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„Anaerobic and aerobic microbial dechlorination of various chlorinated hydrocarbons - characterization of enrichment cultures in microcosms“

verfasst von / submitted by

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Abstract

Tetrachloroethylene (PCE) and trichloroethylene (TCE) are both commonly occurring soil and groundwater contaminants, which is a consequence of their incorrect disposal and historical accidental leakages. 1,1,1-Trichloroethane (1,1,1-TCA) is often present at the contaminated sites as a co-pollutant together with TCE, and is known to inhibit a number of TCE-degrading microorganisms. Hence, their elimination is essential to ensure the successful bioremediation of sites contaminated with a mixture of chlorinated organic compounds.

This study characterized several PCE, TCE and 1,1,1-TCA degrading mixed microbial cultures derived from two contaminated sites in Austria, in comparison with well studied dechlorinating culture KB1. The concentrations of chlorinated hydrocarbons, the intermediate degradation products, and the non-toxic dechlorinated product, ethane, were monitored in the headspace gas of the test bottles.

Results of the anaerobic enrichment experiment with microbial culture enriched from Rohrbach, Lower Austria, showed complete conversion of PCE into ethene within the first 40-50 days since the start of the experiment. However, after a transfer in the bigger volume, dechlorination stopped, and the experiment had to be terminated. Anaerobic enrichment experiments with environmental material from Pöttinger, Upper Austria, showed much slower dechlorination rate, and the intermediate degradation compounds were still detected on the 100th day of experiment. Degradation of TCE and 1,1,1-TCA showed less prominent results. While in anaerobic settings the amount of both TCE and 1,1,1-TCA decreased significantly, they were still present in the headspace of analyzed batches even after the 90th day of experiment. Aerobic degradation experiment indicated almost no reduction in the amount of TCE and 1,1,1-TCA mixture.

Kurzfassung

Tetrachlorethen (PCE) ist aufgrund unsachgemäßer Entsorgung und versehentlicher Verschüttungen ein häufiger Grundwasserschadstoff. 1,1,1-Trichlorethan (1,1,1-TCA) wird häufig als Co-Kontaminant mit Trichlorethen (TCE) gefunden und hemmt einige TCE-abbauende Mikroorganismen. Ihre Entfernung ist daher für eine wirksame biologische Sanierung von Standorten erforderlich, die mit gemischten chlorierten organischen Stoffen kontaminiert sind.

In dieser Studie wurden mehrere PCE-, TCE- und 1,1,1-TCA-abbauende gemischte mikrobielle Kulturen aus zwei kontaminierten Standorten in Österreich im Vergleich zur gut untersuchten Entchlorungskultur KB1 charakterisiert. Im Headspace-Gas der Testflaschen wurden die Konzentrationen chlorierter Kohlenwasserstoffe, der dazwischenliegenden Abbauprodukte und des harmlosen nichtchlorierten Produkts Ethylen überwacht.

Ergebnisse des anaeroben Anreicherungsversuchs mit angereicherter mikrobieller Kultur aus Rohrbach, Niederösterreich, zeigten eine vollständige Umwandlung von PCE in Ethen innerhalb der ersten 40–50 Tage seit Beginn des Versuchs. Nach einem Transfer in das größere Volumen stoppte die Entchlorung jedoch und das Experiment musste abgebrochen werden. Anaerobe Anreicherungsversuche mit Umweltmaterial aus Pöttinger, Oberösterreich, zeigten eine deutlich langsamere Entchlorungsrate und die Zwischenabbauverbindungen wurden noch am 100. Versuchstag nachgewiesen. Der Abbau von TCE und 1,1,1-TCA zeigte weniger ausgeprägte Ergebnisse. Während in anaeroben Umgebungen die Menge an TCE und 1,1,1-TCA deutlich abnahm, waren sie auch nach dem 90. Versuchstag noch im Headspace der analysierten Chargen vorhanden. Das Experiment zum aeroben Abbau zeigte nahezu keine Verringerung der Menge an TCE- und 1,1,1-TCA-Gemisch.

1. Introduction

There are numerous contaminated sites where chlorinated hydrocarbons have leaked into the soil and groundwater, jeopardizing both human well-being and the environment. As these substances are very persistent in the environment, efforts are being made to find in situ for their degradation. Among other approaches, degradation with dechlorinating bacterial cultures has proved itself as one of the most cost-effective. However, it is crucial to accumulate more knowledge about the microorganisms involved, as well as about the requisite environmental conditions. The objective of this work is to contribute to this goal. In this study, bacterial cultures from two contaminated sites in Austria were studied for their ability to decompose chlorinated ethenes. Pollutants investigated in the project were PCE, TCE and 1,1,1-TCA. The project was separated into several branches, starting with anaerobic enrichment culture experiment. The major aim was to stimulate growth of dechlorinating bacteria in enriched media by creating suitable conditions, observe the PCE, TCE and 1,1,1-TCA degradation, select the culture that is the most capable of full and complete dechlorination of pollutants, and potentially isolate it in the pure culture comparable with well studied dechlorinating culture KB1. Another experiment focused on an aerobic cometabolic degradation experiment of a mixture of TCE and 1,1,1-TCA with microbial culture enriched from Pöttinger, Upper Austria.

The final objective of this work was stimulation of the anaerobic and aerobic bacterial dechlorination of chlorinated hydrocarbons (PCE, TCE, 111-TCA) in laboratory conditions and growth of microbial cultures capable of full chlorinated hydrocarbons dechlorination with a further aim of its' application to contaminated sites for CHC reduction on contaminated sites.

2. Chlorinated volatile organic compounds

Chlorinated volatile organic compounds (further Cl-VOCs) are a group of carbon-based chemicals with a C-Cl bond that under normal conditions temperature and pressure are present as liquids, but possess high volatility and evaporate easily. The boiling points of these compounds varies approximately between 50 and 260 °C at the room temperature and normal atmospheric pressure (ATSDR, 2006; Hester & Harrison, 1995; Huang et al., 2014; Li et al., 2009). The bond between carbon and chlorine atoms is strong and gives these compounds high stability, which is seen as a major benefit in agricultural and industrial applications, and at the same time makes them a potential eco-hazard since their molecules tend to stay in the environment for a longer time. Cl-VOCs have been synthesized and widely used in industry for years in diverse commercial products, such as solvents or degreasing agents (Huang et al., 2014). They are difficult to be replaced due to their above-mentioned high stability and non-inflammability. Due to their extensive use, nowadays Cl-VOCs are present in different parts of the environment and are often discovered in contaminated soil, air, groundwater, rivers and lakes (see Fig. 1). Their slow degradation allows them to travel over big distances from the sources of contamination, which are mainly the manufacturing emissions, such as from chemical industries. Release in the environment can also be caused by the improper storage and disposal of Cl-VOC containing household products (Kamal et al., 2016; Li et al., 2009; Moran et al., 2007). Cl-VOCs impact air quality and, along with other VOCs, contribute to air pollution.

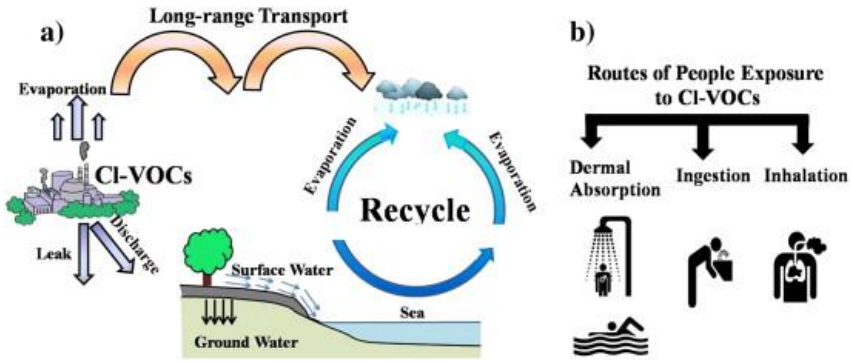

Fig. 1: *"A schematic representation of the fate for Cl-VOCs releasing into environment (a); and the primary routes of human exposure to Cl-VOCs (b)".* (by Huang et al. 2014, used with a permission of Elsevier).

Common ways of human exposure to these chemicals are by accidentally breathing them in or ingesting them, and through having a direct skin contact (Hake & Stewart, 1977). Most Cl-VOCs have been under strict monitoring since they are associated with the risk of cancer. They are included in the catalogue of priority contaminants by governmental administrative bodies of several countries, such as the European Commission (EC), United States Environmental Protection Agency (U.S. EPA) and the Ministry of Environmental Protection (MEP) of China (Huang et al. 2014).

The three chemicals that are going to be used in the study are presented in the Table 1 below.

Substance	**Abbr.**	**Mol. formula**	**CAS RN**	**Density (g/cm3) 20 °C**	**Solubility in water (g/L) 20 °C**	**Carcinogenicity**
1,1,1-trichloroethane	1,1,1-TCA	C2H3Cl3	71-55-6	1.31	<1	Group 3
trichloroethylene	TCE	C2HCl3	79-01-6	1.46	1.28	Group 2A
tetrachloroethylene	PCE	C2Cl4	127-18-4	1.63	0.15	Group 2A

Table 1: Physico-chemical properties and toxicological classifications of the main Cl-VOCs used in this study.

Carcinogenicity rating used in this table was based on CAMEO chemicals, Huang et al., 2014 and the IARC, 1995. Group 2, subdivided into subgroups A and B, catalogues chemicals that likely pose a carcinogenic risk to humans based on limited evidence,

while there is sufficient evidence of their carcinogenicity in animals. Group 2A, to which both PCE and TCE belong, represents a slightly higher level of evidence regarding their potential carcinogenicity in humans. 1,1,1-TCA falls within the Group 3, which categorizes agents as not definitively classifiable as carcinogenic in humans. This classification does not necessarily indicate that these agents are considered non-carcinogenic or safe; rather, it underscores the need for additional research to assess their carcinogenic potential. All of them are still catalogued as priority contaminants by the U.S. EPA and MEP of China, which highlights the importance of continued investigation.

2.1 Perchloroethylene

Perchloroethylene (PCE), or tetrachloroethylene, is a synthetic halogenated hydrocarbon with high stability, good organic solvent properties and low flammability. It is highly volatile; according to calculations by Boutonnet et al., 1998, PCE exhibits a partitioning inclination in the environment with 99.7% in air, 0.3% in water, and less than 0.01% in both soil and sediment. Like many other Cl-VOCs, it has a form of a colorless liquid with a sweet smell and low solubility in water. Some of its physical properties are noted in Table 1. Its' structure is illustrated below.

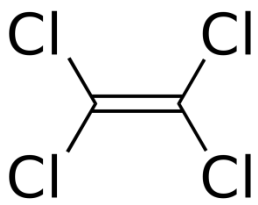

Fig. 2: Perchloroethylene, also known as tetrachloroethene, or tetrachloroethylene.

PCE was first synthesized in 1821 by Michael Faraday, who decomposed perchloroethane under high temperatures (Doherty, 2000), but it was not until the early 20th century that PCE began to be used widely as a dry cleaning solvent due to its non-flammable nature and excellent degreasing capabilities that prevent fabric fibers from swelling and shrinking (Linak et al., 1992; NICNAS, 2001). Some information exists that it was used during the World War II (Norman et al., 1981). PCE

manufacturing achieved its' highest point in the 80s in Japan, some countries in western Europe, and in the United States (Linak et al., 1992). Its use in these applications contributed to widespread contamination of soil and groundwater, especially at industrial sites and military bases (OUSD, 2011). From the 1950s to the 1980s, PCE was used as a degreaser at the Marine Corps Base Camp Lejeune in the Southeast of the United States, and later was found in the drinking water supply among some other chemicals (NRC, 2009). There have been several cases of perchloroethylene contamination in the 21st century, although the extent of their impact is not always clear. One of the examples is the town of Anniston, Alabama, which, according to the Guardian, in 2002 was found to be contaminated with a variety of toxic chemicals, including PCE, that had been released by a nearby chemical plant (O'Hagan, 2018). The contamination was connected with a series of health issues among residents, including cancer and neural system disorders. In the end of the last century, the European CAREX project approximated that around 820,000 workers were exposed in 15 European Union countries. As observed by one study, the majority of exposures took place within dry-cleaning shops (Kauppinen et al., 2000). An update of CAREX for Italy (Mirabelli & Kauppinen, 2005) revealed a minor increase in exposed workers, from 102,500 to 106,300, between 1990-1993 and 2001, respectively. Similarly, another study (von Grote et al., 2006) indicated that the amount of individuals in Germany's dry-cleaning manufactures who suffered from the contact with PCE alone declined substantially from nearly 25,700 in 1975 to about 5,900 in 2001. A recent investigation (Liu et al., 2023) has shown high levels of contamination by PCE in soil samples obtained from the air, soil, and sludge near two tetrachloroethylene factories in China, and the assessment of the risk for workers has suggested potential harmful health effects.

According to the U.S. EPA, 2004, PCE has been the first most frequently detected Cl-VOCs in groundwater all over the U.S. with concentration higher than 5 ppb (Maximum Contaminant Level). Chlorinated ethylenes have been categorized as group 1 pollutant by the International Agency for Research on Cancer (IARC, 1987) and included among the list of priority contaminants by the U.S. EPA (U.S. EPA, 1981). Subjection to PCE can happen through breathing it in, ingesting it, or through having a dermal contact. Health risks associated with tetrachloroethylene exposure depend on the duration and level of exposure. Short exposure to high levels of it were shown

to cause dizziness, migraines, sickness and loss of consciousness, while chronic subjection to lower levels can lead to a variety of health problems, including neurological and reproductive effects, internal organ damage, cancer, and some other health effects (Altmann et al., 1990; Billionnet et al., 2011; Blair et al., 1990; Lynge et al., 1997; Moran et al., 2007). PCE has no known significant natural source, although it has been detected in several types of algae (Abrahamsson et al., 1995). It is also one of the most challenging Cl-VOCs to biodegrade.

2.2 Trichloroethylene

Trichloroethylene (TCE), as PCE, is a chlorinated ethylene, and at normal temperature and pressure is a colorless, sweet smelling, nonflammable liquid. It is highly volatile and easily changes from liquid to a gas state. Its structure is illustrated below.

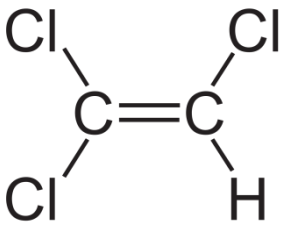

Fig. 3: Trichloroethylene, also known as ethylene trichloride, trichloroethene, and trichlor.

TCE was discovered in 1864 by the German chemist Emil Fisher, and the commercial production of it commenced in Austria around 1905 (Morrison & Murphy, 2015). Since then, TCE has been frequently applied as a solvent for cleaning metal parts, degreasing and dry cleaning fabrics, and as a chemical intermediate in the production of refrigerants, pesticides, and other chemicals. TCE is a common degreaser for metal parts in automotive, aerospace, and other industries (U.S. EPA, 2014).

TCE contamination has been a significant environmental issue for several decades, with numerous incidents of contamination occurring across the world. One of the most

well-known incidents occurred in Woburn, Massachusetts, USA (Heneghan, 2000). During the 1970s, it was discovered that two public city-owned water supply wells in Woburn contained hazardous substances including As, TCE, PCE and chloroform together with smaller amounts of other organic pollutants (Costas et al., 2002). This contamination resulted in elevated incidences of childhood leukemia and other health issues. The case was the subject of a book and a movie, both titled "A Civil Action". TCE was also found in groundwater and soil in several European countries, including Germany, Sweden and Italy (Pirastu et al., 2013; Plepys et al., 2007). More recently, in 2018, the United States Environmental Protection Agency reported that TCE had contaminated the groundwater in several locations across the country, including in Arizona, New Jersey, and Ohio.

Like PCE, TCE is a hazardous substance that was found to be a potential human carcinogen. Exposure can occur through breathing it in, ingesting, or dermal contact with contaminated water, soil, or air. Dermal subjection to TCE can cause skin irritation, while inhaling or ingesting it may lead to symptoms like headaches, impaired coordination, and sickness (Trichloroethylene: toxicological overview, 2021). Chronic exposure to TCE has also been connected with various health effects, including internal organs damage, neurological effects, and reproductive and developmental effects (Morgan & Cassady, 2002; U.S. EPA, 2011).

Due to its hazardous properties, the use of TCE has been restricted in many countries, and efforts have been made to phase it out and find safer alternatives. Starting from the 1980s, the Environmental Protection Agency began regulating trichloroethylene, establishing its' Maximum Contaminant Levels in accordance with the Safe Drinking Water Act (U.S. EPA, 2008). Besides, the Occupational Safety and Health Administration (OSHA) also sets thresholds for the possible exposure to TCE in the air of workplaces. In general, the US. EPA has classified TCE as a hazardous substance and has set limits on its use and disposal to prevent further contamination.

2.3 1,1,1-Trichloroethane

After the peak of extensive manufacturing use of TCE as a solvent in the beginning of the 70s, a seemingly safer alternative, 1,1,1-trichloroethane (1,1,1-TCA), was widely adopted (ATSDR, 2006). 1,1,1-TCA is a colorless, non-flammable liquid with a sweet odor, that belongs to the class of chlorinated ethanes, or polychloroethanes (PCA), which are a group of chlorinated aliphatic compounds. They show poor biodegradability and therefore are persistent in aquatic environments. Its' structure is illustrated below.

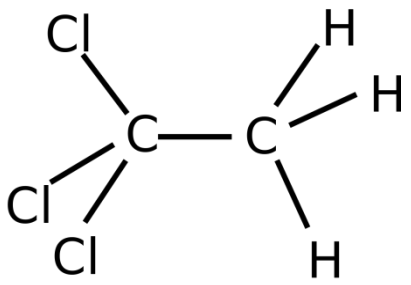

Fig. 4: 1,1,1-Trichloroethane, also known as methyl chloroform

1,1,1-TCA was first synthesized in the early 20th century, and its use expanded over time to include a variety of industrial and consumer applications, such as a cleaning solvent, degreaser, refrigerator and as a propellant in aerosol products (ATSDR, 2006). In the 1960s and 1970s, concerns began to emerge about the potential health and environmental effects of 1,1,1-TCA, particularly its role as a volatile organic compound contributing to the formation of ground-level ozone, a major component of smog. In response to these concerns, many countries began to regulate the use of 1,1,1-TCA in the 1980s and 1990s. In the United States, the use of 1,1,1-TCA as a consumer product was banned in 1995, and its use in industrial applications is heavily regulated under the Clean Air Act and other environmental regulations (Kapp, 2014). Today, 1,1,1-TCA is largely phased out of use in many countries due to its potential health and environmental effects (IPCS, 1990). However, it may still be used in some industrial applications in certain countries where regulations are less strict.

Several compounds from the group of polychloroethanes, to which 1,1,1-TCA belongs, have the potential to induce circulatory and respiratory failure, neurological disorders, and can adversely affect some organs including liver, heart and kidney; a number of those compounds are also potentially carcinogenic (Huang et al., 2014). Although short exposure to significant amount of 1,1,1-TCA can cause central nervous system depression, there is no evidence linking chronic exposure to cancer (Grostern & Edwards, 2006). Nevertheless, 1,1,1-TCA is a substance capable of ozone layer destruction, and its production and utilization have been under the control of Montreal Protocol on Substances that Deplete the Ozone Layer (UNEP, 2000). It can also be added that several accidental spills and inadequate discarding practices have made 1,1,1-TCA a prevalent pollutant found in groundwater. This substance has been identified among other pollutants in approximately 30% of the total sites listed on the National Priorities List (NPL) by the U.S. EPA (Grostern & Edwards, 2006). 1,1,1-TCA is frequently discovered as a co-contaminant with TCE and can impede the degradation process of certain TCE-degrading microorganisms.

3. Microbial dechlorination of Cl-VOCs

Numerous remediation technologies are used for the removal of chlorinated hydrocarbons from the environment. Some of them are based on physical removal of contaminants, such as air-stripping (Li et al., 2012) or adsorption in surfactant solutions with granulated activated carbon (Yang et al., 2009). Despite their effectiveness, these methods do not change the nature of contaminants, which still have to be dealt with after removal. However, methods such as phyto- and bioremediation allow to neutralize chlorinated hydrocarbons by the destruction of the bond between carbon and chlorine atoms during the metabolic activity of plants or microorganisms (Huang et al., 2014).

In the case of contaminated groundwater, where oxygen is often scarce, anaerobic degradation is relevant for the bioremediation of impacted sites. Anaerobic degradation refers to the breakdown of compounds by microorganisms in the absence

of oxygen. Proper monitoring and understanding of the site-specific conditions, together with the selection of appropriate electron donors and acceptors, are crucial for optimizing the anaerobic degradation process and ensuring successful remediation outcomes. A wide group of anaerobic microorganisms shows the capacity to reductively dehalogenate aliphatic and aromatic halogenated hydrocarbons as a part of their respiratory process by using chlorinated hydrocarbons as terminal electron acceptors (Löffler et al., 2013). Energy is captured from reductive dechlorination reactions, in which hydrogen sequentially displaces chlorine (van Agteren et al., 1998). Both metabolic and cometabolic reductive dechlorination is possible.

Knowledge about bacteria capable of aerobically metabolizing Cl-VOCs is still relatively scarce, primarily because these compounds are predominantly degraded under anaerobic conditions in reductive environments. As a result, most research reports and investigations have predominantly concentrated on anaerobic dechlorination mechanisms, given their greater significance for in situ remediation. Nonetheless, some bacteria are also capable to aerobically degrade Cl-VOCs, particularly *cis*-DCE and VC. Notably, a few of these bacteria have been found to adapt to very low oxygen concentrations, indicating their favorable niche within polluted aquifers (Coleman et al., 2002; Fullerton et al., 2014). Several studies have documented growth of a mixed microbial culture in oxic conditions, as well as certain bacterial strains being active in the presence of oxygen with TCE being the only growth substrate (Dey & Roy, 2009; Mukherjee et al., 2012; Schmidt et al., 2014). However, further studies are required for confirmation and better understanding of these processes. It is also important to note that aerobic metabolic biodegradation of single Cl-VOCs has shown itself as more efficient in comparison with the degradation of chloroethene mixtures (Dolinová et al., 2017).

Biodegradation method allows high efficiency (up to 100% conversion into non-toxic hydrocarbons), low cost, in-situ remediation (Grostern & Edwards, 2006; Hoelen & Reinhard, 2004; de de Best et al., 1999). In 2013 bioremediation accounted for 24% among the technologies for the remediation of groundwater (U.S. EPA, 2013, Wang et al., 2022). Up to date, numerous microorganisms with the ability to dechlorinate certain Cl-VOCs have been documented. The genomic databases of those bacteria are also publicly accessible and contain information about the enzymes involved in the degradation processes, which is convenient for their application. However, a relatively

recent publication (Yoshikawa et al., 2017) lists the following disadvantages of the microbial remediation method. Compared to alternative methods like excavation and removal of contaminated soil, bioremediation is a time-consuming process. It is necessary to conduct research to enhance and create bioremediation technologies that can effectively address the multiplex combinations of chemicals that are not uniformly dispensed in the environment, occurring in various states such as solids, liquids, and gases. In real conditions, contaminated soil and groundwater often contains more than one type of chlorinated hydrocarbons, and biodegradation of multiple Cl-VOCs still remains a challenge. Some site factors such as favorable environmental settings for cell growth and reproduction, and appropriate amounts of both nutrients and pollutants have to be considered for a bioremediation to be effective. And sometimes the products generated during biodegradation can potentially be more persistent or harmful compared to the original substance.

3.1 PCE and TCE dechlorination pathways

Anaerobic

Microorganisms engage in the degradation of both PCE and TCE through a sequence of steps that involve increasingly less chlorinated ethenes. This degradation pathway includes *cis*-DCE, *trans*-1,2-dichloroethene (*trans*-DCE), 1,1-dichloroethene (1,1-DCE), VC, and ultimately reaching ethene, ethane and other dechlorination products.

Tetrachloroethylene dechlorination typically involves the reduction of the molecule by various means to break down the chlorine atoms and convert the compound into less harmful forms, such as ethylene (see Fig. 5). A number of methods has been developed and investigated for this aim.

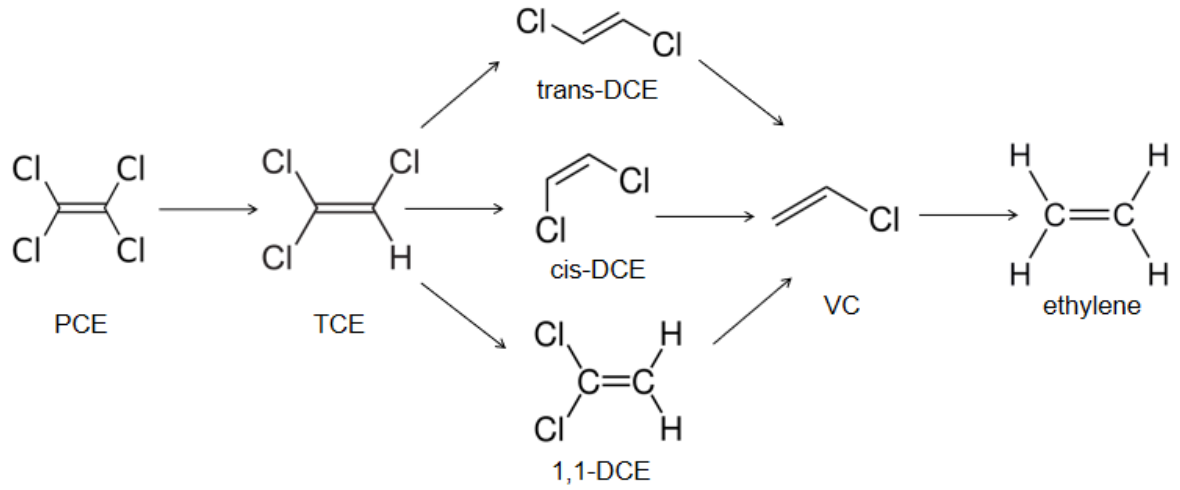

Fig. 5: Tetrachlorethylene (PCE) and trichlorethylene (TCE) anaerobic degradation pathway by *Dehalococcoides* and other genera. *cis*-DCE predominates among other DCE's (modified after Mattes et al., 2010).

A method to reductive tetrachloroethylene dechlorination involves the use of specialized bacteria, such as *Dehalococcoides*, which have the ability to enzymatically dechlorinate chlorinated solvents. These bacteria can selectively remove chlorine atoms from tetrachloroethylene, resulting in the formation of less chlorinated and non-chlorinated compounds. Bioremediation techniques utilizing *Dehalococcoides* bacteria have shown promise for the in-situ treatment of contaminated groundwater and soil.

One approach is a reductive dechlorination using zero-valent iron (ZVI) as a reducing agent. ZVI reacts with chlorinated hydrocarbons, leading to the removal of Cl and the creation of less chlorinated and non-chlorinated compounds (see Fig. 6).

$$Fe^0 \rightarrow Fe^{2+} + 2e^-$$

$$RCl + 2e^- + H^+ \rightarrow RH + Cl^-$$

Fig. 6: Reactions of ZVI corrosion and reduction of aliphatic chlorinated hydrocarbons by ZVI (modified after Yan et al., 2013)

Several studies suggest that zero-valent iron nanoparticles (NZVI) demonstrate higher effectiveness compared to macroscopic ZVI under similar environmental conditions (Deng & Hu, 2002; U.S. EPA 2008). However, some studies have reported that NZVI could have potential toxic impacts on microbes, especially for the gram-negative bacteria (Dong et al., 2016; Saccà et al., 2014). More studies on its ecotoxicity and bioaccumulation are needed.

Aerobic

The knowledge on the aerobic degradation of PCE and TCE are still not as developed, as in case with anaerobic degradation. It's bioremediation was originally thought to be impossible in the presence of oxygen, however, in the 2000's study by Ryoo et al. aerobic degradation of small amounts of PCE by the wastewater bacterium *Pseudomonas stutzeri* was reported (Ryoo et al., 2000). High concentrations of TCE were successfully degraded by a methanotroph strain that used methane and methanole as an only carbon source (Uchiyama et al., 1989), and *Mycobacterium vaccae* grown on propane were able to mineralize smaller amounts of TCE (Vanderberg et al., 1995). Aerobic degradation of PCE and TCE, just as an anaerobic one, involves a series of microbial reactions that break them down into simpler, less toxic compounds (see Fig 7).

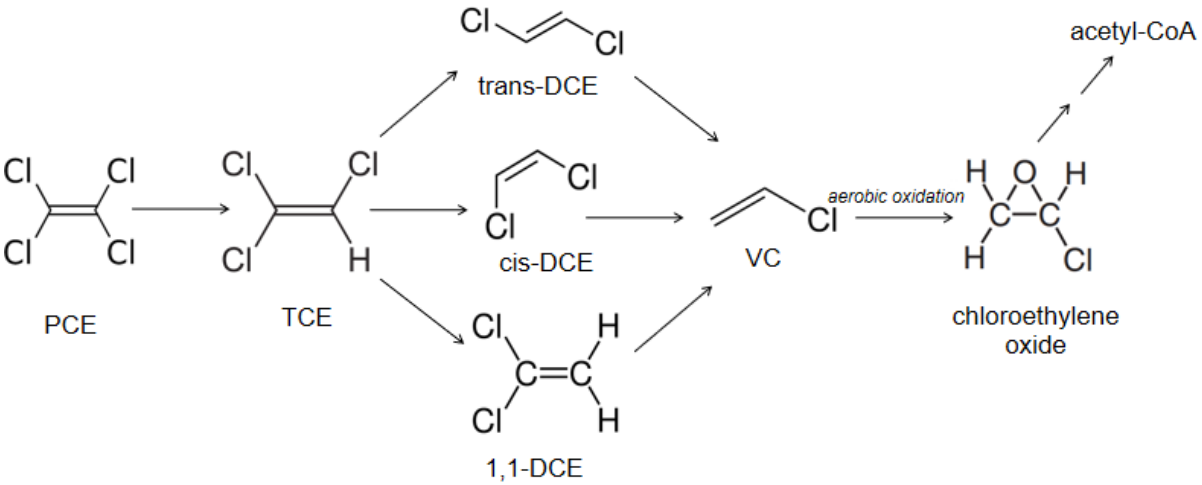

Fig. 7: Tetrachlorethylene (PCE) and trichlorethylene (TCE) aerobic degradation pathway (modified after Mattes et al., 2010).

Initial part of the process resembles the anaerobic one, when PCE is oxidized by aerobic bacteria, typically via enzymes like PCE monooxygenase, which leads to the genesis of trichloroethylene, that in turn is transformed into dichloroethylenes (DCEs), and then to vinylchloride (VC). The next transformation product is chloroethylene oxide, which can form ethylene oxide, or oxirane, a metabolic intermediate in the ethene pathway (Mattes et al., 2010). Ethene-assimilating bacteria mentioned in several studies (Jin & Mattes, 2008; Verce et al., 2000) can then conjugate it into the coenzyme M in a course of a detoxification reaction (Coleman & Spain, 2003).

3.2 1,1,1-TCA dechlorination pathways

Anaerobic

Various anaerobic microorganisms, such as methanogens and sulfate reducers (Adamson & Parkin, 1999; de Best et al. 1999, Scheutz et al., 2011), have the capability to reductively dechlorinate 1,1,1-trichloroethane through cometabolic processes (see Fig. 8).

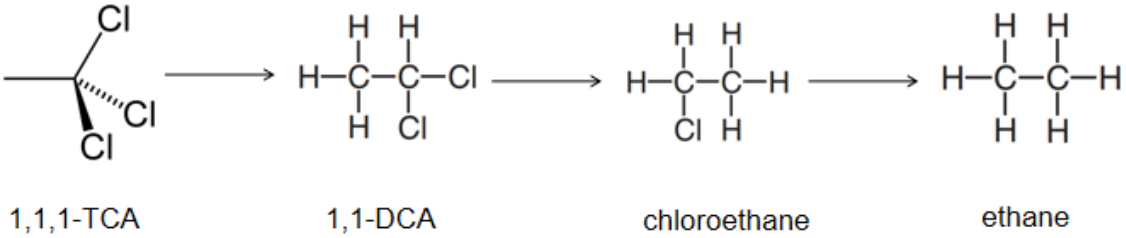

Fig. 8: 1,1,1-trichloroethane (1,1,1-TCA) anaerobic degradation pathway (modified after Mattes et al., 2010).

This typically leads to the formation of partially dechlorinated byproducts, like 1,1-dichloroethane (1,1-DCA) and chloroethane, which can be further degraded into ethane.

Aerobic

The aerobic 1,1,1-TCA degradation still remains relatively unstudied in comparison with anaerobic one, however, some cases have been reported. The aerobic degradation of 1,1,1-TCA can occur through biological processes in the presence of oxygen (see Fig. 9). This process is cometabolic, meaning that it takes place while the microorganisms are metabolizing other compounds.

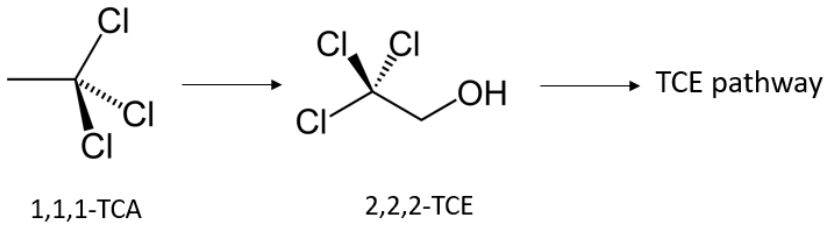

Fig. 9: 1,1,1-trichloroethane (1,1,1-TCA) aerobic degradation pathway by *Methylosinus trichosporium* OB3b and *Mycobacterium spp* (based on Oldenhuis et al., 1989 and Yagi et al., 1999).

As a result, the breakdown of 1,1,1-TCA leads to the formation of byproducts such as 2,2,2-trichloroethanol by methane-oxidizing and some other types of bacteria (Oldenhuis et al., 1989; Yagi et al., 1999). 2,2,2-trichloroethanol is then transformed into 2,2,2-TCE, which undergoes the previously described TCE degradation pathway (see Fig. 9). However, sometimes the byproducts might be trichloroacetic or dichloroacetic acid (Hashimoto et al., 2002).

Figure 10 below illustrates a hypothetical pathway depicting the degradation of both TCE and 1,1,1-TCA by strain TA27, suggested by Hashimoto et al., 2002, and based on previous studies that have documented aerobic bacteria degradation pathways.

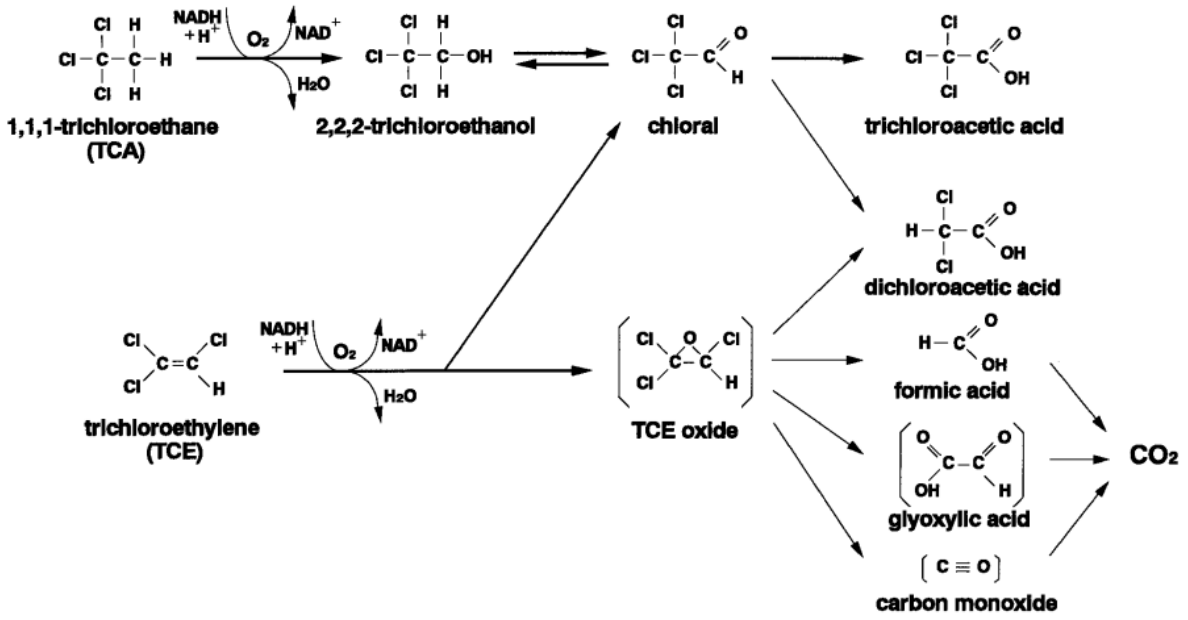

Fig. 10: Simultaneous trichloroethylene (TCE) and 1,1,1-trichloroethane (1,1,1-TCA) aerobic degradation pathway by *Mycobacterium* sp. strain TA27 (from Hashimoto et al., 2002 with a permission of Oxford University Press).

Initially, TCE undergoes conversion into chloral and TCE oxide, facilitated by methane-oxidizing bacteria. Subsequently, TCE oxide and its derivatives transform into carbon dioxide, while chloral can be oxidized into tri- and dichloroacetic acid. 1,1,1-TCA undergoes transformation into 2,2,2-trichloroethanol and then to chloral, which can be further converted into tri- or dichloroacetic acid.

3.3 Bacteria and the enzymes involved

Anaerobic

In this study anaerobic microbial consortium KB-1 (SiREM Lab, Guelph, Ontario, Canada) was used to compare microbial activity and dechlorination rate of bacteria enriched from contaminated sites with well studied culture. According to SiREM Lab, KB-1 is a *"naturally occurring, non-pathogenic microbial culture that contains Dehalococcoides". Dehalococcoides* are well known for their ability to fully convert several Cl-VOCs into ethene. KB-1 is described as a mix of cultures which were enriched

from bacteria discovered in soils and groundwater of sites contaminated with chlorinated hydrocarbons. Importantly, they did not undergo genetic engineering. It has been proven to efficiently and completely break down chlorinated solvents like PCE, TCE, *cis*-1,2-dichloroethene, 1,1-dichloroethene, vinyl chloride, 1,1,1-trichloroethane, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, chloroform, and dichloromethane (SiREM Lab, 2005). This degradation process leads to the formation of non-toxic and environmentally safe byproducts such as ethane, ethane, and acetate. KB-1 is cultivated in a media that consists of various mineral salts and vitamins. To determine the microbial community of KB-1, phylogenetic analysis was carried out by the SiREM Lab, and the results of it are presented in Table 2. Bacteria present in KB-1 have been identified by comparing their 16S rRNA gene sequences to those of well studies organisms. Some features of closely related organisms were compared and helped to recognize the possible traits and properties of the bacteria within KB-1.

Genus
Dehalococcoides sp.
Geobacter sp.
Methanomethlovorans sp.

Table 2: Genus' Identified in KB-1 Microbial Inoculum (SiREM Lab, 2005)

Some other dechlorinating bacteria are presented in Fig. 11 below. While *Dehalococcoides* and *Propionibacterium* are capable of full PCE degradation, other bacteria can only perform part of the dechlorination pathway.

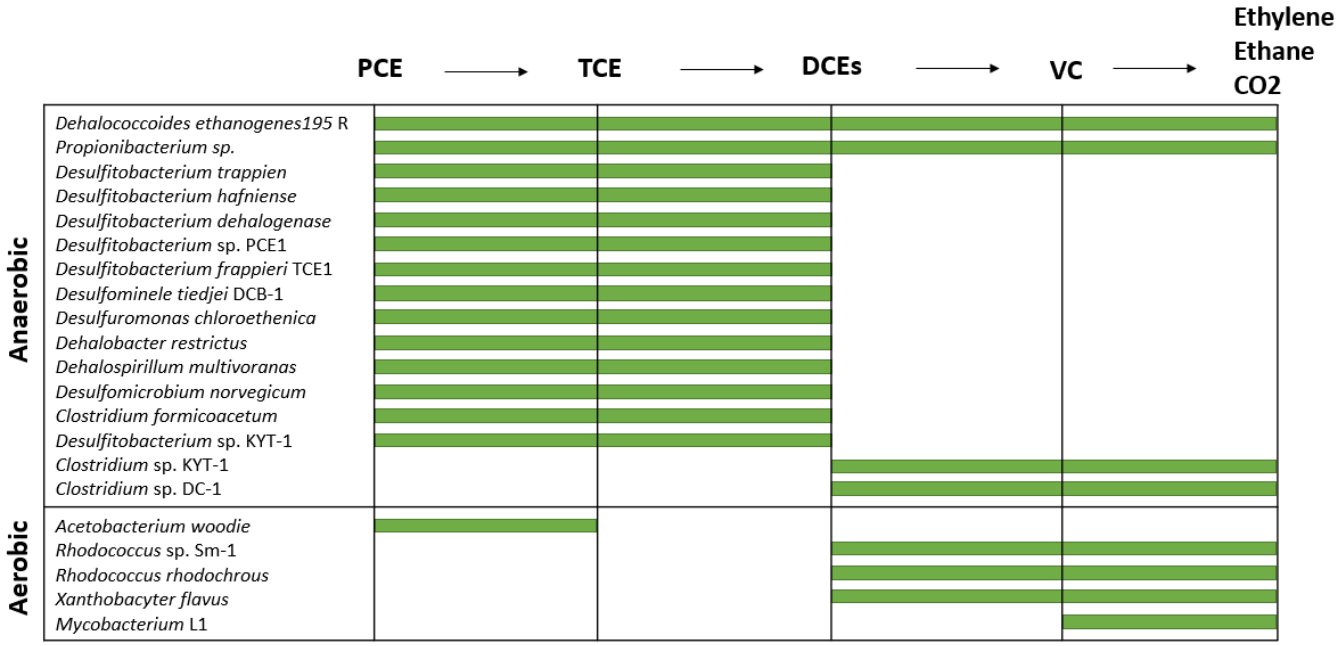

Fig. 11: Isolated Cl-VOCs-dehalorespiring bacteria and dechlorination steps performed (modified after Chang et al., 2012)

As for enzymes involved in the process (see Fig. 12), reductive dehalogenases (RDases) play a crucial role in anaerobic organohalide respiration, as they directly facilitate the cleaving of carbon-chlorine bonds (Futamata et al., 2009). RDases are a group of redox enzymes essential for the anaerobic microbial respiration process that utilizes Cl-VOCs, and that is crucial for their bioremediation (Tang & Edwards, 2013). Microorganisms catalyze dehalorespiring pathways with the help of RDase enzymes, which are usually specifically associated with some certain Cl-VOC substrate (Picott et al., 2022).

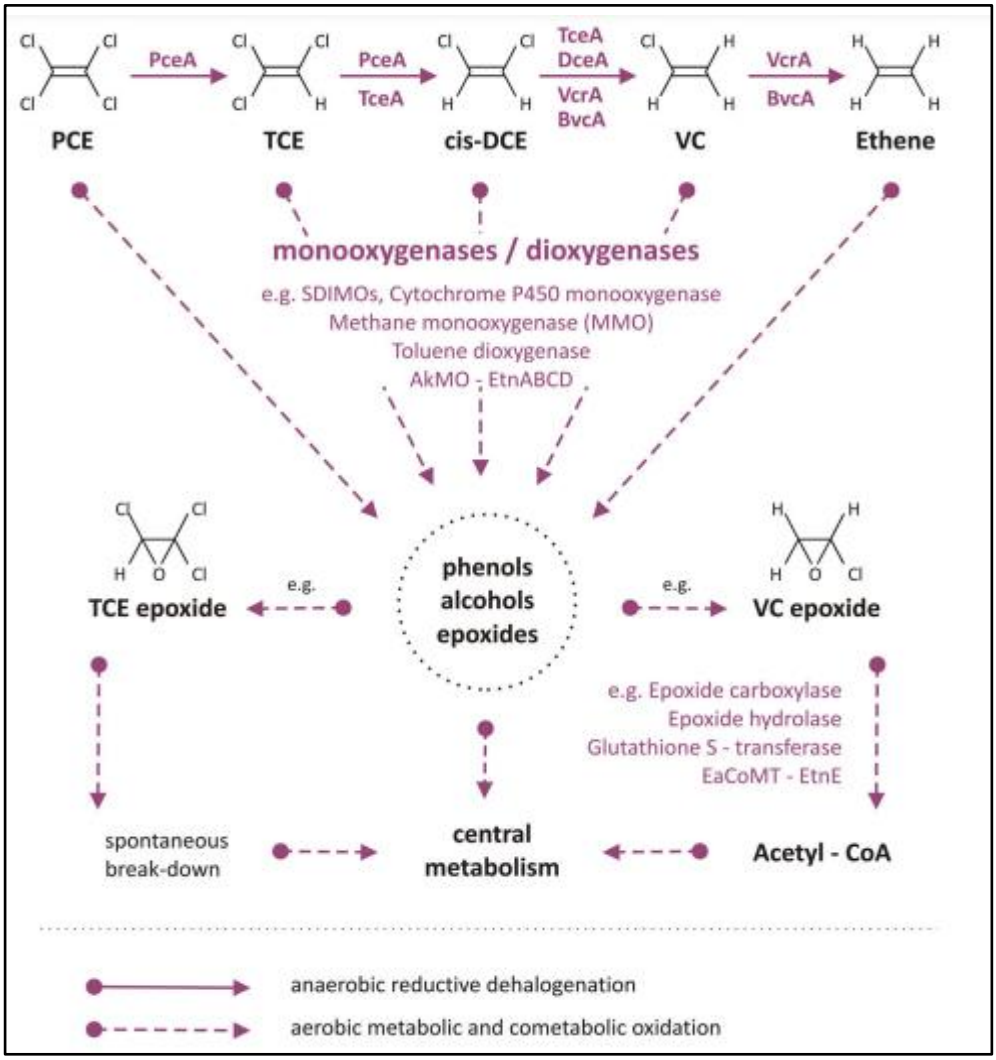

Fig. 12: Enzymes and the intermediate products of Cl-VOCs biodegradation (by Dolinová et al. 2017, used with a permission of Springer Nature)

While most RDases are associated with the cell membrane, certain microorganisms like *Desulfitobacterium*, *Sulfurospirillum* and *Dehalobacter* were found to possess cytoplasmic RDases (Jugder et al., 2015; Maphosa et al., 2012). Different bacterial strains may harbor a range of RDases, each with specific catalytic abilities (Pöritz et al., 2013). Examples include PCE reductive dehalogenase (PCE-RDase), TCE-RDase, and VC-RDase, which perform dechlorination reactions on various chlorinated compounds, eventually leading to ethene production.

The genes responsible for encoding RDases, also known as rdh genes, generally include the rdhA subunit gene, responsible for the catalytic function of the enzyme, and the rdhB gene, which encodes a membrane-anchoring protein (Neumann et al., 1998).

Cobalamin cofactors and iron-sulfur clusters are commonly present in the catalytic subunits of RDases. The expression of RDase genes varies depending on the environmental factors, such as the types of Cl-VOCs present. Nevertheless, the exact correlation between gene expression and the activity of enzyme remains to be fully understood, as documented in several studies (Chow et al., 2010; Jugder et al., 2015; Kranzioch et al., 2015). Bacterial genomes often harbor multiple copies of RDase genes, with over 650 putative rdhA genes known, originating from more than 100 sources, including *Dehalococcoides*. Detection of RDase genes through specific primers can serve as a functional tool used for detecting and assessing dechlorination activity, surpassing the restrictions of some other methods that rely solely on 16S rRNA (Dolinová et al., 2017). The RDase genes responsible for chloroethene reduction include pceA, which encodes PCE reductases found in *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides sp*. strain CBDB1; tceA, which encodes TCE reductases from D. ethenogenes strain 195 and *Dehalococcoides sp*. strain FL2; vcrA, responsible for the VC reductase in *Dehalococcoides sp*. strain VS; and bvcA, encoding the VC reductase in *Dehalococcoides sp*. strain BAV1 (Behrens et al., 2008). Specific primers targeting the above mentioned genes can be employed to determine the growth and activity of chloroethene-degrading microorganisms (Dolinová et al., 2017).

Various types of bacteria, including members of the genera *Pseudomonas* and *Burkholderia* (Ryoo et al., 2000) are known to play a crucial role in the aerobic degradation of PCE. These bacteria possess specialized enzymes that facilitate the breakdown of chlorinated compounds (in detail in Ch. 3.3 Bacteria and the enzymes involved). In one study (Sun et al., 2002) a specific organism (strain TCA1) closely related to *Dehalobacter restrictus* - an organism known for its ability to respire tetrachloroethene (PCE) and trichloroethene (TCE) - was isolated. Strain TCA1 was found to couple the degradation of 1,1,1-TCA and 1,1-DCA to its growth. The prevalence of 1,1,1-TCA-dehalorespiring organisms in various environments is not yet fully understood, but the listed reports demonstrate the prospective of employing microbial activity for remediating the sites where 1,1,1-TCA is present as a pollutant.

Aerobic

Aerobic biodegradation is usually performed with a co-substrate, such as methane, some aromatic compounds, alkanes, alkenes, ammonia or phytochemicals (Ensign et al., 1992; Hamamura et al., 1997; Nelson et al., 1987). These co-substrates are decomposed by oxygenases, which then leads to degradation of chlorinated ethene itself. Numerous aerobic microorganisms have shown ability to co-metabolize certain chlorinated hydrocarbons. *cis*-DCE and VC have been documented to undergo growth-linked oxidation among chlorinated ethenes. In one conducted study (Varzaghani et al., 2019), dechlorination of PCE in relatively high concentrations (0.4–5 mM) was reported by an aerobic bacterial strain, *Sphingopyxis ummariensis VR13*, which was enhanced by the inclusion glucose and yeast extract with acted as an additional substrate. Cometabolic degradation of PCE, TCE and *cis*-DCE in the presence of oxygen has been evidenced by a number of conducted studies (Doughty et al.,2005; Frascari et al. (2006, 2008); Kim et al., 2010). Bacteria that possess the capability to degrade chloroethenes in this manner utilize auxiliary growth substrates, wherein the enzymes initially made for the decomposition of the microbial growth media are involved in breaking down the chloroethenes (Hamamura et al., 1997).

One study lists the most prevalent enzymes responsible for the cometabolic oxidation of Cl-VOCs, which are methane monooxygenase (both soluble and membrane-bound or particulate forms), butane monooxygenase, toluene mono- and dioxygenase (such as toluene-2-monooxygenase, toluene-4-monooxygenase, xylene monooxygenase, and toluene dioxygenase), alkene monooxygenase, and ammonia monooxygenase (Dolinová et al., 2017). Various bacteria express these enzymes, as documented in numerous studies (Halsey et al., 2005; Fox et al.,1990; Saeki et al., 1999; Suttinun et al., 2010). Among these enzymes, it was evidenced in one study that the soluble di-iron monooxygenases (SDIMOs) play a crucial role in initiating hydrocarbon oxidation and are commonly found with a broad range of substrates. SDIMOs comprise a number of groups with the ability to cometabolically oxidize TCE and vinylchloride (Coleman et al., 2006).

Monooxygenases play a key role in the aerobic metabolic degradation of Cl-VOCs by including an atom of O_2 into the growth media in the process of oxidation. Whilst aerobic degradation, microbial organisms predominantly employ epoxidation, which

results in formation of chloroethene epoxides. This reaction is catalyzed by monooxygenases. However, these epoxides in the presence of water tend to show chemical instability and get involved in various chemical reactions rapidly, frequently leading to disruption of cells through the formation of epoxide-macromolecule adducts (Dolinová et al., 2017). Consequently, microbes respond by producing enzymes capable of epoxide transformation, such as epoxyalkane coenzyme M transferase (EaCoMT), epoxide carboxylases, epoxide hydrolases, and glutathione S-transferases, to detoxify the epoxides (Allen et al., 1999; Coleman & Spain, 2003; van Hylckama Vlieg et al., 1998; van Loo et al., 2006)

A variety of microbial strains seem to employ the enzymes listed above during dechlorination of vinylchloride, with alkene monooxygenase (AkMO) playing a significant role in catalyzing VC epoxidation process, which results in formation of chlorooxirane, as a couple of studies show (Hartmans & De Bont, 1992; Verce et al., 2000). The next step is EaCoMT, which facilitates the binding of coenzyme M with the epoxide, forming 2-hydroxyethyl coenzyme M (Coleman & Spain, 2003). The further stage of the vinilchloride dechlorinatio, however, remain not well comprehended.

3.4 Enrichment cultures

Enrichment culture is an isolation technique aimed at creating favorable conditions for the growth of a specific organism of interest while creating an unfavorable environment for any competing organisms. It involves employing specific growth media that promote the growth of the desired microorganism while suppressing others, thereby enriching the sample for the target microbe (Madhuri et al., 2019). Enrichment is typically achieved by introducing nutrients or environmental conditions that exclusively support the growth of the specific organism. This approach allows to amplify a small number of the desired organisms to detectable levels, enabling the detection and identification of microorganisms with diverse nutritional requirements (Liu et al., 2016). Once the microbial enrichment culture is developed, it can be introduced into the contaminated site through different methods. The culture can be injected directly into the groundwater, soil, or aquifer using injection wells or sparging

systems. Alternatively, it can be applied as a bioaugmentation amendment mixed with appropriate carriers. In some cases, additional nutrients, such as carbon sources or electron acceptors (e.g., oxygen or nitrate), may be added to the contaminated area to stimulate microbial activity and improve degradation rates. Throughout the remediation process, the site is closely monitored to assess the progress of chloroethene degradation. Parameters such as microbial activity, degradation rates, and contaminant concentrations are measured to ensure the effectiveness of the treatment. Adjustments to the enrichment culture or nutrient addition may be made based on monitoring results. By promoting the growth of naturally occurring or introduced chloroethene-degrading microorganisms, microbial enrichment remediation offers a sustainable and eco-friendly approach to remediate chloroethene-contaminated sites.

Bioaugmentation technique has repeatedly proved itself effective at enhancing microbial remediation at the contaminated sites. Numerous naturally occurring indigenous microorganisms possess the capability to detoxify various hydrocarbons and their associated contaminants in the environment. In one study (Thavamani et al., 2011) microbes obtained from sites contaminated with various gaseous polycyclic aromatic hydrocarbons couple with toxic heavy metals have shown robust resilience to them, coupled with elevated catabolic activity, increased resistance to metals, enhanced pollutant degradation, and a notable capacity for biodegradation. A more recent study reported that the mixed enriched culture exhibited even greater efficiency in degrading polycyclic aromatic hydrocarbons compared to the individually culturable pure strains (Dhar et al., 2022). In case with chlorinated hydrocarbons, enrichment cultures have also proven to be valuable tools in the remediation of contaminated soils. An anaerobic soil enrichment culture that effectively dechlorinated significant amounts of PCE (ranging from 50 to 58 mg/liter in various environments) was reported (Lee et al., 1997). One study has introduced not 100% pure, but still highly enriched culture obtained from the parent KB-1 mixed culture, that succeeded in reductive dechlorination of TCE, *cis*-1,1 DCE and VC to ethene (Duhamel et al., 2004). Another study effectively implemented a fed-batch fermentation process for production of large volumes (up to 3200 L) of *Dehalococcoides*-containing consortia enriched from various sites under anaerobic conditions, using sodium salt of lactic acid as a source of electron, which results in PCE

or TCE being reduced (Vainberg et al., 2009). The resulting cultures exhibited good storability when refrigerated at 4 °C and maintained their activity in the stored conditions for over 40 days.

However, the success of this technique depends on careful site assessment, appropriate microbial selection, and effective monitoring and optimization during the remediation process. Despite numerous advantages, there are certain limitations to consider. These include relatively slow reaction rates and challenges in managing and controlling microbial activity, which can impact the overall effectiveness of the bioremediation process (Rossi et al., 2021). Special attention should be paid to the old contaminated sites, where the chemical can be present in a separate phase, typically within some hardly penetrable layers of the aquifer, such as zones with high clay content. These layers serve a role of a reservoir for enduring pollutants (Rodrigues et al., 2020). Complexities also occur with the bacterial culture maintenance. In some studies, *Dehalococcoides* consortium displayed reduced growth rates and an increased need for specific conditions when isolated from other organisms. The cultivation and preservation of *Dehalococcoides spp.* have been accomplished by only a handful of laboratories globally (Wang et al. 2014, 2019). The isolation process is challenging due to the intricate nutritional requirements of *Dehalococcoides* genera, which require precise nutrients for growth. Notably, *Dehalococcoides* organisms are unable to biosynthesize vitamin B12, an essential cofactor for dehalogenase enzymes (He et al., 2007). However, the same study shows that this problem can be solved by implementing a co-culture of bacteria capable of B12 production, such as *Acetobacterium woodie*. There have also been some reports when microbes obtained from polluted environments, although successfully adapted to survive within those contaminated settings, did not evolve specifically for efficient contaminant degradation. They often exhibited limited enzyme activity and substrate specificity, leading to suboptimal metabolic competence in the remediation of toxic contaminants (Dvořák et al., 2017; Tahri et al., 2013). Other challenges can also be mitigated by integrating various strategies to enhance the efficiency of the bioremediation approach. These challenges have yet to be overcome.

4. Experiment and Methods:

4.1 Soil and groundwater sampling

Environmental samples from two different locations were used in the experiment.

First site is located in Rohrbach, Upper Austria. Anaerobic bacterial culture was enriched from the local groundwater contaminated with PCE. Groundwater samples were collected from wells at 4 measuring points (GW 8, GW 9, GW 10 and GW 11) which served as monitoring levels. Figure 13 below schematically illustrates the setting of the test field.

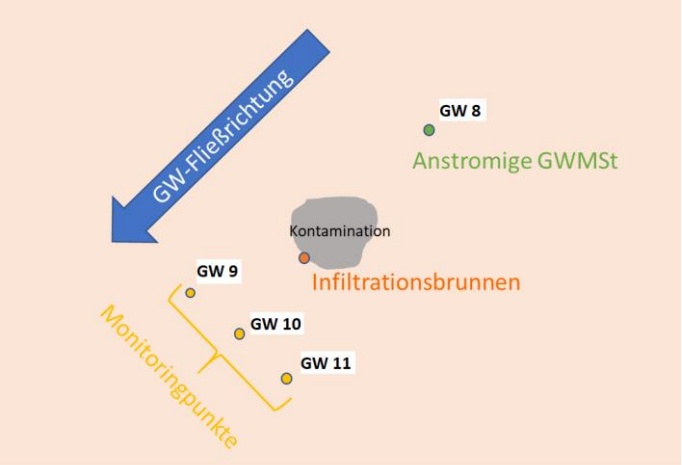

Fig. 13: Schematic representation of the contaminated site in Rohrbach. (with kind permission of Thomas Reichenauer)

For each sample 1L glass bottle and two 120 mL glass batches were filled with groundwater, avoiding letting air in. All 120 mL batches were covered with aluminum seals with a rubber septum using a vial crimper. All 1 L bottles were covered with air-tight lids. The collected groundwater was kept at 4 °C before the start of the laboratory experiments.

Second contaminated site is located at Pöttinger agricultural machinery factory, near Grieskirchen (see Fig. 14).

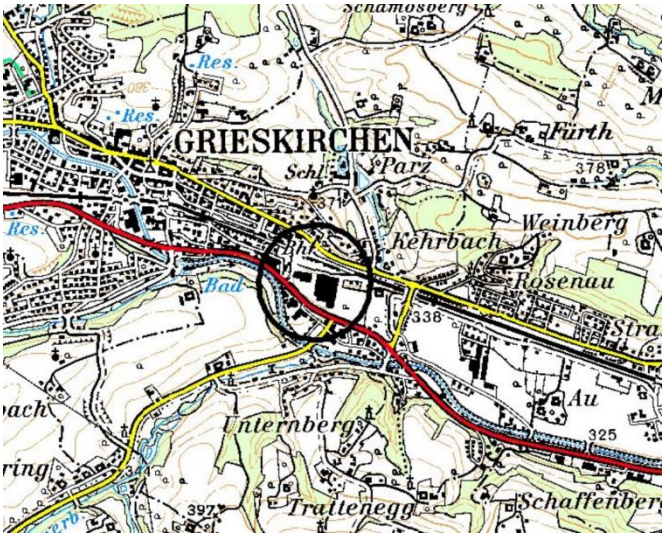

Fig. 14: Location of the second test field in Grieskirchen (with kind permission of Thomas Reichenauer)

Due to usage of various solvents in the machinery production, TCE, 1,1,1-TCA and the products of their dechlorination 1,2-dichloroethene and 1,1-dichloroethene are the major contaminants in the subsoil. Aquifer materials (soil and groundwater) were collected and kindly provided by ESW Consulting WRUSS ZT GmbH. Both groundwater in 1L bottles sealed with airtight caps and soil in plastic buckets were stored at 4 °C prior to the laboratory experiments.

4.2 Samples preparation

4.2.1 Anaerobic degradation batch experiments

Handling of the samples was performed in an Ar-flushed glovebox (SylaTech GmbH, Germany) with an O2 concentration below 40 ppm. Prior to that groundwater was bubbled with N2 gas for 30 minutes to reduce the amount of O2 in the system.

Groundwater from the contaminated sites was used to prepare the nutrient solution with an optimal environment for enrichment cultures. The composition was as follows: molasse from stock solution as a carbon source, Na_2S (sodium sulfide) from stock solution to create a reducing environment, HEPES (zwitterionic sulfonic acid) for extra buffering capacity (pH stabilization) and, finally, resazurin as an anaerobic indicator (see Table 3). 30 mL of media was added to 120 mL serum bottles containing 60 g of

autoclaved quartz sand (0.5-2 mm diameter), which was simulating the aquifer material for the Rohrbach groundwater, and with the soil from Pöttinger in two variations, unsieved and sieved through the 2mm sieve. Since the studied anaerobic bacteria are immobile, they need a material to sit on while growing and performing metabolic activities. For the negative control system, the serum bottles containing autoclaved quartz sand and sterile media prepared with MQ instead of groundwater were used. For the positive control, some serum bottles were injected with the well studied KB-1 culture (see Fig. 15), to compare the rate of dechlorination. The serum bottles were crimped with aluminum seals with a rubber septum and taken out from the glovebox (see Fig. 16).

Substance	**Concentration in stock solutions**	**Amount added**	**Achieved concentration**
Molasse	371,4 g/L	1,98 mL	0,68 g/L
Na_2S	140 g/L	3,96 mL	0,5 g/L
HEPES	Pure powder	12 g	12 g/L
Resazurin	1 g/L	0,495 mL	0,0005 g/L

Table 3: Composition of nutrient solution for a 1L bottle containing 994 mL of groundwater from each sampling point.

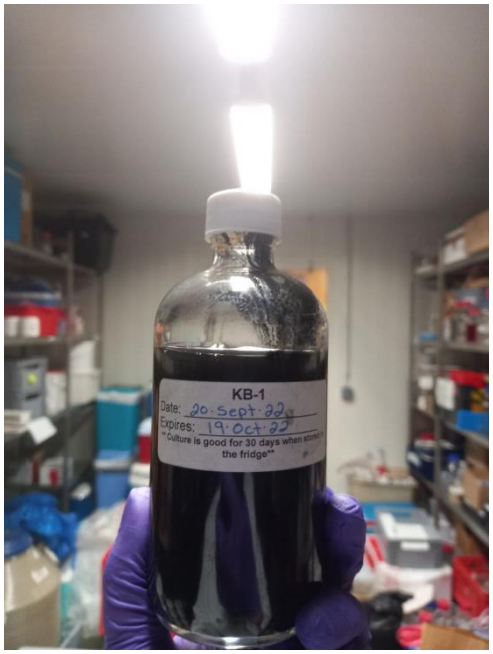

Fig. 15: KB-1 bacterial culture used for positive control system in enrichment culture experiment

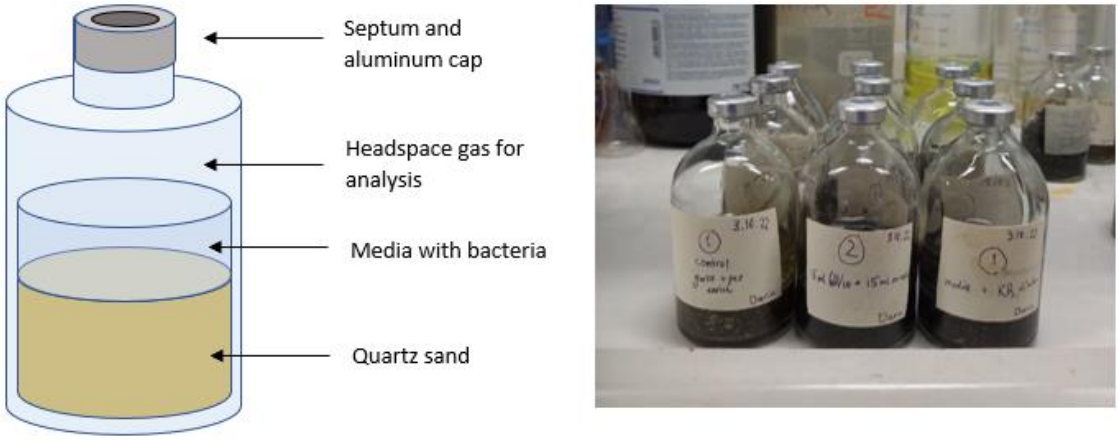

Fig. 16: Schematic outline of and photo of 120 mL serum bottles used for batch experiments. Containing nutrient solution and quartz sand, sealed with aluminum crimps and prepared to be injected with chlorinated hydrocarbons.

Then, for the experiment with tetrachloroethylene, 6 µl of PCE from 100 g/L stock solution (3 mL of neat PCE diluted with 39 mL of ethanol) was injected by a syringe to reach an approximate starting concentration of 20 mg/L (ignoring the amount of PCE that could have been in the groundwater from the site already). For the experiment

with Cl-VOCs mixture 3 µl of TCE and 6 µl of 1,1,1-TCA from 100 g/L stock solutions was injected by a syringe to achieve an approximate initial concentration of 10 and 20 mg/L, respectively.

The batches were then put on a horizontal shaker (80 rpm) at the room temperature without the light exposure to stimulate the flow of the water under the ground. The PCE concentration was measured daily for the first week after the injection, since PCE normally gets degraded within the first few days, and then weekly.

4.2.2 Anaerobic enrichment culture experiment

Among the samples tested, the bacterial cultures enriched from GW 8, 10 and 11 showed the highest dechlorination rate of PCE, and were selected for the further examinations. Following the full dechlorination of the PCE injected in the medium, the inoculum enriched cultures which showed the highest dechlorination rate of PCE were transferred to larger bottles containing 995 mL of fresh growth media (see Fig. 17).

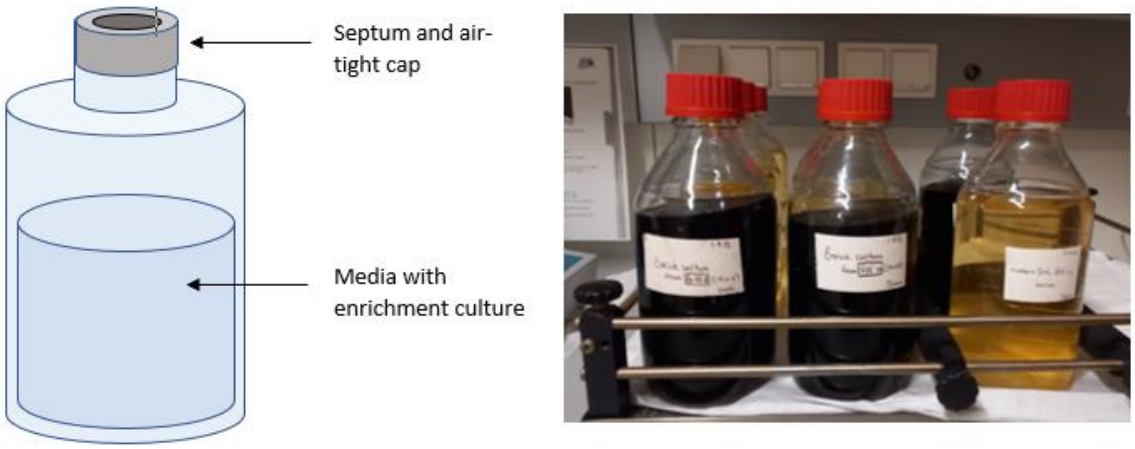

Fig. 17: Schematic outline of 1L test bottles with enrichment culture incubated on a horizontal shaker.

The bottles were then incubated at the room temperature on the horizontal shaker at 80 rpm, at the same conditions as the 120 mL serum bottles, and then measured at the

same frequency as the serum bottles. They were fed once a week with 200 µL of PCE until the number of cells reached the desired value (1*10^7), which would allow to compare them to KB1 and characterize the enriched culture. DNA extraction and PCR were performed every week to monitor the cell growth. However, it was not possible to monitor the dechlorination inside of the bottles for a few weeks, until new caps with septum were ordered. At some point of the experiment, enriched cultures were partially transferred to fresh nutrient solution, and in 1 bottle all 3 microbial cultures were mixed (see Fig. 18).

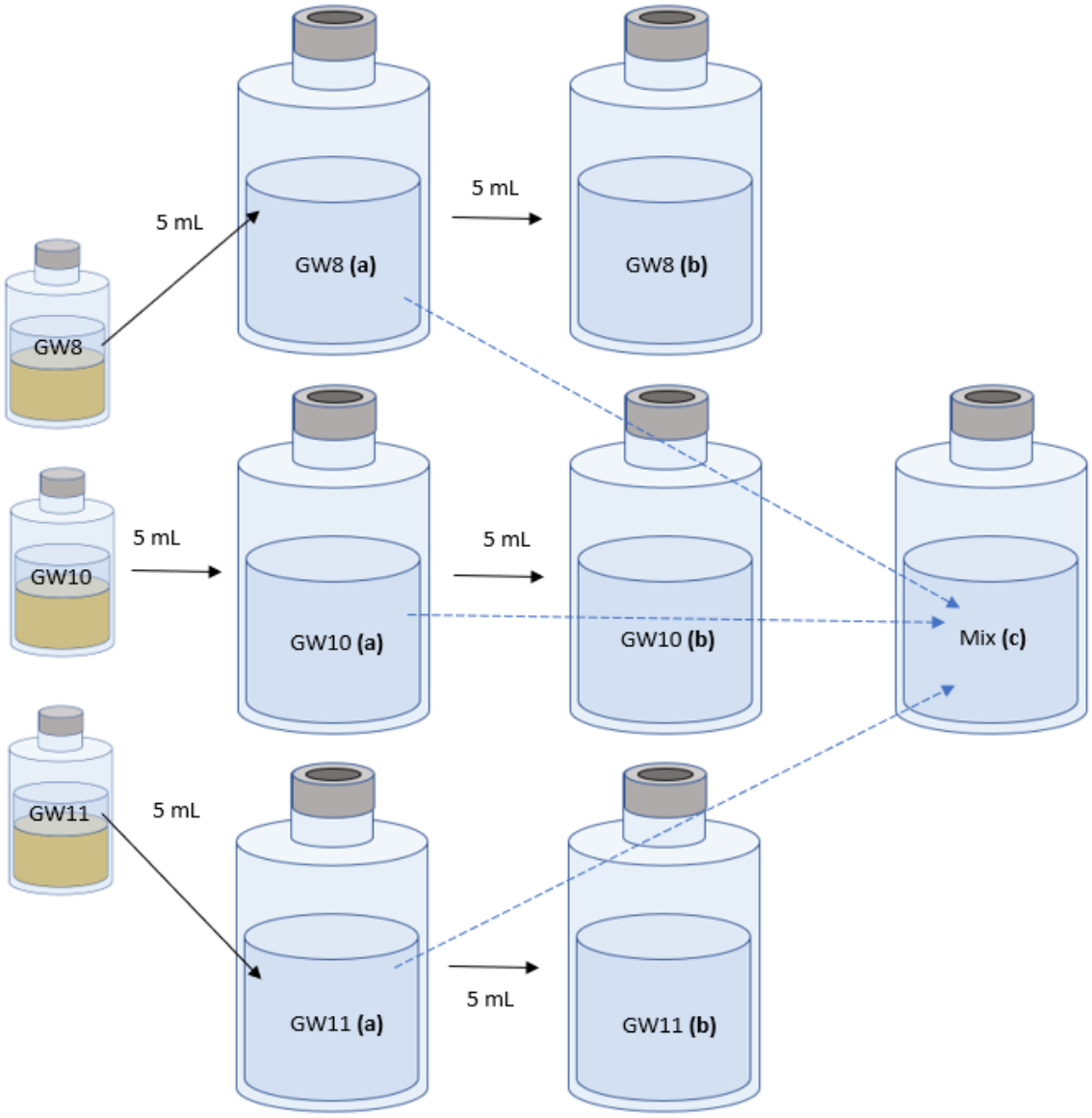

Fig. 18: Scheme of the enrichment culture transfers. 5mL of media from the 120 mL batches was injected in fresh media (a); 5 mL of media (a) is injected in fresh media (b); 5mL of media from each bottle (a) is mixed together in fresh media (c).

All cultures and subcultures continued to be fed and measured. This sub-cultivation was supposed to be replicated up until the point when the culture would presented stable PCE degradation activity, but, unfortunately, the dechlorination seized.

4.2.2 Aerobic degradation batch experiments

First, the bacterial culture was enriched from the contaminated groundwater of the second contaminated site (Pöttinger). Twenty eight samples (50 ml each) were centrifuged repeatedly at 4800 rpm for 15 min under 4°C (Megafuge 16, Thermo Scientific), which was followed by the removal of supernatants. All 28 pellets were then pooled together into 10 ml of contaminated water, and glycerol stocks were prepared.

For the aerobic degradation experiment, the autoclaved 120 mL serum bottles were filled with 60 mL of groundwater from the Pöttinger site. For the control system, the serum bottles containing autoclaved Milli-Q were used. The previously cultivated bacterial culture was then added. Schematic outline of the batch setting is shown on Fig. 19. Serum bottles were crimped with aluminum seals with a rubber septum. 17 µL of butane and propane mixture was injected, to provide the electron source for cometabolic aerobic oxidation. That was followed by 18 µL of TCE and 39 µL of 1,1,1-TCA (both from 100 mg.L stock solutions) to reach the concentration of 0,03 mg/L and 0,065 mg/L respectively, which reflects the contamination levels at the Pöttinger site.

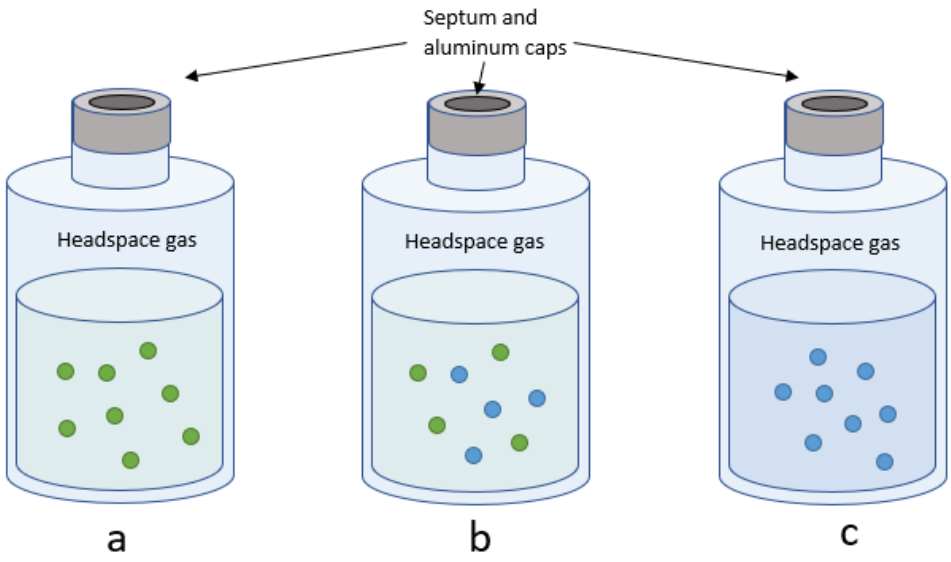

Fig. 19: Schematic outline of 120mL serum bottles used for aerobic batch experiment, filled with a) Groundwater from the contaminated site, b) Groundwater from the contaminated site with the bacteria from the stock, c) Sterile media with the bacteria from the stock. Green dots represent bacteria from the stock, blue dots – bacteria from the groundwater.

The batches were placed on a horizontal shaker (80 rpm) at the room temperature without the light exposure to stimulate the flow of the water under the ground. The headspace composition concentration was measured every 3 days during the first week after the injection, and then weekly.

4.3 Microbial community analyses

Periodic extraction of the microbial consortium DNA was carried out using a PowerSoil DNA isolation kit (QIAGEN GmbH, Germany), precisely following the manufacturer's instructions. The quality and concentration of DNA samples were assesed using the DeNovix DS-11 NanoDrop 2000 spectrophotometer (Thermo Fisher). The DNA products then underwent the preparation for the genomic analysis. The alterations in the microbial consortium structure were examined through the bacterial 16S PCR and the 16sDH specific for *Dehalococcoides* with the Biozym Blue Probe qPCR Kit, which was done with the great help of a colleague Johanna Ley. First, the samples were tested in a 16s qPCR with dilutions (1:10) to see if there is any inhibition, then the 16sDH

specific for *Dehalococcoides* was performed to check the amount of possible bacteria for the degradation experiment. DNA extraction and PCR were performed every week to monitor the cell growth.

4.5 Analytical techniques

The headspace gas composition of the test bottles was measured by injecting 100 µL of headspace gas into a gas chromatograph (Agilent 8860 GC System) provisioned with a flame ionization detector (see Fig. 20). The injection was performed by Gerstel MultiPurpose Autosampler for GC/MS (MPS Autosampler). The temperature of column, injector and detector were 30 °C, 150 °C and 200 °C, respectively. 100 µL injection of propane was used as an internal standard in the batches.

Fig. 20: Gas chromatograph Agilent 8860 and Gerstel MPS autosampler used for the headspace analysis

The output of the analysis in the form of a chromatogram plot (see Fig. 21) with the ordinate axis showing the detector response as peaks with different areas, and the abscissa representing the time (minutes), was a subject for the further examination.

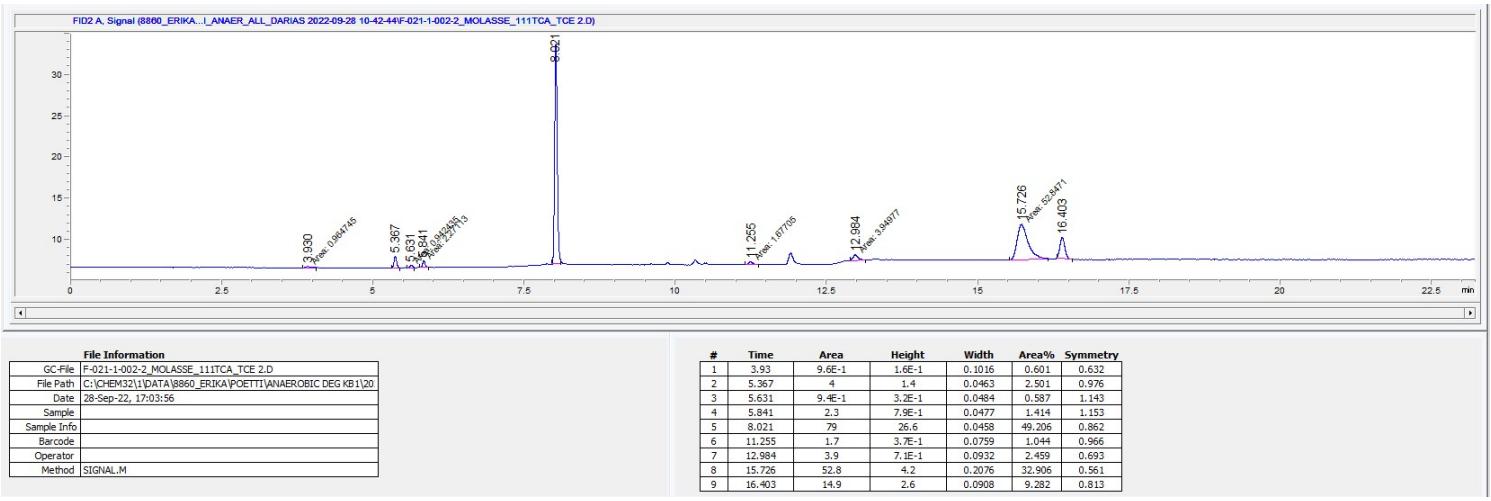

Fig. 21: Example of the chromatogram produced by Agilent 8860 as a result of the analysis of TCE and 1,1,1-TCA anaerobic degradation.

Time point on the X-axis during which a certain compound peaked allowed to identify it using Table 4.

Compound	Retention time (min)
H2	2.14
O2	3.81
Methan	3.88
Ethen	5.34
Ethin	5.60
Ethan	5.81
Propane	8.01

Vinyl chloride	8.99
Butane	9.76
1,1-DCE	11.33
trans-DCE	12.29
cis-DCE	13.32
1,1,1-TCA	15.73
TCE	16.39
PCE	22.30

Table 4: Compounds present in the headspace and retention times used for their detection on the chromatogram.

4.6 Data analysis and mapping

The concentration in the sample headspace was determined by comparing the peak area of the analyte in the sample with the peak area of the standards of a known concentration. Retention time was calculated by a colleague Stefanie Primisser prior to the analysis using analytical standards for all the compounds, and an Excel file prepared by her was used for the final calculations.

Graphs showing changes in concentrations of the compounds during the experiment time were built in RStudio.

5. Results

5.1 PCE anaerobic dechlorination

5.1.1 Enrichment experiment

Comparison of dechlorinating abilities of first-step enrichment cultures from different groundwater collection points in Rohrbach are shown in Fig. 22.

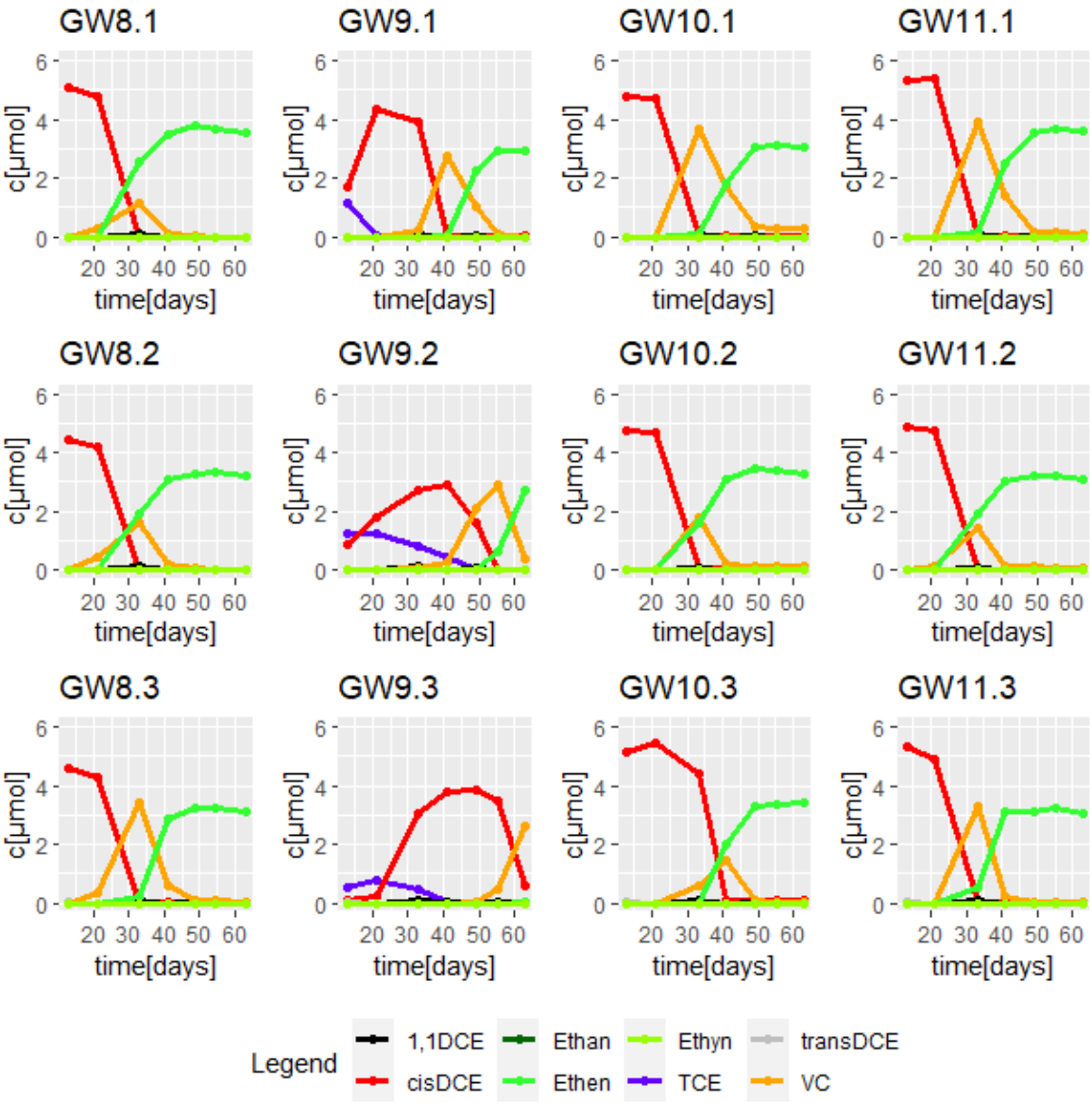

Fig. 22: Comparison of dechlorinating abilities of microbes from groundwater of 4 different wells on Rohrbach. GW 8, 10 and 11 were chosen as the most effective.

Among all of the tested groundwater samples, the ones that were collected from GW 8, 10 and 11 showed the highest dechlorination rate and full degradation of the PCE into ethen within 40-50 days since the start of the experiment. Due to technical inaccuracy, PCE was not measured in the very few days of the experiment, therefore its initial concentration is not visible on the graphs; however, the degradation process can be clearly evaluated. Transformation of PCE mainly occurred during the first week of the experiment. For the GW 8, 10 and 11 samples, TCE was also degraded within the first few days, but in the GW 9 samples it was persistent in the headspace up until day 50.

GW 8, 10 and 11 cultures from the original batches were then transferred to the fresh medium (transfer 1). As can be seen in Fig. 23, the amount of PCE was steadily decreasing, however, no intermediate products of its dechlorination were detected, except from small peaks of 1,1-DCE at the first 3-4 days of the analysis. The internal standard was not tracked in this stage of the analysis due to technical difficulties and absence of fitting equipment (caps with septum for injection), therefore, the chance of the gas phase loss through septum is not excluded. qPCR analysis was only performed on the 1st transfer enrichment on the second week. Results showed that the number of cells in all 3 enrichment bottles decreased in Week 4 in comparison with Week 2 (see Table. 5).

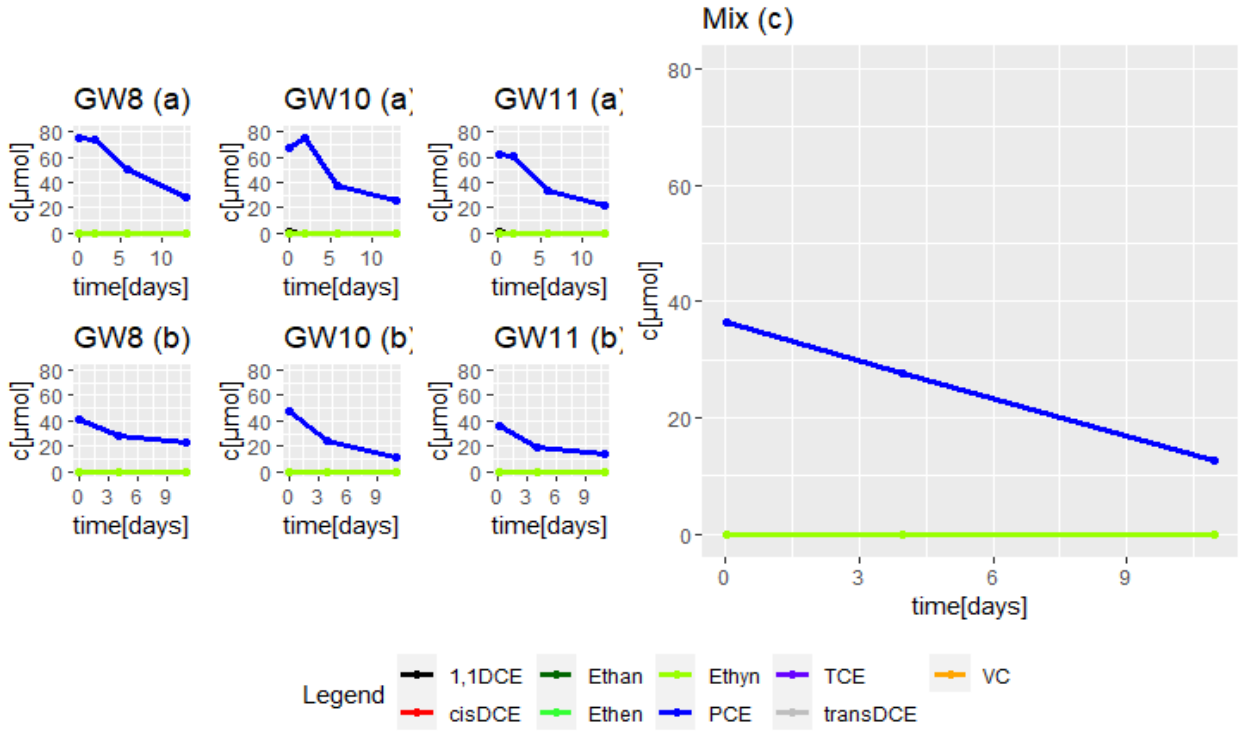

Fig. 23: PCE degradation in fresh media (1L bottles): (a) 1 transfer, (b) second transfer, (c) mixture of microbial culture from GW8, GW10 and GW11.

Despite no observable dechlorination activity, it was decided to perform the second transfer of enrichment cultures. Some bacterial culture containing media from GW10 and GW11 was transferred to smaller 120 mL bottles with quartz sand containing the nutrient solution, and were measured together with controls and batches containing KB-1 culture. As can be seen on Fig. 24 and 25, KB1 culture quickly degraded the PCE present within the first week, while in the batches containing enrichment cultures GW10 and GW11 no dechlorinating activity was observed.

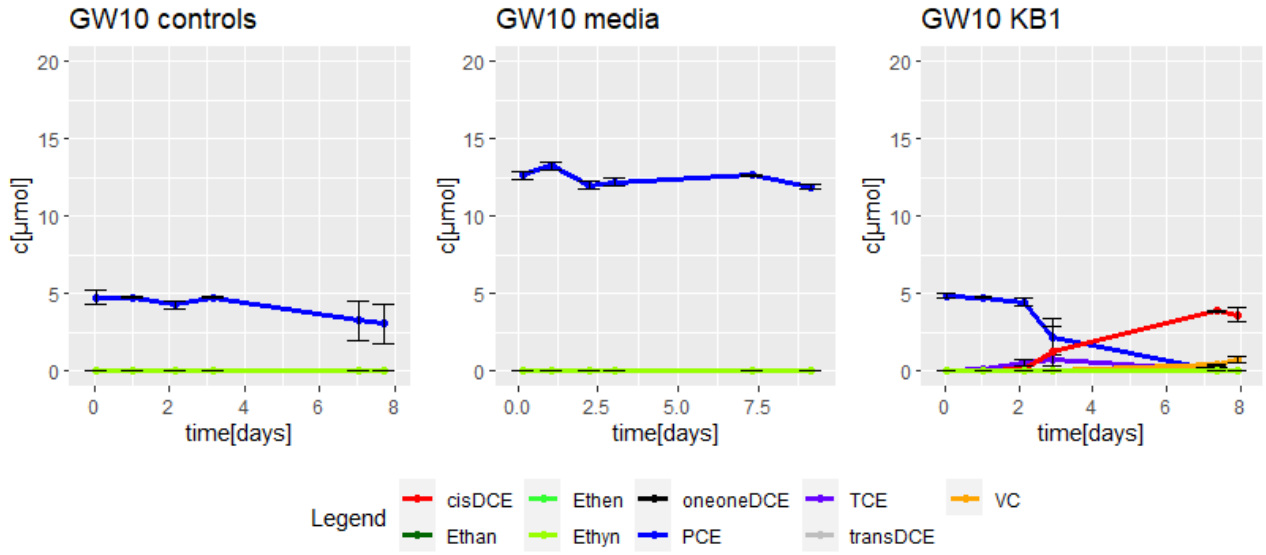

Fig. 24: PCE degradation batch experiment with GW10 cultures after 2d transfer.

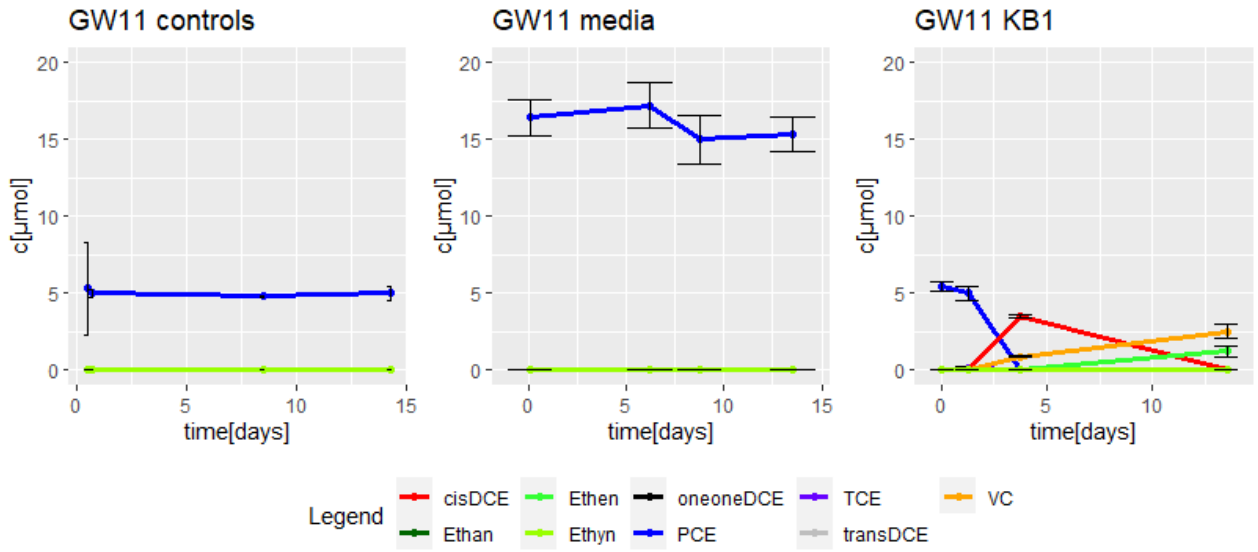

Fig. 25: PCE degradation batch experiment with GW11 cultures after 2d transfer.

It can be seen that control batches have significantly less amount of PCE in them than the enrichment samples. That is explained by the fact that for the controls fresh media injected by 6 µL of PCE was used, while the batches with enrichment culture contained high levels of PCE from the previous experimental step that was not dechlorinated. As can be observed on the graphs, PCE levels remained relatively the same and no dechlorination took place.

Results of the qPCR analysis for the 2d transfer showed that the number of cells in all 3 enrichment bottles with GW 8, GW 10 and GW 11 cultures have decreased significantly in Week 4 in comparison with Week 1 (see Table. 5). Therefore, it was decided to stop the enrichment experiment.

Sample	copies/L			
	Week 1	Week 2	Week 3	Week 4
GW8 (a)	-	1,74E+09	9,30E+08	1,11E+09
GW10 (a)	-	7,78E+09	5,84E+09	8,18E+09
GW11 (a)	-	3,82E+09	5,67E+09	9,28E+09
GW8 (b)	1,22E+07	1,27E+08	1,74E+07	2,08E+06
GW10 (b)	1,97E+08	3,74E+08	8,71E+06	9,05E+06
GW11 (b)	2,33E+07	4,02E+08	2,24E+07	7,78E+06
Mix (c)	8,18E+07	4,51E+08	3,33E+07	1,86E+07

Table 5: Results of the qPCR analysis presenting the number of cells (copies/L) measured weekly. (a) 1 transfer, (b) second transfer, (c) mixture of microbial culture from GW8, GW10 and GW11.

5.1.2 Batch degradation experiment

Similar degradation batch experiment was performed using the soil and groundwater from the second contaminated location, Pöttinger. Comparison of its dechlorinating abilities are shown in Fig. 26.

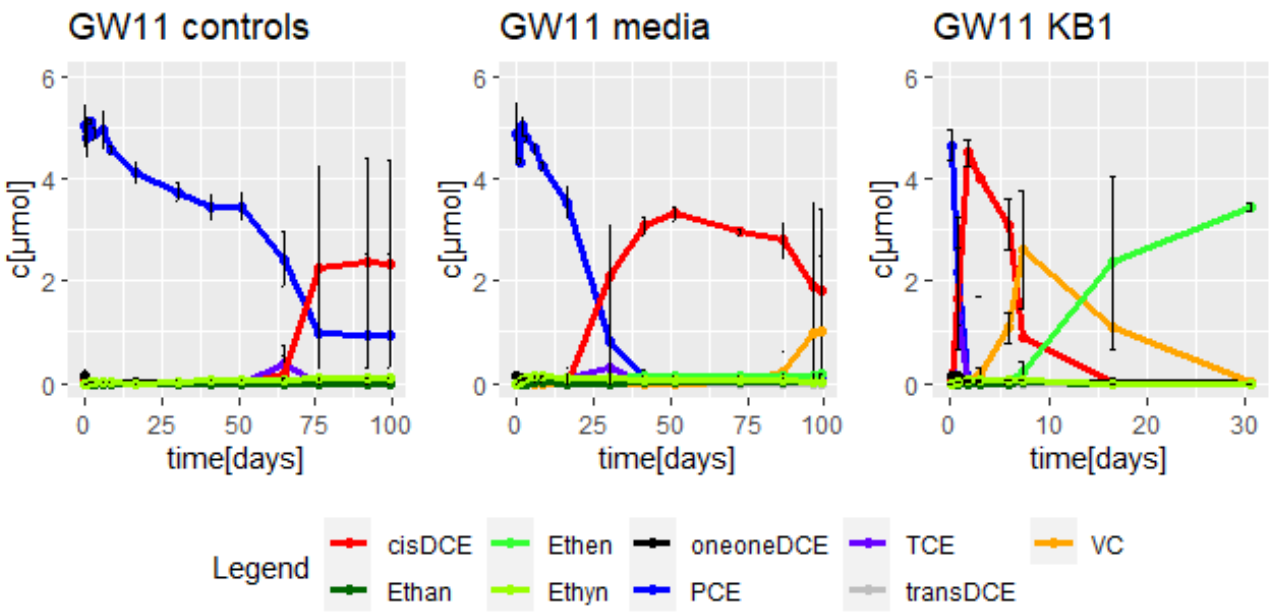

Fig. 26: Comparison of dechlorinating ability of microbes from soil and groundwater sampled from Pöttinger with dechlorinating ability of KB1 culture.

The middle graph presents that microbial culture has fully degraded PCE by day 28-30 of the experiment. However, full dechlorination of intermediate compounds was not observed up to day 100, and the experiment was stopped. Comparatively, batches with KB1 culture added degraded PCE within the first few days, and fully dechlorinated intermediate compounds into ethene by day 30. Unfortunately, it seemed that control batches were contaminated with the microbial culture by the

autosampler at some point of the experiment, therefore PCE degradation in them can be observed starting from day 50.

5.2 TCE and 1,1,1-TCA anaerobic dechlorination

Anaerobic dechlorination of TCE and 1,1,1-TCA mixture by microbial culture sampled in Pöttinger is presented below. Fig. 27 shows dechlorination activity in the batches containing unsieved soil and groundwater from the contaminated site, and Fig. 28 shows changes in the amounts of 1,1,1-TCA in the headspace.

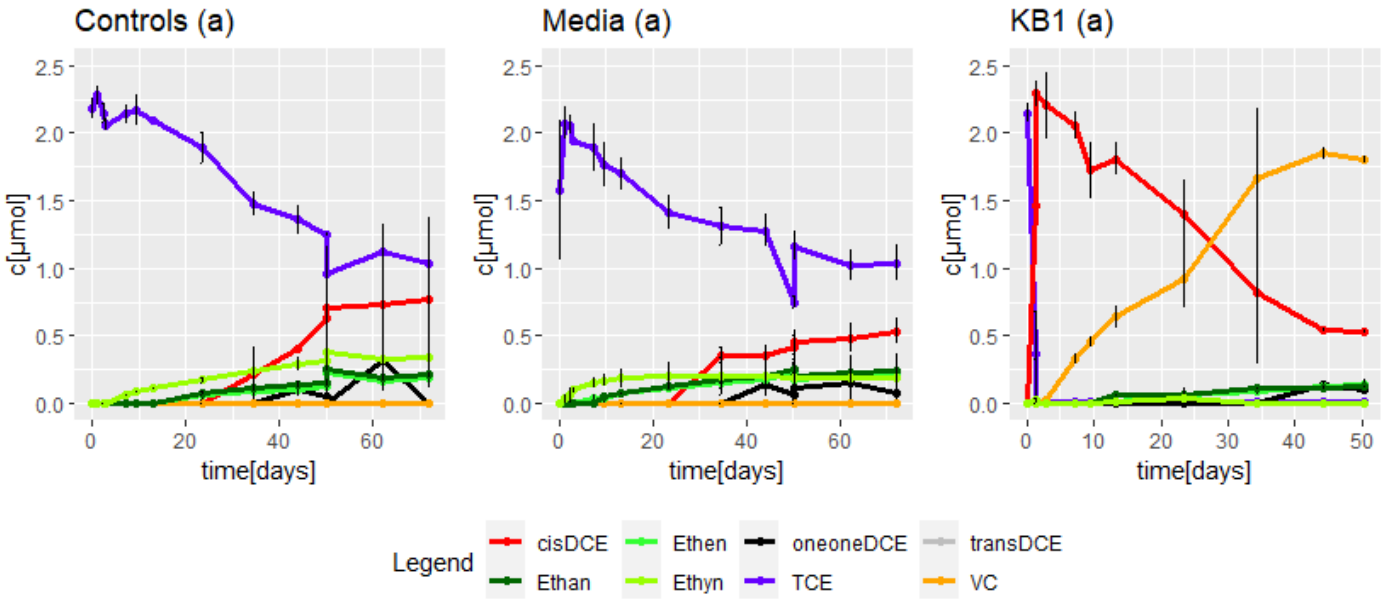

Fig. 27: TCE and 1,1,1-TCA degradation batch experiment with the unsieved soil (a) and groundwater from Pöttinger.

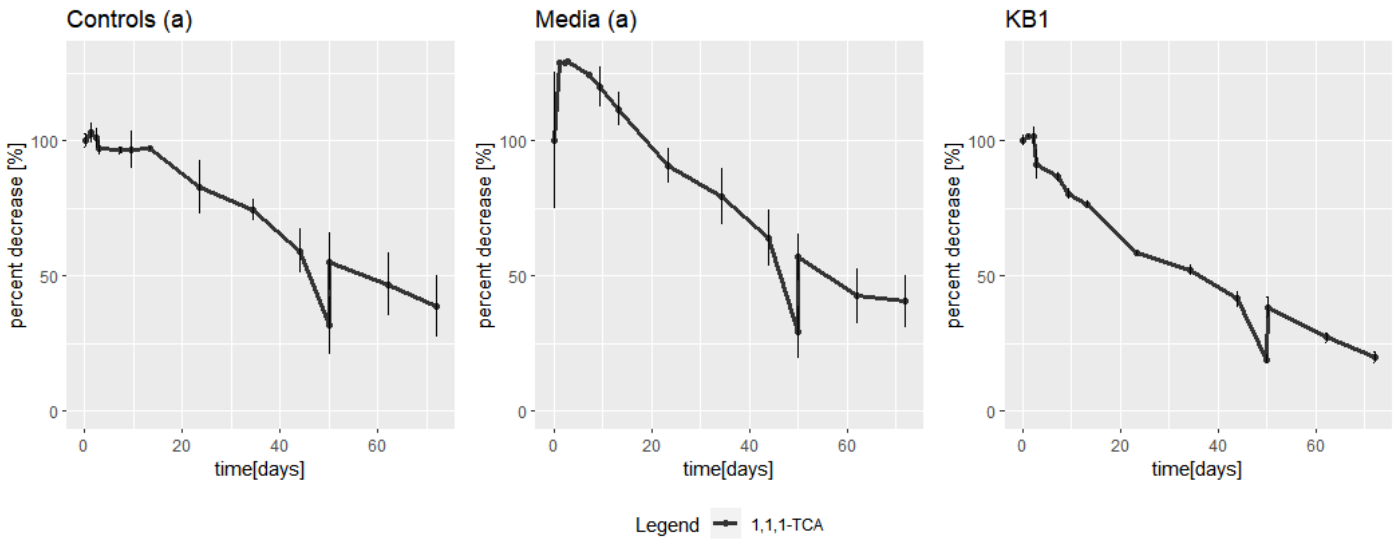

Fig. 28. Areas of chromatogram peaks measured for 1,1,1-TCA in the batches with unsieved Pöttinger soil (a), recalculated into percentage from the original concentration.

While batches containing KB-1 culture, as expected, have shown high dechlorination rate and full degradation of TCE within the first few days, and major decrease in the amount of 1,1,1-TCA, batches containing culture from the contaminated site presented less activity. Within the experiment time range, the amount of TCE decreased almost by half, and the amount of 1,1,1-TCA dropped even more significantly, but they were still present in the headspace on the 70th day of the experiment, and almost no final product ethene was produced. It can also be seen that control batches got contaminated at some point of the experiment, since dechlorination activity has also started happening in them.

On Fig. 29 and 30 results with the similar experiment with the batches containing sieved soil are presented.

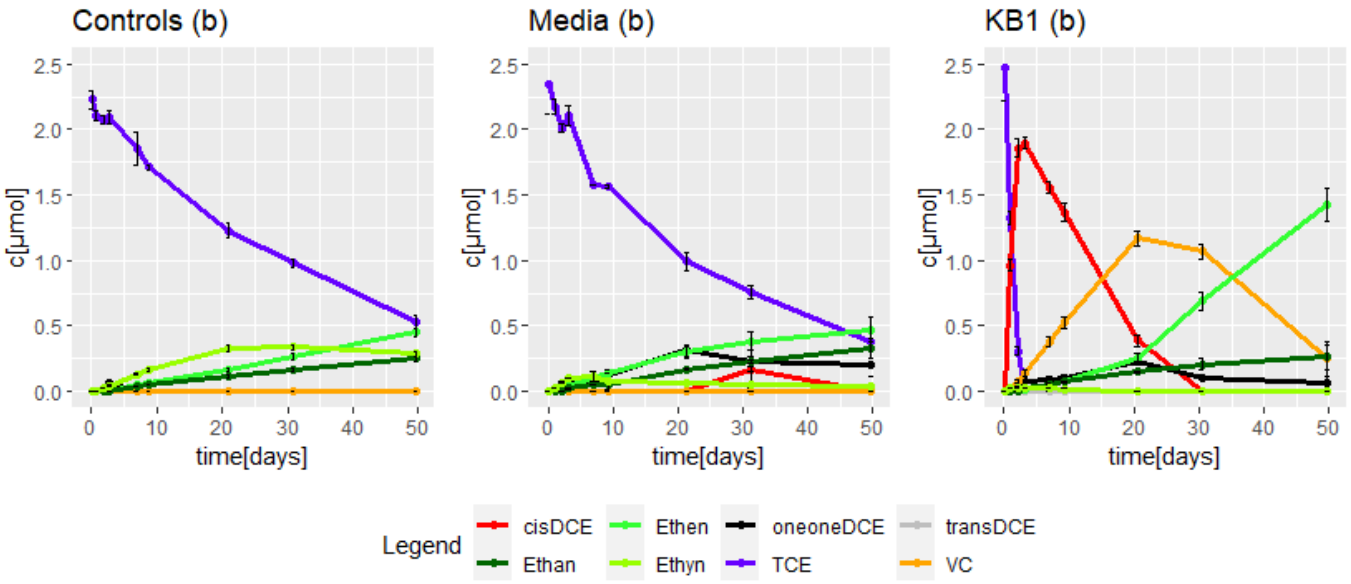

Fig. 29: TCE and 1,1,1-TCA degradation batch experiment with the sieved soil (b) and groundwater from Pöttinger.

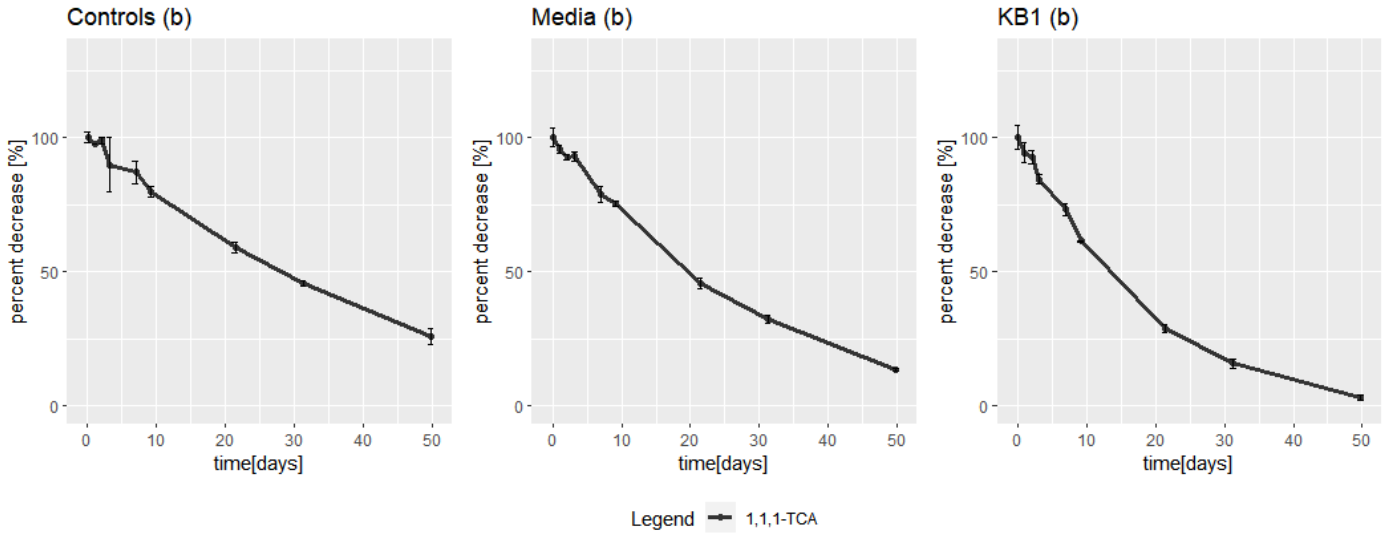

Fig. 30: Areas of chromatogram peaks measured for 1,1,1-TCA in the batches with sieved Pöttinger soil (b), recalculated into percentage from the original concentration.

Batches with the culture from the site presented a slightly faster dechlorination rate than in the experiment with the unsieved soil, and the amounts of both TCE and 1,1,1-TCA have decreased by the last day of the experiment. However, in comparison with KB-1 culture, the speed of contaminants degradation was still slow. In the control batches decrease in the amount of Cl-VOCs and presence of some intermediate products can also be detected, which means microbial activity inside of them.

5.3 TCE and 1,1,1-TCA aerobic dechlorination

Results of the aerobic degradation batch experiment for TCE and 1,1,1-TCA mixture are presented in the tables below using the chromatogram peak area values. In the course of laboratory work the analytes for some of the possible 1,1,1-TCA degradation products were not prepared for the measurement; however, it still possible to track the changes of 1,1,1-TCA amount in the batches, and therefore make an approximate estimation of its' degradation. All the tables contain averaged results for 3 repetitions presented together with their standard deviations (SD).

Time (days)	TCE	SD	1,1,1-TCA	SD	O2	SD	Methane	SD	Butane	SD	Propane	SD	1,1-DCE	SD
0.04	99.03	4.97	284.13	3.59	8.70	0.10	0.54	0.01	0.00	0.00	0.00	0.00	0.00	0.00
3.22	109.13	2.51	302.97	12.52	8.13	0.72	0.77	0.58	11.17	0.50	6.50	0.46	0.00	0.00
14.26	83.90	3.60	246.53	8.71	0.00	0.00	0.00	0.00	166.13	4.55	155.57	3.55	0.83	0.08
16.28	89.27	4.02	259.47	8.22	0.00	0.00	0.00	0.00	162.07	1.88	150.10	1.04	1.00	0.00

Table 6: Areas of chromatogram peaks measured in the control batches for the aerobic degradation experiment.

Time (days)	TCE	SD	1,1,1-TCA	SD	O2	SD	Methane	SD	Butane	SD	Propane	SD	1,1-DCE	SD
0.04	96,.60	4.07	280.00	8.55	8.23	0.23	0.54	0.00	5.50	0.00	1.60	0.00	0.00	0.00
3.22	114.80	6.46	316.17	12.36	7.80	0.26	0.72	0.54	10.53	4.64	4.80	2.01	0.00	0.00
14.26	68.97	4.30	186.87	22.61	0.00	0.00	0.00	0.00	151.40	1.56	145.00	2.79	0.60	0.08
16.28	77.90	4.40	206.43	17.99	0.00	0.00	0.00	0.00	148.10	3.34	140.77	2.37	0.86	0.06

Table 7: Areas of chromatogram peaks measured in the batches containing groundwater from the Pöttinger site for the aerobic degradation experiment.

Time (days)	TCE	SD	1,1,1-TCA	SD	O2	SD	Methane	SD	Butane	SD	Propane	SD	1,1-DCE	SD
0.04	99.97	3.22	275.20	2.95	8.50	0.10	0.62	0.02	7.23	6.05	3.34	2.69	0.00	0.00
3.22	103.40	5.49	286.83	6.09	8.00	0.17	0.94	0.15	9.63	6.42	4.40	2.97	0.55	0.00
14.26	81.53	14.02	216.47	44.84	0.00	0.00	0.00	0.00	156.53	4.77	148.67	3.52	0.83	0.14
16.28	85.23	12.34	227.20	45.50	0.00	0.00	0.00	0.00	153.33	6.34	144.90	6.33	0.85	0.22

Table 8: Areas of chromatogram peaks measured in the batches containing groundwater from the Pöttinger site injected with the previously cultivated bacteria from the stock for the aerobic degradation experiment.

Time (days)	TCE	SD	1,1,1-TCA	SD	O2	SD	Methane	SD	Butane	SD	Propane	SD	1,1-DCE	SD
0.04	91.90	0.00	276.30	0.00	8.60	0.00	0.54	0.00	14.00	0.00	6.40	0.00	0.00	0.00
3.22	89.07	1.05	275.10	16.96	8.63	0.06	0.94	0.07	9.37	2.06	4.37	1.02	0.00	0.00
14.26	77.27	13.91	215.40	69.94	0.00	0.00	0.00	0.00	147.73	7.62	141.43	6.43	0.00	0.00
16.28	70.53	10.83	207.57	60.66	0.00	0.00	0.00	0.00	146.30	8.03	139.70	6.66	0.86	0.21

Table 9: Areas of chromatogram peaks measured in the batches containing sterile media injected with the previously cultivated bacteria from the stock for the aerobic degradation experiment.

As can be seen on the Tables 8-11, both microbial culture from the contaminated site and cultivated bacteria have failed to significantly reduce amounts of TCE and 1,1,1-TCA in the groundwater. Among the identified intermediate products, only 1,1-DCE was detected in negligible amounts. However, it can be noted that by combining two of the microbial cultures together, a slightly higher dechlorination rate was reached (see Table 10). It can also be seen that the amount of butane and propane is not present in the samples in the beginning of the experiment, which is due to technical complications with the clogged syringe. Once the problem was identified, the required amount of the gases was injected, hence it's increase on day 14.

6. Discussion

6.1 PCE anaerobic dechlorination

6.1.1 Enrichment experiment

The anaerobic PCE-degrading capacity test of groundwater samples from the first location in Rohrbach revealed that bacteria present in the environmental sample are capable of full tetrachloroethylene dechlorination into ethylene within approximately 60 days. PCE to TCE conversion occurred within the first few days, which correlates with results obtained in several other studies (Gerritse et al., 1995; Vogel & McCarty, 1985; Yoshikawa et al., 2020). However, the enrichment culture, despite the initial promising dechlorinating activity, did not exhibit significant progress in transforming PCE to less chlorinated compounds at the later stages of the experiment, and failed to yield substantial amounts of ethene. Attempts to increase the number of cells showed failure already in the first transfer to larger volume, since dechlorinating activity was lost completely. Notably the number of cells stopped increasing at the same time frame, which could be explained either by death of cells, or by replacement of bacteria capable of dechlorination by other bacteria competing for hydrogen, such as methanogens (Mueller & Booth, 2016). After the second transfer the accumulation of degradation metabolites was also negligible or not detected. Nonetheless, the PCE concentration has steadily decreased in all samples with enrichment cultures, without any dechlorination products detected. This result suggests several possibilities. During one of the transfers air might have leaked in the sample which would have quickly terminated the dechlorination process and permanently destroyed the ability of the culture to reduce PCE. However, redox-indicator Resazurin, that was present in the media, remained dark and did not turn pink, which would have a been a sign of oxidation. Since in 1L bottles no internal standards were added, it was not possible to exclude the loss of gas phase through the septum. Lastly, only 120 mL batches contained sediment (quartz sand), while 1L bottles contained just the liquid media. That might have affected metabolic activity of organisms. In general, this outcome suggests that the selected microbial enrichment strategies were not effective in promoting the desired biodegradation results. Other reasons for such inhibition or complete loss of dechlorinating ability mentioned in other studies are high PCE

concentrations (>30 mg/L (Yoshikawa et al., 2020)) and excessively elevated (incubation at 45 °C by Fletcher et al., 2011) or lowered (storage at 4° C and −20° C (Natarajan et el. 1985)) temperature, which are not the case for this study.

6.1.2 Batch degradation experiment

Anaerobic batch experiment with soil and groundwater culture collected from the second location in Pöttinger showed slow but steady dechlorination of added PCE. However, even on the day 100 of the experiment toxic intermediate dechlorination products, such as vinyl chloride, were still present in the headspace. Many researchers have observed such incomplete reduction of PCE in a broad range of methanogenic settings (Bouwer & Wright, 1988; Freedman & Gossett, 1989; Parsons & Lage, 1985; Vogel & McCarty, 1985). The resulting buildup of the less chlorinated, but still harmful intermediate compounds, does not allow to call the experiment successful.

6.2 TCE and 1,1,1-TCA anaerobic dechlorination

Anaerobic batch experiment with TCE and 1,1,1-TCA mixture added to soil and groundwater from Pöttinger location showed that the amount of TCE in experiment batches decreased steadily but got stalled at the amount of approximately 0.5 µL. 1,1,1-TCA also decreased significantly, but, since the control batches also showed its' reduction, it's hard to conclude whether it was due to dechlorination or another reason, such as gas loss or measurement inaccuracy. This result, however, corresponds to the one in a conducted study (Wen et al., 2017), where the addition of 1,1,1-TCA inhibited the microbial reduction of TCE by microbial culture. Earlier studies also demonstrated how the 1,1,1-TCA presence negatively affected the removal of PCE (Duhamel et al., 2002; Grostern & Edwards, 2006). So far, there has been some evidence that TCA has the ability to hinder both aceto- and methogenesis in two different studies (Adamson & Parkin, 2000; Vargas & Ahlert, 1987). Such disruption of microbial community structure might be a reason for the negative effect on TCE dechlorination, since both methanogens and homoacetogens have the ability to produce compounds known as corrinoids, which play a an important role of a supplemental factor for the *Dehalococcoides* reproduction and development (Johnson et al., 2009; Wen et al., 2015). In one study (Gander et al., 2002) the ability of FeS to catalyze the partial

reductive dechlorination of 1,1,1-TCA was observed. This allows to suggest that catalytically enhanced dechlorination would show more success in removal of such Cl-VOCs mixture from the environment.

6.3 TCE and 1,1,1-TCA aerobic dechlorination

The aerobic degradation pathways investigated in this thesis showed minimal transformation of TCE and 1,1,1-TCA into less chlorinated ethenes, with no substantial production of ethene. It is apparent that the specific microbial communities investigated did not possess the necessary enzymatic capabilities to efficiently degrade the targeted Cl-VOCs, although, due to several complications and imperfections in the conducted experiment it is hard to make certain conclusions. As discussed in the early chapters of this study, several reports have evidenced the capability of some aerobic bacteria to degrade TCE (Uchiyama et al., 1989; Vanderberg et al., 1995), but the aerobic 1,1,1-TCA degradation still remains relatively uncommon. In a conducted study (Malachowsky et al., 1994), addition of propane has significantly inhibited TCE dechlorination by *Rhodococcus* strains, while 1,1,1-TCA was degraded, and in another study (Yagi et al., 1999) 1,1,1-TCA was also successfully degraded by bacterial strains of *Mycobacterium* spp. in a presence of propane. However, results of the experiment conducted in this study showed no utilization of propane by microbial communities, therefore it most likely had no effect on the dechlorination process. An alternative approach to enhance the aerobic cometabolic degradation exists, that involves the genetic modification of bacterial strains to boost the activity of enzymes and introduce additional systems for detoxifying epoxides. To illustrate, in a conducted study (Rui et al., 2004) an *Escherichia coli* strain was engineered to express an improved version of an aromatic monooxygenase, which exhibited heightened efficiency in breaking down chloroethenes. However, this genetically enhanced strain demonstrated its increased dechlorinating ability to on the example of *cis*-DCE. Therefore, it is early to make conclusions on whether this strategy would be as effective with TCE and 1,1,1-TCA.

7. Conclusions

In summary, the outcomes of this thesis highlight the limitations and complexness of microbial remediation for chloroethenes under the specific laboratory conditions tested. Despite the efforts in exploring the potential of microbial remediation for Cl-VOCs, the results obtained in this thesis indicate limited success in achieving significant degradation of the targeted pollutants. It is just one of many examples of microbes obtained from polluted environments that have failed to evolve specifically for efficient contaminant conversion. Nevertheless, these findings add to the developing body of knowledge in microbial remediation, encouraging further exploration and refinement of remediation strategies for chloroethene-contaminated sites. These results emphasize the complex multiplicity of bacterial processes in degrading Cl-VOCs and underscore the importance of understanding site-specific factors that influence microbial activity. While the study did not achieve the desired remediation outcomes, it contributes valuable information to the field, emphasizing the need for tailored approaches and further investigation into alternative remediation techniques for chloroethene-contaminated environments.

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9. Indexes

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