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*Review*

# Sequential Anaerobic/Aerobic Microbial Transformation of Chlorinated Ethenes: Use of Sustainable Approaches for Aquifer Decontamination

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**Abstract:** Chlorinated ethene contamination is a worldwide relevant health issue. In anaerobic aquifers, highly chlorinated ethenes are transformed by microbially-mediated organohalide respiration metabolism. For this reason, in the last few years, bioremediation interventions have been developed and employed in situ for aquifer decontamination. Biostimulation has been demonstrated to be efficient in enhancing organohalide respiration activity. The use of agrifood wastes that replace engineered substrates as biostimulants permits the low carbon impact of bioremediation treatment as part of a circular economy approach. The present work depicts the effects of available bio-based substrates and discusses their efficiency and impact on microbial communities when applied to contaminated aquifers. As a drawback of anaerobic organohalide respiration, there is the accumulation of more toxic lower-chlorinated ethenes. However, compounds such as dichloroethene (DCE) and vinyl chloride (VC) can be mineralized by metabolic and co-metabolic pathways in aerobic conditions. For this reason, sequential anaerobic/aerobic treatments proposed to stimulate the natural biotransformation activity can achieve complete degradation of chlorinated ethenes. The aim of this work is to provide an up-to-date revision of anaerobic/aerobic microbial transformation pathways towards chlorinated ethenes and to discuss their application in real scenarios and futurable microbial bioelectrochemical systems to remediate contaminated aquifers.

**Keywords:** chloroethenes; microbial bioremediation; organohalide respiration; VC aerobic biodegradation; bio-based substrates



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## 1. Introduction

The pollution of soil, water, and air has been an issue since the last century, with industrial, agricultural, and domestic sectors responsible for widespread contaminations through pollutant discharges or incorrect disposals.

Ensuring access to water of high quality for all people is one of the 17 sustainable development goals of the FAO for 2030 (6. clean water and sanitation) [1]. Groundwater represents approximately 99% of all liquid freshwater on Earth. It is a pivotal reservoir that provides around 25% of the water used for human purposes worldwide, of which 70% is dedicated to crop irrigation [2]. Although the use of groundwater for domestic and industrial purposes is lower (21% and 9%, respectively) [3], aquifers represent the water supply for 50% of the global urban population [2,4]. The impact of each sector on groundwater use is strictly dependent on specific characteristics of the considered country (i.e., population, climate, and economic development) [5].

Groundwater is affected by the contamination of both inorganic and organic compounds. In Europe, 10% of the groundwater is contaminated by chlorinated hydrocarbons, which are the fourth most prevalent contaminants after heavy metals, mineral oils, and aromatic hydrocarbons. Although these compounds predominantly affect groundwater,

they are also present in 8% of all contaminated soil [6]. Tetrachloroethene (PCE) is present in the groundwater of 10 European countries, covering an area of 51,400 $km^2$ [7].

Now, even if the most commonly used environmental remediation strategies are based on chemical and physical treatments of the contaminated matrices (i.e., pump and treat, excavation), in the last 20 years the bioremediation approach based on the exploitation of plant and microbial metabolic capacities has been considered to address the economic and environmental issues related to more invasive approaches and to improve the sustainability of the remediation actions. Bioremediation techniques are cheaper if compared to physicochemical treatments [8], and they have minor impacts on the environment as well as on the health of the workers. In fact, most of the bioremediation treatments are carried out in situ, avoiding direct contact between the workers and the contaminated matrix [9]. In particular, microorganisms play an important role during bioremediation treatments. Indeed, microorganisms use different strategies to convert contaminants into less or non-hazardous compounds (degrade, transform, and accumulate), permitting them to potentially treat all known contaminants [10]. In addition, microbial plasticity allows for remediating matrices contaminated by multiple hazardous compounds that typically affect many polluted sites [11].

This review aims to evaluate the recent outcomes related to microbial dehalogenation of chlorinated hydrocarbons, focusing on anaerobic/aerobic microbial processes in contaminated environments and their exploitation in groundwater bioremediation by different technological approaches.

## 2. Chlorinated Ethenes

Chloroethenes (CEs) are ethene molecules where one or more hydrogens are substituted by chlorine atoms. According to the number of chlorine substitutes, CEs include 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). CEs are colorless liquids or gases (VC is in the form of a gas above 7 °C) with a typical chloroform-like smell and belong to the group of chlorinated volatile organic compounds together with other polychloromethanes and polychloroethanes. These compounds are present in the environment not only because of human activities but also as a result of natural processes such as marine algae metabolism [12,13] and abiotic reactions between humic acids, iron (III), and chlorides [14]. Their concentrations in uncontaminated soils range between 0.001 and 0.1 mg of organic chlorine per $g^{-1}$ of dry soil [15].

Since these compounds are widely used in many industrial sectors, they are among the most frequently detected compounds in several contaminated areas around the world [16]. In particular, PCE and TCE are hardly soluble in water and are non-flammable. For this reason, these compounds have high solvent properties and low fire and explosion potential and are therefore used as solvents for waxes, resins, fats, rubbers, oils, and metal degreasing. They are also commonly found in household products such as dry cleaning solvents and painting products. Due to their higher density than water, PCE and TCE form a dense non-aqueous phase liquid (D-NAPL) that penetrates through permeable groundwater aquifers, forming a contamination plume. Their presence in the environment is mainly due to inadequate disposal methods adopted in the past [17,18].

Because of their wide presence in the environment, human exposure to CEs occurs via different routes, such as dermal absorption, ingestion, and inhalation [19]. In most cases, intoxications are the result of repeated exposures to small doses (chronic exposure), instead of acute narcosis. CEs cause injury to the central nervous, immune, and endocrine systems [20]. Exposure to these compounds shows a significant correlation with cancer. In particular, PCE and TCE are associated with esophageal and cervical cancer and non-Hodgkin's lymphoma. TCE and VC are carcinogenic agents included in Group 1 by the International Agency for Research on Cancer (IARC) [21–23] (Table 1).

**Table 1.** Selected characteristics of chlorinated ethenes [a].

| Compound | | Appearance | Water Solubility at 25 °C (g $L^{-1}$) [a] | Density at 20 °C (g $cm^{-3}$) [a] | Vapor Pressure at 20 °C (kPa) [a] | Autoignition Temperature (°C) [a] | Carcinogenicity [b] | Law Limits (µg $L^{-1}$) Directive 2000/60/EC |
|---|---|---|---|---|---|---|---|---|
| Vinyl chloride (VC) ($C_2H_3Cl$) | | Colorless gas | Slightly soluble | 0.91 | 516.95 | 472° | Group 1 (2012) | 0.5 |
| *cis*-dichloroethene (*cis*-DCE) ($C_2H_2Cl_2$) | | Colorless liquid | 1–5 | 1.28 | 26.66 | 460° | N | - |
| *trans*-dichloroethene (*trans*-DCE) ($C_2H_2Cl_2$) | 1,1-*trans*-DCE | Colorless liquid | 2.5 | 1.213 | 66.5 | 460° | Group 3 (1999) | 0.05 |
| | 1,2-*trans*-DCE | Colorless liquid | <1.0 | 1.25 | 53.33 | 460° | N | 60 |
| Trichloroethene (TCE) ($C_2HCl_3$) | | Colorless liquid | 1.280 | 1.46 | 7.8 | >410° | Group 1 (2014) | 1.5 |
| Tetrachloroethene (PCE) ($C_2Cl_4$) | | Colorless liquid | 0.15 | 1.63 | 1.9 | >650° | Group 2A (2014) | 1.1 |

Notes: N—non-cancerous; [a] CAMEO Chemicals; [b] as determined by International Agency for Research on Cancer.

## 3. Microbial Transformation of Chlorinated Ethenes

Although in the past CEs were considered recalcitrant to biodegradation, the occurrence of the natural production of chlorinated hydrocarbons suggests the presence of microorganisms that are able to transform or degrade these compounds [24].

In fact, in the last few years, one anaerobic pathway (i.e., organohalide respiration, OHR) and two aerobic pathways (i.e., aerobic metabolic and aerobic co-metabolic degradation) were described to lead to the complete dechlorination of CEs. However, while anaerobic dechlorination has been widely studied, very little is known concerning the pathways involved in the aerobic biodegradation of CEs.

CEs with higher numbers of chlorine substituents (i.e., PCE and TCE) have a higher tendency to undergo OHR compared with low chlorinated ethenes (Figure 1). On the other hand, CEs with a low number of chlorine substituents (i.e., DCE and VC) are more easily processed by microbial aerobic oxidation because of their low oxidation state [25].

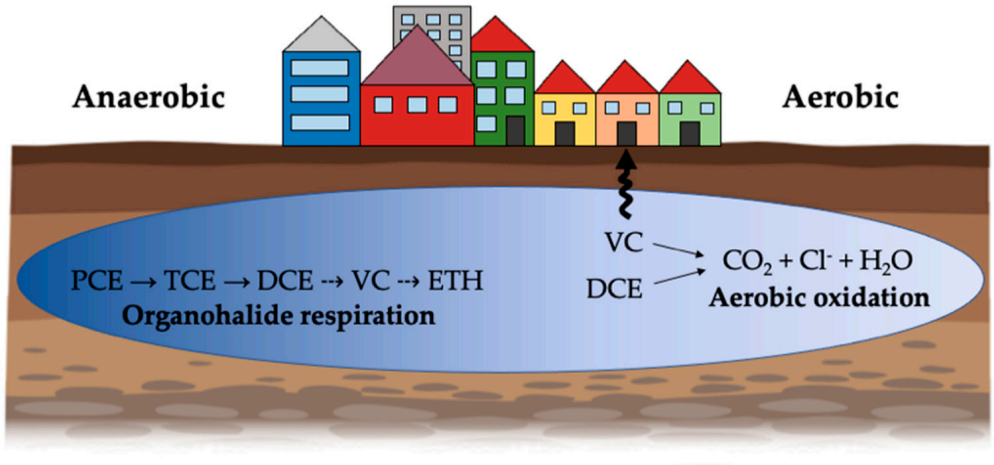


**Figure 1.** CEs degradation pathways. In the anaerobic portion, there has been incomplete reductive dechlorination with the accumulation of DCE and VC (dashed arrows). Following the aerobic portion permits complete mineralization of the pollutants.

Different studies showed an improvement in the efficiency of bioremediation by applying a sequential anaerobic–aerobic biotransformation, thus exploiting the different metabolisms at different redox conditions through indigenous microbial communities [26–28].

## 4. Organohalide Respiration

OHR is a microbial metabolism that takes place in strictly anaerobic conditions, both in marine and groundwater environments [29,30]. Organohalide−respiring bacteria (OHRB) use hydrogen or small organic acids (i.e., lactate or butyrate) as electron donors and CEs as electron acceptors.

Only a few bacterial genera are known to perform anaerobic OHR, including obligate OHRB genera of Chloroflexi *(Dehalococcoides*, *Dehalogenimonas*, and *Dehalobium*) and not-obligate ones of Firmicutes *(Desulfitobacterium*, *Dehalobacter*, and *Clostridium*) and of Proteobacteria *(Comamonas*, *Geobacter*, *Desulfomonile*, *Desulfuromonas*, *Sulfurospirillum*, *Enterobacter*, and *Shewanella*). Two *Dehalococcoides* strains (*Dehalococcoides mccartyi* strains BTF08 and 195, previously *D. ethenogenes* [31]) and one *Dehalogenimonas* strain (*Candidatus* Dehalogenimonas etheniformans strain GP) [32,33] are able to carry out a complete OHR of PCE to ethene [34].

During OHR, chlorine atoms are sequentially replaced with hydrogen atoms, allowing the degradation of PCE to TCE, *cis*-DCE, and VC to ethene (Figure 2).

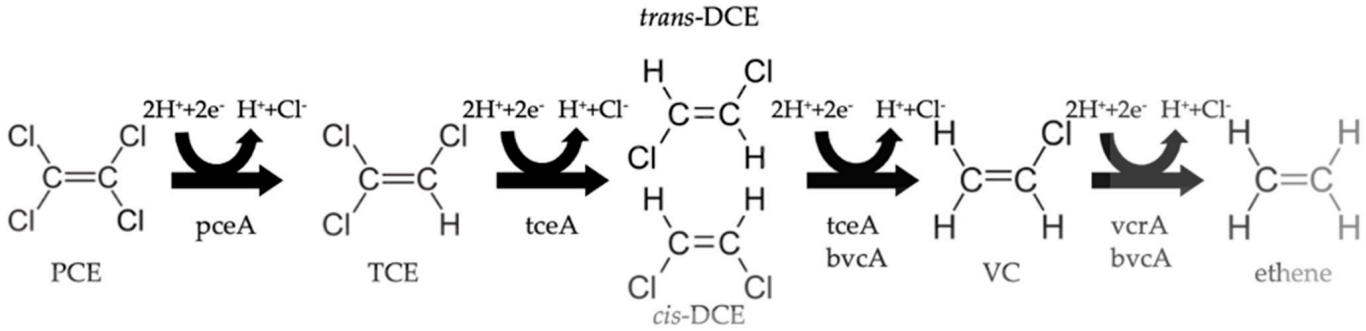


**Figure 2.** Anaerobic respiration pathway: organo−halide dechlorination of PCE to ethene and respective enzymes involved in the process: PCE reductase pceA, TCE reductase tceA, DCE/VC reductases bvcA, and vcrA. Modified from [35].

The dechlorination steps from *cis*-DCE to ethene are less energetically favored. Indeed, dechlorination of DCE to VC and of VC to ethene showed a $\triangle$G of $-121.1$ kJ/mol$^{-1}$ and $-118.4$ kJ/mol$^{-1}$, respectively, compared to the $\triangle$G values of the first two steps (PCE to TCE and TCE to cis-DCE): $-156.8$ kJ/mol$^{-1}$ and $-147.4$ kJ/mol$^{-1}$ [36]. Minor effective dechlorination leads to the accumulation of DCE and VC in the contaminated sites [24,37–39].

Bacterial genes involved in OHR fall within the class of reductive dehalogenase homologous genes (*rdh* or RDases), which include tetrachloroethene reductive dehalogenase (*pceA*), trichloroethene reductive dehalogenase (*tceA*), and vinyl chloride reductase (*bvcA* and *vcrA*). These genes encode reductases involved in the degradation of PCE to TCE (*pceA*), TCE degradation to DCE or DCE to VC (*tceA*), DCE to VC and VC to ethene (*bvcA*), and the degradation of VC to ethene (*vcrA*, Figure 2). Most of the RDases use corrinoids (coenzyme B12) as a cofactor [38,40]. It has been shown that 8.1–34 pg L$^{-1}$ of cobalamin supports the dechlorination activity of *Dehalococcoides* [41].

Bacterial OHR activity is supported by a plethora of other microorganisms that, with their activity, provide $H_2$ and corrinoids, as well as oxygen removal. In fact, it has been shown that when *Dehalococcoides* is grown in co-culture with *Desulfovibrio vulgaris* Hildenborough and *Methanobacterium congolense*, its VC respiration increases [42]. A syntrophic relationship develops between fermentative bacteria (i.e., *Clostridium* spp.), which produce $H_2$ that is consumed by OHRB, efficiently decreasing $H_2$ concentration and acting as an inhibitor of fermentation [43]. In some cases, methanogens can couple methanogenesis with fermentation, for example, producing $H_2$ from acetate in the case of *Methanosarcina* [36].

Members of the genera *Acetobacterium*, *Desulfovibrio*, *Spirochaetes*, *Sedimentibacter*, *Pelosinus*, and *Geobacter* synthesize corrinoids [44–49]. Sulfate-reducing bacteria and

methanogens could synthesize coenzyme $B_{12}$ with beneficial effects on OHR [50]. Microorganisms that use $O_2$ for respiration or that have an oxygen detoxification pathway indirectly protect strictly anaerobic OHR bacteria [51]. On the other hand, bacterial OHR decreases the concentration of CEs, which can inhibit fermentation activities.

Ultimately, OHR is anaerobic microbial teamwork, and the exploitation of all the microorganisms directly and/or indirectly involved in the dechlorination is crucial for in situ groundwater decontamination.

OHR bacteria compete with methanogens, dissimilatory sulfate-reducing bacteria, and acetogens for the use of energy sources [52–54] due to sharing ecological niches with the same range of redox potential: OHR occurs between −210 and −470 mV [55], methanogenesis from −175 to −400 mV, and sulfate reduction from −50 to −250 mV.

The competition between OHR bacteria and methanogenic archaea was demonstrated by the decreased methane production and concomitant increase of TCE dechlorination and *Dehalococcoides* 16S rRNA gene copies after the addition of methanogen inhibitors [56].

OHR bacteria are favored when hydrogen is present at low concentrations [57], the ORP range is between −210 and −470 mV, and the pH is between 6.8 and 7.8 [35]. Particularly, pH lower than 7 and ORP higher than −210 mV slow down the bacterial dechlorination activity [58]. These aspects are crucial when planning bioremediation strategies based on feeding the anaerobic trophic chain of organic carbon degradation.

## 5. Aerobic Pathways of Lower Chlorinated Ethenes Biodegradation

Aerobic biodegradation of higher CEs such as PCE has not been detected yet, and that of TCE has been rarely described [59]. On the contrary, aerobic biodegradation of lower CEs can be carried out by two different mechanisms: metabolic and co-metabolic.

### *5.1. DCE and VC Metabolic Degradation*

By integrating a multi-omic approach, enzyme assays, and compound-specific isotope analysis, Jennings and colleagues [60] hypothesized two pathways for *cis*-DCE oxidation: (i) glutathione S-transferase (GST)-catalyzed dehalogenation; and (ii) monooxygenase-catalyzed epoxidation. In the GST-catalyzed dehalogenation (Figure 3), the two chlorine atoms of *cis*-DCE are replaced with glutathione (GSH) and a hydroxyl group, respectively, yielding glycolate as the final product. In the monooxygenase-catalyzed epoxidation (Figure 3), *cis*-DCE is oxidized, resulting in the formation of glycolate as the final product. Glycolate can be further transformed into glyoxylate, which is converted to succinate through the glyoxylate cycle, finally entering the TCA cycle (Figure 3).

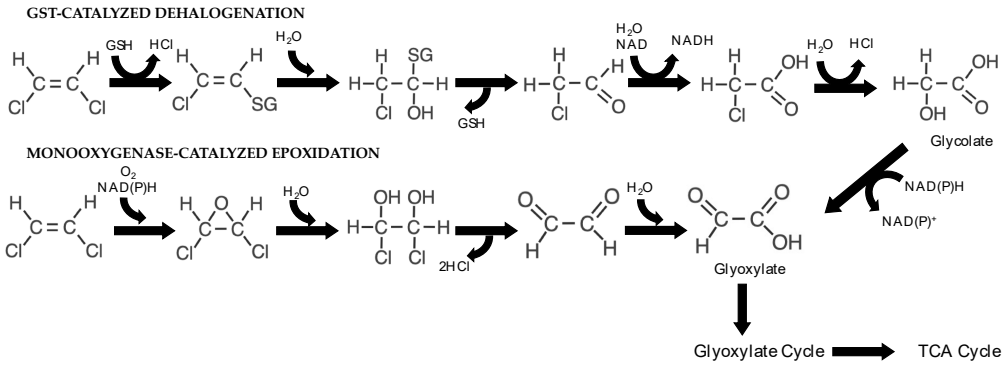


**Figure 3.** *cis*-DCE oxidation pathways (adapted from [61]). In the glutathione S-transferase (GST)-catalyzed dehalogenation, one chloride is replaced by glutathione (SG).

*Polaromonas* sp. strain JS666 has been demonstrated to use *cis*-DCE as the sole carbon and energy source [61]. Genome analysis of strain JS666 [62] revealed the presence of genes for degradative enzymes of different compounds, such as CEs (chloroethane and chloroacetate), aromatic hydrocarbons, and genes involved in metal resistance.

In the VC oxidation pathway (Figure 4), the first enzyme of the pathway is an alkene monooxygenase (AkMO) that, with the addition of one oxygen atom, converts its substrates (VC and ethene) into aliphatic epoxides (i.e., epoxyethane and chlorooxirane). AkMO is composed of four subunits that are encoded by the genes *etnA*, *etnB*, *etnC*, and *etnD* [63,64]. The second enzyme is epoxyalkane:coenzyme M transferase (EaCoMT), encoded by the *etnE* gene. EaCoMT mediates the conjugation of toxic epoxides to coenzyme M, thus decreasing their toxicity [65]. Based on predicted analyses of DNA regions flanking the *etnABCD* and *etnE* genes, it has been hypothesized that the last compound of the degradative pathway enters the TCA cycle [63,65].

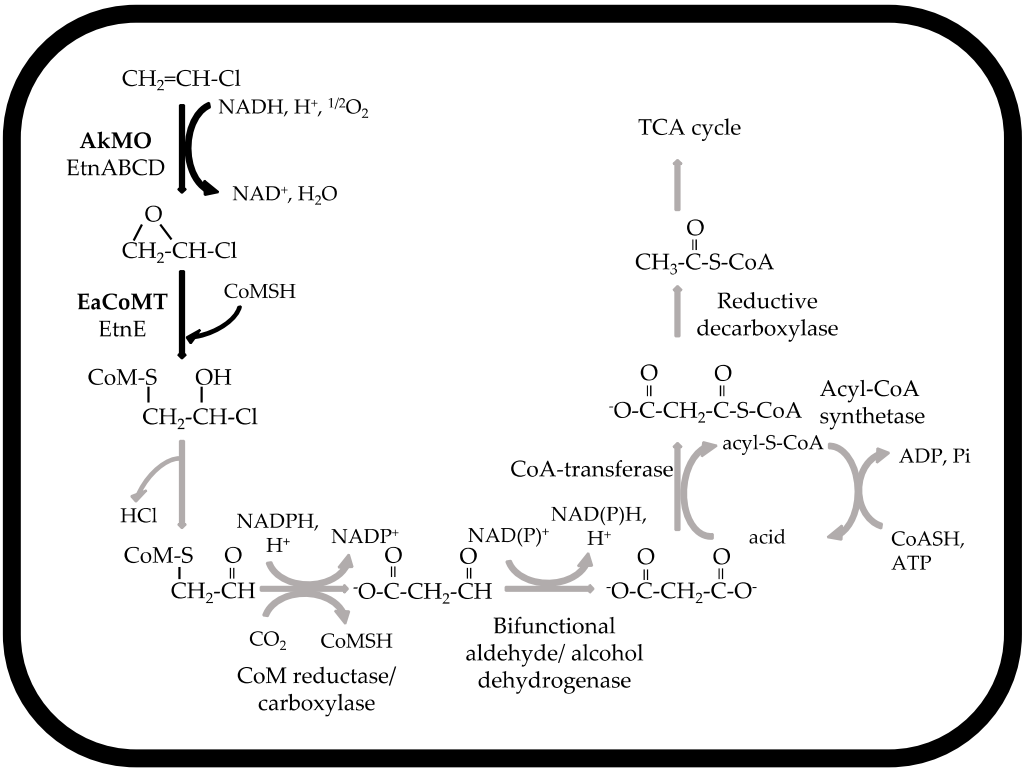


**Figure 4.** Aerobic oxidation pathway of VC and ethene. Grey arrows: hypothetical reactions (modified from [65]).

*etnABCDE* genes are located within the same operon on a large linear transmittable plasmid that can be lost by bacterial cells after one day of VC starvation [63,66].

Although, in general, these enzymes can oxidize both VC and ethene, different isoforms show different affinity for the two molecules. Jin and colleagues [67] observed that when ethene-oxidizing bacteria were exposed to VC as the sole carbon and energy source, two mutations (i.e., W243G and R257L) were selected in the *etnE* genes. It was assumed that these two mutations permitted easy access of chlorooxirane, which is larger than epoxyethane, to the active site of the enzyme. Moreover, Illumina sequencing of the 16S rRNA gene in enrichment cultures set up from the same groundwater showed the selection of different microbial communities based on the sole carbon source of incubation, clearly indicating that different bacterial species show different affinity to VC or ethene [68]. The lower presence of alternative carbon sources in the groundwater induced a higher level of variability between microcosms amended with VC and ethene. The presence of VC induced an increase in Proteobacteria, in particular *Pseudomonas*, while *Actinobacteria* (in particular, *Nocardiodes*) and *Acidobacteria* were enriched in ethene microcosms.

Only eight bacterial genera are able to grow on VC. All these strains were isolated from contaminated soils, activated sludge, and waters by enrichment culture procedures where VC was added as the sole carbon and energy source (Table 2).

**Table 2.** Bacterial strains that are able to use VC as a carbon and energy source in aerobic conditions.

| Genus | Species/Strains | Isolation Sources | References |
|---|---|---|---|
| *Mycobacterium* | *aurum* strain L1 | Contaminated soil | [69] |
| | strains JS60 | Contaminated groundwater | [66] |
| | strains JS61 | Activated sludge | [66] |
| | strains JS616 | Sediment of industrial site | [66] |
| | strains JS617 | Activated carbon of pump and treatment plant | [66] |
| *Rhodococcus* | *rhodochrous* | PCE degrading enrichment culture | [70] |
| *Nocardioides* | sp. strain JS614 | Soil of industrial site | [66] |
| *Pseudomonas* | *aeruginosa* strain DL1 | Activated sludge | [71] |
| | *aeruginosa* strain MF1 | VC degrading enrichment culture | [72] |
| | *putida* strain AJ | Hazardous waste site | [73] |
| *Ochrobactrum* | sp. strain TD | Hazardous waste site | [73] |
| *Ralstonia* | sp. strain TRW-1 | Chloroethene degrading enrichment culture | [74] |
| *Brevundimonas* | sp. | Contaminated groundwater | [75] |
| *Rhodoferax* | sp. | Contaminated groundwater | [75] |

*etnC*, *etnE,* and 16S rRNA genes of the bacterial species that carry *etnC* and/or *etnE* genes, phylogenetic trees were constructed (Figure 5) to show the variability of these enzymes. All sequences of the two enzymes of interest belonged to only three genera: *Mycobacterium* (renamed *Mycolicibacterium* by Gupta and colleagues) [76], *Nocardioides,* and *Rhodococcus*. In the *Mycolicibacterium* genus, several strains were isolated and sequenced (Figure 5). *etnE* showed a higher level of sequence variability than *etnC*. The phylogeny of *etnC* was different with respect to the 16S rRNA gene phylogeny, likely confirming that horizontal gene transfer occurred by plasmid exchange in the three genera.


**Figure 5.** Phylogenetic trees of EtnC (**a**), EtnE (**b**), and 16S rRNA genes (**c**). Protein sequences found in the NCBI (National Center for Biotechnology Information) database through the BLASTp (Basic Local Alignment Search Tool protein in proteins) program based on the protein sequences of the enzymes EtnC and EtnE of *Nocardioides* sp. JS614 (accession numbers AAV52081.1 and AAV52084.1 on GenBank) were used. Branch length corresponds to the number of substitutions, and under each phylogenetic tree, the legend of the scale is reported. Sequences were analyzed with MEGA X software [77]. Sequence alignment was based on the Muscle algorithm [78], and phylogenetic trees were constructed using the Maximum Likelihood method with the JJT matrix model [79]. Sequences of methane monooxygenase were used as outgroups for the functional genes (WP_040789699, WP_005572960, MSQ68733, WP_083042312, WP_059039071 e WP_066161679), while *Lactobacillus xujianguonis* strain HT111-2 (NR_174278.1), *Shinella yambaruensis* (AB285481.1), *Brucella melitensis* (AY594216.1), *Phyllobacterium leguminum* strain ORS 1419 (NR_042396.1), *Chromobacterium aquaticum* strain CC-SEYA-1 (EU109734.1), *Francisella hispaniensis* FSC454 (NR_116944.1), *Woeseia oceani* strain XK5 (NR_147719.1), *Bombiscardovia* sp. DCY76 (JX863369.1), *Saccharothrix yanglingensis* (GQ284639.1), *Euzebya tangerine* (AB478418.1), *Moorella perchloratireducens* (EF060194.1), *Dethiobacter alkaliphilus* AHT 1 (EF422412.2), *Synechococcus elongatus* (D83715.1), *Cyanobacterium aponinum* strain PCC 10605 (NR_102443.1), and *Halomicronema hongdechloris* C2206 (NR_177001.1) were used as outgroups for the 16S rRNA gene tree.

VC biodegradation was detected in subtoxic conditions at a dissolved oxygen concentration of 0.04 mg $L^{-1}$ [80], making this process relevant also in anaerobic conditions where possible microoxic conditions can occur [66,81]. In situ, the limiting factor for VC and *cis*-DCE aerobic biodegradation would be more likely the presence of pollutant mixtures. The biodegradation of *cis*-1,2-DCE was observed to be decreased by the presence of TCE, 1,1-DCE, *trans*-1,2-DCE, and VC [82].

### *5.2. Co-Metabolic Aerobic Degradation*

In some microorganisms, the degradation of CEs is carried out by enzymes that originally evolved to degrade other compounds but that also show affinity for CEs, a process called co-metabolism. In this case, bacterial cells do not use CEs as a carbon or energy source. Usually, these enzymes are monooxygenases [83]. Aerobic co-metabolic degradation was reported for all CEs, and it was coupled to the degradation of different co-substrates such as ammonium, cumene, ethane, ethene, isoprene, phenol, propane, methane, and toluene [84,85]. Low concentrations of PCE can be degraded in aerobic conditions by *Pseudomonas stutzeri OX1* and *Sphingopyxis ummariensis* VR13 [86,87]. *Rhodococcus* sp. PB1 can degrade CEs while growing on propane [88]. TCE can be oxidized by *Pseudomonas putida* strain F1 in the presence of toluene through toluene dioxygenase [84]. Butane monooxygenase from *Pseudomonas butonovora* can degrade DCE in the presence of butane [85]. TCE and all DCEs are degraded by *Comamonas testosteroni* RF2 in the presence of phenol and sodium lactate [89].

In methanotrophic bacteria, the oxidation of VC and TCE is catalyzed by methanol monooxygenases (MMO), which mediate the conversion from methane to methanol [90,91]. Two MMOs are known: one in the membrane, particulate MMO (pMMO), and the other one in the cytosol, soluble MMO (sMMO), encoded by the *pmoA* and *mmoX* genes, respectively. pMMO is more frequently expressed than sMMO by methanotrophs. Both MMOs can degrade CEs, but sMMO shows a lower specificity for methane, so it can degrade a wider range of substrates, including CEs, and at a higher rate with respect to pMMO [90]. However, it was demonstrated that sMMO can be inhibited by high concentrations of CEs (>50 μM) [91,92]. Facultative methanotrophs (i.e., *Methylocystis*, *Methylocapsa*, and *Methylocella*) were discovered, suggesting a possible co-metabolic degradation of CEs with co-substrates other than methane such as acetate, pyruvate, and ethanol [93–95]. In two different studies [96,97], the influence of methane and ethene on VC degradation was analyzed. In both studies, the contemporary presence of ethene and methane increased VC oxidation. In Freedman and colleagues' study [96], the presence of ethene improved VC degradation better than methane, also after prolonged incubation. On the other hand, in Findlay and colleagues' study [97], methane was found to be the best compound for VC degradation. In both studies, inocula were from two different contaminated sites, so these different results can be explained by the different composition of the microbial community or the hydrogeochemical characteristics of the site. The mechanisms that influence VC's co-metabolic degradation rate are still little known.

## 6. Bio-Based Substrates for Stimulation of OHR Bacteria

In order to enhance anaerobic OHR bacterial activity, the addition of fermentable reducing substrates, such as alcohols, organic acids, emulsified vegetable oil, complex organic materials (e.g., molasses), and plant-based materials (wood chips, corn cobs) is widely used [98]. The production of reducing equivalents (i.e., $H_2$) and of carbon sources (such as acetate) decreases the oxidative redox potential (ORP) and feeds OHR bacteria in groundwater, respectively [99]. The injection of reducing substrates through injection wells into the contamination plume creates an active in situ bioreactor called a permeable reactive bio-barrier system.

In addition to the hydrogeological setting and concentration and nature of the contaminants, substrate distribution, microbial competition, operational scale, timeline, and

available budget are also important factors to take into consideration when choosing an appropriate reducing substrate.

Several substrates are engineered in order to obtain an effective product with the desired degree of solubility, fluidity, fermentation, and pH stability [100]. Soluble substrates such as molasses are easily distributed in aquifers but also quickly biodegrade, thus requiring frequent injections. On the other hand, insoluble substrates (e.g., butyrate and propionate) are slowly fermented and release lower levels of $H_2$, thus favoring OHRB over methanogens [51]. Since hydrogeological settings affect the distribution of electron donors, accurate site characterization and monitoring of substrate dispersion areas far from injection wells are fundamental to ensuring that single or multiple injections at periodic intervals of 1–5 years maintain their efficacy [100].

Different substrates have been tested for OHR bacterial stimulation at the microcosm scale, whereas the scientific literature at the field scale is still scarce. Small organic acids have been proven to be suitable to enhance the complete dechlorination of highly CE to ethene in microcosms at different levels. Lactate has been proven to be effective in long-time incubation experiments (i.e., 125 days) [101], whereas in shorter ones (i.e., 40 days), it led to VC accumulation [102]. Formate promoted complete dechlorination after 78 days of incubation, and its addition determined a lower bacterial growth and a lower impact on microbial community diversity compared to lactate and citrate [103]. Gamma-polyglutamic acid, while enhancing complete dechlorination of TCE to ethene and *Dhc* gene copy numbers, also increased anaerobic respirations coupled to nitrate, ferric ion, and sulfate reduction [104]. Inorganic compounds such as nanozero-valent iron (nZVI) are efficient substrates for abiotic [105] and microbial dechlorination [106]. The addition of nZVI to a microbial dechlorinating consortium, in addition to the degradation of original chlorinated compounds, led to the detection of both ethene and ethane in a lysimeter chamber system, deriving from biotic and abiotic dechlorination, respectively [106]. The combination of both organic and inorganic substrates determined the dechlorination of *cis*-DCE and VC while increasing the abundance of OHR bacteria in a pilot plant of contaminated groundwater with added poly-3-hydroxybutyrate and ZVI [107].

Nowadays, the circular economy model promotes the study of bio-stimulation treatments using substrates from agri-food, industrial, and biorefinery wastes. Among promising substrates, molasses were found to increase the dechlorination rate of higher CE while causing accumulation of *cis*-DCE and VC [108] at both the microcosm and field scales. Chen and colleagues [98] observed at a microcosm scale that molasses induced a higher dechlorination rate with respect to acetate and soybean oil, but the rapid $H_2$ release induced a higher production of methane. Wood mulch and whey enhanced complete microbial dechlorination to ethene but increased the production of methane after 90 days of incubation [109,110] and sulfide [111], respectively. At the field scale, whey addition coupled with the addition of a dechlorinating bacterial consortium allowed the completion of the process [112].

Anaerobic chain elongation (i.e., conversion of ethanol and acetate into butyrate and caproate) has been recently postulated [113] as a new possible mechanism to lead to complete dechlorination in anaerobic conditions due to the production of $H_2$ during the final fermentation steps. Bacteria with a chain-elongation activity are poorly studied, with only *Clostridium kluyveri* being known to perform this process [114].

## 7. Bioelectrochemical Systems

Bioelectrochemical systems (BES) combine biological and electrochemical processes to generate electricity, hydrogen, and organic molecules interesting for technological applications. BES is composed of two electrodes (i.e., anode and cathode) that act either as electron donors or acceptors for microorganisms to carry out reduction and oxidation reactions: the anode plays as an electron acceptor from microbial cells, and the cathode as an electron acceptor. Subsequently, this transfers the electron to cathodic cells, thus obtaining the concomitant reduction of an alternative electron acceptor.

The development of BES for bioremediation applications has rapidly evolved in the past decade. In BES, the capacity of some microorganisms to transfer electrons to external donors or acceptors (i.e., electrodes or conductive minerals) can be exploited to overcome the lack of natural electron acceptors/donors at the contaminated sites that slow down the natural attenuation of hydrocarbons, chlorinated compounds [115–117], and PAHs [118].

Microorganisms can use different mechanisms to perform external electron transfer. In direct interspecies electron transfer, the microbial cells directly exchange electrons using electrically conductive structures such as cell membrane nanotubes and pili, allowing the transfer of an electron between microorganisms belonging to the same species or different species (for example, between *Geobacter* and *Methanosaeta*) [119]. In the absence of these conductive structures, small electroactive molecules such as quinones and cytochromes, as well as abiotic conductive materials such as biochar and granular activated carbon, humic acids, and manganese, can act as electron carriers between different microorganisms or between microorganisms and electrodes, a process called mediated electron transfer [119,120]. Mobile electron shuttles or biofilm matrices play as a mediator for the electron transfer also in electrochemically inactive bacteria [121]. *Shewanella*, *Lactococus*, *Pseudomonas*, and *Klebsiella* can produce electron shuttles, for example, flavins and phenazines [120]. Biofilms can contain outer membrane *c*-type cytochromes or flavins that increase electron transfer [120]. Biofilm EPS might play a dual role, both anchoring shuttle molecules that promote electron transfer [122] and inhibiting electron transfer due to the insulating nature of polysaccharides [123].

Bioelectrochemical systems can be used sequentially in anaerobic/aerobic treatments in order to achieve complete dechlorination of chlorinated hydrocarbons to ethene (Figure 6) [116,124].

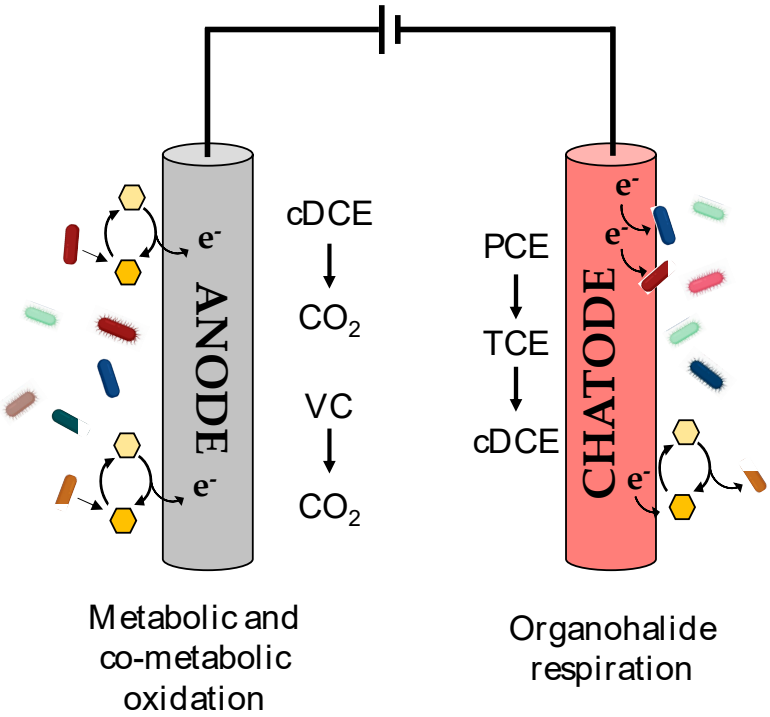


**Figure 6.** Illustration of a bioelectrochemical cell with aerobic and anaerobic biotransformation of chloroethene mechanisms.

On the cathodic side, the dechlorination of PCE and TCE occurs [124]. Hydrogen production at the cathode and cathodic polarization are important factors to promote reductive dechlorination with respect to methanogenesis [116,125]. However, only a low concentration of $H_2$ allows OHRB to outcompete methanogens [116,125]. Optimum cathodic

polarization for dechlorination of TCE is −250 mV, whereas values lower than −650 mV increase methanogenic activity [125].

In some cases, molecules acting as low-redox mediators are required for the electron transfer between the cathode and the microorganisms [126].

The accumulation of lower chlorinated ethenes (*cis*-DCE and VC) on the cathodic side can be solved with the addition of an anodic chamber for their oxidation to $CO_2$ by exploiting the production of $O_2$ through water electrolysis [127]. The electrically-stimulated biomineralization of *cis*-DCE and VC was reported for the first time in 2009 by Lohner and Tiehm [128]. Biodegradation of lower chlorinated ethenes is more efficient if oxygen is generated at the electrode surface than if it is supplied through spiking [129]. To induce the production of oxygen at the electrode surface, the voltage of the anode should be +1.5 V versus the SHE (standard hydrogen electrode) [129,130].

The use of two separate tubular bioelectrochemical reactors preserves the complete mineralization of PCE through sequential reductive/oxidative processes and allows for cost reduction by avoiding the use of ion exchange membranes [130].

Only *Geobacter*, *Anaeromyxobacter*, and *Shewanella* are known to be OHRBs that are able to accept electrons directly from the cathode [131–133]. In bioelectrochemical systems, *Dehalococcoides maccartyi* was reported in high amounts in the catholyte, suggesting an indirect electrochemical enhancement of the dechlorination activity [127]. Indeed, the characterization of cathodic biofilm revealed that, together with known OHRB, other microorganisms were present (e.g., *Lactococcus*, *Bacillus*, and *Pseudomonas*), with a hypothetical role in the electron transfer [134]. Bradley [24] and Wang and colleagues [135] proved that pure species showed a lower dechlorination efficiency if compared to the bacterial consortium. Meng and colleagues [136] showed that in a cathodic chamber set up with pristine paddy soil, the presence of *D. mccartyi* NIT01 promoted also the presence of *Desulfosporosinus* in both the cathodic biofilm and the catholyte (23.2% and 70.6%, respectively), but in the biofilm *Pseudomonas* (8.7%) and *Clostridium* (6.9%) were also present in high amounts. The absence of *D. mccartyi* NIT01 facilitated the presence of *Clostridium* (41.6% and 21.8%) and *Cellulomonas* (25.9% and 18.2%) in biofilm and catholyte. In the open-circuit voltage systems, these bacterial species were not detected [136]. These studies showed that the presence of only OHRB in the BES was not enough to determine an enhancement of OHR activity. In addition, the microbial community colonizing the electrode surface and the one living in the surrounding electrolytic solution were different, demonstrating that in these two compartments different mechanisms occur and that each microorganism has a characteristic role.

The mineralization of *cis*-DCE in the anodic compartment was shown to be carried out by different species of the genus *Bacillus* (*B. weihenstephanensis*, *B. mycoides*, *B. cereus*, and *B. thuringiensis*) in co-metabolism with ethene [129]. The analysis of the anodic microbial community showed the absence of known VC-degrading bacteria (e.g., *Mycobacterium*), while *Dechloromonas* was abundant in the liquid medium and the silica biofilm [127].

The microbial communities that are enriched in BES are affected by the type of CEs and by the cathodic potential. For example, the addition of PCE to wastewater collected from a wastewater treatment plant increased the relative abundance of *Lactococcus*, *Bacillus*, and *Pseudomonas* [134]. The addition of a carbon source (i.e., glucose, sodium acetate, and sodium bicarbonate) to the cathode affected the composition of the microbial community and its PCE dechlorination activity [137].

According to Dell'Armi and colleagues [138], the dechlorination efficiency reached by BES and by the addition of fermentable substrates such as lactate was comparable.

Different challenges have to be addressed to use BES at the field scale [139]. A possible system configuration was proposed by Palma and colleagues [140] where BES is integrated with biobarriers, creating a "bioelectric well" that can be installed directly in wells. Currently, the application of this technology at the field scale is still infeasible. However, increasing interest and research on BES permitted the assumption of rapid development of an appropriate in situ configuration.

## 8. Environmental Halogenomics: Detection and Distribution of Halo-Bacteria

The determination and subsequent monitoring of CEs bio-attenuation in situ were achieved through multidisciplinary analyses that included the measurement of contaminants concentrations, stable isotope analysis, and molecular biology techniques [141,142].

Molecular biology techniques improve the analysis of bacterial populations involved in environmental biodegradation processes that are hardly assessed only by traditional microbial cultivation methods by considering all the interactions among microorganisms (syntrophic processes) in the microbial communities. Through the analysis of DNA, RNA, and proteins, it is possible to understand the composition of the microbial community as well as the effects of the different treatments used for stimulating bacterial metabolism. Specific phylogenetic and functional quantitative Real-Time PCR biomarkers as well as high throughput sequencing (both Illumina and Shotgun) have been used to monitor and characterize halobacteria, either in situ or at microcosm scales.

The quantification of *Dehalococcoides* has been used to determine the minimum bacterial concentration ($10^5$ copies $mL^{-1}$) necessary to achieve an efficient TCE dechlorination in a reactor where reducing substrates (acetate, soybean oil, and molasses) were added [98,143]. At the field scale, Wu and colleagues [144] demonstrated that different dechlorination rates of two monitoring wells at the same site were correlated to different concentrations of *Dehalococcoides*. Particularly, the well activated by a biostimulation treatment showed a higher amount of *Dehalococcoides* 16S rRNA gene copies ($10^8$ copies $L^{-1}$) with respect to the unamended one (16S rRNA gene copies of $10^6$ $L^{-1}$). This correspondence is not always respected. In many field-based experiments, *Dehalococcoides* and *Dehalococcoida* biomarker quantification is not consistent with biodegradation rates, thus indicating that OHR can be carried out by a wider range of microorganisms [109,145]. In contaminated groundwater, four known OHRBs (*Dehalococcoides*, *Desulfitobacterium*, *Dehalobacter*, and *Dehalogenimonas*) were quantified [146]. *Dehalococcoides* was present in higher copies per $mL^{-1}$ than the other bacteria ($10^7$), although they showed a copy number per mL between $10^4$ and $10^5$ [146].

RDase genes were used as functional biomarkers for monitoring OHR activity. In a lower chlorinated ethenes-contaminated aquifer, *tceA*, *vcrA*, and *bvcA* were quantified in four wells [147]. Quantification showed a higher level of variability. *tceA* was present between $10^3$ and $10^4$ copies $mL^{-1}$, except in a well where it presented with a concentration of $10^1$ copies $mL^{-1}$. *vcrA* and *bvcA* concentrations were between $10^2$ and $10^4$ copies $mL^{-1}$ and $10^2$ and $10^3$ copies $mL^{-1}$, respectively. The well with a lower concentration of *tceA* ($10^1$) showed a lower concentration of ethene [147].

Biostimulation (the addition of vegetable oil) increased the concentration of these functional biomarkers by one order of magnitude, increasing from $10^3$ copies $L^{-1}$ to $10^4$ copies $L^{-1}$ [148]. The treatment induced a higher increase in functional biomarkers than *Dehalococcoides* 16S rRNA gene copies, which were present in higher copies in unamended wells than functional biomarkers. Biostimulation coupled with bioaugmentation (*Clostridium butyricum*, a hydrogen-producing bacteria) amplified the increase of *tceA*, *vcrA*, and *bvcA* copies, with a rise of two orders of magnitude [148].

Aquifer depth seems not to affect the presence of dechlorination functional biomarkers. In aquifers at 14 m and 1.5–2 m below ground level, OHR biomarkers were in the range of $10^2$–$10^4$ gene copies $L^{-1}$ in both conditions [147,148].

The microbial community composition in a contaminated aquifer can be very heterogeneous among different sampling points. Yoshikawa and colleagues [147] reported the composition of four wells of the microbial community, coupled in pairs, in a lower chlorinated, ethenes-contaminated aquifer. *Proteobacteria* were present in high relative abundance in all wells. *Actinobacteria* were present in higher amounts in the wells with lower insoluble iron concentrations (1.3–3.7 mg $L^{-1}$) and higher soluble iron concentrations (4.3–5.3 mg $L^{-1}$). Two wells with lower ORP (−248 mV and −264 mV) showed a higher relative abundance of *Firmicutes*, while the other two, with higher ORP (−183 and −186), presented a higher relative abundance of *Thaumarchaeota.* With a relative abundance of ~30%, *Euryarchaeota* were predominant in wells with a lower concentration of chloroethene

but a higher amount of ethene [147]. The presence of a high relative abundance of *Proteobacteria*, *Actinobacteria*, and *Thaumarchaeota* was also reported by Jin and colleagues [145] in three wells of the chloroethene-contaminated aquifer. In the microbial community of this analyzed aquifer, *Bacteroidetes* and *Parcubacteria* were also detected in high amounts of ~60% and ~20%, respectively [145]. The effect of anaerobic biostimulation treatment (the addition of vegetable oils) on the bacterial community was analyzed by monitoring five wells. The three injection wells located close to each other showed a similar final bacterial community composition with an increase of organic acid and hydrogen-producing bacteria (*Propionispora*, *Sporomusa*, *Dysgonomonas*, and *Veillonellaceae*), OHRB and OHR-involved bacteria (*Shewanella*, *Bacteroides*, *Citrobacter*), and iron and sulfate-reducing bacteria (*Desulfovibrio*) [148].

Hellal and colleagues [149] compared qPCR data with 16S rRNA gene Illumina sequencing data and showed that there was no correlation between the presence of known OHRB 16S rRNA genes and functional biomarkers, suggesting the presence of other OHRB that were not characterized yet.

Aerobic VC mineralization was monitored through the genes encoding enzymes involved in metabolic (*etnC* and *etnE*) and co-metabolic (*pmoA* and *mmoX*) pathways. Jin and Mattes [150] tested different PCR primers for the quantification of *etnC* and *etnE* in three different CE-contaminated aquifers where VC was from 0.8 to 46 μg $L^{-1}$. They found that *etnC* and *etnE* were present in all the samples with around $10^3$–$10^5$ gene copies $L^{-1}$, regardless of VC concentration, with *etnE* being more abundant than *etnC*. In VC-contaminated groundwater, the *etnC* and *etnE* transcripts ranged from $10^3$ to $10^4$ transcript copies $L^{-1}$, and the *pmoA* transcript was $10^3$–$10^5$ transcript copies per L, more abundant than the *mmoX ones* ($10^3$–$10^4$ transcript copies per $L^{-1}$) [151]. 16S rRNA gene libraries were consistent with functional biomarkers, with a higher relative abundance of methanotrophic genera (belonging to *Alphaproteobacteria* and *Gammaproteobacteria*) than VC/ethene degrading bacteria (only *Mycobacterium* was detected). The highest numbers of methanotrophic bacteria were detected in wells with higher oxygen and VC concentrations [151].

The coexistence of anaerobic and aerobic degradation of VC in aerobic and anaerobic growth conditions was examined at a microcosm scale by Atashgahi and colleagues [152]. In addition, the microcosms were also set up with two different soils, one with a higher organic carbon load and the other with a lower organic carbon load. The presence of the three VC degradation pathway expressions of biomarkers for both anaerobic (i.e., *bvcA*, *tceA*, and *Dehalococcoides* 16S rRNA gene) and aerobic (i.e., *etnC*, *etnE*, and *pmoA*) processes/bacteria was investigated by RT-qPCR. In soil with a high organic carbon load, where oxygen has limited penetration in the sediments, OHR, aerobic co-metabolism, and metabolic oxidation were all present. On the other hand, in microcosms containing low levels of organic carbon sediment, aerobic conditions led to a decrease in *Dehalococcoides*. This study suggested the importance of pedologic analysis to determine the possible VC biodegrading activity [152].

The microbial community of contaminated groundwater was characterized during a biostimulation treatment with the addition of oxygen [153]. *Proteobacteria* was the predominant phylum in all the analyzed wells (20.1–90.2% relative abundance). Different VC-oxidizing bacteria belong to this phylum (*Ralstonia*, *Rhodoferax*, and *Pseudomonas*). Methanotrophs' relative abundance showed high variability between 2.5% and 39.3% in different wells. In the first 10 OTUs, 3 methanotrophs were present (*Methylosinus* sp., *Crenothrix* sp., and *Methylococcus* sp.). Even if oxygen was added, the *Chloroflexi* phylum was detected with a relative abundance of about 8% [153]. Regarding the Archaeal community, the predominant phylum was *Euryarchaeota*, and methanogens were between 23.8 and 85.6% of total Archaea [153].

The simultaneous presence of biomarkers for the three VC transforming pathways (OHR, metabolic oxidation, and co-metabolic oxidation) was investigated at the field scale. Changes in the composition of in situ microbial communities during oxygen injection treatments were monitored at a site where enhancement of reductive dehalogenation produced an accumulation of VC [153]. In the groundwater, *vcrA* genes for VC reductase were absent,

whereas *bvcA* genes were present in $10^1$ and $10^5$ gene copies $L^{-1}$. *etnC* and *etnE* genes and transcript amounts were stable during the monitored time (from $10^4$ to $10^5$ transcript copies $L^{-1}$). *pmoA* ($10^6$–$10^8$ gene copies $L^{-1}$ and $10^4$–$10^7$ transcript copies $L^{-1}$) and *mmoX* ($10^3$–$10^7$ gene copies $L^{-1}$ and $10^3$–$10^5$ transcript copies $L^{-1}$) genes and transcripts were present in high amounts. Even with the oxygen injection treatment, the presence of *bvcA* and OHRB showed that a low anaerobic reductive dechlorination activity was carried out.

Richards and colleagues [154] quantified *etnC* and *etnE*, *pmoA* and *mmoX*, and *vcrA* and *bvcA* in chloroethene-contaminated aquifer soil at different depths. *etnC* and *etnE* ($10^7$ and $10^6$ gene copies $L^{-1}$, respectively) were always present in a higher amount than *pmoA*, *mmoX*, *vcrA*, and *bvcA* in all considered wells ($10^5$, $10^3$, $10^4$, and $10^5$ gene copies $L^{-1}$, respectively). Aerobic biomarker concentration decreased in the deeper soil portion, particularly co-metabolic biomarkers, but they were still present 5 m below ground level. On the other hand, anaerobic biomarkers were not affected by depth. These three biomarker groups were present in coexistence in 48% of the sample [154]. All 6 VC biotransformation biomarkers were quantified in 35 wells of 16 contaminated groundwater plumes affected or not by biostimulation with whey addition [155]. *etnC* and *etnE*, *pmoA*, and *mmoX* were detected simultaneously in 90% of groundwater samples. Considering all biomarkers, coexistence was established in 78% of the sample, which increased to 88% if only biostimulated sites were considered [155]. Biomarkers were then related to physical-chemical parameters detected in the contaminated groundwaters through Pearson correlation. *etnC* and *etnE* showed a positive correlation with *cis*-DCE and VC concentrations. On the other hand, *pmoA* and *mmoX* were not correlated with these compounds but with methane and Fe. OHR biomarkers (*vcrA* and *bvcA*) showed a strong positive correlation ($p$-values = 0.001) with *Dehalococcoides*, *Dehalogenimonas*, and *Dehalobacter* gene copies. They were negatively correlated with ORP value and $SO_4^{-2}$ concentration, due to optimal activity conditions common to OHR and sulfur reduction [155].

The coexistence of anaerobic and aerobic biomarkers emphasizes the heterogeneity of aquifer composition that accommodates aerobic and anaerobic microenvironments, allowing the simultaneous activity of the three different pathways.

While OHR and VC oxidation were efficiently monitored at the field scale through different molecular biomarkers, specific *cis*-DCE biomarkers are not known yet [80].

The results of these studies show that it is important to conduct a molecular analysis of the microbial population to understand which bacteria are responsible for the degradation of VC. In this case, because of the stability of the number of genes characteristic of aerobic oxidation and the increase of the methanotroph community, it is possible to speculate that VC degradation was carried out by these microbial species.

## 9. Conclusions

Chlorinated ethenes are persistent contaminant compounds that are hardly degraded by physical and chemical treatments. For these reasons, their bacterial degradation is a valid alternative to remediating contaminated sites.

The accumulation of less chlorinated compounds (i.e., VC and *cis*-DCE) from OHR poses serious health problems, especially when their vapors enter the vadose zone and reach the surface. For this reason, it is important to characterize deeper microorganisms that are able to achieve complete dechlorination of CEs or determine the more favorable conditions to enhance the dechlorination of lower chlorinated ethenes. On the other hand, a greater knowledge of the aerobic degradation of DCE and VC can be a valid support for reaching complete detoxification of CEs.

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## Abbreviations

| | |
|---|---|
| 1,1-DCE | 1,1-*trans*-dichloroethene |
| 1,2-DCE | 1,2-*trans*-dichloroethene |
| AkMO | alkene monooxygenase |
| BES | bioelectrochemical systems |
| *bvcA* | vinyl chloride reductase |
| CEs | chloroethenes |
| *cis*-DCE | *cis*-dichloroethene |
| D-NAPL | dense non-aqueous phase liquid |
| EaCoMT | epoxyalkane:coenzyme M transferase |
| GSH | glutathione |
| GST | glutathione S-transferase |
| MMO | methanol monooxygenase |
| nZVI | nano zero valent iron |
| OHR | organohalide respiration |
| OHRB | Organohalide respiring bacteria |
| ORP | oxidative redox potential |
| PCE | tetrachloroethene |
| pceA | tetrachloroethene reductive dehalogenase |
| pMMO | particulate MMO |
| RDases | reductive dehalogenase homologous genes |
| sMMO | soluble MMO |
| TCE | trichloroethene |
| tceA | trichloroethene reductive dehalogenase |
| VC | vinyl chloride |
| vcrA | vinyl chloride reductase |

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