolated from the pellet using the DNA PowerSoil® Isolation kit (Qiagen, Germany) in triplicate for each sample. Nucleic acids were quantified by using Spectrophotometer Power Wase XS2 (BioTEK Instruments, US).

Real time quantitative PCR

Gene copy number of 16S rRNA genes of total Bacteria and Archaea, *Geobacteraceae* 16S rRNA (Geo), *Dehalococcoides* 16S rRNA genes (*Dhc*), trichloroethylene reductase gene (*tceA*) and vinyl chloride reductase gene (*vcrA*) were quantified through Real Time quantitative (qPCR). Primer sets for each target gene are described in Table S1. The thermal protocol for 16S rRNA total bacteria gene was applied according to Fierer et al., 2005. The

thermal protocol for 16S rRNA Archaea gene was: initial denaturation for 15 min at 95°C, 40 cycles of 1 min at 95°C, 30 sec at 60°C, and 1 min at 72°C. Melting curve was set from 60°C to 95°C with an increment of 1.6°C/sec for 5 sec. The thermal protocol for *Dhc* gene was: initial denaturation for 15 min at 95°C. 40 cycles of 1 min at 95°C, 40 sec at 58°C, and 40 sec at 72°C. Melting curve was set from 58°C to 95°C with an increment of 1.6°C/sec for 1 min. The thermal protocol for *tceA* and *vcrA* genes was: initial denaturation for 15 min at 95°C. 40 cycles of 1 min at 95°C, 40 sec at 58°C, and 60 sec at 72°C. Melting curve was set from 58°C to 95°C with an increment of 1.6°C/sec for 1 min. Each reaction mixture contained 1x of Titan HotTaq Probe qPCR Mix (Bioatlas science of life, Estonia), 0.25 µM of forward and reverse primers and 10 ng of DNA and PCR-grade water (AppliChem, Germany) to a final volume of 20 µL. Standard curves were set up through amplification of plasmids carrying insert of each target gene (SM).

Illumina MiSeq Sequencing of 16S rRNA genes

Groundwater microbial community was characterized at the beginning (February 2016) and at the end (January 2018) of the bioremediation intervention by Illumina sequencing of Bacteria and Archaea 16S rRNA genes. Sequencing was performed on DNA isolated from sample PZ16, by DNA Services (DNAS) facility, Research Resources Center (RRC), University of Illinois at Chicago (UIC). Primer pairs CS1_341F/CS2_806R and CS1_ARC344F/CS2_ARC806R were used for Bacteria and Archaea 16S rRNA genes, respectively. The primers contained 5′ common sequence tags (known as common sequence 1 and 2, CS1 and CS2) as described previously (Moonsamy et al., 2013).

Sequence analyses were performed with QIIME2 (Caporaso et al., 2010). Merge of forward and reverse reads were carried out using PEAR (Zhang et al., 2014). In order to have high quality data, trim steps were used with a quality threshold of p = 0.01. Primer sequences and ends with ambiguous nucleotides were trimmed. Reads with internal ambiguous nucleotides were

discarded. Less than 300 bp reads were discarded. To remove chimeric sequences USEARCH algorithm were used. Amplicon Sequence Variants (ASVs) were created in accordance with a similarity higher than 97% using UCLUST method. ASVs were aligned to the GreenGenes database (http://greengenes.lbl.gov).

Statistical analyses

Statistical analyses were performed using base and accessory packages of the program R (R Core Team, 2015). Microcosms chemical data were analyzed with analysis of variance (aov) (Chambers et al., 1992) to determine significant differences ($p \leq 0.05$) among different thesis and incubation times of chloroethenes degradation.

Data from qPCR quantification were analyzed through aov (Chambers et al., 1992) to determine significant differences in markers genes among different piezometers and over time ($p \leq 0.05$).

Analysis of Illumina data was carried out through quantile-adjusted conditional maximum likelihood (qCML) with EdgeR package (Robinson et al., 2010; McCarthy et al., 2012). Common dispersion and tagwise of sequences were determined (Chen et al., 2014). Pairwise comparisons were performed to highlight species significantly different in the two time points ($p \leq 0.05$) (Robinson and Smyth, 2008).

RESULTS

Chemical groundwater characterization

In February 2016, all chlorinated ethenes were present in high amount in the landfill with concentrations of 4-5 orders of magnitude higher than Italian law limits (Table1). VC concentrations was 70'800.00 µg L^{-1} instead of law limits of 0.5 µg L^{-1}. Dissolved oxygen concentration was 1.46 mg L^{-1}. Redox potential was -181.50 mV and pH was neutral (6.66). The higher concentrations of

organohalides were located in the center of the plume. Distal zone of the plume showed a lower concentration of these compounds (Figure 3).

Table 6 Chemical parameters of landfill in February 2016

		Law limits D. Lgs. 152/06	Landfill
PCE	µg L^{-1}	1.1	34’100.00
TCE	µg L^{-1}	1.5	49’050.00
1,1 DCE	µg L^{-1}	0.05	4’265.00
1,2 DCE	µg L^{-1}	60	27’650.00
VC	µg L^{-1}	0.5	70’800.00
ethene	µg L^{-1}	-	17’705.00
Eh*	mV	-	-181.50
pH		-	6.66
DO*	mg L^{-1}	-	1.46

*DO= dissolved oxygen, Eh=Redox potential

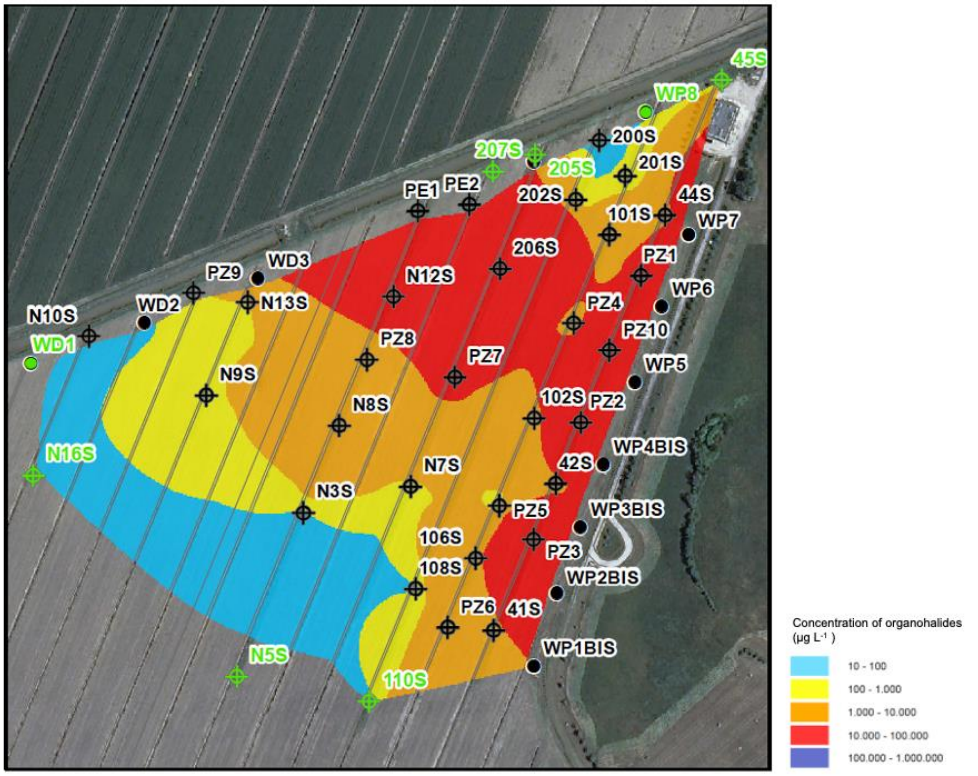


Figure 5 Concentrations of organohalides present in February 2016 in the plume due to leak of landfill

Characterization of active microbial community

Euryarchaeota was the main part of active archaeal community (66%) (Figure 4). 60.14% of them belonging to *Methanomicrobia* class and 3.35% to *Methanobacteria*. *Crenarchaeota* relative abundance was of 24.1%. *Parvarchaeota* was present only for 5.77% of the total active archaeal community.

Active bacterial community was characterized from a high present of *Firmicutes* and *Proteobacteria* (39.85% and 49.14% respectively). Indeed, all genus with a relative abundance above 1% belong to these two phyla. In *Firmicutes* phylum, *Fusibacter* was present with a relative abundance of 31.66% of the total bacterial community. *Coprococcus* and *Sedimentibacter* relative abundance was 1.15 and 4.81%, respectively. In addition, in

Proteobacteria phylum, *Xanthomonadaceae* and *Caulobacteraceae* relative abundance was of 8.23 and 7.96%, respectively. *Mycoplana* and *Thermomonas* were both present at about 2%. Indeed, *Alcaligenaceae* and *Phyllobacteriaceae* showed a relative abundance of only about 1.5%. There were also *Actinobacteria*, *Bacteroidetes* and *Chloroflexi* but they were present in lower relative abundance, 3.91%, 2.48% and 2.69% respectively. Active OHRB in the landfill showed low relative abundance, below to 1% except for *Shewanella* that was present with a relative abundance of 11.39% (Figure 5).

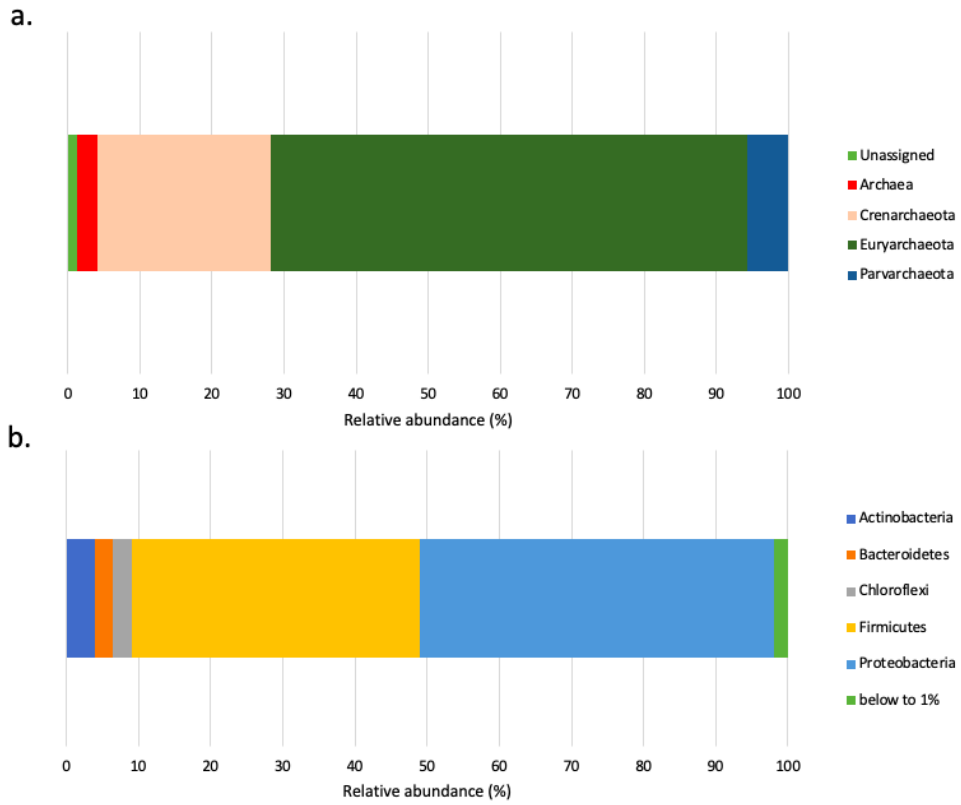


Figure 4 Active microbial population relative abundance of landfill. Illumina sequencing of 16S rRNA of Archaea (a) and Bacteria (b)

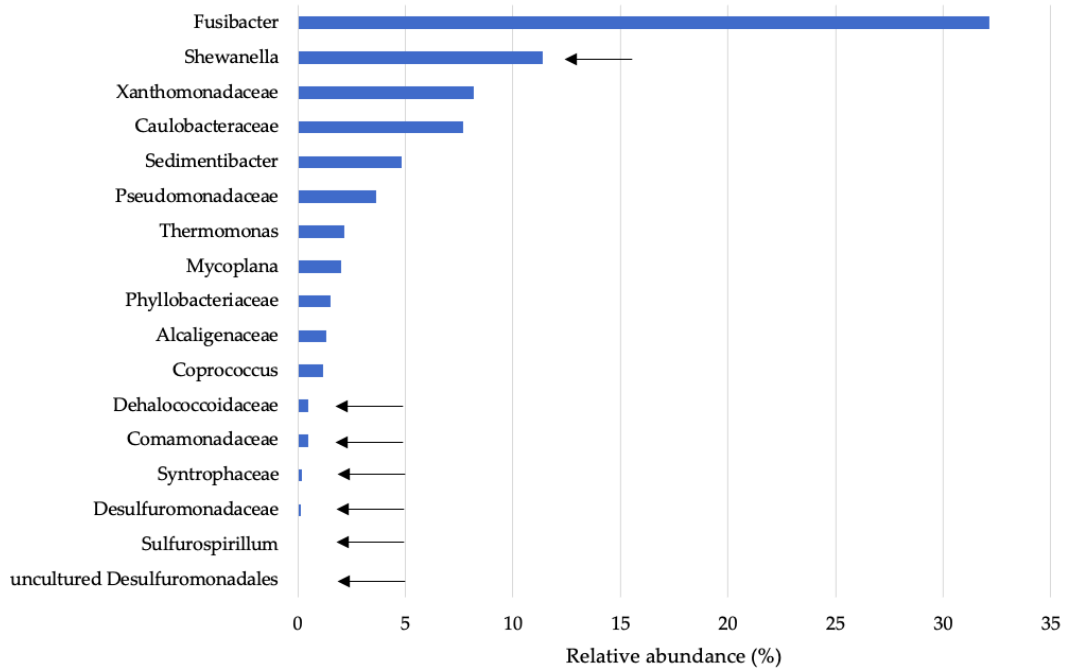


Figure 5 Active bacteria present in landfill with a relative abundance above 1% at genus level and OHRB. Arrows indicate OHRB genera or family

Functional genetic characterization of microbial community in the landfill

OHR biomarkers were quantified at gene (DNA) and transcript (RNA) level. In February 2016, biomarkers for OHR were in high copies in the aquifer, between 10^6 and 10^7. *Dehalococcoides* (Dhc) gene and transcript copies were present in 10^5 and 10^6, respectively, with a difference of only 1 order of magnitude (Figure 6). Instead, functional genes *tceA* and *vcrA* gene and transcript copies showed a higher gap between them. Indeed, *tceA* DNA copies were higher of 3 orders of magnitude respect RNA copies (DNA was 10^7 and RNA was 10^4), and in *vcrA* target the difference was of 4 orders of magnitude (10^6 and 10^2, gene and transcript, respectively).

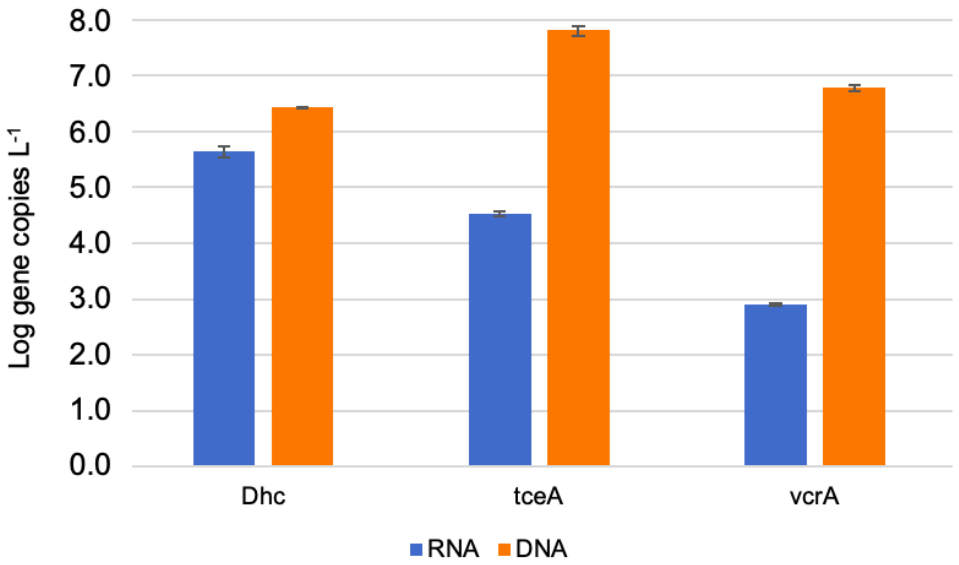


Figure 6 DNA and RNA gene copies abundance of Dehalococcoides, tceA and vcrA in the landfill in February 2016

Determination of natural OHR in the aquifer

Anaerobic microcosms were set up with groundwaters from different piezometers in order to highlight the ability of indigenous microbial communities to perform reductive dehalogenation of chlorinated ethenes and gaining information on natural attenuation processes at the site. Moreover, the effect of the addition of a reducing substrate was considered, with the aim to determine the possibility of accelerating the bioremediation process in the aquifer.

GC-MS analyses at time zero of incubation showed that significantly higher concentrations of highly chlorinated ethenes (PCE and TCE) were present in Pz16 and Pz3 microcosms, than in Pz13 and Pz10, which conversely displayed higher concentrations of low chlorinated ethenes (DCE and VC) (Figure 7, $p<0.05$ in figure S2 in supplementary material). In accordance with initial field monitoring, the distribution of different chlorinated solvents at the site is not homogeneous, evidencing that groundwaters from Pz16 and Pz3

derive from the proximity of the contamination source, whereas those in Pz13 and Pz10 are at the plume fringe.

After 6 month-incubation, PCE disappeared in all microcosms, going from 22% to zero also in the highest contaminated sample Pz16. During the time course of incubation, TCE slightly decreased in Pz16 where DCE was concomitantly formed. TCE accumulated in Pz13 without further dechlorination to DCE. In Pz3 and Pz10, where negligible TCE concentrations were present, a transient accumulation of DCE (18%) was observed in Pz3 after 6 month-incubation, then decreasing to 3% after 12 month-incubation. In Pz10, DCE reduction proceeded to vinyl chloride, which was accumulated to 23% at the end of the incubation time. Vinyl chloride accumulated also in Pz3 (12%) and in Pz13 (5%), whereas in Pz16 it disappeared with concomitant formation of ethene. These data point out that the ability of the aquifer microbial communities to conduct reductive dehalogenation reactions was present and active at the different sampling points, although not homogeneously distributed at the site. In most of the samples, dehalogenation was not complete, with accumulation of vinyl chloride.

The addition of the reducing substrate had different effects on the reductive dehalogenation process in the different microcosms. In Pz16, the addition of the substrate resulted in an increase of TCE, DCE and VC degradation after 6 months incubation, although after 12 months an accumulation of 5% vinyl chloride was observed in the microcosm added of the substrate. In Pz13, the addition of the substrate promoted the degradation of TCE (12.45 and 8.8 %, in the absence and the presence of substrate, respectively) and to a lesser extent that of DCE (19 to 17.9% in the absence and the presence of substrate, respectively). Vinyl chloride degradation in Pz13 microcosm was not affected by substrate addition. In Pz10 and Pz3 microcosms, the addition of the substrate increased the degradation of TCE, DCE and vinyl chloride already after 6 months incubation. After 12 months, vinyl chloride was degraded to a significantly higher extent in Pz10 and Pz3 augmented microcosms (5%) than

in the not amended ones (23 and 12.4%, respectively) (Figure 7, $p<0.05$ in figure S3 in supplementary material).

In sterilized microcosms, a statistically negligible decrease of chloroethenes was observed (data not shown).

Overall, it is possible to envisage that the addition of the reducing substrate could represent a strategy to accelerate and improve reductive dehalogenation reactions, although the production of vinyl chloride should be carefully monitored at the site.

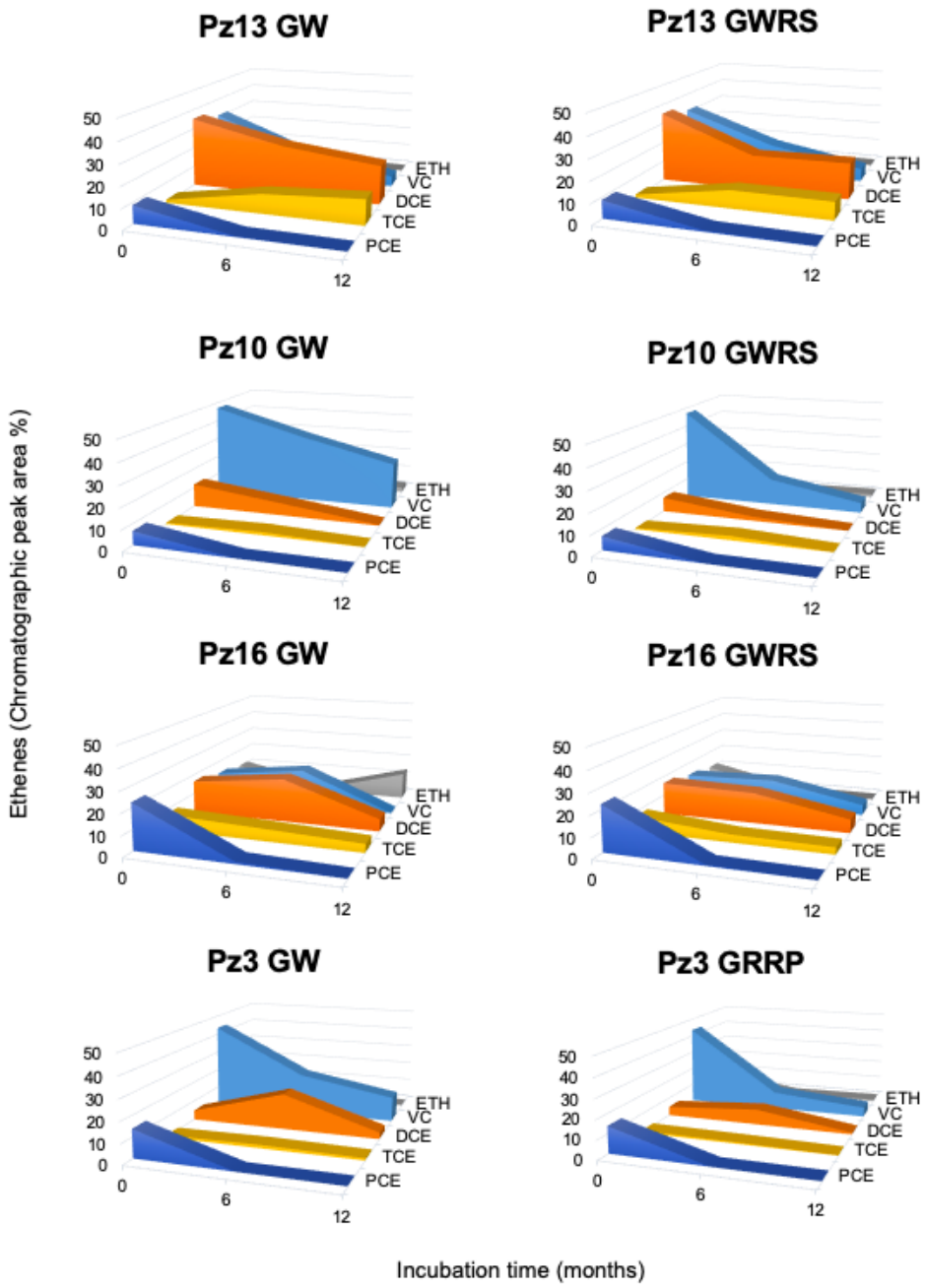


Figure 7 Chloroethenes relative abundance in four piezometers (Pz13, Pz10, Pz16 and Pz3) during one year (T12) of incubation in different conditions: without reducing substrate (GW) and with reducing substrate (GWRS). PCE = blue, TCE = yellow, DCE = orange, VC = light blue, ethene = grey.

Chemical field monitoring

In light of the characterization of the indigenous microbial community and of the results obtained with the laboratory experiments, in May 2016 a permeable reactive bio-barrier was built, aimed at the injection of a reducing substrate in the aquifer to promote OHR at the site.

According to the chemical analysis, the concentration of high CE decreased downstream injection wells in all transects in all time points (Table S2, Figure 7). Generally, upstream injection wells PCE degradation was higher up to two orders of magnitude with respect to downstream injection wells (Figure 1). Downstream injection barrier, TCE concentration was lower of up to 3 orders of magnitude. In addition, 1,1-DCE and 1,2-DCE also decreased downstream injection wells from one to two orders of magnitude (from 10^4 to 10^3/10^2). On the other hand, VC concentration decreased downstream injection point only in the transect Pz13-Pz10 (Figure 8. a-b). In transect Pz16-Pz3 (Figure 8. c-d), VC concentration remained unaltered. Ethene concentration, in the order of 10^4-10^3 µg L^{-1}, did not decrease downstream injection wells in all transects for the whole monitored period. The degradation rate of total CE from May 2016 to September 2018 was of 23.74 kg day^{-1}.

CEs and ethene concentrations in Pz22 were in the same order of magnitude respect to Pz13, upstream the injection wells. On the other hand, the concentration of low CE (1,2 DCE, VC e ethene) was different in Pz25 and Pz16. 1,2 DCE and VC concentration was lower in landfill of one order of magnitude. Moreover, ethene concentration was higher of two order of magnitude in Pz16 than in the landfill piezometer (Table S1).

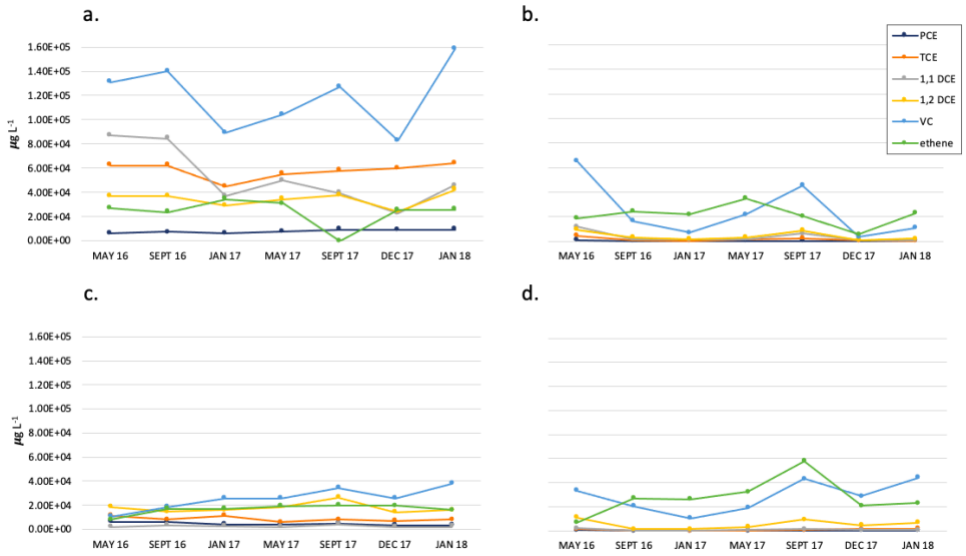


Figure 8 Chloroethenes concentrations during 20 months in the four piezometers: Pz13 (a), Pz10 (b), Pz16 (c) and Pz3 (d).

In all site, pH remained stable with value around 6.5 to 7.5. Oxygen concentration was always lower than 1 mg L^{-1} except in Pz13 and Pz16 in May 2016.

Redox potential (Eh) of the site was higher in the landfill with a difference between the two analyzed piezometers. Pz22 showed a lower Eh, that it means a more reducing condition. In anaerobic bio-barrier, Pz13 had a Eh similar to landfill piezometers. Indeed, Pz10 and Pz3 Eh was very low. In particular in the second piezometers Eh reached value of -300 mV (Table S1).

Field monitoring of microbial populations involved in OHR

In all piezometers, total bacterial and archaeal 16S rRNA genes ranged from 10^8 to 10^{11} and 10^2 to 10^7 gene copies per L, respectively (Figure S4). Members of the family *Geobacteraceae* and *Dehalococcoides* accounted for 10^2 to 10^6 copies of 16S rRNA genes per L. Genes involved in the degradation of TCE and DCE (*tceA*) and of VC (*vcrA*) varied between 10^4 to 10^7 copies per L (figure 9-10). *tceA* was higher than *vcrA* copies per L.

In all piezometers, Archaea increased significantly in December 2017 and January 2018 of two order of magnitude in Pz13 and Pz10 (from 10^5 to 10^7 and from 10^4 to 10^6, respectively) and of one order of magnitude in Pz16 and Pz3 from 10^5 to 10^6. Phylogenetic and functional genes showed the same trend upstream and downstream injection wells during all sampling time. An initial increased and decreased period was followed by a stable balance. Then, all genetic markers increased in January 2018.

In both transect, *tceA* and *vcrA* showed an increase downstream injection wells of anaerobic biobarrier of one or two order of magnitude. On the other hand, members of the family *Geobacteraceae* were more abundant upstream injection wells. Transect Pz13-Pz10 showed a higher statistically relevant difference between upstream and downstream piezometers (Figure 9). Opposed, in Pz16-Pz3 transect, genetic markers increased was mostly downstream injection wells (Figure 10).

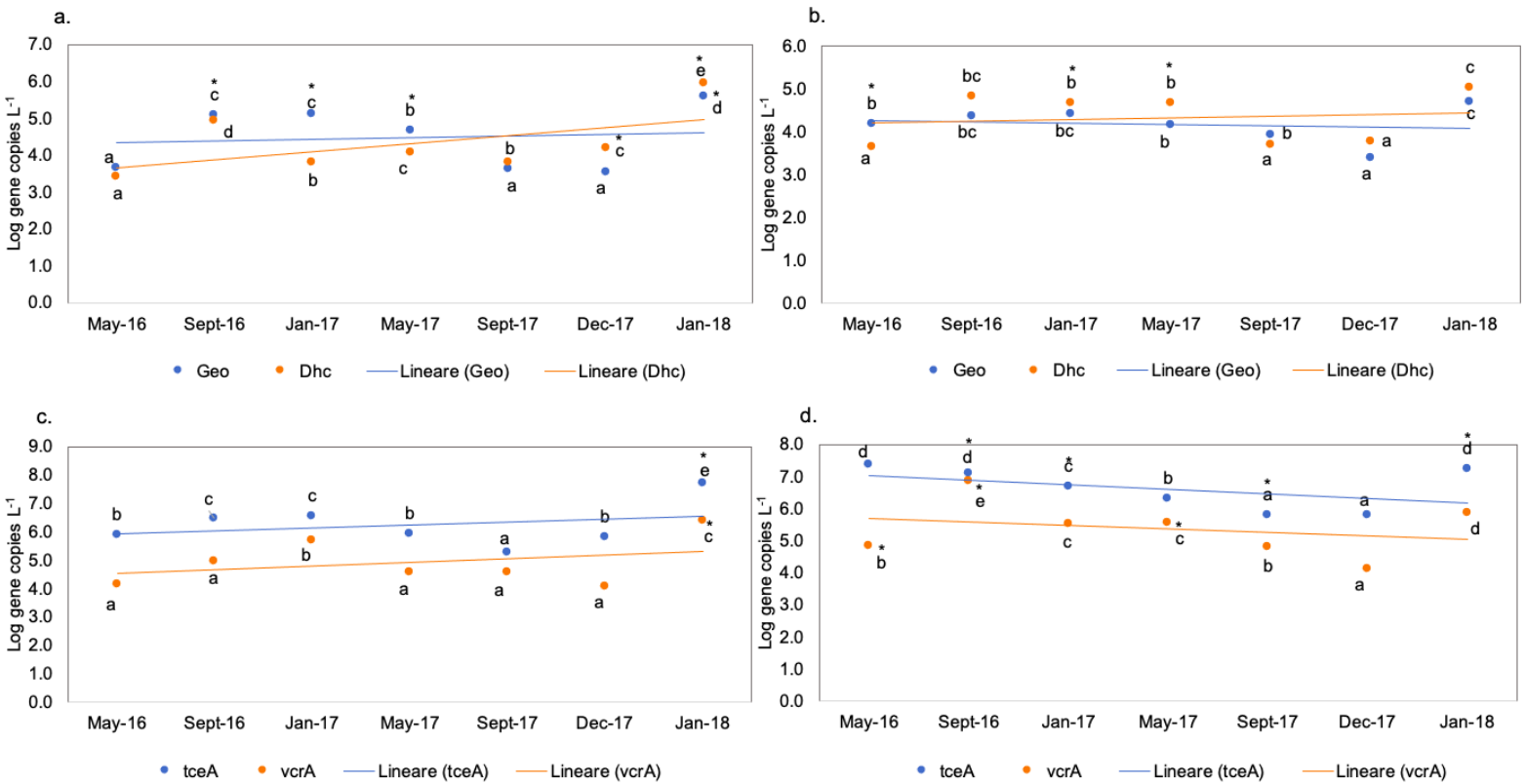


*Figure 9 Gene copy abundance of Geobacteraceae and Dehalococcoides in Pz13 (a) and Pz10 (b), and tceA and vcrA in Pz13 (c) and Pz10 (d) during time. * indicates significant difference between piezometers of transect upstream and downstream injection wells of anaerobic bio-barrier. Lowercase letters indicate significant or not significant difference between different time (Tukey's test, $\rho \leq 0.05$).*

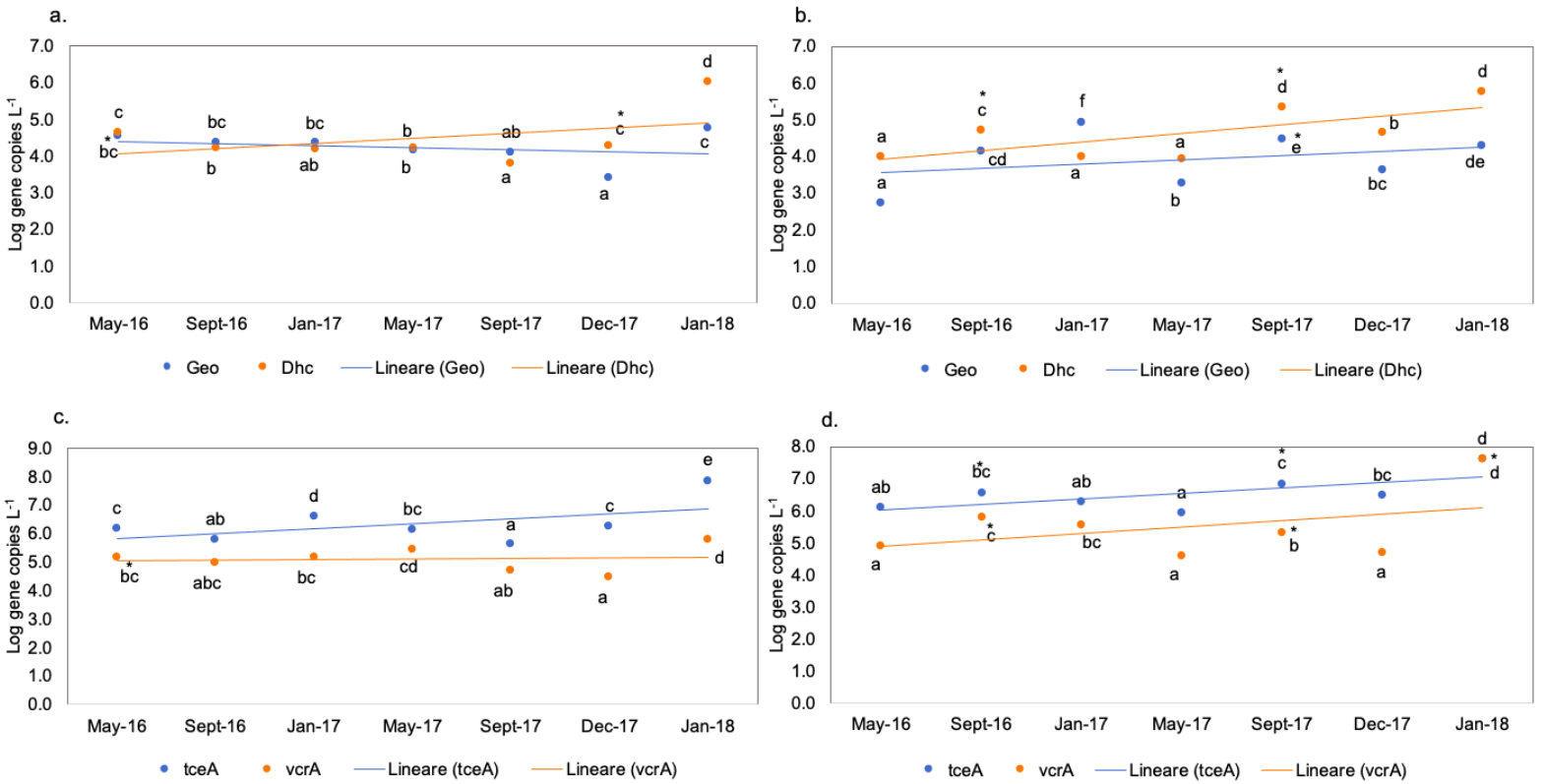
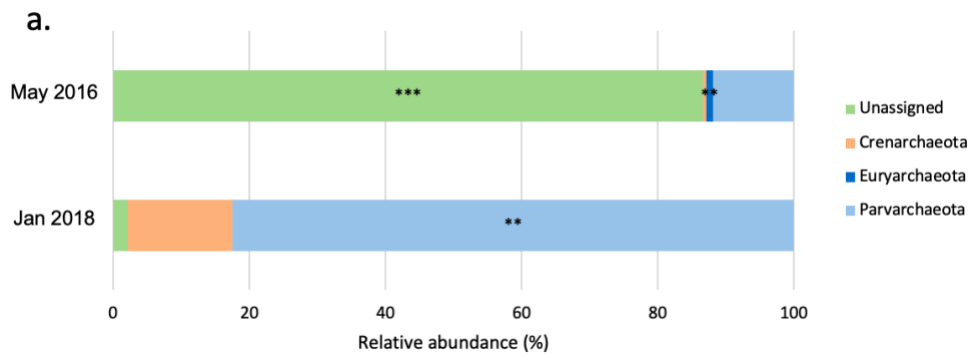


*Figure 10 Gene copy abundance of Geobacteraceae and Dehalococcoides in Pz16 (a) and Pz3 (b), and tceA and vcrA in Pz16 (c) and Pz3 (d) during time. * indicates significant difference between piezometers of transect upstream and downstream injection wells of anaerobic bio-barrier. Lowercase letters indicate significant or not significant difference between different time (Tukey's test, $\rho \leq 0.05$).*

Effect of substrate addition on microbial community of the aquifer

The effect of reducing substrate addition on the microbial community, was analyzed in Pz16 sample at the beginning and after 20 months of treatment (Figure 12.a). In May 2016, the Archaea kingdom mostly comprised Unassigned sequences (86.6%), that were reduced to 2% in January 2018.Statistical analyses of Illumina data (Figure 12.b) revealed that the treatment increased the relative abundance of archaeal phylum *Parvarchaeota* (from 11.9% to 82.5%). In particular, most of this variation was accountable to order YLA114 that passed from 0.03% to 79.74%. Also *Crenarchaeota* phylum increased at class level (*Miscellaneous Crenarchaeota Group* (MCG) from 0.43% to 7.22%), and at genus level (*Candidatus* Nitrososphaera 8.24% in January 2018). *Euryarchaeota* accounted for approximately 1% of the total archaeal community at the beginning of treatment, but they were not detected in January 2018.



b.

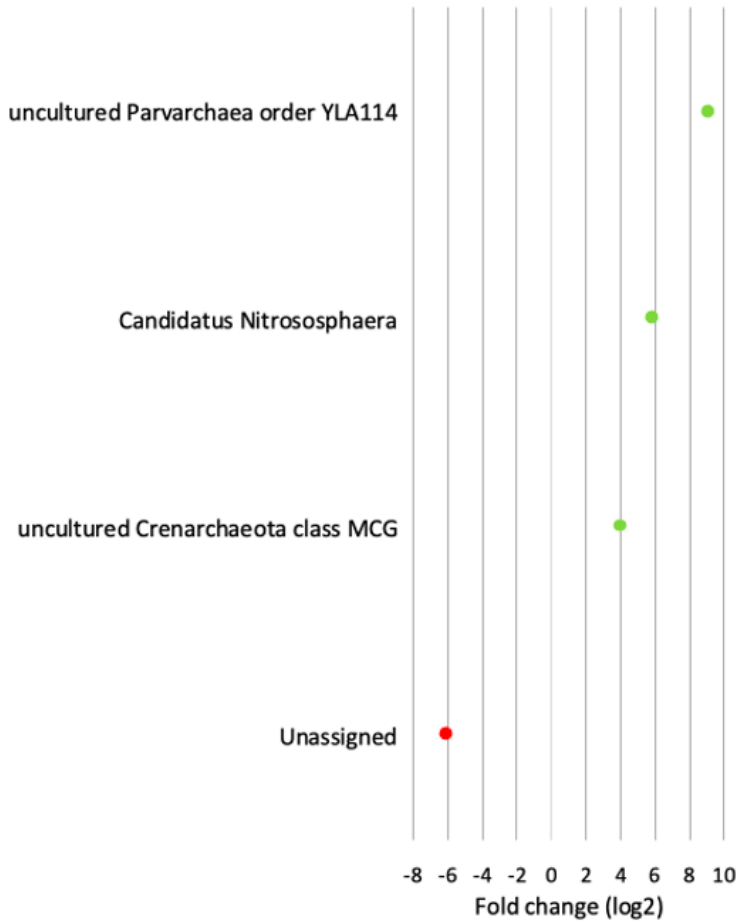


*Figure 12 (a) Archaeal relative abundance at phylum level in anaerobic bio-barrier after 20 months of treatment. * indicates significant variation of relative abundance during 20 months (b) Statistically relevant changes of archaeal relative abundance at genus level during 20 months of treatments.*

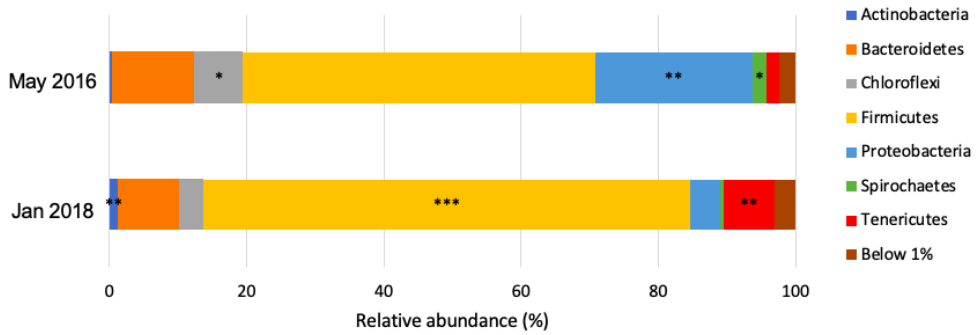
Treatment affected also bacterial community composition (Figure 13.a). *Actinobacteria* showed an increase passing from 0% to 1%. *Firmicutes* and *Tenericutes* relative abundance increased from 51.3% to 70.9% and from 1.8% to 7.5%, respectively. On the other hand, *Proteobacteria, Chloroflexi,*

and *Spirochaetes* relative abundance decreased relevantly of 16, 3 and 1.5 points, respectively.

Uncultured *Bacteroidales* relative abundance increased from 1.2% to 5.97% and particularly ML635J-40 family increased significantly. Mollicutes class and its RF39 order could be found only in January 2018 (2%). *Dehalobacterium* and *Desulfosporosinus* relative abundance increased from 1.97% to 6.77% and from 21.97% to 44.4%, respectively (Figure 13.b). Fermentative *Ruminococcaceae* bacteria slightly increased.

Proteiniclasticum, *Dehalogenimonas, Porphyromonadaceae* family and *Arcobacter* relative abundant decreased. In particular, *Dehalogenimonas* was no longer present in January 2018 and *Porphyromonadaceae* family and *Arcobacter* decreased to near 0%. At the beginning of the treatment, *Pseudomonas*, *Treponema*, C1_*B004* and *T78* genera of *Anaerolinaceae* family were present with a relative abundance of above 2% and slightly decreased after the treatment. Uncultered *Tenericutes* order *ML615j*-28, uncultured *Bacillales* and *Acidaminobacteraceae* relative abundance was about 1% then decreasing in January 2018. In particular, *Acidaminobacteraceae* was not longer detected after 22 months.

a.



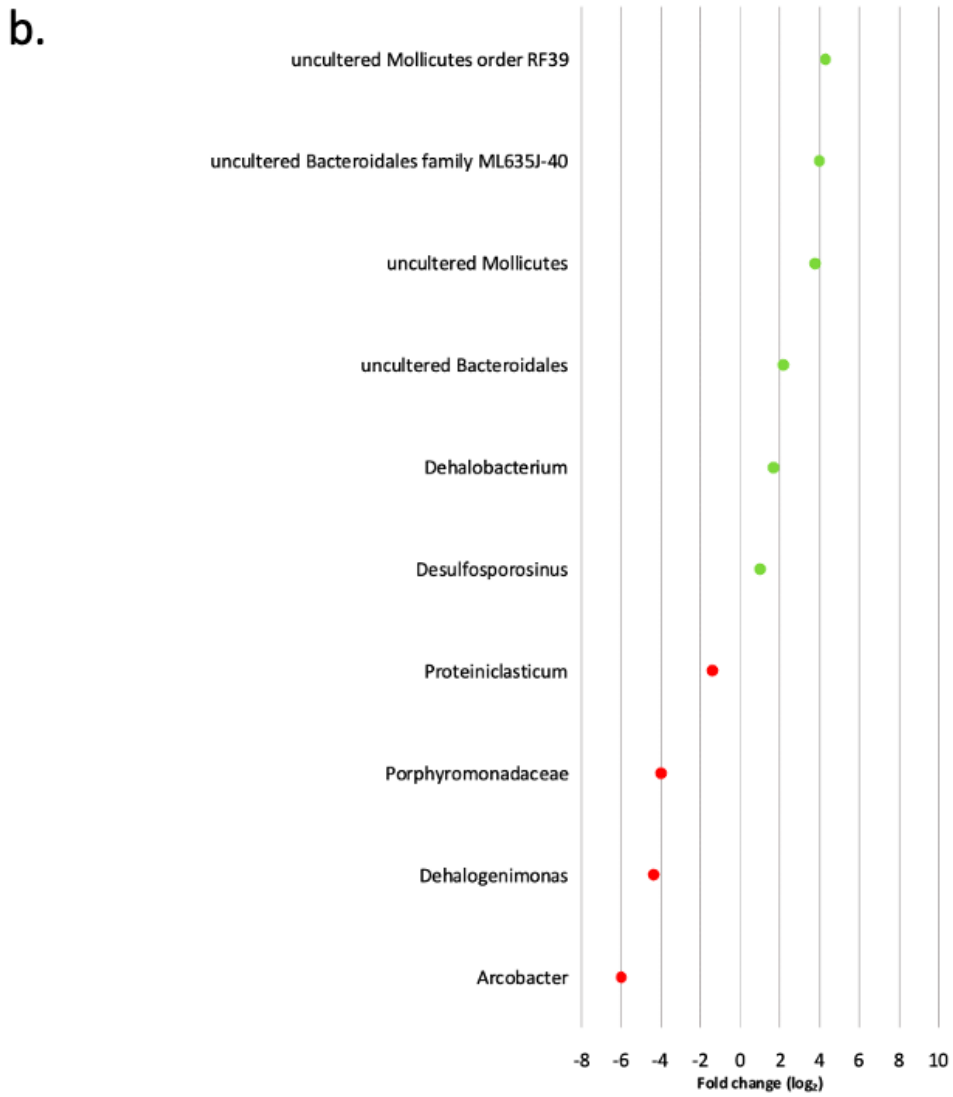


*Figure 13 (a) Bacterial relative abundance at phylum level in anaerobic bio-barrier after 20 months of treatment. * indicates significant variation of relative abundance during 20 months (b) Statistically relevant changes of bacterial relative abundance at genus level during 20 months of treatments.*

Some bacterial genera were present with a relative abundance above 1% in at least one of the two times, but they were not affected by treatment (figure 14). *Sedimentibacter* relative abundance did not relevantly change but it was present at 4.17 and 3.48% in the two times. Regarding, OHRB genera and

families present in aquifer, *Sulfurospirillum* relative abundance increased from 0.03 % to 0.27 %. *Geobacter* and *Shewanella* decreased of 1.1 and 3.5 points, respectively.

Among OHRB, rare families and genera were detected: *Comamonadaceae*, *Syntrophaceae, Enterobacteriaceae, Desulphuromonadaceae, Dehalococcoides, Dehalobacter, Clostridium, Desulfitobacterium* (Figure 14). They were mostly affected by the treatment, with the exception of *Clostridium* (2.4%), *Dehalobacter* and *Desulfitobacterium* that did not change after 20 months.

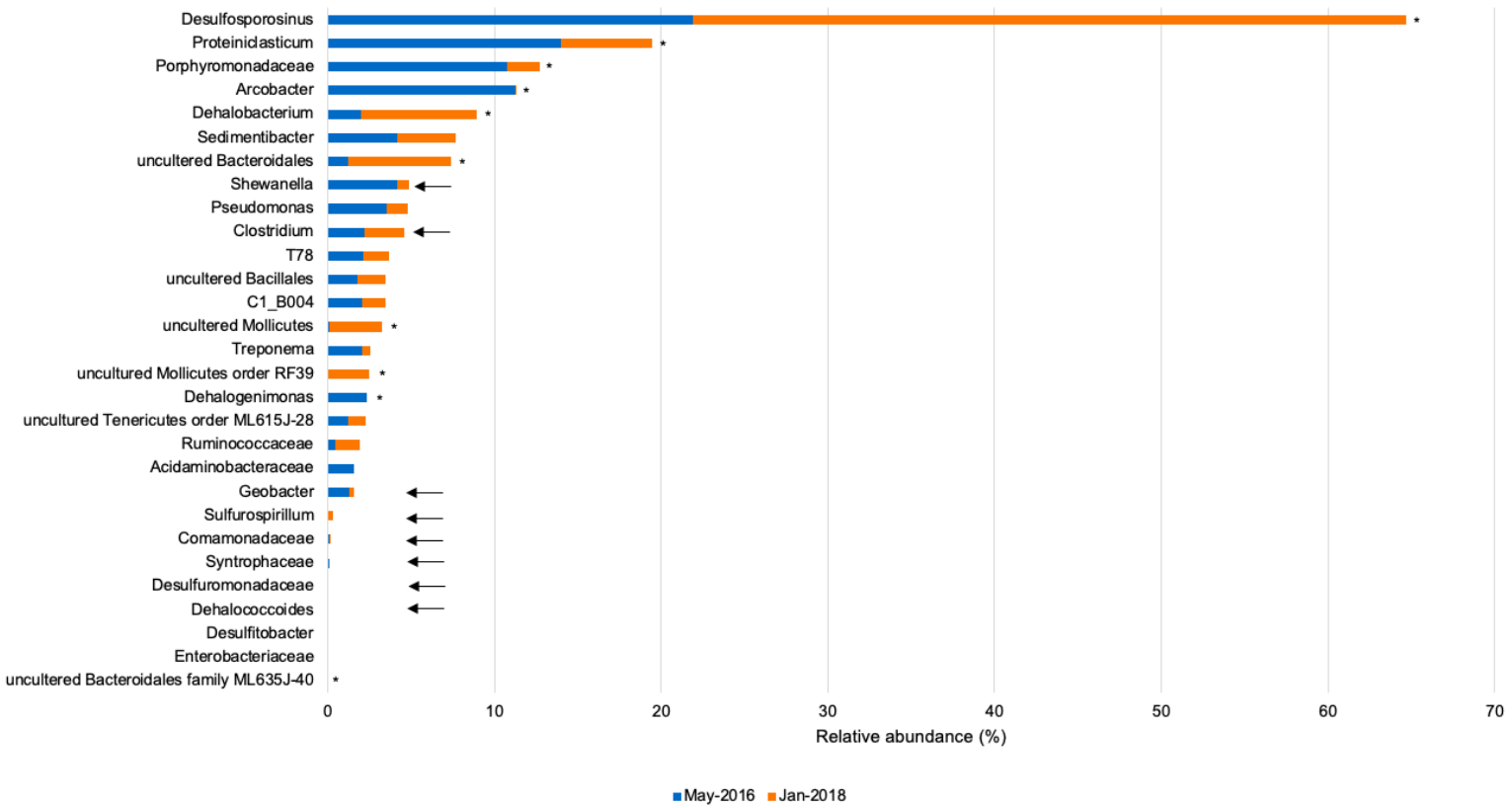
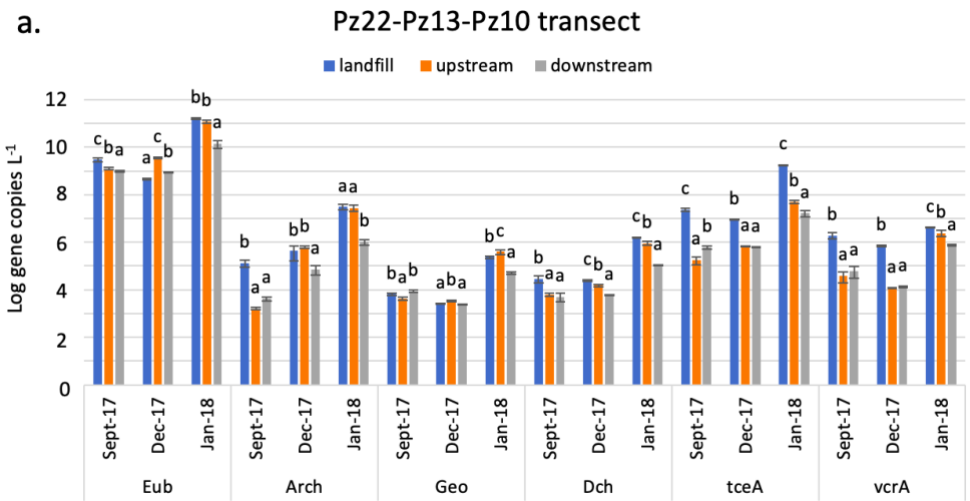


*Figure 14 Bacteria present in anaerobic bio-barrier with a relative abundance above 1% at genus level and OHRB. * indicates significant variation of relative abundance during 20 months. Arrows indicate OHRB genera or family*

Field monitoring of OHR functional biomarkers in landfill transects

In the landfill and in the corresponding piezometers near the bio-barrier, the quantification of all gene targets was significantly different. In transect Pz22-Pz13-Pz10 (Figure 15-a), Eubacteria and Archaea gene copies L^{-1} were equal or higher in the landfill than in the piezometers. *Geobacteraceae* were equal to downstream injection wells piezometer for the first two times, then increased in the upstream injection wells. In transect Pz25-Pz16-Pz3 (Figure 15-b), Archaea and *Geobacteraceae* were significantly higher. In transect Pz22-Pz13-Pz10, *Dehalococcoides* was significantly more abundant in the landfill than in piezometers, being the opposite in the other transect. *tceA* and *vcrA* genes were significantly higher in the landfill with respect to the piezometers in both transects.

According to these data, the presence of the permeable reactive bio-barrier significantly affected the concentration of some OHR genetic markers.



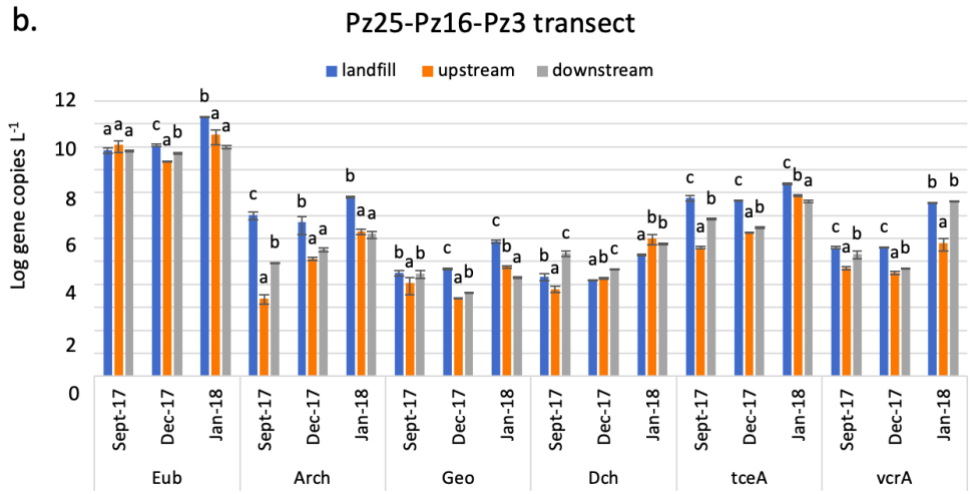


Figure 15 Gene copy abundance of total bacteria, archaea, Geobacteraceae, Dehalococcoides, tceA and vcrA, in the landfill, upstream and downstream injection wells of anaerobic bio-barrier in transect Pz22-Pz13Pz10 (a) and Pz25-Pz16-Pz3 (b). Lowercase letters indicate significant difference between different time points (Tukey's test, $\rho \leq 0.05$).

DISCUSSION

Site characterization

In the landfill, high concentrations of chloroethenes were present and redox potential was near the optimal Eh for OHR activity.

Phylogenetic and functional OHR biomarkers were present in high amount, although differences between RNA- and DNA-based analysis evidenced that active OHR bacteria were lower of four orders of magnitude, as expected.

In landfill, active Archaea community was characterized mainly by methanogenic microorganisms (*Euryarchaeota*, in particular *Methanomicrobia* genus) and anaerobic aromatic degrader (*Crenarchaeota*). *Firmicutes* and *Proteobacteria* were the main members of active bacterial community present in the landfill. In previous studies, *Fusibacter* (*Firmicutes* phylum) and *Mollicutes* were detected in microcosms amended with PCE and

TCE enrichment cultures, respectively, but it is not clear their role (Lee et al., 2011; Macbeth et al., 2004). *Sedimentibacter* and *Coprococcus* can have also organic acids producing role in soil amended with crop residues (Tan et al., 2019).

Witthin the *Proteobacteria* phylum, *Shewanella* showed the higher relative abundance than the other active OHRB present in the landfill. *Xanthomonadaceae*, *Caulobacteraceae, Phyllobacteriaceae* and *Alcaligenaceae* are aerobic polycyclic aromatic hydrocarbons degraders (Viñas et al., 2005; Farber et al., 2019; Obi et al., 2017; Hatayama et al., 2008) and *Thermomonas* is associated with *Xanthomonadaceae* but its role is not characterized (Kaplan et al., 2004). *Mycoplana* is an anaerobic aromatic hydrocarbons degrader (Brinda et al., 2013).

At microcosms level, degradation rate of highly chlorinated compounds was enhanced by the addition of the reducing substrate. The data suggested that, although in the presence of a native microorganisms able to conduct natural attenuation processes, the addition of reducing substrate is a feasible strategy to accelerate and improve reductive dehalogenation reactions, as it was reported in other works with different substrate as sludge cake/cane molasses, and lactate, butyrate and hydrogen (Kao et al., 2003; Aulenta et al., 2005). Beside this, the production of vinyl chloride should be carefully monitored, since the acceleration of reductive dehalogenation reactions led to an increment of vinyl chloride accumulation.

Organohalide respiration affected by reducing substrate

Contaminated compounds had a different distribution along anaerobic bio-barrier at field scale since the beginning of the treatment. Piezometer Pz13 presented higher quantities of CE than other transects, attributable to non-linear hydrologic flow course from the landfill. In all piezometers, VC accumulation was present as reported in several works (Fellen et al., 2001; Aeppli et al., 2010; Imfeld et al., 2011). Indeed, highly chlorinated ethenes were efficiently degraded. pH, oxygen concentration and Eh were optimal for

microbial OHR activity, in particular near anaerobic bio-barrier. These data were in accordance with laboratory-based microcosms experiment outputs and highlight the importance to find solution for enhancing vinyl chloride biodegradation, thus preventing the accumulation of the carcinogenic intermediate in the contaminant plume.

Impact of biostimulation treatment on aquifer microbial community

Treatment affected mainly Archaea amount than total bacterial amount because addition of reducing substrate promoted the increase of fermentative microorganisms, that in the site belong in high percentage to Archaea kingdom according to NGS data. Other gene targets did not follow a linear increase, but they remain in the same orders of magnitude except for the last sample time, where a significant increase was measured. Functional markers *tceA* and *vcrA* were more abundant than phylogenetic markers (*Geobacter* and *Dehalococcoides*), thus suggesting the presence of other OHRB carrying these functional genes.

Reducing substrate addition modified the microbial community composition at the site. In general, in the anaerobic portion of the field, methanogens presence, in particular *Euryarchaeota,* decreased in the aquifer archaeal community. On the other hands, fermentative archaea relative abundance increased. For *Parvarchaeota,* included in the superphylum DPANN (Rinke et al., 2013), glycolysis capacity and acetaldehyde fermentative activity were hypothesized from metagenomic study (Chen et al., 2018). In addition, also bacteria with a fermentative activity showed an increase of their relative abundance. Among these, *Firmicutes* can play the role of fermentative bacteria in chlorinated contaminated site (Ziv-El et al., 2011; Liu et al., 2017; Němeček et al., 2017). In particular, *Desulfosporosinus* ferments lactate (Spring et al., 2006) and *Proteiniclasticum* is a proteolytic and fermentative bacterium (Zhang et al., 2010). *Porphyromonadaceae* family, *Spirochaetes* and T78 belong to *Anaerolinaceae* family involved in fermentation activity (Sakamoto, 2014; Jiang et al., 2019). *Ruminococcaceae* has a role in trophic

chain during acetogenesis phase (Ozbayram et al., 2018). Our data find response in the literature and evidence that the addition of a reducing substrate constituted by molasses fuels the trophic chain of anaerobic of organic compounds, thus enhancing reducing power in the system.

Known OHRB were present in low amount. *Geobacter* decreased during 20 months of treatment, as evidenced by qPCR data of *Geobacteraceae* 16S rRNA gene copies L^{-1} number. *Clostridium* and *Shewanella* constituted an exception, but they did not show benefit from the treatment. Nevertheless, some bacteria present in the aquifer showed a possible correlation with OHR activity. *Desulfosporosinus* is not a known OHRB but it belongs to the *Peptococcaceae* family in which different strains of *Desulfitobacterium* are known to carry out OHR (Atashgahi et al., 2016) and many OHRB are sulfur-reducing bacteria. *Dehalobacterium* were founded in contaminated area halogenated pollutants and it can ferment dichloroethane (Trueba-Santiso et al., 2017). In this perspective, the analysed aquifer might host new *Desulfosporosinus* strains involved in OHR processes. Future investigations with specific probes will be dedicated to ascertain its role.

It was shown that OHRB for their reductive activity need specific physic-chemical conditions. In some cases, these requirements cannot be met by OHRB themselves, but they need other microorganisms. *Actinobacteria* and *Proteobacteria* can protect strictly anaerobic OHRB as *Dehalococcoides* from oxygen and its free radical damage (Hug et al., 2012; Liu et al., 2017). Cobalamin (B12), a corrinoid, is very important for OHR activity, but some OHRB are not able to produce it themselves (Wei et al., 2016). *Spirochaetes* (*Treponema*) and *Sedimentibacter* are corrinoids cofactor producing bacteria (Ziv-El et al., 2011; Maphosa et al., 2012), that were retrieved in the studied aquifer and might have a protective role towards OHRB. Mollicutes was found in TCE enrichment culture but its role was not determined (Macbeth et al., 2004).

Aquifer was affected also by the presence of the high quantity of hydrocarbons. *Miscellaneous Crenarchaeota Group* (MCG), *Crenarcheota*

phylum retrieved at the site, are uncultivated Archaea present in different environments. They can use wide substrates as carbon source, suggesting an important role in carbon biogeochemical cycling (Zhou et al., 2018). Furthermore, genes involved in anaerobic degradation of aromatic compounds (Meng et al., 2014) and putative methane-metabolizing genes (Vanwonterghem et al., 2016) were identified in their genome. Order YLA114 (*Parvarchaeota)* are not well characterized but they were found in crude oil reservoir and hydrocarbon degradation role was suggested (Shelton et al., 2016). *Bacteroidetes* and *Firmicutes* (*Acidaminobacteraceae*) are present in hydrocarbons degrading communities with degradation role or other roles not entirely characterized (Militon et al., 2010; Yang et al., 2014; Koo et al., 2015; Yang et al., 2016). In phylum *Tenericutes,* there were different characteristic bacteria of hydrocarbons contaminated site but they role in these areas is unknown (Ding et al., 2015; Gao et al., 2018). *Comamonadaceae* family was also detected in microcosms amended with diesel (Jung et al., 2014). *Nitrososphaera,* an aerobic ammonium-oxidizing Archaea, can degrade polyaromatic hydrocarbons (Campeão et al., 2017; Li et al., 2019). Sun et al. (2014) determined a toluene-assimilating activity of *Desulfosporosinus* in methanogenic conditions. *Arcobacter* are widely present in petroleum contaminated site and they act as nitrate-reducing and sulfide-oxidizing bacteria (Sette et al., 2007). In microcosms study it has been reported that *Arcobacter* were affected by the presence of PCE (Hermon et al., 2019).

High number of unassigned Archaea can be due to the presence of slow growth bacteria, hardly investigated, promoted by environment inhospitable characteristics of this aquifer, with high concentrations of contaminant compounds (Comeau et al., 2012). Indeed, in January 2018, this class of Archaea decreased significantly. ML615J-28 order of *Tenericutes* phylum is not yet characterized as environmental bacteria.

Economic considerations on sustainability of this type of action will be compared with other remediation intervention.

CONCLUSIONS

Bacterial OHR efficiently reduced chloroethenes present in the aquifer. Reducing substrate addition effects were clear in organohalide respiration of chloroethenes at microcosms scale, although the site hosted an already well adapted OHR microbial community able to perform natural attenuation. In situ and in microcosms VC accumulation was observed. On site molecular and chemical analyses evidenced that the biostimulation treatment initially increased OHRB activity which later reached a balance, being fueled by increment of fermentative bacteria. Beside this, the decrease of chloroethenes obtained in the aquifer can only be explained by the presence of still unexplored OHRB and further analyses are in progress.

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SUPPLEMETARY MATERIALS

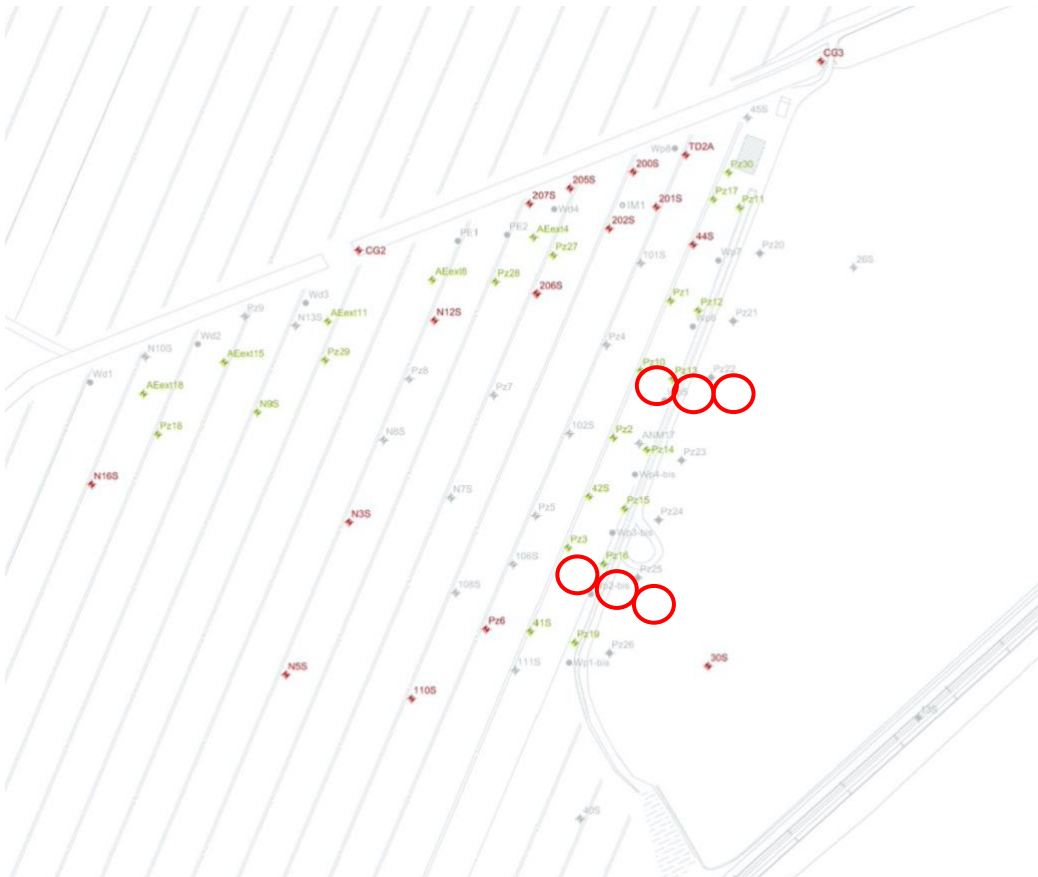

Figure S6 Site map. Analyzed monitoring piezometers are marked with red circles. TAUW Italia

Table S1 List of primers used

Target gene	Primers	Sequences	Product size (bp)	References
Bacteria 16S rRNA	Eub338f	ACT CCT ACG GGA GGC AGC AG	180	Fierer et al., 2005
	Eub518r	ATT ACC GCG GCT GCT GG		
	27f	AGAGTTTGATCMTCGTCCCTC	1465	Edwards et al., 1989 Lane et al., 1991
	1492r	TACGGYTACCTTGTTAGGCTT		
Archaeal 16S rRNA	Arc787F	ATT AGA TAC CCS BGT AGT CC	272	Yu et al., 2005
	Arc1059R	GCC ATG CAC CWC CTC T		
Geobacteraceae 16S rRNA	Geo546F	AAGCGTTGTTCGGAWTTAT	294	Cummings et al., 2003 Sandford et al., 2007
	Geo840R	GGCACTGCAGGGGTCAATA		
Dehalococcoides 16S rRNA	Dhc1154f	CAC ACA CGC TAC AAT GGA CAG AAC	132	Krzmarzick et al., 2012
	Dhc1286r	GAT ATG CGG TTA CTA GCA ACT CCA AC		
tceA	TceA1270F	ATC CAG ATT ATG ACC CTG GTG AA	66	Jonhson et al., 2005
	TceA1336R	GCG GCA TAT ATT AGG GCA TCT T		
vcrA	Vcr1022F	CGG GCG GAT GCA CTA TTT T	71	Ritalahti et al., 2006
	Vcr1093R	GAA TAG TCC GTG CCC TTC CTC		

Eubacteria PCR protocol

PCR was carried out in a 25 μL reaction mixture with primers 27F-1492R (Table S1) 0.3μM and 1x Taq PCR Master Mix (QIAGEN) following this protocol: 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 40s, and elongation at 72°C for 1 min and 40s; the final elongation was performed at 72°C for 10 min.

Eubacteria standard plasmid construction

Eubacteria 16S rRNA was amplified with primer 27F-1492R (Table S1) using 1x Taq PCR Master Mix (QIAGEN) to a final volume of 25μL. PCR products were cloned in pCR™2.1-TOPO® vector using the TOPO®TA Cloning® Kit (Invitrogen) according protocol**.** Plasmid of clones were extracted with UltraClean™ 6 min Mini Plasmid Prep Kit™ (MO BIO) and quantified through spectrophotometry.

Table S2 Plasmids used to set up standard curves for quantification of genetic target through qPCR

Source organism	Name of the clone	Vector (plasmid)	Metabolic gene quantified	Reference
Dehalococcoides ribotype BTF08	BTF08-1492 [a.]	pGEM-T Easy	Dhc	Kaufhold et al., 2012 Maymó-Gatell et al., 1997
Geobacter lovleyl strain SZ	16S-Geo [a.]		Geo	Pöritz et al., 2013
Methanobacterium formicicum	[b.]		Arch	Rago et al., 2015
Dehalococcoides ribotype BTF08	vcrA-BTF08 [a.]		vcrA	Kaufhold et al., 2012 Maymó-Gatell et al., 1997
Dehalococcoides ethenogenes 195	tceA-195 [a.]		tceA	Sung et al., 2006

Clone provided by:
[a.] Dr. Ivonne Nijenhuis, Department of Isotope Biogeochemistry of the Helmholtz Center of Environmental Research (UFZ) in Leipzig, Germany
[b.] Rago et al., 2015

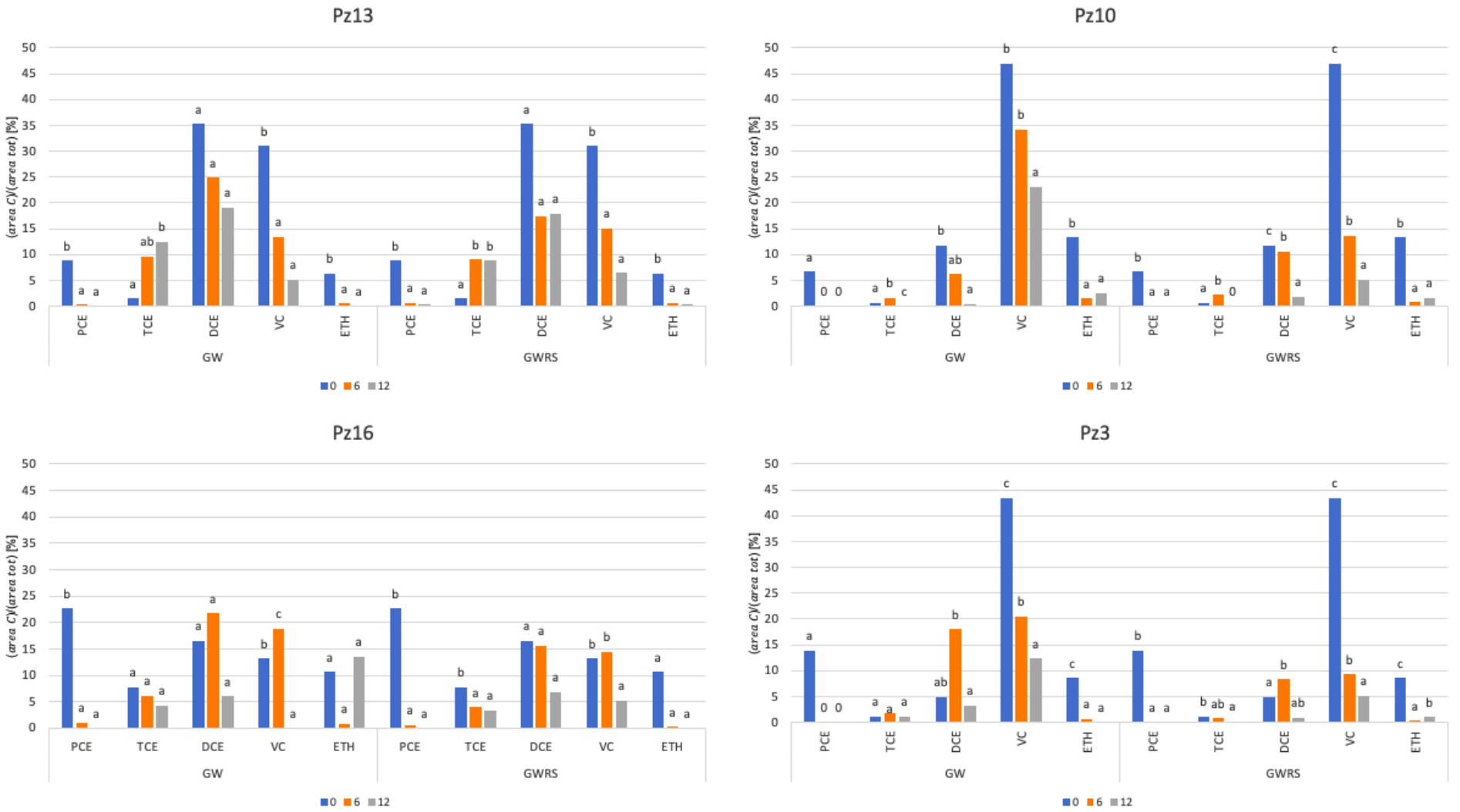

Figure S2 Comparison of chloroethenes relative abundance during the time in the same microcosms. Piezometers: Pz13, Pz10, Pz16 and Pz3.

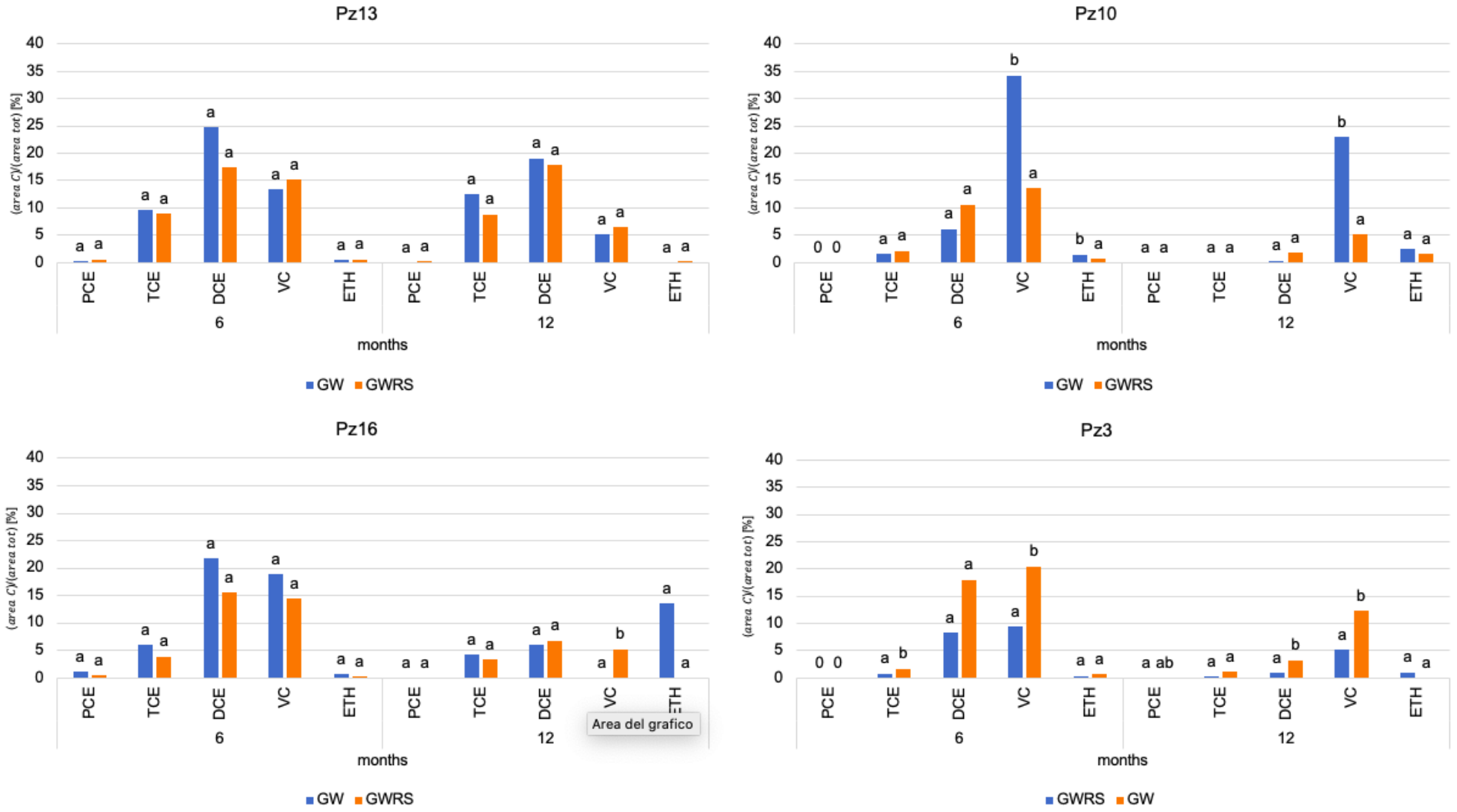

Figure S3 Comparison of chloroethenes relative abundance in microcosms with (GWRS) or without reducing substrate (GW). Piezometers: Pz13, Pz10, Pz16 and Pz3.

Table S2 Chemical data of six piezometers analyzed

		PCE ($\mu g\ L^{-1}$)	TCE ($\mu g\ L^{-1}$)	1,1 DCE ($\mu g\ L^{-1}$)	1,2 DCE ($\mu g\ L^{-1}$)	VC ($\mu g\ L^{-1}$)	ethene ($\mu g\ L^{-1}$)	Eh* mV	pH	DO* $mg\ L^{-1}$
Law limits D.Lgs. 152/06		1.1	1.5	0.05	60	0.5	-	-	-	-
Pz13	MAY 16	6400.00	62000.00	87000.00	37100.00	131000.00	26900.00	-243.00	6.68	1.66
	SEPT 16	7300.00	62000.00	84000.00	36800.00	140000.00	23500.00	-174.00	6.79	0.06
	JAN 17	6300.00	45000.00	37000.00	28800.00	89000.00	34400.00	-235.00	6.43	0.25
	MAY 17	7800.00	55000.00	50000.00	34300.00	104000.00	30900.00	-162.00	7.31	0.23
	SEPT 17	9100.00	58000.00	39000.00	37400.00	127000.00	1.60	-117.00	6.32	
	DEC 17	8800.00	60000.00	22800.00	23800.00	83000.00	25300.00	-185.00	7.39	0.90
	JAN 18	9000.00	64000.00	46000.00	42200.00	159000.00	25800.00	-139.00	6.40	0.16
Pz10	MAY 16	900.00	4500.00	12000.00	9400.00	66000.00	18700.00	-233.00	6.39	0.04
	SEPT 16	360.00	640.00	1780.00	3130.00	16600.00	24400.00	-261.00	6.83	0.07
	JAN 17	61.00	73.00	1130.00	1670.00	7000.00	21600.00	-220.00	7.73	0.13
	MAY 17	108.00	1490.00	1780.00	2990.00	22200.00	35000.00	-278.00	7.39	0.15
	SEPT 17	390.00	2400.00	6100.00	8710.00	45000.00	20300.00	-262.00	7.37	0.27
	DEC 17	280.00	470.00	680.00	825.00	3600.00	5620.00	-218.00	7.57	0.47
	JAN 18	171.00	249.00	920.00	1930.00	10800.00	22700.00	-230.00	6.60	0.15
Pz16	MAY 16	6200.00	11100.00	2130.00	18200.00	10600.00	8510.00	-120.00	6.63	1.26
	SEPT 16	5900.00	8300.00	3300.00	14500.00	18600.00	16600.00	-87.00	6.70	0.05
	JAN 17	4100.00	11300.00	2300.00	16300.00	25300.00	16800.00	-68.00	6.51	0.19
	MAY 17	4000.00	6100.00	2140.00	18600.00	25300.00	19300.00	-192.00	7.40	0.12
	SEPT 17	4800.00	8500.00	3900.00	26400.00	34000.00	19800.00	-295.00	6.23	
	DEC 17	3020.00	7000.00	1490.00	14300.00	25700.00	19500.00	-250.00	7.39	0.90
	JAN 18	3360.00	8000.00	2190.00	16400.00	38000.00	16300.00	-254.00	6.49	0.07

Pz3	MAY 16	870.00	1580.00	2130.00	10600.00	33000.00	6490.00	-288.00	6.52	0.15
	SEPT 16	74.00	197.00	236.00	1610.00	20400.00	26400.00	-351.00	6.90	0.03
	JAN 17	48.00	83.00	380.00	1610.00	10100.00	26000.00	-294.00	7.78	0.11
	MAY 17	71.00	261.00	830.00	2930.00	19000.00	32300.00	-268.00	7.36	0.17
	SEPT 17	290.00	500.00	1330.00	9500.00	43000.00	58000.00	-300.00	7.10	0.31
	DEC 17	282.00	1430.00	760.00	4240.00	28600.00	20600.00	-309.00	7.52	
	JAN 18	167.00	1770.00	830.00	6550.00	44000.00	23100.00	-321.00	6.48	0.07
Pz22	SEPT 17	7300.00	43000.00	22800.00	17000.00	72000.00	19800.00	-126.00	7.4	1.2
	JAN 18	8200.00	47000.00	51000.00	29900.00	142000.00	20200.00	-128.00	6.5	0.2
Pz25	SEPT 17	2860.00	7300.00	1370.00	5640.00	6200.00	980.00	-85.00	7.5	0.9
	JAN 18	2950.00	7500.00	2490.00	8900.00	12800.00	1256.00	-65.00	6.6	0.1

*DO= dissolved oxygen, Eh=Redox potential

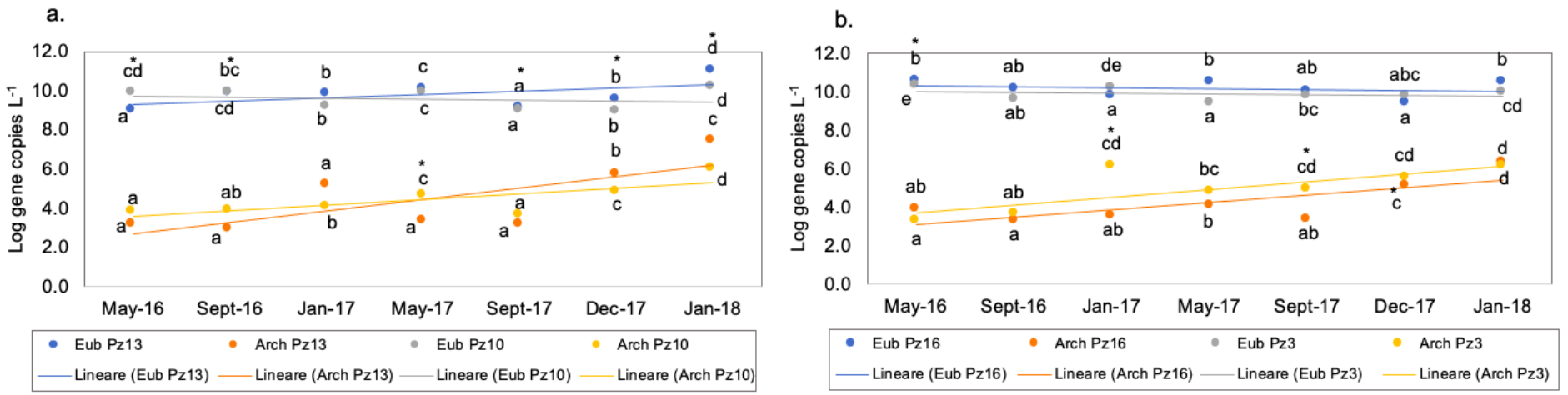

*Figure S4 Gene copy abundance of bacterial and archaeal 16S rRNA in Pz13/ Pz10 (a) and Pz16/Pz3 (b). * indicates significant difference between piezometers of transect upstream and downstream injection wells of anaerobic bio-barrier. Lowercase letters indicate significant or not significant difference between different time (Tukey's test, $\rho \leq 0.05$).*

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CHAPTER 2

VINYL CHLORIDE BIODEGRADATION IN CONTAMINATED AQUIFER UNDERGOING STIMULATION TREATMENTS

Authors: Martina Bertolini[1], Sarah Zecchin[1], Patrick R. Richards[2], Timothy E. Mattes[2] and Lucia Cavalca[1]

[1] Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente (DeFENS), Università degli Studi di Milano Via Celoria 2, I-20133 Milano, Italy
[2] College of Engineering, University of Iowa, 4112 Seamans Center for the Engineering Arts and Sciences, Iowa City, IA, USA

ABSTRACT

Vinyl chloride is a chlorinated ethene. It is a wide contaminant of soil and water because it is a product of degradation of higher chlorinated ethenes, but it is also frequently used in industrial sector for the production of PVC. It is very toxic for human because of its carcinogenicity. Vinyl chloride is hardly transformed in anaerobic conditions by microorganisms creating accumulation in chlorinated solvents contaminated area where reductive dehalogenation is promoted via biostimulation treatments. Nevertheless, it is efficiently biodegraded in microaerobic and aerobic conditions by bacteria. The first enzyme involved in its oxidation is alkene monooxygenase (AkMO), encoded by *etnABCD*.

In this work, aerobic oxidation of vinyl chloride present in a contaminated aquifer was investigated in order to trace microbial activities involved in the decrease of vinyl chloride. From molecular analyses conducted on aquifer DNA, it was possible to ascertain the presence vinyl chloride degrading microorganisms. Chemical monitoring at the site confirmed that a natural degradation of vinyl chloride was already present in the aquifer. Laboratory-

scale experiments confirmed that vinyl chloride bacterial populations were active at the site.

INTRODUCTION

Chloroethenes are widely used in industrial activity as solvents. Moreover, these compounds are present also in several daily use products, such as paint products or dry-cleaning solvents. Because of their toxicity and carcinogenicity, they are strictly normed, and their degradation is extensively studied. Tetrachloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE), and vinyl chloride (VC) can be transformed in different conditions by microorganisms. In anaerobic conditions, they are electron acceptors of organo-halide respiration (OHR) performed by organo-halide respiring bacteria (e.g., *Dehalococcoides*) that allow to transform these toxic compounds in ethene, a safe molecule (Mattes et al., 2010; Adrian and Löffler, 2016). Nevertheless, this respiration can efficiently dechlorinate highly substituted chloroethenes but not DCE and VC, that generate accumulation in anaerobic contaminated environments (Futagami et al., 2008). The degradation of low-chlorinated compounds can be achieved in aerobic conditions. In particular, VC can be degraded by metabolic or co-metabolic pathways. Cometabolic degradation of VC is performed by methanotrophs bacteria (Findlay et al., 2016). Metabolic pathway proceedes via the initial activity of two enzymes: alkene monooxygenase (AkMO) (encoded by *etnABCD* genes) and Epoxyalkane:coenzyme M transferase (EaCoMT) (encoded by *etnE* gene). Only these two enzymes have been currently characterized, and it was assumed that the final product of this pathway is acetyl-CoA, compounds involved in TCA cycle (Coleman et al., 2003; Mattes et al., 2005). This pathway is used by ethene (also called etheneotrophs) and VC degrading bacteria. The gene for the enzymes AkMO and EaCoMT are located on the same large plasmid (Danco et al., 2004). The rapid loss of VC degrading capacity after a VC starvation period has led to hypothesize

plasmid loss in absence of the compound (Coleman et al, 2002a). Oxidative degradation of VC and ethene can occur in presence of very low amount of oxygen (0.5-1.8 mg L^{-1}) (Coleman et al., 2002a; Fullerton et al., 2014). The most studied VC degrading bacteria genera are: *Mycolicibacterium*, *Nocardioides* and *Rhodococcus* (Mattes et al., 2010).

The contaminated site considered in this work, is characterized by a VC accumulation due to incomplete anaerobic OHR of chloroethenes present in the contaminated aquifer. In this work, VC degradation activity of microorganisms present at the site was analyzed. Furthermore, effects of stimulating treatment on degrading microbial populations were monitoring through genetic biomarker (*etnC*).

MATERIALS AND METHODS

Site description and sampling

The site of interest is located in Dogaletto (Venice, North Italy) near the important petrochemical plant of Porto Marghera. The landfill, present in the site, was used for the collection of industrial waste. The landfill surface covers about 16 hectares with a total waste mass of 1’700’000 tons. The site is delimited by the Venice lagoon to the South-side and by an irrigation channel to the North-side (Figure S1). Through drainage pump system an adequate water level is provided inside the channel.

A continuous leaching of the landfill contaminated the underlying aquifer with a multiple contamination: BTEX, chlorinated compounds and hydrocarbons. From 1995, pump and treat (P&T) method was chosen as solution to remediate the area. In 2016, two permeable reactive biobarriers were set up to assist P&T method and, in the future, replace it (Figure S1). The anaerobic biobarrier was about 390 m long and consisted of 39 wells 10 m deep where water was recirculated and added of reducing substrate to enhance OHR. The aerobic biobarrier is about 500 m long and 10 m deep, with 53 recirculation

wells and 43 injection wells that allow addition of urea, ammonium phosphate and O_2 to stimulate microbial aerobic degradation.

Five monitoring piezometers (N12S, 206S, AEext4, AEext8 and AEext11 in Figure 1) were sampled for three times in 9 months (December 2019, June 2020 and September 2020). For microbial degradation analyses, groundwater from the five piezometers collected in July 2019. For 3 piezometers (AEext4, AEext8 and AEext11) downstream the biobarrier groundwater was sampled at two profundity: one 5 meters from filed plan (superficial) and the other one 1.5 meters from the bottom of the well (depth).

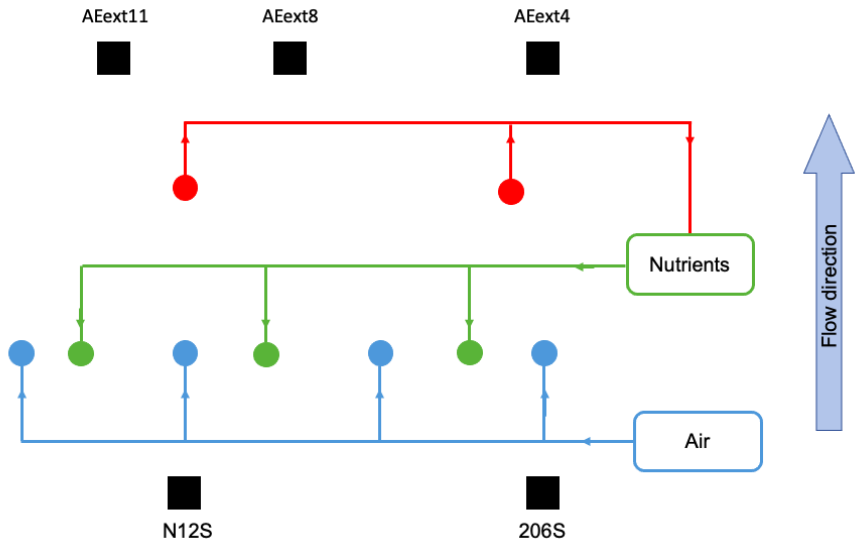

Figure 7 Monitoring piezometers, and extraction and injection wells positions near the aerobic biobarrier

Chemical analysis

Chlorinated ethenes were quantified through gas chromatography mass spectrometry (GC-MS) following EPA8260D 2018 method for volatile organic compounds. Groundwater collected for chemical analysis was sampled following equilibrium based static headspace preparation (EPA5021A 2014 method) to carry out the analysis in headspace.

Ethene and VC biodegradation tests

Microcosms were set up to determine ethene and VC biodegradation activity of microbial population present in the aquifer in July 2019. For each piezometer, two vials were set up, one with VC and one with ethene. In 160mL vial, 25 mL of minimal salts medium (MSM) (Coleman et al., 2002b) and 25 mL of groundwater were added. In the used groundwater, contaminated compounds were undetectable because of stripping process during vial set up. Vials were closed with rubber stoppers and crimp caps. 205 μM of VC (99.5%) (SynQuest labs, USA) and ethene (99%) (Praxair Surface Technology, USA) were added. Subsequent molecule spiking was carried out when the molecules were totally degraded, and their amount increased each next addition (410 μM, 620 μM, 825μM). After addition of higher VC or ethene concentration, enrichment culture was transferred in fresh MSM medium (50%, vol/vol), 25 mL of enrichment culture and 25 mL of MSM. Abiotic control samples were set up with sterile MSM and the addition of VC and ethene. Enrichment cultures were incubated inverted on an orbital shaker (200rpm) at room temperature in the dark. Enrichment cultures were monitored for 60 days, and VC and ethene concentrations were analyzed through gas chromatography with flame ionization detector (GC-FID) for eight successive incubation times (0, 13, 20, 27, 34, 41, 53 and 59 days). Gas chromatographic apparatus (Agilent 6890, Agilent, USA) was equipped with a Supelco 1% SP-1000 Carbopack B, 6ft x 1/8 inch diameter column (Sigma-Aldrich, Germany). Inlet temperature was 200°C and the oven isothermal program was used at 90°C. Carrier gas was nitrogen (Praxair Surface Technology, USA) with flow rate of 30 mL min^{-1}. FID temperature was 250°C, and hydrogen and air flows were 40 mL min^{-1} and 450 mL min^{-1}, respectively.

Determination of Ethene degrading bacteria

Most Probable Number (MPN) method was used to quantify ethene/VC degrading bacteria in aquifer samples in June 2020 in two depths. In 50 mL vials, 9 mL of mineral medium MSM were added with 1 mL of groundwater

after a serial dilution (from 10^{-1} to 10^{-6}) in triplicate for each dilution. 1 mL of ethene (Sapio, Milano, Italy) was added in each vial. Control vials were set up without the addition of ethene. Vials were closed with butyl rubber stoppers and crimp caps, and they were incubated at 30°C in the dark under shaking conditions. After 30 days, growth turbidity was detected, and quantification of degrading bacteria were estimated through MPN table for three replicates (McCrady 1918).

DNA extraction, real time quantitative PCR and standard curve construction

From each piezometer (N12S, 206S, AEext4, AEext8 and AEext11) 20L of groundwater were collected and filtrated on mixed cellulose ester filters (MediaKap™ ME2M-050-18S ∅ 0.2 µm) (Cole-Parmer, USA) using peristaltic pump apparatus (Masterflex L/S Economy Variable-Speed Drive, 20 to 600 rpm with Masterflex L/S Easy-Load Head for High-Performance Tubing, PSF/CRS) (Cole-Parmer, Vernon Hills, Illinois, Stati Uniti). DNA was extracted from pellet obtained from filters by using DNA PowerSoil® Isolation kit (MOBIO, Carlsbad, CA, USA). For each piezometer, three DNA extractions were performed. Nucleic acids were quantified by using Spectrophotometer Power Wase XS2 (BioTEK Instruments, USA).

16S rRNA genes of total bacteria and *etnC* (gene that encodes for one of subunit of AkMO) were quantified through real time quantitative PCR. In a final volume of 20 µL, 1x of Titan HotTaq Probe qPCR Mix (Bioatlas science of life, Estonia), 0.3 µM of forward and reverse primers (Table S1), 10 ng of DNA and PCR-grade water (AppliChem, Germany) were used. For the total bacteria quantification were used Eub338f-Eub518r primers and the following qPCR program: 15 min at 90°C, 40 cycles of 95°C for 1 min, 53°C for 40 sec and 72°C for 1 min, followed by 1 min at 72°C. Melting curve from 53°C to 95°C with an increment of 1.6°C sec^{-1} for 5 sec.

EtnC genes were quantified by using RTC-f and RTC-r primers (Table S1) and the qPCR protocol brought back here: 10 min at 95°C, followed by 40 cycles

at 95°C for 15 sec and 60°C for 1 min, then melting curve from 60°C to 95°C with an increment of 1.6°C sec^{-1} for 5 sec.
Standard curve, used to determine absolute quantification of the genes, was designed on amplification of plasmid carrying a target of each gene (SM).

Statistical analysis

R program (R Core Team, 2015) was used to analyzed qPCR data. Analysis of variance (aov) (Chambers et al., 1992) and TukeyHSD (Miller, 1981; Yandell, 1997) functions were selected in order to determine significant differences ($p \leq 0.05$) in biomarkers (16S rRNA eubacteria and *etnC*) expression between different piezometers, and treatment effects during the time.

RESULTS

Chemical data at field scale

In the aquifer, at the aerobic biobarrier level, chloroethenes were present in very different concentrations (Table S2). PCE and TCE were low. Indeed, PCE was never higher than 7.70 µg L^{-1} and TCE than 25.70 µg L^{-1}, both values registered in December 2019 in AEext4. On the other hand, in 206S piezometer, PCE and TCE were always under Italian law limits (D.Lgs. 152/06). 1,1-DCE was always lower than the other DCE isomer, 1,2-DCE (Table S2). The difference was of one or two order of magnitude. Concentration of VC in all piezometers was higher than Italian law limit of 0.5 µg L^{-1}. (D. Lgs. 152/06). Particularly, VC concentrations in the different piezometers were different with a gap of one order of magnitude between the higher (206S in December 2019), 39000 µg L^{-1}, and the lower 82 µg L^{-1} (AEext8 in June 2020) (Figure 2). In December 2020, VC was higher in 206S than in AEext4, being 39000 and 17100 µg L^{-1}, respectively. Instead, in the other two times VC was higher in AEext4. In June 2020, VC concentration

was lower than one in September 2020. VC increase was due to the decrease of DCE concentration that was dechlorinated to VC. The other transect showed a different situation. VC concentration was always lower in piezometers downstream the biobarrier (AEext8 and AEext11). A progressive decrease of VC concentration was observed in all the piezometers, from December 2019 to September 2020.

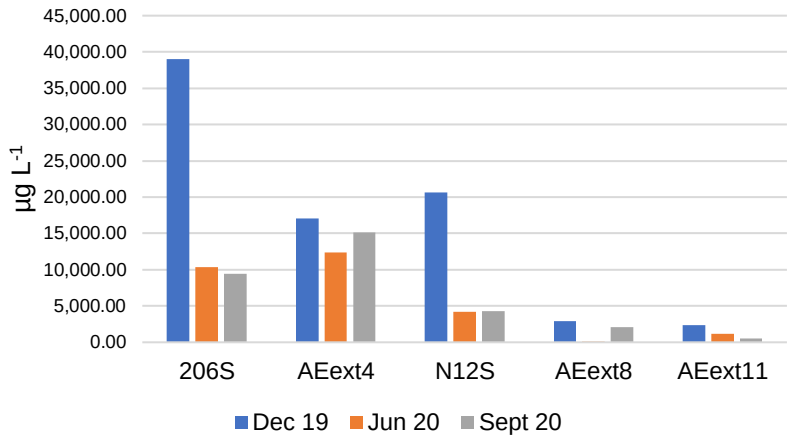

Figure 8 VC concentrations in the five piezometers near aerobic biobarrier in three sampling times

Aquifer temperature remained unchanged, like the pH, always between 6.5 and 7.8. Dissolved oxygen in the aquifer was generally low, under 1 mg L^{-1}. Indeed, in AEext11, dissolved oxygen was 3.08 and 2.64 mg L^{-1} in June 2020 and September 2020, respectively.

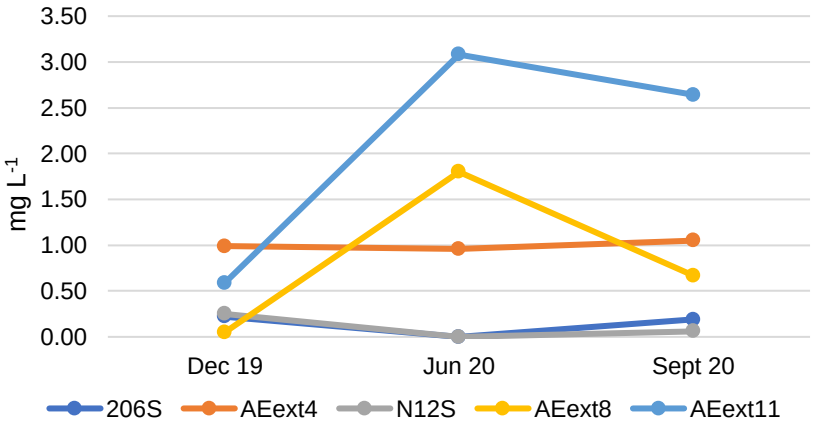

Figure 9 Dissolved oxygen concentration in piezometers in December 2019, June 2020 and September 2020

Determination of ethene and VC biodegradation

Ethene was completely degraded after two weeks incubation in microcosms set up with all piezometers, except in AEext4 (Figure 4a). In this case, ethene was complete degraded after 27 days since first compound addition. AEext8 and AEext11 microbial communities were able to completely degrade ethene after 7 days from all the spiking points. Instead, N12S and 206S needed more time (27 days) to degrade the second addition, performed at 13th day. On the other hand, N12S and 206S recovered faster the degradation activity after subsequent transplants and AEext8 and AEext11 needed about 10 days to degrade ethene (Figure 4).

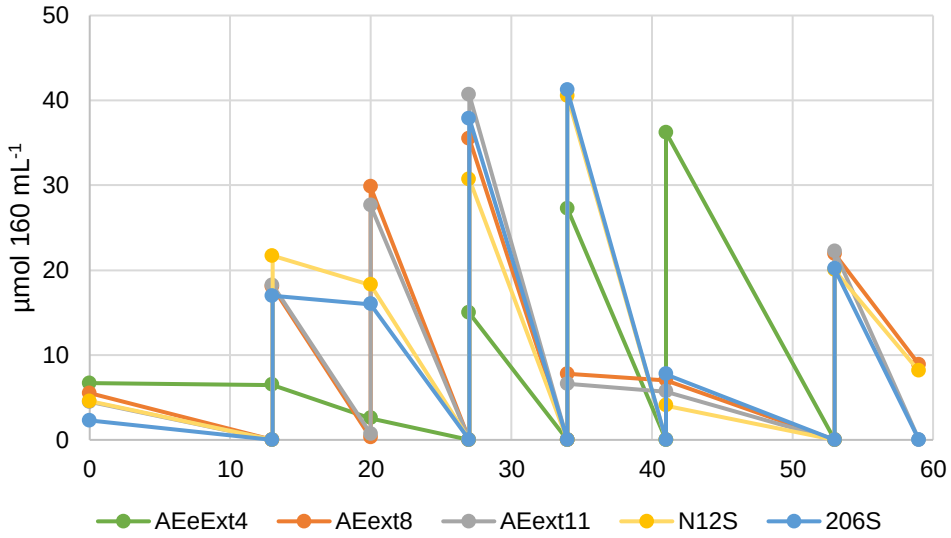

Figure 10 Quantification of ethene through GC-FID in biodegradation test conducted with enrichment cultures

After testing the presence of ethenotrophic bacteria at the site, their VC biodegradative ability was ascertained. In the VC degradation trials, AEext8 groundwater consumed VC faster than the other piezometers. After 20 days, it completely degraded VC present in the vials. AEext11 and 206S needed 27 days to oxidate completely the VC. After 34 days of incubation, also VC in N12S microcosms disappeared. Instead, in AEext4 vial, VC degradation started only after 53 days. After the initial acclimation, AEext8, AEext11, N12S and 206S showed a good VC degradation rate.

In control vial, neither ethene nor VC concentrations decreased (data not shown).

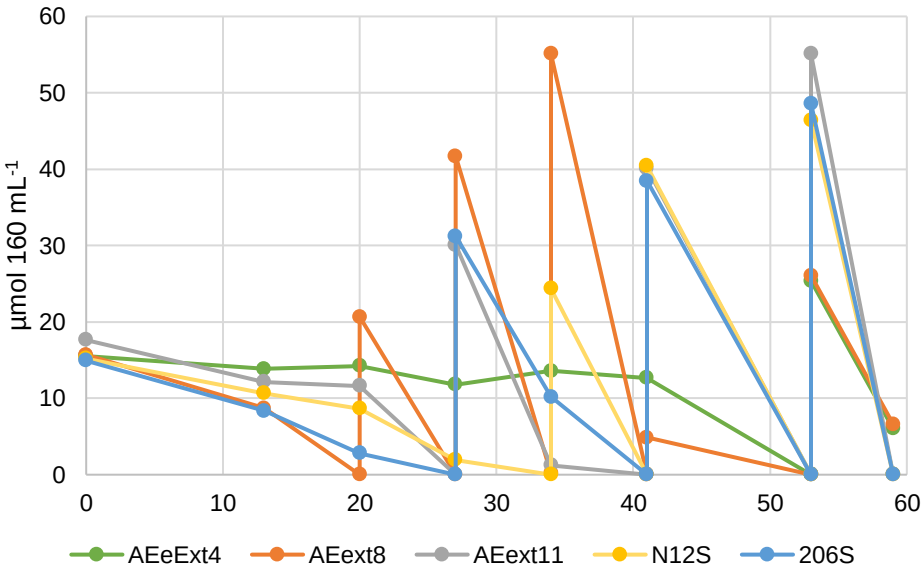

Figure 11 Quantification of VC through GC-FID in biodegradation test set-up with enrichment cultures

Ethene degrading bacteria quantification

In June 2020, Ethene degrading bacteria were present in the aquifer from 10^3 to 10^6 MPN mL^{-1}. In the first transect, there was no difference between upstream and downstream the biobarrier. Indeed, 206S and AEext4 showed the same amount of degrading bacteria (10^3 MPN mL^{-1}). Ethene degrading bacteria were present in higher amount in the second transect (N12S/AEext8/AEext11). N12S etheneotrophs were lower than AEext11 etheneotrophs, but they were higher than AEext8 ones. Degrading bacteria value did not change in AEext4 based on depth of sampling point (Table1). On the other hand, in AEext8 and AEext11, superficial point (5m from filled plan) showed a lower value than depth point (1.5 m from bottom of the well). The difference was of one order of magnitude: 9.00 x 10^3 MPN mL^{-1} and 7.00 x 10^4 MPN mL^{-1} in AEext8, and 2.00 x 10^5 MPN mL^{-1} and 1.10 x 10^6 MPN mL^{-1} in AEext11. In AEext 8, in depth area, in September 2020, MPN value did not change, it remained 10^4 MPN mL^{-1}.

Table 7 Quantification of ethene degrading bacteria through MPN mehod (MPN/mL) in June 2020

Piezometer		MPN mL^{-1}
206S		4.00×10^3
AEext4	Superficial 5m f.p.	7.00×10^3
	depth 1.5m b.w.	4.00×10^3
N12S		7.00×10^4
AEext8	Superficial 5m f.p.	9.00×10^3
	depth 1.5m b.w.	7.00×10^4
AEext11	Superficial 5m f.p.	2.00×10^5
	depth 1.5m b.w.	1.10×10^6

f.p.= from filed plan
b.w.=from bottom of the well

Quantification of aquifer total bacteria and *etnC* biomarkers

In aerobic biobarrier, total bacteria were present with value from 10^6 to 10^8 gene copies mL^{-1}. *EtnC* was in the range of 10^0-10^4 gene copies mL^{-1} with only two times outside this range: in June 2020, N12S *etnC* gene copies were 10^3, and in September 2020 they were 10^7 in AEext11. Eubacterial 16S rRNA was higher in December 2019 in all piezometers and it decreased in the other two times of approximately two order of magnitude (Figure 6a). In 206S, total bacteria gene copies decreased from 10^{11} in December 2019 to 10^9 in September 2020. Even if the trend is equal in all piezometers, there were some differences between the two transects. 206S/AEext4 showed higher gene copies upstream the biobarrier. Instead, in N12S/AEext8/AEext11 transect, gene copies were higher downstream biobarrier.

EtnC gene copies were higher in December 2019 in the piezometers 206S, N12S and AEext8, instead in AEext4 and AEext11 they were higher in September 2020 (Figure 6b). As total bacteria, VC functional biomarkers were

higher downstream biobarrier in transect N12S/AEext8/AEext11. Rather, in 206S/AEext4 transect, *etnC* in piezometers downstream the biobarrier was higher only in September 2020 and it was higher upstream the biobarrier in June 2020.

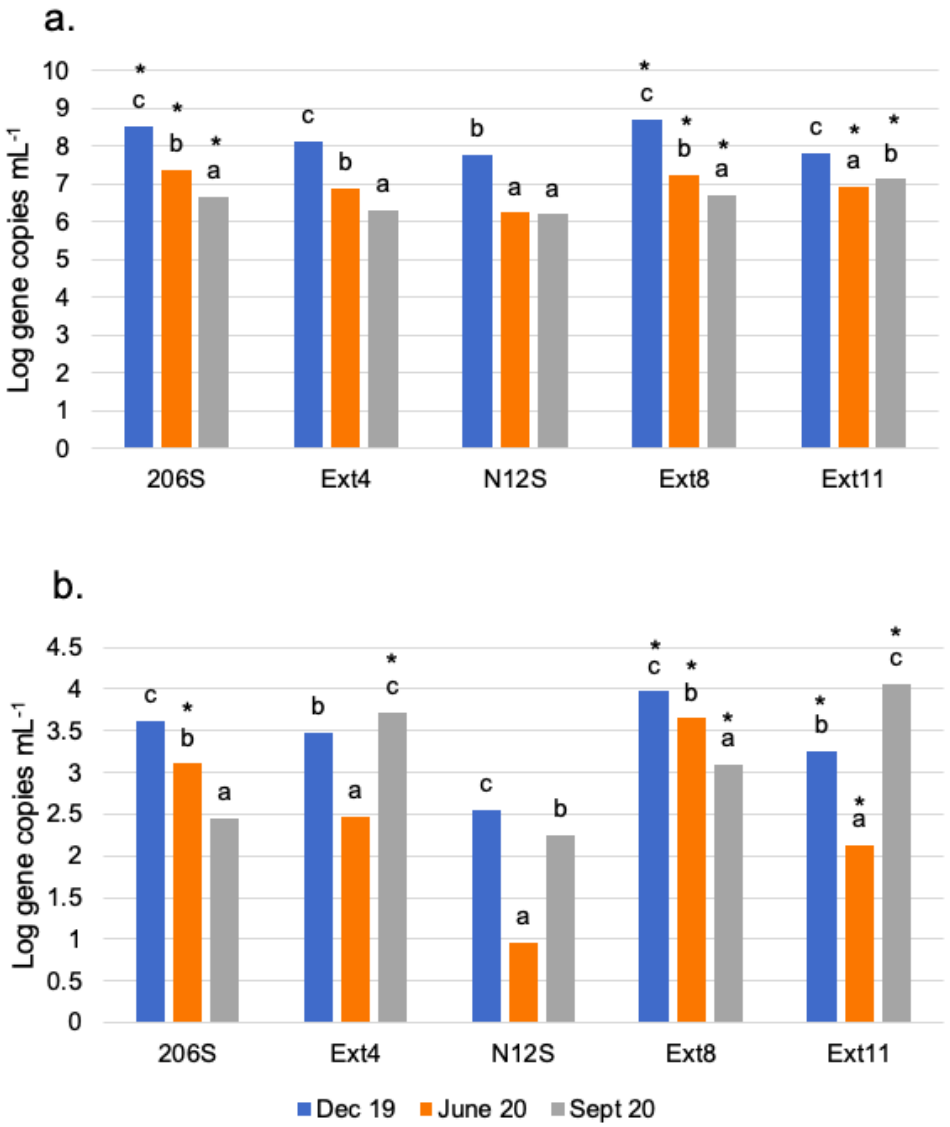

*Figure 12 Gene copy abundance of total bacteria (a) and etnC (b) in aerobic biobarrier during the time. * indicates significant difference between piezometers of transect upstream and downstream the aerobic biobarrier (N12S/AEext4 and 206S/AEext8/AEext11). Lowercase letters indicate significant or not significant difference between different time (Tukey's test, $\rho \leq 0.05$).*

DISCUSSION and CONCLUSIONS

In aerobic biobarrier, VC accumulation was present in all piezometers with two or five order of magnitude higher than Italian law limits (D.Lgs. 152/06). However, VC concentrations was very different between the different zones of the site. DCE was present with their isomer in significant different concentrations, even if, in our knowledge, different degradation rate between the two isomers was not reported. Although the aquifer showed a low amount of dissolved oxygen, aerobic biodegradation activity of VC and ethene was present, particularly in AEext8 and AEext11, with a higher rate if compared to previous microcosm studies (Liu et al., 2018). In accordance with this, it was reported that aerobic oxidation of VC can also occur in aquifer at low oxygen conditions (Fullerton et al., 2014). The presence of VC degrading bacteria was also confirmed by MPN data of ethene degrading bacteria that were present in high amount in the studied aquifer. Furthermore, *etnC* gene in the environmental DNA was present in the same amount as reported by Liu et al. (2016) in a different contaminated site affected by different remediation techniques. *etnC* gene copies number mL^{-1} was generally high, but it was lower than the number of degrading bacteria obtained by MPN method. This might be explained with the fact that bacteria grown in MPN microcosms could possess catabolic genes different than *etnC*, or that the primers used in the present study might not encopass all the different sequence variants present in the microbial population. During the time course of the biostimulation treatment with the addition of air and mineral nutrients at the aerobic bio-barrier, the *etnC* gene copies increased in most of studied piezometers as previously reported in Davis et al., 2009 and in Kurt et al., 2014 at microcosms scale. In conclusion, aquifer showed an autoctone VC degradation activity that can be efficacy improved with the ongoing stimulation treatments.

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SUPPLEMENTARY MATERIALS

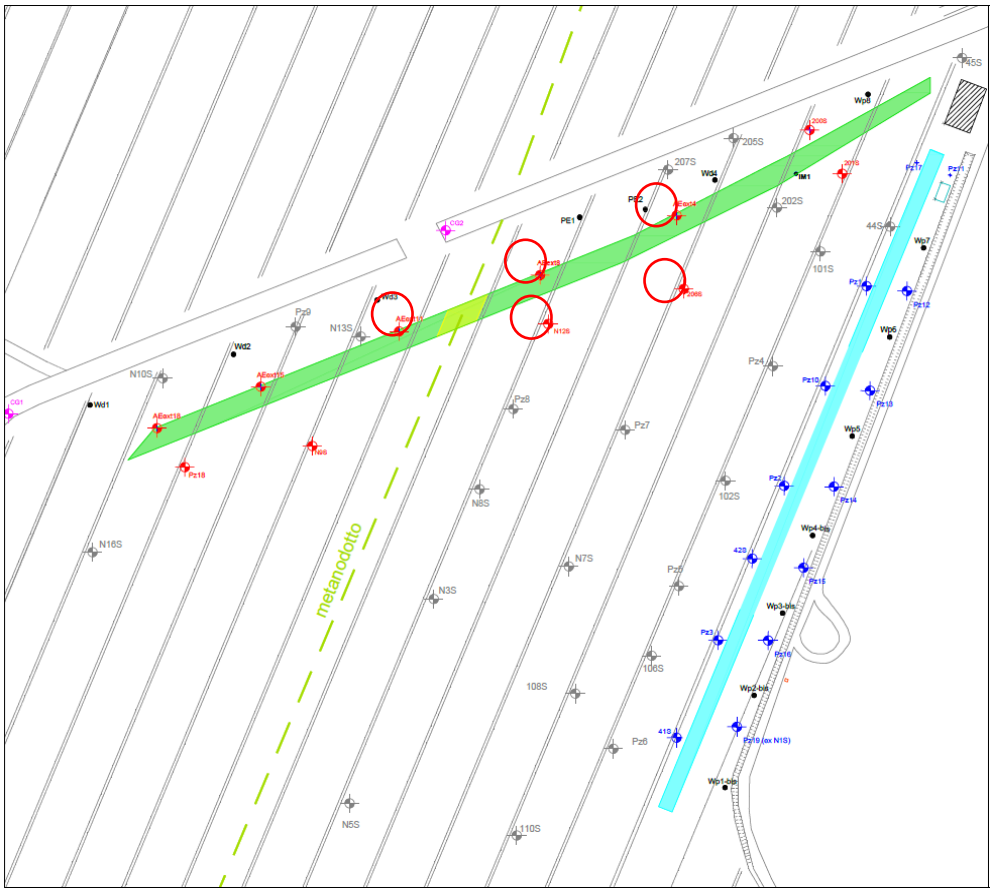

Figure S13 Site map with anaerobic biobarrier (light blue) and aerobic biobarrier (green). In red circle five analyzed monitoring piezometers are highlighted.

STANDARD CURVE SET UP

Plasmids were constructed followed TOPO®TA Cloning® Kit (Invitrogen) protocol. 27f and 1492r, and JS614-etnCF and JS614-etnCR primers (Table S1) were used to amplified total bacteria 16S rRNA and *etnC,* respectively. Taq PCR Master Mix (QIAGEN) was used for the amplification of both target in a final volume of 25 μL with following program. Eubacteria: 15 min at 95°C, 36 cycles of 45 sec at 95°C, 55°C for 45 sec and 72°C for 75sec, followed by

10 min at 72°C. *etnC*: 3 min at 95°C, 35 cycles of 95°C for 30 sec, 30 sec at 68°C and 1 min at 72°C, followed by 10 min at 72°C.
Plasmids were extracted with UltraClean™ 6 min Mini Plasmid Prep Kit™ (MO BIO) and quantified through spectrophotometry.

Table S8 Primers used for PCR of the standard curve plasmids insert and for qPCR

Target gene	Primers	Sequences	Product size (bp)	References
16S rRNA	**27f**	AGAGTTTGATCMTCGTCCCTC	1465	Edwards et al, 1989
	1492r	TACGGYTACCTTGTTAGGCTT		Lane et al, 1991
	Eub338f	ACT CCT ACG GGA GGC AGC AG	180	Fierer et al., 2005
	Eub518r	ATT ACC GCG GCT GCT GG		
etnC	**JS614-etnCF**	GCGATGGAGAATGAGAAGGA	1138 bp	Jin and Mattes, 2010
	JS614-etnCR	TCCAGTCACAACCCTCACTG		
	RTC-f	ACCCTGGTCGGTGTKSTYTC	106 bp	Jin and Mattes, 2010
	RTC-r	TCATGTAMGAGCCGACGAAGTC		

Table S9 Chemical data of piezometers near aerobic biobarrier. Italian law limits of each chloroethene were reported.

			Law Limit D.Lgs. 152/06	Dec 19	Jun 20	Sept 20
206S	PCE	µg L^{-1}	1.1	0.27	0.11	0.45
	TCE	µg L^{-1}	1.5	0.40	0.15	0.48
	1,2 DCE	µg L^{-1}	60	570.00	79.00	270.00
	1,1 DCE	µg L^{-1}	0.05	0.24	0.15	< RL
	VC	µg L^{-1}	0.5	39'000.00	10'400.00	9'400.00
	DO*	mg L^{-1}		0.22	0.00	0.19
	Eh*	mV		-21.00	-96.60	-241.70
	Temperature	°C		15.09	14.68	15.82
	pH	-		6.87	6.75	6.74
AEext4	PCE	µg L^{-1}	1.1	7.70	0.31	2.99
	TCE	µg L^{-1}	1.5	25.70	4.30	12.10
	1,2 DCE	µg L^{-1}	60	220.00	44.00	22.00
	1,1 DCE	µg L^{-1}	0.05	43.00	42.00	< RL
	VC	µg L^{-1}	0.5	17'100.00	12'400.00	15'100.00
	DO*	mg L^{-1}		0.99	0.96	1.05
	Eh*	mV		-145.00	-170.00	-187.00
	Temperature	°C		15.03	15.66	15.93

	pH	-		6.79	6.74	6.67
N12S	PCE	µg L^{-1}	1.1	7.10	1.27	5.80
	TCE	µg L^{-1}	1.5	18.40	3.90	15.50
	1,2 DCE	µg L^{-1}	60	800.00	89.00	340.00
	1,1 DCE	µg L^{-1}	0.05	13.90	3.40	13.80
	VC	µg L^{-1}	0.5	20'600.00	4'200.00	4'300.00
	DO*	mg L^{-1}		0.25	0.00	0.06
	Eh*	mV		-154.00	-164.30	-158.70
	Temperature	°C		14.88	14.97	15.22
	pH	-		6.78	6.87	6.76
AEext8	PCE	µg L^{-1}	1.1	3.40	0.39	8.50
	TCE	µg L^{-1}	1.5	21.40	1.23	12.70
	1,2 DCE	µg L^{-1}	60	91.00	1.80	36.00
	1,1 DCE	µg L^{-1}	0.05	17.50	0.47	5.20
	VC	µg L^{-1}	0.5	2'950.00	82.00	2'070.00
	DO*	mg L^{-1}		0.05	1.80	0.67
	Eh*	mV		-9.00	-108.00	-138.00
	Temperature	°C		15.20	15.61	14.94
	pH	-		6.82	7.08	6.72
AEext11	PCE	µg L^{-1}	1.1	3.10	0.99	3.30
	TCE	µg L^{-1}	1.5	12.90	6.10	23.40

1,2 DCE	µg L^{-1}	60	48.00	6.80	16.00
1,1 DCE	µg L^{-1}	0.05	0.24	0.23	0.36
VC	µg L^{-1}	0.5	2'350.00	1'210.00	530.00
DO*	mg L^{-1}		0.59	3.08	2.64
Eh*	mV		-90.00	-120.00	-150.00
Temperature	°C		15.29	14.57	15.16
pH	-		7.01	7.86	6.93

*DO= dissolved oxygen, Eh=Redox potential

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CHAPTER 3

AROMATIC COMPOUNDS BIODEGRADATION IN A CONTAMINATED AQUIFER SITE

Authors: Martina Bertolini[1], Sarah Zecchin[1], Lucia Cavalca[1]

[1] Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente (DeFENS), Università degli Studi di Milano Via Celoria 2, I-20133 Milano, Italy

ABSTRACT

Presence of aromatic hydrocarbons in the environment is a global issue. Their release in ecosystem is mainly due to vehicle exhausts and industrial activity emissions. Several bacteria are known to be able to mineralize these compounds in aerobic conditions through different oxidation steps catalyzed by different oxygenase enzyme systems (e.g., toluene/benzene monooxygenase, *tbmD*). For this reason, bioremediation techniques that rely on bacterial metabolism are considered efficient methods to remediate contaminated site. This study was focused on the determination of BTEX and chlorobenzene biodegradation in contaminated aquifer in microaerobic conditions. Chemical, microbial and molecular analysis were used in order to analyzed oxidation of these compounds by microbial community present in contaminated area. Bacteria were isolated from the aquifer, identified and tested for their ability to degrade BTEX and chlorobenzene. *tbmD* coding for a toluene/benzene monooxygenase was used as biomarker to detect the degrading pathway both within the aquifer and in bacterial isolates.

INTRODUCTION

BTEX (Benzene, Toluene, Ethylbenzene and Xylenes) and chlorobenzene are monoaromatic Volatile Organic Compounds (VOCs). They are widespread contaminant compounds: in 2011, BTEX were responsible of 15% of all contaminated groundwaters in Europe (Van Liederkerke et al., 2014). They are widely used as solvents and in petrochemical production, indeed they are present in many petroleum products. The cause of their presence in water and soil is due to accidental spills. Contamination of air by these compounds is caused by industrial activity emissions and vehicle exhausts (El-Naas et al, 2014). Because of their volatility and their high solubility, BTEX are very dangerous contaminants for environment.

Benzene is carcinogenic, included in group 1 of AIRC Monographs. Toluene affects central nervous system and in particular respiratory tract, due to the fact that main exposure of this compounds is through inhalation (ATSDR, Georgia). Chlorobenzene can determine disturbances of central nervous system.

BTEX and chlorobenzene can be efficiently degraded by microorganisms in anaerobic and aerobic conditions. In aerobic conditions, aromatic ring is oxidated by a monooxygenase or dioxygenase (Kahng et al, 2001; Furukawa et al., 1993) becoming a catechol structure. Then, catechol aromatic structure is linearized to protocatechuic acid, that enters into Krebs cycle intermediates (Harayama et al, 1993). In toluene and benzene degradation pathway, the monnoxygenase of the first step is Toluene/Benzene-2-Monoxygenase (TBMD) (Johnson et al., 1995). Even if, this degradation pathway is mainly met in aerobic conditions, there is different example of hydrocarbons degradation in hypoxic conditions, with an oxygen concentration of 2 mg L^{-1} or less (Kukor et al., 1996).

Pseudomonas strains are frequently identified in oil refineries for their ability to degrade BTEX (Di Martino et al., 2012) and chlorobenzene (Wenderoth et al., 2003).

Because of the toxicity of these compounds for human and opportunity to use microorganisms to make them harmless, in this study, an aquifer affected by mixed contamination was examined to analysis degradation of these compounds at filed scale through microbiology and molecular techniques.

MATERIALS AND METHODS

Field site description and sampling

Contaminated site is in Dogaletto (Venice, North Italy). In the area, a former landfill for the collection of industrial waste from the near petrochemical plant of Porto Marghera is present. The landfill surface covers about 16 hectares with a total waste mass of 1'700'000 tons. The area is delimited by the Venice lagoon to the South-side and by an irrigation channel to the North-side (Figure S1). Through drainage pump system an adequate water level is provided inside the channel.

The underlying groundwater is affected by continuous landfill leaching. Contamination is mainly composed by chlorinated compounds, hydrocarbons and BTEX.

In order to remediate the area, until 2016, a hydraulic barrier pump and treat (P&T) method was used. In 2016, two permeable reactive barriers were set up to assist P&T method and then replace it (Figure S1). The anaerobic barrier is about 390 m long and 10 m deep with 39 wells for water recirculation and stimulation substrates. The aerobic barrier is about 500 m long and 10 m deep, with 53 recirculation wells and 43 injection wells that allow addition of urea, ammonium phosphate and O_2 to stimulate microbial aerobic degradation.

In six sampling campaigns, between May 2016 and September 2020, five monitoring piezometers (N12S, 206S, AEext4, AEext8 and AEext11) near aerobic barrier were sampled for chemical and microbiological analysis. Groundwater for classical microbiological analysis was sampled at two profundity: one 5 meters from field plan (superficial) and the other one 1.5

meters from the bottom of the well (deep). The piezometers were located two upstream the barrier and two downstream, coupled in transects (Figure 1). Sampled groundwater was stored in tanks in cooler bag in the dark and stored in laboratory at 4°C until filtrations.

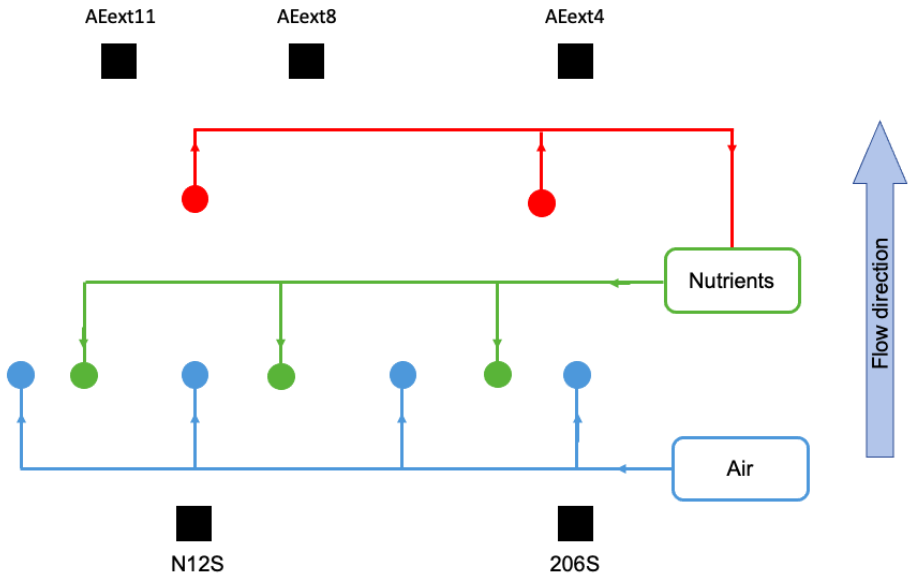

Figure 14 Monitoring piezometers and extraction and injection wells positions in aerobic barrier

Chemical Analysis

Groundwater was sampled following equilibrium based static headspace preparation (EPA5021A 2014 method) in order to quantify benzene, toluene, chlorobenzene and total hydrocarbons. Analysis were performed by AGROLAB Group. In order to quantify benzene, toluene and chlorobenzene gas chromatography mass spectrometry (GC-MS) was used following EPA8260D 2018 method for volatile organic compounds. Total hydrocarbons quantification was carried out with EPA8015C 2007 method through gas chromatography with flame ionization detector (GC-FID).

Most Probable Number (MPN)

Degrading bacteria present in each piezometer in different times were quantified through Most Probable Number (MPN). Three compounds were tested: toluene, benzene and chlorobenzene.

In 50 mL vial, 9 mL of mineral medium M9 were added with 1 mL of groundwater after a serial dilution (from 10^{-1} to 10^{-6}) in triplicate for each dilution. Compounds were added with a concentration of 400 ppm. For each piezometer, control vials were set up without any compounds. Vials were closed with Teflon stoppers and crimp caps and incubated at 30°C for 30 days. After 30 days, growth turbidity was detected, and quantification of degrading bacteria were estimated through MPN table for three replicates (McCrady 1918).

Enrichments culture set up

Groundwater of AEext4, AEext8 and AEext11 piezometers of January 2018 was used to set up enrichment cultures on three different compounds: benzene, toluene and chlorobenzene. In 500 mL bottles with Teflon cap, 100mL of mineral medium M9 (Kunz et al., 1981) were added with 100mL of groundwater and contaminant molecule with a concentration of 400ppm, three for each piezometers and molecules. Control bottles without compounds were prepared. Cultures were incubated at 30°C.

After 14 days, 10 mL of culture were transferred in new bottle with 90 mL of mineral medium M9. A third transfer was carried out after 14 days.

Strains isolation and identification

In September 2017, groundwater of all six piezometers were plated on Tryptic Soy Agar (TSA) (Difco, Detroit, USA). Isolated strains were tested for their ability to growth on toluene as sole carbon source in microtiter plates with mineral medium M9 and a toluene concentration of 300ppm. Toluene degrading strains were identified through 16S rRNA sequencing. From each isolated strain, DNA were extracted through Ultraclean Microbial DNA

Isolation Kit (MOBIO) according protocol. Then, 16S rRNA were amplified with primers 27f (Edwards et al., 1989) and 1492r (Lane et al., 1990) (Table S1) in concentration of 0.6 µM. PCR reaction was performed with 1x TAQ PCR Master Mix (QIAGEN) in 25 µL of total volume with 2 µL of DNA. PCR thermocycler conditions were: 15 min at 95°C, 36 cycles of 45 sec at 95°C, 55°C for 45 sec and 72°C for 75sec, followed by 10 min at 72°C. PCR products were sequenced by GATC (Germany) through Sanger sequencing method. NCBI (National Center for Biotechnology Information) 16S database BLAST (Basic Local Alignment Search Tool) program was used for taxonomic identification of 16S rRNA.

In January 2018, strains were isolated from AEext11 toluene enrichment cultures on TSA 0.1X plates. Plates were incubated at 30°C.

Isolated strains were tested for their growth ability on four molecules; toluene and three other compounds present in high concentrations in the aquifer: benzene, chlorobenzene and n-hexane. The last one was chosen in order to test the ability of bacteria in aerobic degradation of aliphatic compounds. Even in this case 16S rRNA were amplified for each isolated strain and then sequenced through Sanger sequencing method (GATC (Germany)).

All isolated strains were tested for the presence of gene *tbmD* through PCR. In total volume of 25µL 1x TAQ PCR Master Mix (QIAGEN) was used with primers TBMDf and TBMDr (Table S1) at 0.6 µM concentration and 2 µL of DNA. PCR program: initial denaturation for 15 min at 95°C. 40 cycles of 1 min at 95°C, 40 sec at 58°C, and 20 sec at 72°C.

Environmental DNA extraction and real time quantitative PCR

20L of groundwater from piezometers N12S, 206S, AEext4 and AEext8 for all sampling time was filtrated on mixed cellulose ester filters (MediaKap™ ME2M-050-18S ø 0.2 µm) (Cole-Parmer, USA) using peristaltic pump apparatus (Masterflex L/S Economy Variable-Speed Drive, 20 to 600 rpm with Masterflex L/S Easy-Load Head for High-Performance Tubing, PSF/CRS) (Cole-Parmer, Vernon Hills, Illinois, Stati Uniti). Filters were stored at -20°C

until DNA isolation. DNA was extracted from the pellet using the DNA PowerSoil® Isolation kit (Qiagen; Germany) in triplicate for each piezometer. Nucleic acids were quantified by using Spectrophotometer Power Wase XS2 (BioTEK Instruments, US).

Real time quantitative PCR was used to quantify 16S rRNA genes of total bacteria and *tbmD*. Each reaction mixture contained 1x of Titan HotTaq Probe qPCR Mix (Bioatlas science of life, Estonia), 0.3 µM of forward and reverse primers (Table S1), 1 µL of DNA and PCR-grade water (AppliChem, Germany) to a final volume of 20 µL. Eubacteria qPCR program: 15 min at 90°C, 40 cycles of 95°C for 1 min, 53°C for 40 sec and 72°C for 1 min, followed by 1 min at 72°C. Melting curve from 53°C to 95°C with an increment of 1.6°C/sec for 5 sec. *tbmD* qPCR program was the same of PCR one with addition of the melting curve: from 58°C to 95°C with an increment of 1.6°C/sec for 5 sec. For absolute quantification standard curve was set up with amplification of plasmid carrying a target of each gene (SM).

Statistical analysis

qPCR data were analyzed through base and accessory packages of R program (R Core Team, 2015). Analysis of variance (aov) (Chambers et al., 1992) TukeyHSD (Miller, 1981; Yandell, 1997) were used to determine significant differences ($p \leq 0.05$) between different piezometers and treatment effects during the time.

Amplification of environmental TBMD and cloning

*tbm*D was amplified from environmental DNA extracted from AEext4 and AEext8 in January 2018. PCR was performed in total volume of 25 µL with 1x TAQ PCR Master Mix (QIAGEN) and 0.6 µM primers TBMD-f and TBMD-r (Hendrickx et al., 2006). PCR program starts with denaturation time of 3 min at 95°C, 32 cycles of 95°C for 60 sec, 58°C for 60 sec and 72°C for 2min, then elongation time of 10 min at 72°C.

PCR products were cloned in pCR™2.1-TOPO® vector according to TOPO®TA Cloning® Kit (Invitrogen) protocol. Plasmids were extracted with UltraClean™ 6 min Mini Plasmid Prep Kit™ (MO BIO). Then, 16 *tbmD* plasmids were sequenced through Sanger sequencing method by GATC (Germany). BLAST similarity searching based on NCBI database was used to compare obtained sequences with those already present in GenBank database.

RESULTS

Groundwater chemical data

Aquifer in aerobic barrier showed a constant pH in all piezometers, between 6.4 and 7.5 (Table S2). Even if, in this part of the site, aerobic degradation pathways were stimulated, oxygen concentration in groundwater was low, under 1 mg L^{-1} expect in piezometer AEext8 and in AEext4 and AEext11 at the last times. Nevertheless, downstream the barrier, from December 2019, oxygen concentration increase was achieved. Furthermore, aquifer was in reductive conditions as it was possible to see from reduction potential (Eh) values, negative in most of the times (Table S2).

Total hydrocarbons were the compounds that were present in higher amount. Chlorobenzene was present in high concentrations especially in N12S (Figure 2). Instead, toluene concentration was in low amount in all piezometers. In 206S and AEext4 piezometers, compounds concentrations were similar. On the other hand, all the compounds were higher in N12S piezometer upstream aerobic barrier than in AEext8 and AEext11 piezometers that are placed downstream the barrier.

Degradation activity of microbial community

Degrading bacteria of chlorobenzene, benzene and toluene were present in low amount in the aquifer. MPN mL^{-1} were around from 10^0 to 10^1 in superficial

and depth (1.5 from bottom of the well) areas (Table 1). Higher values were present in September 2017. In the other times, a decrease took place for all the molecules in all piezometers. In the first two sampling times, September 2017 and January 2018, degradation activity was different among piezometers. Instead, when MPN values decreased, also the difference among piezometers decreased. Chlorobenzene degrading bacteria showed an equal distribution in the two examined areas. The same was true also for benzene degrading bacteria. Only in December 2019, they were present in different amount in the two areas. In AEext8 bacteria were present in higher amount in superficial zone but in AEext11 bacteria higher amount was in depth zone. In the case of toluene, degrading bacteria were present in superficial part of the aquifer in higher or equal number than in depth area.

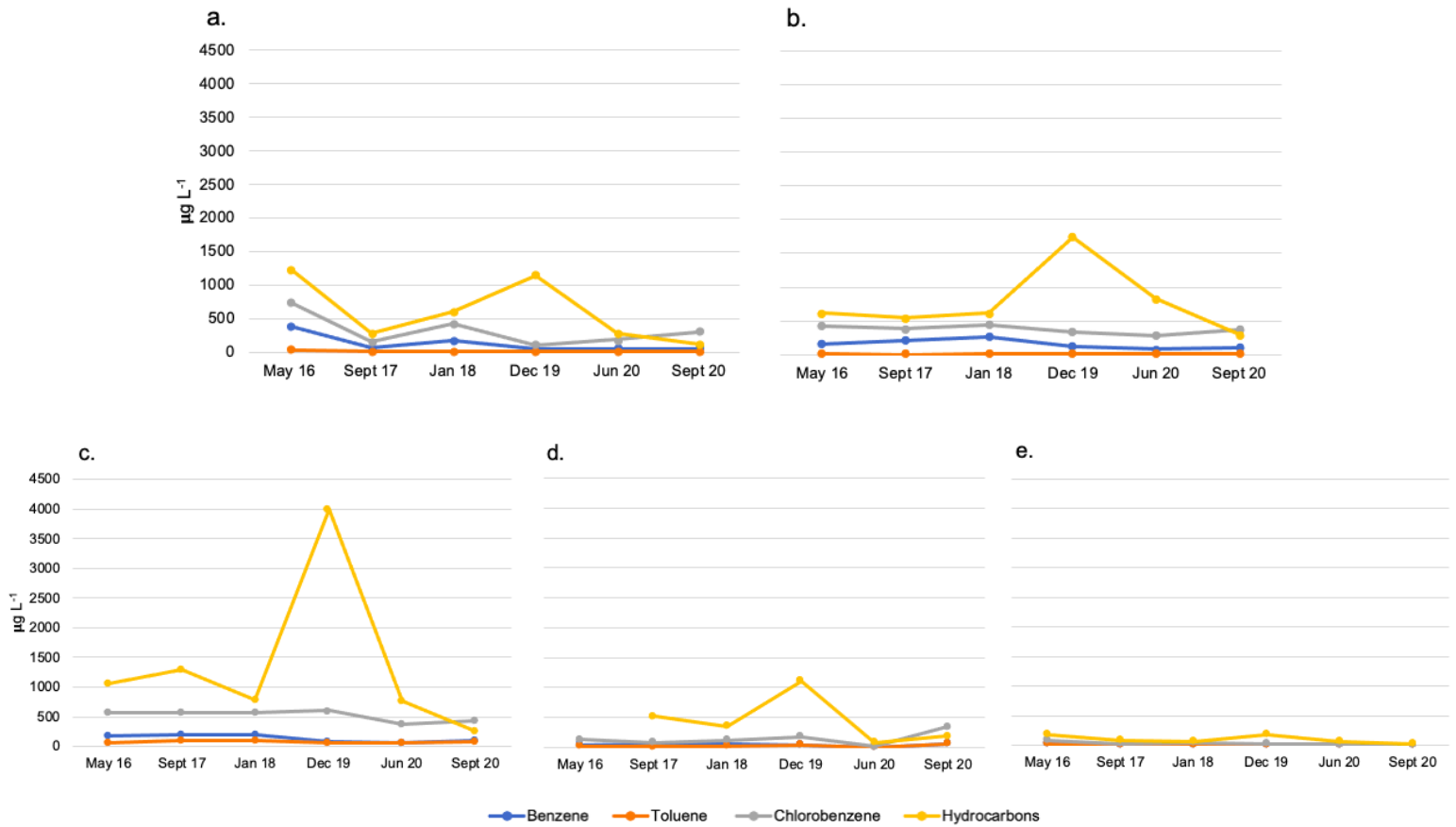

Figure 15 Benzene, toluene, chlorobenzene and total hydrocarbon concentrations present in aerobic barrier from May 2016 to September 2020. 206S (a), AEext4 (b), N12S (c), AEext8 (d) and AEext11 (e).

Table 1 Quantification of degrading bacteria of benzene, toluene and chlorobenzene (400 ppm) through MPN method (MPN mL^{-1})

Sampling	Compounds	AEext4		AEext8		AEext11	
		Superficial 5 m f.p.	Depth 1.5 b.w.	Superficial 5 m f.p.	Depth 1.5 b.w.	Superficial 5 m f.p.	Depth 1.5 b.w.
Sept 2017	Benzene	n.d.	$2.50x10^{1}$	n.d.	$4.50x10^{1}$	n.d.	$9.50x10^{1}$
	Toluene	n.d.	$4.50x10^{1}$	n.d.	$9.50x10^{0}$	n.d.	$4.50x10^{1}$
	Chlorobenzene	n.d.	$2.50x10^{0}$	n.d.	$2.00x10^{0}$	n.d.	$4.50x10^{1}$
Jan 2018	Benzene	n.d.	$9.50x10^{0}$	n.d.	$1.50x10^{1}$	n.d.	$9.50x10^{0}$
	Toluene	n.d.	$1.50x10^{0}$	n.d.	$7.50x10^{0}$	n.d.	$2.00x10^{0}$
	Chlorobenzene	n.d.	$4.50x10^{0}$	n.d.	$9.50x10^{0}$	n.d.	$4.00x10^{1}$
Dec 2019	Benzene	$2.50x10^{0}$	$2.50x10^{0}$	$4.50x10^{0}$	$2.50x10^{0}$	$2.50x10^{0}$	$4.50x10^{0}$
	Toluene	$2.50x10^{0}$	$2.50x10^{0}$	$1.50x10^{1}$	$0.90x10^{1}$	$4.50x10^{0}$	$2.50x10^{0}$
	Chlorobenzene	$2.50x10^{0}$	$2.50x10^{0}$	$0.00x10^{0}$	$0.00x10^{0}$	$9.00x10^{-1}$	$2.50x10^{0}$
Jun 2020	Benzene	$2.50x10^{0}$	$2.50x10^{0}$	$2.50x10^{0}$	$0.00x10^{0}$	$0.00x10^{0}$	$0.00x10^{0}$
	Toluene	$2.50x10^{0}$	$2.50x10^{0}$	$2.50x10^{0}$	$0.00x10^{0}$	$0.00x10^{0}$	$0.00x10^{0}$
	Chlorobenzene	$2.50x10^{0}$	$2.50x10^{0}$	$2.50x10^{0}$	$0.00x10^{0}$	$2.50x10^{0}$	$0.00x10^{0}$
Sept 2020	Benzene	$1.25x10^{0}$	$0.83x10^{1}$	$0.83x10^{1}$	$0.00x10^{0}$	$0.83x10^{1}$	$0.83x10^{1}$
	Toluene	$1.25x10^{0}$	$0.83x10^{1}$	$0.83x10^{1}$	$0.00x10^{0}$	$0.83x10^{1}$	$0.83x10^{1}$
	Chlorobenzene	$1.25x10^{0}$	$0.83x10^{1}$	$0.83x10^{1}$	$0.00x10^{0}$	$0.83x10^{1}$	$0.83x10^{1}$

f.p.= from filed plan
b.w.=from bottom of the well

Bacterial growth on BTEX and chlorobenzene

Aquifer microbial community sampled in January 2018 grew better in toluene presence (Table2). Benzene was the molecule that was hardly used as carbon source by microbial community. Between the three piezometers, AEext11 showed a better degrading activity for all three molecules. AEext4 did not growth on benzene and it showed a mild growth on toluene. In AEext11, sequential transfers improved microbial growth. On the other hand, in AEext4 and AEext8, microbial community was positively affected by transfers.

Table 2 Bacterial growth determination in enrichment cultures.

Sample	Transfer	Benzene	Toluene	Chlorobenzene
AEext4	I	-	+/-	+
	II	-	-	+/-
	III	-	-	-
AEext8	I	+/-	+	+/-
	II	-	+/-	-
	III	-	+/-	-
AEext11	I	+	+	+
	II	+	++	+
	III	+	++	+

Characterization of isolated strains

147 strains were isolated from TSA plates and 18 of them was able to use toluene as sole carbon source (Table 3). The most represented genus was *Rhodococcus*. Indeed, 10 strains belonged to this genus. *Pseudomonas* genus was identified in 4 strains. Even if these strains can grow on toluene as sole carbon source, only *Pseudomonas veronii* strain R02 has Toluene/Benzene-2-Monoxygenase in its genome.

Toluene AEext11 enrichment culture was selected because it showed a better growth with respect the other piezometers on all tested molecules. 28 strains were isolated. The most represented species was *Arthrobacter oryzae strain KV-651* with 7 strains (Table 4).

Only 3 species had *tbmD* even if other species were able to growth on toluene and benzene. Not all bacteria isolated from toluene enrichment culture were able to growth on this molecule when they were isolated (Table 4).

Brevibacterium casei NCDO 2048, Nocardia coeliaca strain DSM 44595 and *Pseudomonas reidholzensis* strain ID3 could efficiently growth on all four molecules. *Lysinibacillus fusiformis* strain NBRC 15717, *Microbacterium ginsengiterrae* strain DCY37 and *Staphylococcus* strain ATCC 15305 could not growth on any molecules. 7 strains can growth on benzene, and 5 on toluene. Bacteria that could degrade toluene cannot always degrade also benzene. Chlorobenzene could be used as sole carbon source by 4 strains. 6 strains could degrade aromatic compounds but also aliphatic compounds, in this case n-hexane (*Arthrobacter, Brevibacterium, Microbacterium, Nocardia* and *Pseudomonas).*

In the two isolation steps, there were some genera present in both steps: *Rhodococcus*, *Pseudomonas*, *Microbacterium* and *Staphylococcus.*

Table 10 Identification of isolated strains from aquifer in aerobic barrier. It was reported the capacity of growth with toluene as sole carbon source, the presence of tbmD gene in genome and the number of strains beloved to single species (N°).

GenBank	Identification	Toluene	*tbmD*	N°
KY020327	*Rhodococcus* sp. strain OB0511_154-F5	+	-	6
KY945839	*Rhodococcus* sp. strain HBUM200062	+	-	4
KP257571	*Pseudomonas stutzeri* strain ss1	+/-	-	3
AJ313025	*Cellulomonas* sp. MN 60.3	+	-	1
KX665599	*Microbacterium* sp. strain P1	+	-	1
KP980625	*Micrococcus luteus*	+	-	1
CP018420	*Pseudomonas veronii* strain R02	+	+	1
AB735691	*Staphylococcus equorum* strain: NCCP-722	+	-	1

Table 11 Identity determination of isolated strains from toluene enrichment culture. It was reported the capacity of growth with benzene, toluene, chlorobenzene and n-hexane as sole carbon source, the presence of tbmD gene in genome and the number of strains beloved to singol species (N°).

Accession number	Species	Benzene	Toluene	Chlorobenzene	n-hexane	*tbmD*	*N°*
NR_041545.1	*Arthrobacter oryzae strain KV-651*	+/-	+	-	+/-	+	7
NR_041996.1	*Brevibacterium casei DSM 20657*	+/-	-	-	-	-	5
NR_156954.1	*Microbacterium tumbae strain T7528-3-6b*	+/-	-	-	-	-	5
NR_119071.1	*Brevibacterium casei NCDO 2048*	+	+	+	+	-	2
NR_117266.1	*Microbacterium suwonense strain M1T8B9*	-	-	+/-	+/-	-	2
NR_112569.1	*Lysinibacillus fusiformis strain NBRC 15717*	-	-	-	-	-	1
NR_116483.1	*Microbacterium ginsengiterrae strain DCY37*	-	-	-	-	-	1
NR_104776.1	*Nocardia coeliaca strain DSM 44595*	+	+	+	+	-	1
NR_114911.1	*Pseudomonas extremaustralis strain 14-3*	+	-	-	+	+	1
NR_157777.1	*Pseudomonas reidholzensis strain ID3*	+	+	+	+	+	1
NR_115708.1	*Rhodococcus jialingiae strain djl-6-2*	-	+	-	-	-	1
NR_074999.2	*Staphylococcus saprophyticus strain ATCC 15305*	-	-	-	-	-	1

Functional biomarker (*tbmD*) quantification

Total bacteria gene copies in aerobic barrier were around 10^5 to 10^8 gene copies mL^{-1} (Figure 3.a-b). AEext8 eubacterial 16S rRNA copies were higher than in N12S piezometers upstream the barrier. In general, AEext8 showed higher amount of this gene target copies than the other piezometers. Between 206S and AEext4 there were some relevant differences, but they were not characteristic of position in the site.

tbmD amount showed more variability. Indeed, range went from 10^0 to 10^5 gene copies mL^{-1} (Figure 3.c-d). Treatment effects could be seen only in the last times in AEext8 piezometers.

For both genetic targets, there was not a linear trend during the time. Indeed, gene copies showed statistically relevant fluctuation with a clear increase in December 2019.

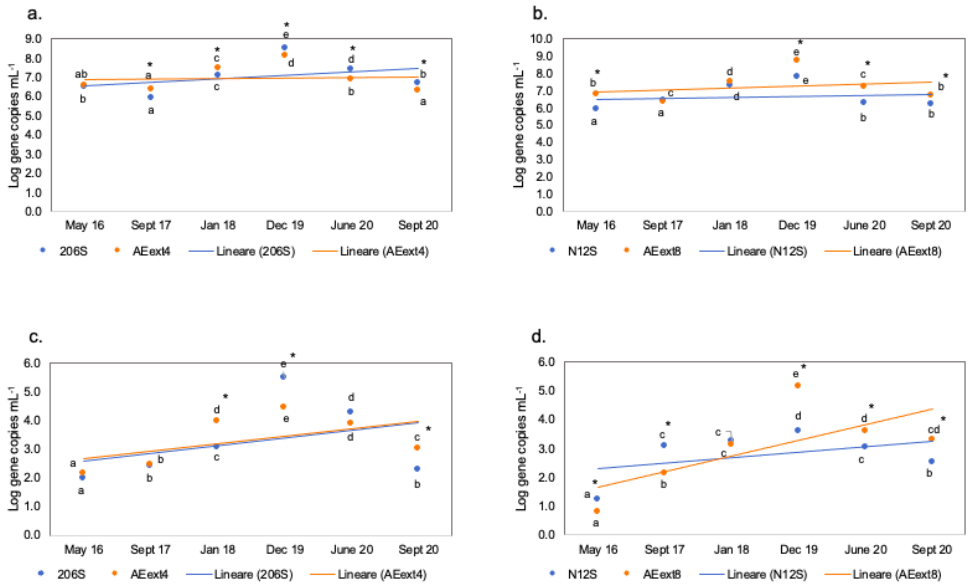

*Figure 16 Gene copy abundance of total bacteria (a, b) and tbmD (c, d) in aerobic barrier during the time in piezometers 206S and AEext4 (a, c), and N12S and AEext8 (b, d). * indicates significant difference between piezometers of transect upstream and downstream the aerobic barrier. Lowercase letters indicate significant or not significant difference between different time (Tukey's test, $\rho \leq 0.05$).*

Environmental *tbmD* Identification by clone library

All analyzed sequences showed homologies with a monooxygenase/hydroxylase involved in aromatic hydrocarbons reactions (toluene/benzene and phenol) (Table 5).

2 sequenced *tbmD* showed a high similarity to *tbmD,* belonged to specie that we isolated before: *Pseudomonas veronii* strain R02. 5 sequences were successfully aligned with *Pseudomonas* genus enzyme, in two different species: *P. mendocina* strain PC12 and *P. veronii* strain R02. *Cupriavidus metallidurans* CH34 *tbmD* was the most frequent homologue sequence. Indeed, 6 sequences had similarity of 79% to that monooxygenase. The other *tbmD* sequences were recognized as hydroxylase of *Pseudoxanthomonas* sp. BD-a59 and *Delftia* sp. Strain LCW; respectively, 3 and 2 amplification products.

Table 12 genus to which belong sequenced tbmD in identity order

	Identity %	Access Number	N°	References
Pseudomonas mendocina strain PC12 phenol hydroxylase large subunit gene	99	AY875732.1	3	Heinaru et al., 2000
Pseudoxanthomonas sp. BD-a59 isolate TBMD hydroxylase alpha subunit	90	EU734588.1	*3*	*Unpublished*
Delftia sp. Strain LCW phenol hydroxylase large subunit gene	85	MF804845.1	*2*	*Unpublished*
Cupriavidus metallidurans CH34, complete genome	79	CP000352.1	*6*	*Unpublished*
Pseudomonas veronii strain R02 chromosome, complete genome (phenol 2-monooxygenase)	78	CP018420.1	2	Montes Vidal et al., 2017

DISCUSSION

BTEX degradation activity

According MPN data, in aquifer was present a degradation activity also at low concentration of oxygen in accordance with a previous study (Kukor et al., 1996). In particular, degrading bacteria were higher in September 2017 and January 2018 in all piezometers. The amount of degrading bacteria was not different in the superficial and depth area of aquifer. MPN data were not correlated with chemical data. Indeed, even if in AEext4 concentration of chlorobenzene was higher in September 2017, chlorobenzene degrading bacteria were higher in AEext11. Furthermore, degrading bacteria decrease of all compounds was not followed by a substantial decrease of compounds concentrations in aquifer.

In all times, *tbmD* quantification showed a higher value than toluene/benzene degrading MPN bacteria value. This suggested that not all bacteria with *tbmD* could growth in synthetic mineral medium in MPN vials. *tbmD* quantification values did not follow fluctuations of MPN data.

Higher amount of *tbmD* was present in December 2019 for all piezometers, but at that time, benzene/toluene degrading bacteria amount (MPN mL^{-1}) was lower than previous times. This suggested the presence of other microbial pathways for the benzene and toluene degradation.

BTEX degrading bacteria

Data reached from isolation of strains from groundwater and toluene enrichment culture showed that *tbmD* gene was not present in all bacteria that can growth on toluene as sole carbon source. Therefore, it can be assumed the presence in the aquifer of other metabolic pathways involved in toluene degradation. Bacteria (*Lysinibacillus fusiformis strain NBRC 15717, Microbacterium ginsengiterrae strain DCY37* and *Staphylococcus strain ATCC 15305)* that after isolation could not growth on any selected compounds suggested the presence in enrichment culture of bacteria that had a role in bacterial community different from direct degradation activity.

Even if the two groups of isolated strains came one from groundwater and the second one from a toluene enrichment culture, the isolated strains shared similar genera (*i.e. Rhodococcus*, *Pseudomonas*, *Microbacterium* and *Staphylococcus*).

Rhodococcus, *Pseudomonas, Microbacterium, Arthrobacter* and *Brevibacterium* are genera well known as BTEX degrading bacteria (Cavalca et al., 2004; El-Naas et al., 2014; Feng et al., 2020). *Staphylococcus* is not a characteristic BTEX degrading bacterium, but it is known as phenol degrading one (Senthilvelan et al., 2014), aromatic molecule derived from benzene. Furthermore, sequence homology data of environmental DNA showed that *tbmD* sequences found at the site were monooxygenases with a high similarity to monooxygenase for phenol degradation. Gholami-Shiri et al. (2017) isolated *Lysinibacillus fusiformis* from oily sludge and it showed a degrading activity of BTEX and in particular toluene. *Micrococcus*, *Nocardia* and *Cellulomonas* were reported as petroleum degrading bacteria by Roy et al. (2002) and Logeshwaran et al. (2018).

The sequenced *tbmD* genes were similar to monooxygenase of *Pseudomonas*, *Pseudoxanthomonas, Delftia and Cupriavidus*. *Pseudoxanthomonas* sp. BD-a59 is the only known strain of this genus to show BTEX degrading activity also because of its difficulty to growth on mineral medium (Choi et al., 2013). The phenol hydroxylase of *Delftia* sp. strain LCW can catalise degradation of benzene (Vásquez-Piñeros et al., 2018). *Cupriavidus metallidurans* CH34 genome was sequenced (Janssen et al., 2010) and it was verified at genome level its BTEX degrading activity (Espinoza Tofalos et al., 2018).

CONCLUSIONS

In the aquifer, BTEX and chlorobenzene degrading microbial community was present, even if oxygen concentration was low. Degrading bacteria quantification (MPN) was not correlate with BTEX and chlorobenzene

concentrations in aquifer. Quantification of *tbmD* gene showed a different trend respect to cultivable degrading bacteria present, suggesting the presence of bacteria unable to growth in microcosms.

Pseudomonas and *Rhodococcus* were the main genus involved in BTEX degradation in the aquifer. Moreover, in the aquifer the genera involved in degradation of these compounds were well defined. Isolated *Brevibacterium, Nocardia* and *Pseudomonas* strains were able to degrade all tested compounds: benzene, toluene and chlorobenzene. The lack of correlation of *tbmD* and MPN data, and presence of isolated bacteria that can use toluene as sole carbon source without carrying *tbmD* allows to assume that in the aquifer different degradation pathways were present.

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SUPPLEMENTARY MATERIALS

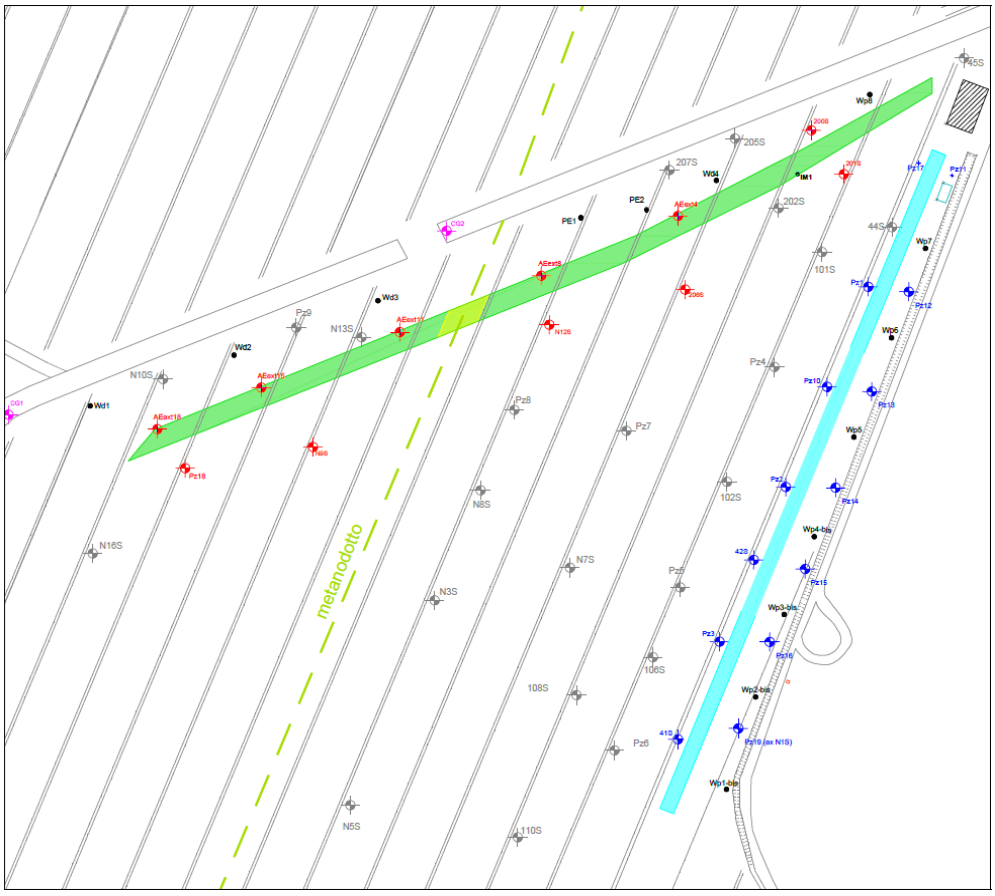

Figure S17 Site map

Construction of standard plasmids

Standard plasmids for the two gene target were constructed through TOPO®TA Cloning® Kit (Invitrogen). Genes were amplified with primer 27F-1492R, for total bacteria, and previous primers TBMD-f and TBMD-r, for functional gene. PCR was performed with 1x Taq PCR Master Mix (QIAGEN) to a final volume of 25μL, 0.6 μM forward and reverse primers. PCR programs used were the same of previous PCR. PCR products were cloned in pCR™2.1-TOPO® vector**.** Then, plasmids were extracted with UltraClean™ 6 min Mini Plasmid Prep Kit™ (MO BIO) and quantified through spectrophotometry.

208 *Table S1 Primers used in PCR and qPCR*

Target gene	Primers	Sequences	Product size (bp)	References
16S rRNA	**27f**	AGAGTTTGATCMTCGTCCCTC	1465	Edwards et al, 1989
	1492r	TACGGYTACCTTGTTAGGCTT		Lane et al, 1991
	Eub338f	ACT CCT ACG GGA GGC AGC AG	180	Fierer et al., 2005
	Eub518r	ATT ACC GCG GCT GCT GG		
tbmD	**TBMD-f**	GCCTGACCATGGATGCSTACCTGG	640	Hendrikcx et al, 2006
	TBMD-r	CGCCAGAACCACTTGTCRRTCCA		

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211 *Table S2 Chemical data*

			Law Limit D.Lgs. 152/06	May 16	Sept 17	Jan 18	Dec 19	Jun 20	Sept 20
206S	Benzene	µg L^{-1}	1	390	66.00	172	42.00	51.00	53.00
	Toluene	µg L^{-1}	15	32	6.80	14	5.50	12.70	10.40
	Chlorobenzene	µg L^{-1}	40	750	148.00	420	102.00	183.00	296.00
	Hydrocarbons	µg L^{-1}	350	1240.00	283.00	602.00	1'140.00	280.00	120.00

	DO*	mg L^{-1}	-	0.75	0.14	0.02	0.22	0.00	0.19
	Eh*	mV	-	-133	-118.00	-23.1	-21.00	-96.60	-241.70
	Temperature	°C	-	13.98	16.91	13.15	15.09	14.68	15.82
	pH	-	-	6.46	7.13	6.81	6.87	6.75	6.74
AEext4	Benzene	µg L^{-1}	1	164	204.00	253	123.00	80.00	109.00
	Toluene	µg L^{-1}	15	17.3	8.70	17.5	16.70	13.30	12.20
	Chlorobenzene	µg L^{-1}	40	420	380.00	440	330.00	285.00	370.00
	Hydrocarbons	µg L^{-1}	350	612.00	538.00	613.00	1'730.00	830.00	290.00
	DO*	mg L^{-1}	-	0.04	0.15	0.11	0.99	0.96	1.05
	Eh*	mV	-	-190	-63.40	-81.3	-145.00	-170.00	-187.00
	Temperature	°C	-	14.39	17.83	13.21	15.03	15.66	15.93
	pH	-	-	6.57	6.70	6.81	6.79	6.74	6.67
N12S	Benzene	µg L^{-1}	1	172	207.00	197	72.00	63.00	91.00
	Toluene	µg L^{-1}	15	62	92.00	92	62.00	68.00	86.00
	Chlorobenzene	µg L^{-1}	40	570	570.00	570	600.00	370.00	430.00
	Hydrocarbons	µg L^{-1}	350	1'060.00	1'290.00	785.00	4'000.00	770.00	260.00
	DO*	mg L^{-1}	-	1.18	0.05	0.01	0.25	0.00	0.06
	Eh*	mV	-	-273	-191.00	-63.8	-154.00	-164.30	-158.70
	Temperature	°C	-	14.52	17.05	13.77	14.88	14.97	15.22
	pH	-	-	6.89	6.82	6.78	6.78	6.87	6.76
AEext8	Benzene	µg L^{-1}	1	39	51.00	53	47.00	2.26	67.00

	Toluene	µg L^{-1}	15	21.5	14.90	19.9	36.00	1.55	61.00
	Chlorobenzene	µg L^{-1}	40	128	81.00	118	176.00	13.40	340.00
	Hydrocarbons	µg L^{-1}	350		524.00	356.00	1'110.00	79.00	188.00
	DO*	mg L^{-1}	-	1.2	2.03	1.81	0.05	1.80	0.67
	Eh*	mV	-	-242	-34.50	-4.3	-9.00	-108.00	-138.00
	Temperature	°C	-	14.32	20.45	13.21	15.20	15.61	14.94
	pH	-	-	6.8	7.16	7.32	6.82	7.08	6.72
AEext11	Benzene	µg L^{-1}	1	251	154.00	103	75.00	46.00	49.00
	Toluene	µg L^{-1}	15	253	68.00	65	43.00	38.00	34.00
	Chlorobenzene	µg L^{-1}	40	790	301.00	380	174.00	143.00	145.00
	Hydrocarbons	µg L^{-1}	350	1'780.00	820.00	616.00	1'790.00	650.00	200.00
	DO*	mg L^{-1}	-	0.02	0.59	0.49	0.59	3.08	2.64
	Eh*	mV	-	-101	-29.70	-41.2	-90.00	-120.00	-150.00
	Temperature	°C	-	14.28	19.97	14.04	15.29	14.57	15.16
	pH	-	-	6.5	6.76	6.86	7.01	7.86	6.93

212 *DO= dissolved oxygen, Eh=Redox potential

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GENERAL CONCLUSIONS

Bioremediation is an effective method in order to remediate contaminated area. In particular, the use of permeable reactive biobarriers has increased due to their low environmental impact and their low maintenance cost. Moreover, this technique is safer for operators because they do not come in direct contact with contaminants.

In the studied site organohalide respiration of chloroethenes in anaerobic conditions and degradation of vinyl chloride and BTEX in aerobic conditions were investigated.

In anaerobic conditions, a natural microbial community that was able to reductively dechlorinate chloroethenes was present in the aquifer. Moreover, biostimulation treatment based on the addition of reducing substrate improved OHR activity in groundwater.

Laboratory based experiments determined the natural attenuation potential of aquifer microbial community and forecasted its possible increment via application of the bioremediation technique at the site.

Field monitoring mirrored batch experiments. In particular, treatment effects were more pronounced on lower chlorinated compounds, whereas PCE and TCE were marginally reduced by the treatment (Table1). On the other hand, lower chlorinated ethenes showed a significant decrease. Both isomers of dichloroethene decreased after 4 years of treatment by one order of magnitude, approaching the law limits. Moreover, vinyl chloride even decreased of two orders of magnitude, passing from 111’000 µg L^{-1} in June 2014 to 19'600 µg L^{-1} in May 2016 and 4'300 µg L^{-1} in September 2020, still representing an issue for the site.

Table 13 Chloroethenes concentrations in June 2014 and in June 2020 after about four years of treatments (started in May 2016) at the end of contamination plume.

	Law limits D.Lgs. 152/06	MAY 2016	SEPTEMBER 2020

PCE	µg L^{-1}	1.1	9.20	5.80
TCE	µg L^{-1}	1.5	180.00	15.50
1,2 DCE	µg L^{-1}	60	1'550.00	340.00
1,1 DCE	µg L^{-1}	0.05	150	13.80
VC	µg L^{-1}	0.5	19’600	4'300.00

Total concentrations of organohalide compounds decreased in aquifer plume. In September 2020, higher concentrations of contaminants were placed only near the landfill. Instead, the rest of the plume showed lower concentrations respect to May 2016. Total organohalide compound concentration decreased of one order of magnitude in 4 years and half (from 10’000-100’000 to 1’000-10’000) in the contaminant plume at the aerobic barrier. Near the landfill at the anaerobic barrier, contaminants mass that was degraded every day before and after the installation of biobarrier was calculated. Before the installation of the biobarrier, cancerogenic chlorinated aliphatic compounds were degraded 11.11 kg day^{-1}, during the activity of treatment (from May 2016 to September 2020), degradation rate increased until 17.72 kg day^{-1}. Regarding degradation of total chlorinated aliphatic compounds, the increase was from 20.73 kg day^{-1} to 23.74 kg day^{-1}. In two years and half of treatment, 11’000 kg of cancerogenic chlorinated aliphatic compounds and 15’000 kg of total chlorinated aliphatic compounds were degraded.

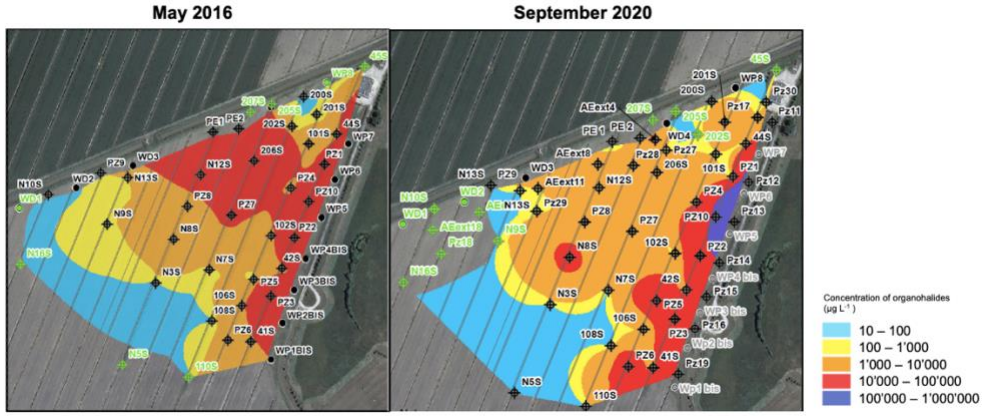

Figure 18 Concentration of organohalide compounds in May 2016 (before biobarriers installation) and in September 2020 (after about four years and half of treatment) in aquifer plume

Natural organohalide activity present in the aquifer, determined in this study, was improved from the addition of reducing substrate, in particular in dechlorination of lower chloroethenes, as chemical data showed. Improvement of OHR activity was supported also by biomarkers monitoring at filed scale. Genes coding for enzyme involved in respiration increased their copies number L^{-1}. Reducing substrate addition changed the microbial community composition, inducing an increase of fermentative bacteria. Known organo-halide respiring bacterial species were present in low numbers at the site, suggesting the possible presence of previously uncharacterized OHR bacterial species at the site, thus deserving of further investigation.

The accumulation of increasing concentration of vinyl chloride in the plume has represented an issue during the bioremediation process. In order to envisage the possibility to lower its concentration, this study determined that at the end of the plume, microbial community possessed natural degradation activity of lower chlorinated ethenes, that was enhanced in the last two years of aerobic treatment. Similarly, also BTEX, chlorobenzene and aliphatic hydrocarbons benefited of this intervention.

It was calculated that the completely remediation of the aquifer by permeable biobarrier will be achieve in 30 years.

Evaluation on analogues sites where permeable reactive biobarriers were used as remediation intervention showed lower cost than chemical-physical techniques. Economic valuation will be made in the future on this site.

FUTURE DIRECTIONS

Starting from data collected during my PhD, different future prospects can be examined.

Illumina data showed a low presence of known OHRB before the treatment started and after one year and half of treatment. The absence of OHRB is not supported by field scale chemical data of the aquifer that showed an efficacy degradation of chloroethenes, especially of higher chlorinated ethenes. These differences suggest the presence of OHRB not yet characterized. Further work will be carried out for isolation and identification of possible new OHRB, present in the aquifer, as well as for their degradation characteristics.

In permeable reactive biobarrier, as said before, selection of appropriate reducing substrate to stimulate OHR activity is crucial. Reducing substrate has to support dechlorination of chloroethenes including lower chlorinated ethenes. In addition, it should have low environmental footprint, such as wastes of food and agricultural productions. Reducing substrate has to be easily used at filed scale and it has to be cheap. Through future microcosms and field scale experiments a new reducing substrate that can reach out all these requests can be selected.

The VC aerobic degradation pathway is still poorly understood. Only two enzymes were characterized: AkMO and EaCoMT. Metatranscriptomic analysis will help in the identification of new enzymes involved in the vinyl chloride oxidation pathway, thus leading to a better understanding of mechanisms that can be implemented in contaminated environments.

APPENDICES

1. National and international conferences

Oral presentations:

- M. Bertolini, S. Zecchin, L. Cavalca, *Microbial populations involved in the degradation of chlorinated ethenes in contaminated aquifer*, Cortona Procarioti 2018, 17-19/05/2018, Cortona

Poster presentations:

- M. Bertolini, S. Zecchin, L. Cavalca, Biodegradation of chlorinated ethenes and aromatic compounds in contaminated aquifer, XXIII Workshop on the developments in the Italian PhD research on food science, technology and biotechnology, 19-21/09/2018, Oristano
- M. Bertolini, S. Zecchin, M. Colombo, S. Foiani, L. Cavalca, Microbial bioremediation of aquifer affected by chloroethenes and petroleum hydrocarbon contamination, Bageco 15, 15th Symposium on Bacterial Genetics and Ecology, 26-30/05/2019, Lisbon
- G.P. Beretta, L. Cavalca, M. Bertolini, S. Zecchin G. Buscone, L. Ledda, L. Ferrari, G. Carnevale, G. Bozzetto, J. Terreni, *Anaerobic and aerobic bioremediation of chlorinated solvents and hydrocarbons plumes from an old landfill in the Venice lagoon environment,* Flowpath National Meeting on Hydrogeology, 12/06/2019, Milano
- M. Bertolini, S. Zecchin, L. Cavalca, Microbiologia dei siti contaminati: valutazione della bioattenuazione nei processi di risanamento, Sicon: SITI CONTAMINATI: esperienze negli interventi di risanamento, 12-14/09/2019, Brescia

Conference proceedings:

- M. Bertolini, S. Zecchin, L. Cavalca, Biodegradation of chlorinated ethenes and aromatic compounds in contaminated aquifer, XXIII Workshop on the developments in the Italian PhD research on food science, technology and biotechnology, 19-21/09/2018, Oristano
- M. Bertolini, S. Zecchin, L. Cavalca, Microbiologia dei siti contaminati: valutazione della bioattenuazione nei processi di risanamento, Sicon: SITI CONTAMINATI: esperienze negli interventi di risanamento, 12-14/09/2019, Brescia

2. Training courses

- 03-07/09/2018. *Summer school on computational analysis from genomic diversity to ecosystem structure*, Organized by SIMTREA, Firenze.
- 26-29/10/2020. *Introduction to statistics in R*, Organized by Physalia Courses

3. Publication

Bertolini, M., Zecchin, S., Markantonis, M., Cavalca, L., Microbial degradation of chloroethenes: a review, submitted to *Microorganisms*