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**EVALUATION OF AEROBIC AND ANAEROBIC
BACTERIAL DECHLORINATION POTENTIAL OF A
1,2-DICHLOROETHANE CONTAMINATED AQUIFER**

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SUMMARY

Abstract	5
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Chapter I: General introduction

1.1. Chlorinated solvents	7
1.1.1. Physicochemical properties and applications	7
1.1.2. Origin and diffusion in the environment	8
1.1.3. Exposure to CAHs and effects on human health	9
1.2. Remediation approaches	11
1.2.1. Conventional active cleanup technologies	12
1.2.2. Monitored Natural Attenuation (MNA)	13
1.2.3. Bioremediation	14
1.2.3.1. <i>Principles of bioremediation</i>	14
1.2.3.2. <i>Bioremediation of CAHs</i>	18
1.3. Biodegradation of chlorinated solvents	19
1.3.1. Reductive dechlorination	19
1.3.1.1. <i>Reductive dehalogenases</i>	21
1.3.1.2. <i>Organohalide-respiring bacteria</i>	22
1.3.2. Cometabolic anaerobic reductive dechlorination	24
1.3.3. Direct anaerobic oxidation	24
1.3.4. Direct aerobic oxidation	25
1.3.5. Aerobic cometabolism	27
1.4. 1,2-dichloroethane	28
1.5. Aim of the project	31

Chapter II - Evaluation of the catabolic potential of a 1,2-dichloroethane contaminated aquifer

2.1. Introduction	33
2.1.1. Methods to study the catabolic potential	33
2.1.2. Methods to study CAHs dechlorination potential	35
2.1.2.1. <i>Metagenomic approach and other molecular tools</i>	35
2.1.2.2. <i>Biostimulation treatment tests in microcosm</i>	37
2.1.3. Aim of the work	38
2.2. Materials and Methods	39
2.2.1. Groundwater samples	39
2.2.2. Groundwater filtration and DNA extraction	40
2.2.3. 16S rRNA metagenomic sequencing	41

2.2.4. Microcosms setup	41
2.2.4.1. Anaerobic microcosms setup and enrichment cultures	42
2.2.4.2. Aerobic microcosms setup and enrichment cultures	45
2.2.5. Chemical monitoring	47
2.2.5.1. <i>Headspace Sampling Gas Chromatography - Mass spectrometry (HS-GC/MS) analysis</i>	47
2.2.5.2. <i>Chloride release assay</i>	49
2.2.6. Analysis of the bacterial communities	49
2.2.6.1. <i>Automated Ribosomal Intergenic Spacer Analysis (ARISA)</i>	49
2.2.6.2. <i>16S rRNA metagenomic sequencing of microcosms</i>	50
2.2.6.3. <i>Search for known OHRB genera with specific primer sets</i>	50
2.2.7. Search for catabolic genes in microcosms and enrichment cultures	51
2.2.7.1. <i>Search for reductive dehalogenase genes in anaerobic microcosms</i>	51
2.2.7.2. <i>Search for haloalkane dehalogenase genes in aerobic enrichment cultures</i>	52
2.3. Results and discussions	53
2.3.1. Groundwater microbial communities	53
2.3.2. Preliminary monitoring of September 2020 microcosms	58
2.3.2.1. <i>1,2-DCA removal analysis in September 2020 microcosms</i>	59
2.3.2.2. <i>Biological monitoring of September 2020 microcosms</i>	59
2.3.3. Anaerobic microcosms (May 2021) and enrichment cultures	64
2.3.3.1. <i>1,2-DCA removal analysis in anaerobic microcosms</i>	64
2.3.3.2. <i>Anaerobic 1,2-DCA dechlorinating bacterial communities characterization</i>	66
2.3.3.3. <i>Detection of reductive dehalogenase genes</i>	71
2.3.4. Aerobic microcosms and enrichment cultures	72
2.3.4.1. <i>Aerobic dechlorinating microcosms</i>	72
2.3.4.2. <i>Aerobic 1,2-DCA dechlorinating bacterial communities characterization</i>	74
2.3.4.3. <i>Detection of haloalkane dehalogenase gene dhla</i>	79
2.4. Conclusions	81

Chapter III - Isolation and characterization of aerobic dechlorinating bacteria

3.1. Introduction	83
3.1.1. Aerobic 1,2-DCA dechlorinating bacteria	83
3.1.2. Aim of the work	85
3.2. Materials and methods	86

3.2.1. Bacterial isolations from the aerobic enrichment cultures	86
3.2.2. Growth in liquid medium and biomass collection	86
3.2.3. Chloride release assay	87
3.2.4. DNA extraction from isolates	87
3.2.5. Selection of representative R2A isolates	87
3.2.6. Identification of isolates	88
3.2.7. Search for <i>dhlA</i> gene	89
3.2.8. Whole genome sequencing	89
3.3. Results and discussions	90
3.3.1. Isolation of 1,2-DCA dechlorinating cultures	90
3.3.2. Biodegradation abilities of the cultures BIIS1ox	91
3.3.3. Detection of the haloalkane dehalogenase gene <i>dhlA</i> in dechlorinating cultures	92
3.3.4. Identification of dechlorinating isolates	93
3.3.5. Isolation in pure culture on nutrient medium	94
3.3.6. Whole genome sequencing of selected dechlorinating consortia	96
3.3.6.1. <i>Community profiling of the aerobic BIIS1ox dechlorinating consortia</i>	96
3.3.6.2. <i>Detection of catabolic genes in the aerobic dechlorinating consortia</i>	99
3.4. Conclusions	102
Chapter IV - Poly-β-hydroxybutyrate as electron donor: a pilot study in column	104
Degradation of 1,2-dichloroethane in real polluted groundwater by using enriched bacterial consortia in aerobic and anaerobic laboratory-scale conditions	106
Chapter V - Biopolymeric scaffolds for enhanced bioremediation	
5.1 Introduction	134
5.1.1. Carriers in bioremediation and sampling	134
5.1.2. Aim of the work	136
5.2. Materials and methods	137
5.2.1. Production and characterization of scaffolds	137
5.2.2. 1,2-DCA uptake test	138
5.2.3. Sterilization of biopolymeric scaffolds	138
5.2.4. Adhesion tests with a model biofilm-forming microorganism	138
5.2.5. Groundwater samples collection and filtration	139

5.2.6. Microbial adhesion test in 1,2-DCA contaminated groundwater in microcosms	139
5.2.7. Field exposure experiment	140
5.2.8. Scanning electron microscopy	141
5.2.9. DNA extraction	141
5.2.10. 16S rRNA metagenomic sequencing	141
5.3. Results and discussions	142
5.3.1. 1,2-DCA uptake by biopolymeric scaffolds	142
5.3.2. Bacterial biofilm formation on biopolymeric scaffolds	142
5.3.3. Scaffolds colonization by groundwater microbial communities	143
5.3.3.1. <i>Scaffold-colonizing groundwater microbial communities in microcosm</i>	145
5.3.3.2. <i>Scaffold-colonizing microbial communities in the aquifer</i>	151
5.4. Conclusions	155
Final conclusions	156
References	158
Attached A	174

Abstract

Chlorinated solvents, belonging to the class of chlorinated aliphatic hydrocarbons (CAHs), are synthetic organohalide chemicals frequently found as contaminants of groundwater and soil, due to their widespread use in several industrial processes and improper disposal methods. These compounds pose serious health threats because of their toxic and sometimes carcinogenic effects. Among these compounds, 1,2-dichloroethane (1,2-DCA) is one of the most common aquifer contaminants, considered toxic and classified as a possible human carcinogen. Remediation approaches toward these contaminants include conventional cleanup technologies based on physical/chemical methods, and bioremediation, considered an eco-friendly and low-cost alternative. Bioremediation relies on biodegradation of CAHs, that is carried out by specialized bacteria under anaerobic and aerobic conditions. Biodegradation processes include direct pathways, associated with the growth of specialized microorganisms, and indirect (or cometabolic) pathways, fortuitously mediated by non-specific enzymes produced by microorganisms to grow on other substrates. In contaminated aquifers, chlorinated solvents, including 1,2-DCA, are generally biodegraded by anaerobic specialized bacteria called OHRBs (Organohalide Respiring Bacteria) in a reductive process called dehalorespiration. Biostimulation with appropriate amendments can allow the exploitation of these processes in bioremediation strategies.

The aim of this work is to investigate the intrinsic biodegradation potential of a CAHs (mainly 1,2-DCA) contaminated aquifer by evaluating the response of the autochthonous microbial communities to anaerobic and aerobic biostimulation treatments in microcosm, to provide knowledge in the perspective of eventual site-specific bioremediation strategies. The preliminary microbiological characterization of the autochthonous bacterial communities of the aquifer, performed by 16S rRNA Illumina sequencing, revealed a notable difference between the bacterial communities from different sampling sites of the aquifer. Low relative abundances of known anaerobic and aerobic dechlorinating taxa were observed, suggesting a poor intrinsic bioremediation potential of the site. In order to enrich the dechlorinating components of the autochthonous bacterial communities, different anaerobic and aerobic biostimulation treatments in microcosm were tested, using groundwater samples and appropriate culture media amended with 1,2-DCA and additional substrates, if required, to stimulate different biodegradative processes. Chemical monitoring of 1,2-DCA concentration by Gas Chromatography-Mass Spectrometry, revealed high removal efficiency in different anaerobic and aerobic microcosm conditions. The analysis of microcosms bacterial communities, performed by 16S rRNA Illumina sequencing, revealed

the enrichment in anaerobic or aerobic dechlorinating taxa. Reductive dehalogenase (*dcaA*) or haloalkane dehalogenase (*dhlA*) genes were detected in anaerobic and aerobic microcosms, respectively, by PCR probing the metagenomic DNA extracted from microcosms. Isolation attempts from the aerobic biostimulation treatments led to the achievement of stable aerobic 1,2-DCA dechlorinating oligospecific consortia, that were analyzed by a *Next Generation Sequencing* approach.

The enriched dechlorinating bacterial communities obtained from biostimulation trials were used in a pilot study to test the feasibility of a polyhydroxybutyrate-based Permeable Reactive Barrier (PRB) and of an aerobic bioremediation approach in two column simulations fed with real contaminated groundwater. Both approaches led to an effective 1,2-DCA removal.

Finally, innovative biopolymeric tridimensional scaffolds were tested to evaluate their suitability as carriers either for the immobilization of groundwater autochthonous bacteria or for bioaugmentation with specialized dehalogenating biofilms to be eventually exploited in enhanced bioremediation. Laboratory scale studies and a preliminary field experiment confirmed scaffold could uptake 1,2-DCA and be colonized by a bacterial biofilm from groundwater bacterial communities, also made up of dechlorinating taxa.

CHAPTER I

General introduction

1.1. Chlorinated solvents

1.1.1. Physicochemical properties and applications

Chlorinated solvents are synthetic organohalide chemicals belonging to the class of chlorinated aliphatic hydrocarbons (CAHs). These chemicals consist in hydrocarbons with one or more halogen elements made up of chlorine atoms covalently bonded. Based on their chemical structure, CAHs usually encountered in the environment can be classified into three main groups: chloromethanes, chloroethanes and chloroethenes. This classification however does not encompass all types of CAHs that are likely to be found in contaminated sites, that may include others as chlorinated propanes, but the same physical and chemical principles can easily be extended to all chlorinated solvents.

Chlorinated solvents are mostly found in the form of colorless liquids at room temperature (Stroo & Ward, 2010). Physicochemical properties of CAHs (Tab. 1.1) are influenced by the number of chlorine atoms. Most CAHs are characterized by higher density than water and low water solubility. The solvents aqueous solubility defines the maximum extent of dissolution of a chlorinated solvent into water at a given temperature. Generally, as the number of chlorine atoms increases, density increases, and aqueous solubility generally decreases. They are considered moderately hydrophobic according to octanol-water partitioning coefficient, that generally increases as the number of chlorine substituents increases. Finally, CAHs are relatively volatile compounds. When an aqueous chlorinated solvent is in contact with air, the gas-phase concentration of the solvent is proportional to its concentration in water, with an equilibrium described by Henry's Law, referred to dilute solutions of chlorinated solvent in water.

Because of their stable nature, desirable chemical properties and low production costs, chlorinated solvents have been massively used since the 1950s in several industrial processes, including metal degreasing and surface cleaning, production of plastics, paints and coatings, pharmaceuticals and cosmetics, adhesives, agrochemicals and pesticides, electronic components and synthetic textile fibers; they are employed in textile processing and food packaging, as coating resins, refrigerants, hydraulic fluids in industrial equipment,

and they constitute the intermediates in the manufacture of other chlorinated solvents (Fracari et al., 2015).

Name	Acronym	Chemical structure	Boiling point (°C)	Solubility in water at 25°C (mg L ⁻¹)	Density at 20°C (g cm ⁻³)	Henry's Law Constant (KH) (x 10 ⁻³ atm m ³ mol ⁻¹)	Log octanol/water partitioning coefficient
CHLOROMETHANES							
Chloromethane	CM		-24.2	5,235	0.92	9.6	0.91
Dichloromethane	DCM		39.6	13,2	1.33	1.7	1.25
Trichloromethane (chloroform)	CF		61.2	8,2	1.49	3.8	1.97
CHLOROETHANES							
Chloroethane	CA		12.3	5,7	0.89	1.8	1.43
1,1-Dichloroethane	1,1-DCA		57.3	5,06	1.18	6.2	1.79
1,2-Dichloroethane	1,2-DCA		85.3	8,6	1.25	1.2	1.48
1,1,1-Trichloroethane	1,1,1-TCA		74	1,49	1.34	14.5	2.49
1,1,2-Trichloroethane	1,1,2-TCA		113.8	4,39	1.44	0.96	2.38
1,1,2,2-Tetrachloroethane	1,1,2,2-TeCA		145.2	2,85	1.59	0.44	2.39
CHLOROETHENES							
Vinyl chloride	VC		-13.9	2,7	0.91	79.2	1.38
1,1-Dichloroethene	1,1-DCE		31.6	2,25	1.21	23.0	2.13
cis-Dichloroethene	c-DCE		60.1	3,5	1.28	7.4	1.86
trans-Dichloroethene	t-DCE		48.7	6,26	1.26	6.8	1.93
Trichloroethene	TCE		88	1	1.46	11.7	2.53
Tetrachloroethene	PCE		121.3	200	1.62	26.3	2.88

Table 1.1. Main physicochemical parameters of CAHs (non-comprehensive).
Modified from (Stroo & Ward, 2010; Tobiszewski & Namieśnik, 2012)

1.1.2. Origin and diffusion in the environment

As a consequence of the widespread use of chlorinated solvents in several industrial processes and improper disposal methods that lead to unintentional release into the environment, these chemicals are frequently found as contaminants of groundwater and soil (Fig 1.1). Physicochemical properties of CAHs strongly influence the fate and diffusion of these pollutants in the environment. CAHs belong to a group of compounds referred to as Dense Non-Aqueous Phase Liquids (DNAPLs), characterized by low water solubility and

density higher than water. When released into the environment, the peculiar physical-chemical characteristics of these compounds cause them to typically form an organic separate phase sinking through permeable zones of soils and aquifers until a non-permeable zone is reached (Mercer & Cohen, 1990). This downward migration leads to the contamination of deep soils and groundwater. During the vertical migration driven by gravity, that is also accompanied by lateral spreading due to the effect of capillary forces, residual liquid can be trapped in the pore spaces or be sorbed to the surface of the soil particles (U.S. EPA, 1999). Moreover, because of the limited but environmentally significant aqueous solubility, a notable amount of chemicals can also be found dissolved in water, and the slow dissolution can act as a long-term source of contamination of flowing groundwater (Mercer & Cohen, 1990).

All these aspects result in the contamination of large volumes of the subsurface environment.

Finally, most chlorinated solvents also have sufficient vapor pressure to allow for volatilization into soil gas, leading to vapor phase plumes of contamination.

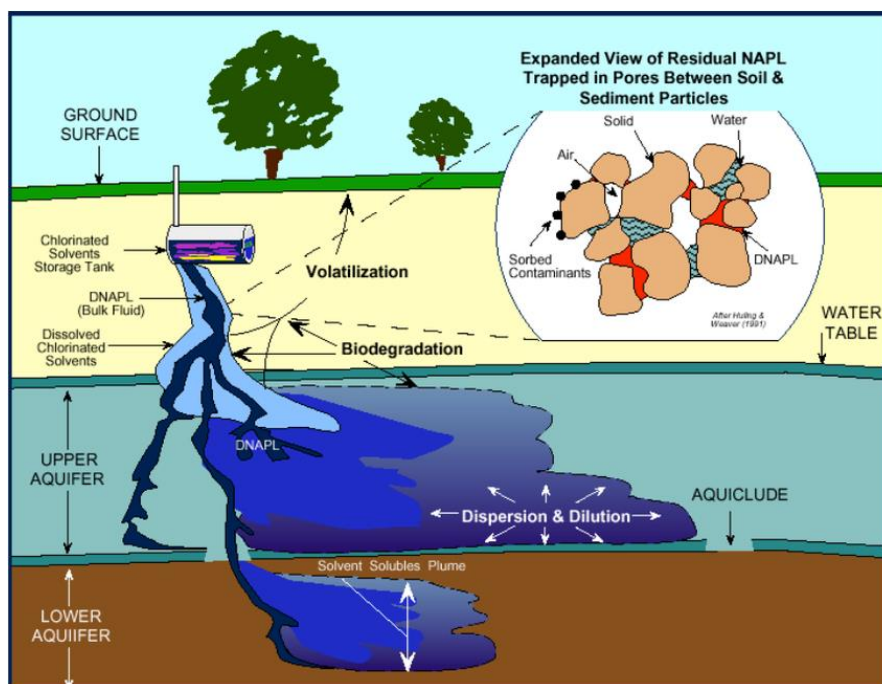


Figure 1.1. Schematic representation of DNAPLs distribution in subsurface soils and aquifers.
From (U.S. EPA, 1999)

1.1.3. Exposure to CAHs and effects on human health

Exposure to chlorinated solvents can occur through occupational use, but general population can be exposed to the presence of these contaminants in the ambient air, where they can be found as a result of their volatilization, in contaminated drinking water or food

ad from accidental environmental spills. Some chemicals, such as dichloromethane, can also be found in paint strippers, hairspray aerosols, and room deodorants. inhalation is the main route of exposure, but dermal contact and ingestion can potentially occur (Harper et al., 2022; McDermott & Heffron, 2013).

Based on animal and human studies on toxicology and metabolism, exposure to CAHs has been associated with several short- or long-term adverse health effects (McDermott & Heffron, 2013).

The severity of the damage following exposure depends on factors including the specific chemical(s), its concentration, and the length and frequency of the exposure.

Short-term effects include nausea, vomiting, eye and skin irritation and narcotic effects, as well as effects on the central nervous system, blood, liver, heart, and lungs. Long-term exposure can result in fatigue, irritability, and mental and memory disturbances, liver and kidneys damage toxicity and carcinogenicity (Jordan et al., 2021).

Toxic effects include nervous system, kidney, liver, immune system, male reproductive system toxicity, genotoxicity, embryonic or fetal mortality, prenatal growth inhibition (Chiu et al., 2013; Harper et al., 2022). As an example, trichloroethene (TCE), one of the most common CAHs in the environment, shows potential human health hazard. In particular, strong evidences of nephrotoxicity, male reproductive toxicity, and immunotoxicity were reported based both on human and laboratory animal studies (Chiu et al., 2013).

Many CAHs are reported as carcinogens or suspected carcinogens. Some examples include vinyl chloride (VC), classified as a (group 1) carcinogen by the International Agency for Research on Cancer (IARC), and trichloroethene (TCE) and tetrachloroethene (PCE), classified as probable carcinogens (group 2A) and defined as “dangerous” according to the EU Water Framework Directive.

As CAHs show, as described, relatively high aqueous solubilities, groundwater contamination can reach levels potentially harmful to human health. Maximum legal contamination levels of chlorinated solvents in environment and water bodies are regulated by EU Directives in Europe. In Italy, threshold concentration of contamination (CSC), established to qualify a site as polluted or not, are generally in the order of few micrograms per liter, ranging from 0.15 to 3 µg/L with particular reference to the carcinogenic ones

(Legislative Decree 152/2006, Part IV, Title V - Bonifica di siti contaminati, Attached 5)
(Tab. 1.2).

Carcinogenic CAHs	CSC (µg/L)
Chloromethane	1,5
Trichloromethane	0,15
Vinyl Chloride	0,5
1,2-dichloroethane	3
1,1-dichloroethene	0,05
Trichloroethene	1,5
Tetrachloroethene	1,1
Hexachlorobutadiene	0,15
Total CAHs	10
Non carcinogenic CAHs	CSC (µg/L)
1,1-dichloroethane	810
1,2-dichloroethene	60
1,2-dichloropropane	0,15
1,1,2-trichloroethane	0,2
1,2,3-trichloropropane	0.001
1,1,2,2-tetrachloroethane	0,05

Table 1.2. Threshold concentration of contamination (CSC) of CAHs in environment according to Italian law (Legislative Decree 152/2006)

1.2. Remediation approaches

Chlorinated solvents pollution is recognized as a serious environmental issue, threatening human health and ecosystems. The persistence of these compounds in the environment is due to the stability of carbon–halogen bonds in the molecules. In the last 30 years a great effort was put in the development and implementation of containment and removal technologies in order to achieve law-based cleanup goals in contaminated sites. The challenges in chlorinated solvents clean-up are mainly due to the peculiar properties of these compounds and their presence in the environment in the form of DNAPLs (Kueper et al., 2014).

The application of DNAPLs remediation strategies can be very difficult because of the several aspects affecting the diffusion of the contamination plume in the subsurface, such as the volume of released DNAPLs, the area and duration of release, the specific properties of the compounds and the characteristics of subsurface stratification and of hydraulic gradient

of the particular site under examination (Luciano et al., 2010). Therefore, the unicity of each specific site in terms of geology, hydrogeology, geochemistry, the nature and extent of contamination makes it challenging to predict the efficacy of any particular technology. Moreover, achieving remediation goals may require not only treating the contamination plume, as traditional approaches usually do, but also intervening on DNAPLs pools that act as a long-term source zone of contamination (Kueper et al., 2014).

In summary, the remediation strategy to be implemented at a site depends on a number of factors including the contaminant type, remedial objectives, the budget, the geologic and hydrogeologic framework and the characteristics of the source zone and contaminant distribution (vapor, sorbed, aqueous phase and DNAPLs form)(Kueper et al., 2014).

Generally, chlorinated solvents removal can occur through physical, chemical or biological processes, or a combination of those, that can be exploited in the implementation of a variety of *in situ* technologies or a combination of *in situ/ex situ* strategies. The main remediation approaches currently employed can be grouped as:

1. conventional technologies;
2. monitored natural attenuation;
3. bioremediation.

1.2.1. Conventional active cleanup technologies

Conventional active cleanup technologies include several remediation strategies exploiting physical or chemical processes. Some examples are here reported and briefly described (Stroo & Ward, 2010):

- Pump & Treat (P&T) system was the first adopted technology for the remediation of contaminated groundwater and it is still the most widely exploited strategy in Italy. P&T consists in extracting contaminated groundwater and transferring it to an *ex situ* water treatment system, where other remediation technologies will be employed, such as volatilization by air stripping. Treated groundwater is then discharged to surface waters or may be reintroduced into the aquifer (Stroo & Ward, 2010). This method is usually employed to control and manage the contamination plumes, but complete plume remediation is difficult to achieve, as pumping does not affect the source of contamination in short time frames. Given the persistence of chlorinated solvents in the subsurface, and the presence of DNAPLs pools acting as a slow-

release source of contaminant plumes, pump-and-treat systems should operate indefinitely, resulting in high long-term management costs (Kueper et al., 2014).

- Soil Vapor Extraction (SVE) is an *in situ* technology used for volatile contaminants. Contaminated air is removed through a vacuum pump and directed to an *ex situ* treatment system to remove contaminants from the air. The air movement also favors the vaporization of the DNAPL and its removal.
- In situ air sparging exploits volatilization of the compounds from contaminated groundwater by injection of pressurized air.
- Cosolvent and surfactant flushing consist in using cosolvents and surfactants that increase the solubility of DNAPLs to ease their extraction from groundwater.
- In situ thermal technologies include a variety of methods that generate high temperatures in the subsurface to increase volatilization and contaminants desorption to air.
- In situ chemical processes can be divided into oxidative and reductive processes. Oxidative processes are based on the use of chemical oxidants leading to the degradation of chlorinated solvents into carbon dioxide and chloride. Most widely used oxidants include ozone, permanganate and “Fenton’s reagent”, a mixture of hydrogen peroxide and ferrous iron. Reductive processes are mostly based on the use of zero-valent iron (ZVI), that has the capability of to remove chloride from chlorinated compounds and replacing the chlorides with hydrogen. This technology is usually associated with the installation of permeable reactive barriers (PRBs), in situ treatment zones positioned to passively capture a contaminant plume.

Conventional cleanup technologies such as pump-and-treat are usually time-consuming and expensive procedures, and they are often ineffective in the complete remediation of the site within a reasonable time frame. A promising, cost-effective alternative to conventional remediation technologies is represented by natural attenuation strategies.

1.2.2. Monitored Natural Attenuation (MNA)

At some sites, under proper conditions, naturally occurring processes may be sufficient to meet all the remedial goals. The reliance on these naturally occurring processes to achieve site-specific remedial objectives, within the context of a controlled and monitored site cleanup approach, is referred to as “monitored natural attenuation (MNA)”. Natural attenuation is typically defined as a variety of physical, chemical, or biological processes that under favorable conditions allow to reduce the mass, toxicity, mobility, volume, or

concentration of contaminants in soil or groundwater, without human intervention (U.S. EPA, 1999). It should be underlined that this approach, considered as a form of *in situ* technology, is only intended to act on the dissolved, sorbed or vapor phases of contamination, while it is not expected to remediate the DNAPLs form. The processes MNA relies on, indeed, include: all the forms of mobilization of the contaminant from the dissolved form (dispersion, dilution, sorption/desorption, volatilization); chemical reactions, such as the breakdown of chlorinated solvent vapors in the air or dissolved solvents in surface water or soil, caused by exposure to sunlight; biodegradation processes mediated by specialized microorganisms.

The difference between MNA and other “active” remedies is that it relies on processes and conditions that are preexisting at the site. Therefore, the efficacy of remediation needs to be monitored and defined through appropriate site characterization. Evidences supporting the suitability of MNA include groundwater and/or soil chemistry data showing a significant trend of decreasing contaminant mass or concentration over time; when relying on biological degradation processes, data from field or microcosm studies (conducted in or with actual contaminated site matrices) demonstrating the occurrence of biodegradation of the contaminant of concern. This approach can be taken into account when the achievement of remediation goals is expected to occur within a limited time frame comparable to other more active methods (U.S. EPA, 1999). Nevertheless, the particular stability and persistence of chlorinated compounds reduces their biodegradability and efficacy of natural attenuation.

When MNA is not acceptable as remediation strategy, other active technologies need to be designed and implemented at the site.

1.2.3. Bioremediation

1.2.3.1. Principles of bioremediation

Bioremediation is an eco-friendly and cost saving alternative to conventional chemical and physical clean-up technologies. Since the past decades, research in this field has been rapidly increasing and several advances in bioremediation techniques were done (Azubuike et al., 2016).

Bioremediation can be defined as a process which relies on biological mechanisms to reduce (degrade, detoxify, mineralize, or transform) the concentration of pollutants (below concentration limits established by regulatory authorities) or transform them into an innocuous state in order to clean up contaminated sites. It uses naturally occurring bacteria,

fungi or plants that convert contaminants to less harmful compounds to human health and/or the environment through reactions that take place as a part of their metabolic processes, thanks to the notable ability, that such organisms have developed, to survive under a wide variety of conditions (Azubuike et al., 2016; Stroo & Ward, 2010; Vidali, 2001). In particular, here we primarily refer to microorganisms.

The control and optimization of bioremediation processes depend on several factors including:

- the characteristics of the contaminants (nature, depth and degree of pollution) and their availability to the microbial population;
- the existence of a microbial population capable of degrading the pollutants, as biodegradation relies on some specialized microorganisms;
- the environmental factors such as temperature, pH, presence/ absence of oxygen or other electron acceptors and nutrients, the type of soil or, in the case of groundwater, hydrogeochemistry of the site.

All these factors drive the selection of the most suitable bioremediation technique for a specific site or goal. Therefore, fundamental steps prior to the implementation of a bioremediation strategy are: the characterization of the pollutants; the microbial characterization of the site; the hydrogeochemical characterization of the site.

Bioremediation may just consist in monitoring intrinsic biodegradation, or it may include active interventions to deliberately alter the environment to increase the efficiency of desired biological processes. Intrinsic bioremediation refers to naturally occurring biological processes mediated by the autochthonous microbial communities in the polluted site. This strategy is the Monitored Natural Attenuation (MNA), as already described, when based on biological processes. When active strategies to improve biodegradation are adopted, the term Enhanced Bioremediation is used (Azubuike et al., 2016); the two major approaches to enhance bioremediation are biostimulation and bioaugmentation.

Biostimulation is a strategy applied to meet the environmental requirements of autochthonous microorganisms. It generally involves adding nutrients, energy sources or oxygen to stimulate growth and activity of the degrading organisms already existing in the site, as they could not be present in numbers required for bioremediation purposes (Vidali, 2001).

In some cases, enhanced bioremediation consists in adding to the contaminated sites selected indigenous or exogenous microorganisms with the ability to degrade specific pollutants to improve the biodegradation capacity or its rate. This process is known as bioaugmentation (Vidali, 2001).

With regards to site of application, bioremediation techniques can be categorized as: *ex situ* or *in situ* (Fig. 1.2).

Ex situ techniques are those applied to soil and groundwater after they are removed from the site via excavation (soil) or pumping (water) and transported to another aboveground site for treatment. Some examples of techniques used for contaminated soils are: landfarming, that consist in spreading soil on ground surface and tilling it; biopile, that consists in piling soil and adding nutrients and aeration; windrow that consists in piling and periodically turning soil. Bioreactors, used for the treatment of both contaminated soil and water pumped up from a contaminated plume, engineered containment vessel in which raw materials are converted into specific products and bioprocess parameters can be controlled and manipulated to provide optimum growth conditions (Azubuike et al., 2016).

In situ techniques consist in treating polluted substances at the site of pollution. They are generally less expensive although they can require on-site installation of some equipment to improve microbial activities. These techniques include MNA and various Enhanced Bioremediation methods that consist in: delivering oxygen into the subsurface environments to increase activities of indigenous microbes, while minimizing volatilization and release of contaminants to the atmosphere (bioventing); injecting air under pressure to provide oxygen and simultaneously cause upward movement of volatile organic compounds (biosparging); combining vacuum-pumping and bioventing to recover products from the site first and complete remediation process by air injection as in bioventing method (bioslurping).

Finally, Permeable Reactive Barrier (PRB) is a physical method for remediating contaminated groundwater with some elements of bioremediation. In PRB technique, biological reactions play an enhancement role acting in conjunction with other mechanisms (degradation, precipitation and sorption) of contaminant removal. It consists in a submerged reactive barrier, mostly made up of zero-valent iron (ZVI), where pollutants become trapped while polluted water flows through it, under its natural gradient, and undergo a series of reactions, resulting in clean water. The permeability of the barrier, indeed, allows the flow of water but not pollutants (Obiri-Nyarko et al., 2014). Other designs and reactive media can be applied to a barrier. For example, it was reported that coupling ZVI with a biodegradable

polymer, polyhydroxybutyrate (PHB), that acts as a slow-releasing electron donor, promoted biological activity when used as a barrier, resulting in enhanced removal of chlorinated compounds (Baric et al., 2014).

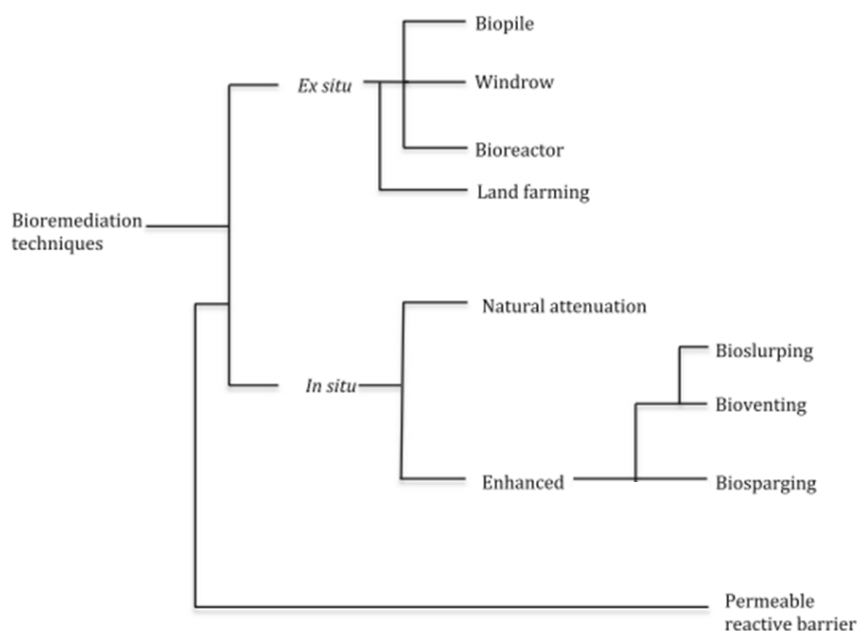


Figure 1.2. Bioremediation techniques. Modified from (Azubuike et al., 2016)

Bioremediation shows several advantages in respect to conventional remediation techniques, as it is usually less expensive and environmentally friendly because it relies on natural processes and allows the complete degradation of a wide variety of contaminants, even reducing costs and threats to human health associated to transportation of the contaminants in the case of in situ techniques. Nevertheless, as every remediation technology, bioremediation also has some drawbacks as it is a relatively slow technology if compared to other conventional treatments, and it depends on the presence of suitable environmental growth conditions, in case of in situ approaches. Moreover, some concerns derive from the risk that some biodegradation products may be more persistent or toxic than the parent compound. Finally, bioremediation can only be applied to compounds that are susceptible to rapid and complete degradation (Vidali, 2001).

So far biodegradation has been applied to a wide variety of pollutants including hydrocarbons, polyaromatic hydrocarbons (PAHs), BTEX (benzene, toluene, ethylbenzene, and xylene), plastics, pesticides, heavy metals, wastewater and chlorinated compounds.

1.2.3.2. Bioremediation of CAHs

Chlorinated organic compounds are recalcitrant to microbial attack, but some specialized microorganisms are able to degrade them both under aerobic and anaerobic conditions by several mechanisms, mediated by specific enzymes, that lead to the sequential removal of chlorine atoms from the compound. CAHs degradation is usually referred to as dechlorination, and specialized microorganisms are defined dechlorinating.

The possibility to exploit these biodegradative pathways made bioremediation one of the most common and successful remedial approaches for restoring contaminated aquifers (Adrian & Löffler, 2016). Moreover, its flexibility allows to use it in combination with several other technologies (Stroo & Ward, 2010)

Among bioremediation techniques applied to chlorinated solvents-contaminated aquifers, *in situ* bioremediation is the predominant approach because it is relatively easy to implement and control under field conditions and it is a relatively inexpensive technology in terms of operation and maintenance costs. Moreover, anaerobic conditions required for some dechlorination processes can be obtained in the aquifer, allowing the treatment of those compounds, such as perchloroethene (PCE), that can only be degraded anaerobically (Stroo & Ward, 2010).

In particular, the most widely used approach is *in situ* biostimulation, relying on the addition of chemicals to support the nutritional needs of specialized indigenous microorganisms and to enhance *in situ* activity and to create an anaerobic environment required for some dechlorination processes. Biostimulation can potentially lead to the complete detoxification of dissolved CAHs *in situ* with little impact on site infrastructure. On the other hand, the drawback of potential accumulation of intermediate dechlorination products and an extended timeframe for remediation can limit its applications. Substrates are generally provided by direct injection, dissolved in water if in liquid form or by biosparging if in gaseous form (Stroo & Ward, 2010).

In some cases, biostimulation alone is not sufficient to achieve complete degradation of chlorinated solvents. Therefore, bioaugmentation approaches can be adopted if native dechlorinating species capable of complete dechlorination of CAHs are not present, or they are poorly distributed, or cannot be properly stimulated. Moreover, bioaugmentation may be used to reduce the time to achieve effective biodegradation of chlorinated solvents in respect to biostimulation, that may require time for autochthonous microbial populations to acclimate and grow under the anaerobic conditions induced by substrate addition.

Bioaugmentation may be applied as a preventive measure when biostimulation alone is expected to take a long time or not to lead to complete CAHs degradation. When this happens, dechlorinating cultures can be added at the same time as substrate addition.

The predominant biodegradation pathways exploited for CAHs bioremediation are anaerobic reductive dechlorination, aerobic cometabolism and direct aerobic oxidation (Frasconi et al., 2015; Stroo & Ward, 2010).

1.3. Biodegradation of chlorinated solvents

Chlorinated solvents, as previously stated, are recalcitrant to microbial degradation because of their xenobiotic nature and their stability. Nevertheless, under suitable environmental conditions, these compounds can be biodegraded by some specialized microorganism capable of metabolic pathways mediated by specific dehalogenating enzymes that lead to carbon–chlorine bond breakage and the simultaneous release of chloride ions (Cl^-). Biodegradation can occur both by direct (or metabolic) processes, coupled to the growth of the microorganism, and by cometabolic processes in which the microorganisms do not gain energy or carbon for growth. Both types of processes can occur under aerobic and anaerobic conditions.

The main processes occurring under anaerobic conditions are reductive dechlorination and cometabolic reductive dechlorination. Under aerobic conditions, hydrolytic and oxidative direct degradation and cometabolic processes can occur. The type of degradative process taking place depends on the specific catabolic capabilities of different microorganisms, on the environmental conditions, such as redox potential and the presence of required nutrients or substrates, and on the nature and degree of chlorine substitution of the compound.

In general, highly chlorinated CAHs, recalcitrant to aerobic degradation, are degraded through reductive reactions, while less chlorinated compounds can be degraded through oxidative processes (Adrian & Löffler, 2016).

1.3.1. Reductive dechlorination

Reductive dechlorination, also known as organohalide respiration or dehalorespiration (in particular chlororespiration or dechlororespiration for chlorinated compounds), is a respiratory process in which specialized microorganisms, called organohalide-respiring bacteria (OHRBs), use CAHs as terminal electron acceptors of a respiratory chain, harnessing energy from the process. Depending on the microorganism, the electron donor is

usually H_2 , in most cases, or acetate for a limited number of chlororespiring microorganisms. In environmental samples, under mixed culture conditions, complex carbon substrates such as butyrate, lactate, benzoate, propionate, methanol, ethanol, formate and glucose have been shown to support chlororespiration, thanks to the presence of non-dechlorinating, fermentative species that produce H_2 or acetate from fermentable substrates (Stroo & Ward, 2010).

Two types of organohalide respiration of CAHs exist: hydrogenolysis and dichloroelimination. Hydrogenolysis is a reductive process in which the halogen atoms are sequentially replaced by hydrogen atoms. Dihaloelimination implies the elimination of two vicinal chlorine atoms, resulting in the formation of an unsaturated C=C bond (Adrian & Löffler, 2016).

All chlorinated ethenes, ethanes and methanes can potentially be degraded by reductive dechlorination.

As an example, the respiration pathways of some ethenes and ethanes are shown in Fig.1 .3. Among chloroethenes, tetrachloroethene (PCE) and trichloroethene (TCE) are the most susceptible to anaerobic dechlorination and OHRBs capable of degrading PCE and TCE can be considered ubiquitous in subsurface environment (Stroo & Ward, 2010). Most microorganisms can reductively dechlorinate PCE and TCE to cis-1,2-dichloroethene (cis-DCE) or to vinyl chloride (VC), but not all can dechlorinate these compounds beyond cis-DCE or VC. VC is generally degraded more slowly than the parent compounds as the result of a slower cometabolic processes. As a consequence, VC tends to accumulate, for example in the environment or in treatment systems. This is a common concern because VC is considered more toxic than the parent compounds, and a known human carcinogen. Nevertheless, some *Dehalococcoides mccartyi* strains were found able to completely dechlorinate PCE to harmless ethene (Fig. 1.3) (Löffler et al., 2013). For this reason, *D. mccartyi* is an important organism for bioremediation and most research about CAHs degradation focus on this species.

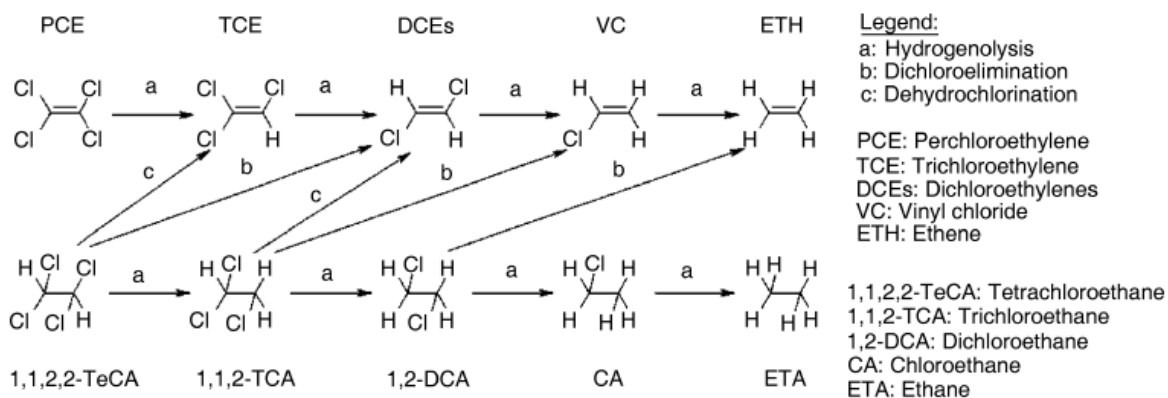


Figure 1.3. Dehalorespiration of some ethenes and ethanes by hydrogenolysis and dichloroelimination. From (Aulenta et al., 2006a)

1.3.1.1. Reductive dehalogenases

The key enzymes in dehalorespiration are reductive dehalogenases, referred to as RDase if characterized, otherwise RdhA.

RdhAs are membrane associated and oxygen sensitive enzymes. They are characterized by several specific conserved motifs, including two iron–sulfur cluster-binding motifs and a twin-arginine signal motif for translocation to or across the cell membrane. RdhAs belong to a family of cobalamin (B₁₂)-dependent enzymes, as they harbor in their active center a corrinoid co-factor (usually cobalamin, B₁₂, but also other variants were observed), thought to be essential for enzymatic function. It is not certain if all RdhA proteins share a common evolutionary origin (Hug et al., 2013).

Reductive dehalogenase genes (or reductive dehalogenase homologous genes, *rdh*, if the encoded protein has not yet been biochemically characterized) are typically made up of an operon containing the gene *rdhA* for the catalytically active enzyme, *rdhB* encoding a putative membrane-anchoring protein, and one or more associated genes. The nomenclature of reductive dehalogenase genes as homologues is based on the conservation of shared motifs and on the absence of evidence of convergent evolution (Hug et al., 2013).

OHRBs can contain one or multiple *rdhA* genes and the range of halogenated electron acceptors used depends on the reductive dehalogenases present within an organism, enrichment culture or contaminated site.

Some examples of *rdhA* genes are here reported. The *pceA* genes, identified from *Sulfurospirillum*, *Desulfitobacterium*, *Dehalobacter* and *Geobacter*, encode for enzymes

active on the chlorinated ethenes PCE and TCE; *dcaA* from *Desulfitobacterium* and *Dehalobacter* encode for enzymes active on 1,2-DCA; *tceA*, *bvcA* and *vcrA* genes from *D. mccartyi* encode for enzymes TceA catalysing the dechlorination of TCE; BvcA active on TCE, DCE isomers, VC and 1,2-DCA; VcrA dechlorinating TCE, DCE isomers and VC to ethene and dihaloelimination of 1,2-DCA (Hug et al., 2013; Hug & Edwards, 2013).

The *rdhA* genes family is characterized by significant diversity and complexity and a wide environmental distribution. Within the known, characterized *rdhA* genes, sequence identity above 90% does not always correspond to shared substrate specificities and, vice versa, reductive dehalogenases with shared functions do not necessarily have high sequence similarity. For this reason, search for novel *rdhA* genes from environmental samples is performed by using degenerate primers that allow the detection of *rdhA* genes with low sequence similarity to those present in reference databases (Hug et al., 2013; Hug & Edwards, 2013).

1.3.1.2. Organohalide-respiring bacteria

Known organohalide-respiring bacteria (OHRBs) are spread among several phyla. Their distinctive trait is the ability to produce reductive dehalogenase enzymes (RDase) enabling them to use different organohalides as terminal electron acceptors. Species found capable of obtaining energy from reductive dehalogenation can be divided into obligate and facultative groups based on whether reductive dehalogenation is their only energy-gaining metabolism. Each species has different dehalogenases and dechlorinating abilities, depending on the substrate specificity of the reductive dehalogenase enzymes. A synthetic overview of OHRB genera, degrading in particular chlorinated compounds, and their family, phylum and chlorinated compounds degraded is reported in Tab. 1.3.

The obligate OHRBs, that are not able to utilize non-halogenated compounds as terminal electron acceptors, include *Dehalobacter* within the phylum Firmicutes, and *Dehalococcoides* and *Dehalogenimonas* within the phylum Chloroflexi (Adrian & Löffler, 2016).

Facultative OHRBs are characterized by a more versatile metabolism and they can grow on a wide range of electron acceptors nitrate, nitrite, fumarate, thiosulphate and fermentable organic acids (Maphosa, 2010; Türkowsky et al., 2018). They mostly belong to Proteobacteria, such as *Anaeromyxobacter*, *Desulfuromonas*, *Desulfovibrio*, *Desulfomonile*, *Geobacter*, (Deltaproteobacteria), *Sulfurospirillum* (Epsilonproteobacteria), *Shewanella*

(Gammaproteobacteria), and to Firmicutes, such as *Desulfitobacterium*. These groups also include non-dechlorinating species.

	Phylum	Family	Genus	Electron acceptors
OBLIGATE	Chloroflexi	Dehalococcoidaceae	<i>Dehalococcoides</i>	1,2-DCA; DCE; TCE; PCE; VC
			<i>Dehalogenimonas</i>	1,2-DCA; 1,1,2-TCA; 1,1,2,2-TeCA
		Unclassified Chloroflexales	<i>Dehalobium</i>	TCE; PCE
	Firmicutes	Peptococcaceae	<i>Dehalobacter</i>	TCM; 1,1-DCA; 1,2-DCA; 1,1,1-TCA; TCE; PCE
FACULTATIVE			<i>Desulfitobacterium</i>	1,1-DCA; 1,2-DCA; 1,1,1-TCA; TCE; PCE; Chlorophenols
	Gammaproteobacteria	Shewanellaceae	<i>Shewanella</i>	PCE
	Deltaproteobacteria	Cystobacteraceae	<i>Anaeromyxobacter</i>	Chlorophenols
		Geobacteriaceae	<i>Geobacter</i>	1,2-DCA; 1,1,2-TCA; TCE; PCE
		Desulfuromonadaceae	<i>Desulfuromonas</i>	TCE; PCE
		Syntrophaceae	<i>Desulfomonile</i>	Chlorophenols
		Desulfovibrionaceae	<i>Desulfovibrio</i>	Chlorophenols
	Epsilonproteobacteria	Campylobacteraceae	<i>Sulfurospirillum</i>	TCE; PCE

Table 1.3. Phyla, family and genera of obligate and facultative dechlorinating OHRBs and chlorinated compounds used as electron acceptors. Ref: (Adrian & Löffler, 2016; Türkowsky et al., 2018)

Obligate and facultative OHRBs differ in their genome size and number of *rdhA* genes. In general, the obligate OHRBs tend to have smaller genomes (1.3– 3.2 Mbp) and a high number of *rdhA* genes (e.g. *Dehalococcoides mccartyi* strains can host up to 38 *rdhA* genes on a single genome), consistent with an extreme specialization in organohalide-respiration; their versatile (facultative) counterparts tend to have larger genomes (2.9–6.5 Mbp) and fewer *rdhA* genes (1–7) (Türkowsky et al., 2018). Moreover, facultative OHRBs can use a wider variety of electron donors, including H₂, acetate, formate, pyruvate, lactate, butyrate, than the obligate OHRBs, that are limited to H₂ (Maphosa, 2010)

Notably, there is no correlation between phylogenetic affiliation and specificities for used chlorinated substrates (Hug et al., 2013).

The diversity of OHRBs is not yet fully described and new organohalide-respiring genera will probably be discovered. Detailed physiological and genetic characterization is challenging, as most OHRBs are notoriously difficult to culture and do not grow to high

density, many *Dehalococcoides* spp., for example, are difficult to maintain in pure culture and can grow only in a microbial community or in strict co-culture or consortia (Maphosa, 2010). Indeed, the activity of many OHRBs depends on H₂ provided by the fermentative metabolism of other non-dechlorinating microorganisms, or on the production of vitamin B₁₂ from other members of the microbial community when de novo synthesis pathways for vitamin B₁₂ or other corrinoids are lacking in the OHRB itself (Adrian & Löffler, 2016). This is also the reason why inocula for bioaugmentation with *Dehalococcoides* spp. usually consist in mixed cultures (Stroo & Ward, 2010).

1.3.2. Cometary anaerobic reductive dechlorination

In cometary reactions, the degradation of the chlorinated compound is mediated by an enzyme or cofactor produced during microbial metabolism of another compound. The electrons flow is mainly directed to a primary electron acceptor and only a small fraction of the electron flow is fortuitously used on the chlorinated compound. During this process, the microorganism does not yield any energy or growth, therefore sufficient primary substrate is required to support growth of the microorganisms to sustain the cometary process (Adrian & Löffler, 2016). Cometary metabolism is a slow process, carried out by anaerobes like methanogens, acetogens, or sulfate-reducing bacteria thanks to highly reducing corrinoids and other cofactors like vitamin B₁₂, coenzyme F₄₃₀, or hemes that fortuitously dechlorinate a wide variety of halogenated compounds. Examples are the cometary dechlorination of 1,2-DCA, 1,1,2-TCA and PCE by pure cultures of methanogens such as *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri*; the tetrachloromethane cometary metabolism by the sulfate-reducing *Desulfobacterium autotrophicum* (Egli et al., 1987; Fathepure & Boyd, 1988); 1,2-DCA cometary degradation by a strain of the homoacetogen *Acetobacterium* sp. (De Wildeman et al., 2003). Cometary reductive dechlorination is thought to be less significant relative to halofermentation due to slower rates of reaction and because it does not often lead to complete dechlorination. Moreover, the contribution of cometary processes in reductive dechlorination reactions can be hard to address, as it occurs as a fortuitous transformation of a compound by enzymes used for other beneficial purposes (Atashgahi et al., 2018).

1.3.3. Direct anaerobic oxidation

In some cases, organochlorines with few chlorine substituents can be used as carbon or energy source under anaerobic conditions. So far, few microorganisms are known to have this pathway. Examples are homoacetogenic bacteria utilizing aliphatic chlorinated

methanes such as chloromethane (*Acetobacterium dehalogenans* strain MC) and dichloromethane (*Dehalobacterium formicoaceticum* strain DMC) as a carbon source for growth, and a betaproteobacterial strain growing on 1,2-DCA under denitrifying conditions (Atashgahi et al., 2018).

1.3.4. Direct aerobic oxidation

Low-chlorinated CAHs, showing a limited degradation under anaerobic conditions can be degraded under aerobic conditions. Compounds such as chloromethane (CM), dichloromethane (DCM), 1,2-dichloroethane (1,2-DCA), cis-1,2-dichloroethylene (c-DCE), trans-1,2-dichloroethylene (t-DCE), and vinyl chloride (VC) can be used as carbon and electron sources by some microorganism by a direct oxidation process leading to carbon dioxide, chloride ions and biomass. The range of used substrates depends on the microorganism; some strains can utilize more than two types of CAHs. VC-degrading bacteria are widely spread and belong to genera *Mycobacterium*, *Pseudomonas*, *Nocardioides*, *Ochrobactrum*, *Ralstonia*; one strain of *Polaromonas* sp. was described capable of c-DCE utilization and only one strain of *Stenotrophomonas* sp. is known to degrade TCE; strains from *Pseudomonas* and *Polaromonas*, as well as *Xanthobacter* spp., *Ancylobacter* spp. and *Starkeya* are known as 1,2-DCA degraders; DCM degrading genera include *Methylobacterium* and *Bacillus*; CM degradation is reported in *Hyphomicrobium* and *Methylobacterium* (Xing et al., 2022). Nevertheless, only certain strains of these bacteria have the metabolic capability to degrade chlorinated pollutants.

The degradation pathway for aerobic degradation depends on the specific CAH and the degrading microorganism and not all pathways have been described so far. Different mechanisms and enzymes are involved.

In most cases, aerobic organisms produce specific dehalogenases that selectively act on carbon-chlorine bonds, while in other cases cytochrome P450 monooxygenase are involved (Atashgahi et al., 2018; Nishino et al., 2013).

Among dehalogenases involved in aerobic process, at least two groups can be recognized: haloalkane dehalogenases, that hydrolyze halogenated alkanes, and haloacid dehalogenases, active on short-chain halogenated carboxylic acids.

Haloalkane dehalogenases (HLDs) are the key enzymes in direct aerobic oxidation of CAHs. These enzymes are hydrolytic dehalogenases belonging to the α/β -hydrolase fold family. They are characterized by broad and different substrate specificities and the main catalytic

mechanism is a two-step process made up of a S_N2 nucleophilic substitution and subsequent hydrolysis by addition of water, although mechanisms details can differ among different enzymes. So far, three archetypal HLDs have been characterized: LinB, a haloalkane dehalogenase from *Sphingomonas paucimobilis*, DhaA from *Rhodococcus* sp. and *Pseudomonas* sp., DhmA from *Mycobacterium* spp., and DhIA from *Xanthobacter* spp. and *Ancylobacter aquaticus* (Nagata et al., 2015).

As an example of different pathways of direct CAHs utilization as C and energy sources, 1,2-DCA biodegradation is reported (Fig. 1.4). Under aerobic conditions 1,2-DCA can be biodegraded via either a hydrolytic pathway or a monooxygenase-mediated pathway.

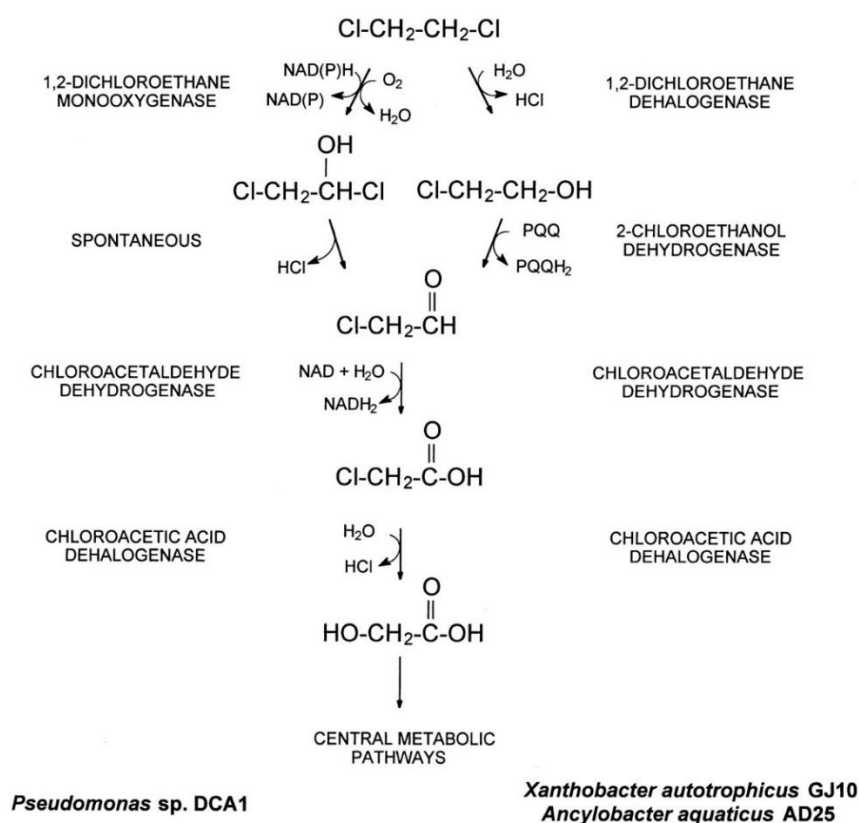


Figure 1.4. Hypothetical hydrolytic and monooxygenase-mediated pathways for 1,2-DCA aerobic direct biodegradation. From (Hage & Hartmans, 1999).

The hydrolytic pathway is carried out by bacteria including *Xanthobacter* and *Ancylobacter* (Janssen et al., 1985; Van Den Wijngaard et al., 1992). The initial reaction is catalyzed by the haloalkane dehalogenase DhIA which converts 1,2-DCA to 2-chloroethanol. Subsequent degradation involves two sequential oxidations catalyzed by alcohol and aldehyde dehydrogenases to yield chloroacetate. Chloroacetate is cleaved by a second hydrolytic dehalogenase (haloacid dehalogenase, DhIB) to glycolate (Song et al., 2004).

So far, the haloalkane dehalogenase DhIA, the key enzyme in this reaction, is the only known hydrolytic haloalkane dehalogenase that operates in 1,2-DCA degrading bacteria; it, is encoded by the strongly conserved, plasmid-borne *dhlA* gene. It was originally discovered in the nitrogen-fixing hydrogen bacterium *X. autotrophicus GJ10*, enriched with 1,2-DCA as the sole carbon source, and it was later found identical in other *Xanthobacter*, *Ancylobacter* and *Starkeya* isolates with different geographical origins (Germany, Netherlands, South Africa, Korea, and Australia), located on different plasmids and associated with different insertion sequences (Janssen et al., 2005; Munro et al., 2016). Instead, the haloacid dehalogenase DhIB is constitutively expressed by the gene *dhlB*, located on the chromosome (Janssen et al., 1985).

The oxidative pathway for 1,2-DCA degradation, proposed for *Pseudomonas* and *Polaromonas* was not confirmed. Nevertheless, a pathway involving a cytochrome P450 monooxygenase in the initial step was proposed (Hage & Hartmans, 1999; Nishino et al., 2013). The hydrolytic and oxidative pathways could differ in the initial steps and then converge.

Direct aerobic degradation could be potentially exploited for *in situ* CAHs bioremediation, but its application is hampered by the limited number of strains capable to perform it, and of CAHs potentially degradable through this process (Fracari et al., 2015).

1.3.5. Aerobic cometabolism

As in the anaerobic cometabolic reduction, aerobic cometabolism (AC) of CAHs consists in the fortuitous oxidation of the chlorinated molecule by microbial enzymes produced for the uptake of another compound, used as a growth substrate. This process does not provide benefits for the microorganism in terms of energy or growth. Non-specific enzymes may accidentally cleave carbon-halogen bonds or facilitate dechlorination by generating labile structures that undergo spontaneous decomposition in aqueous medium. In most cases these enzymes are monooxygenases produced by methanotrophs, nitrifiers, butane-, ethene-, toluene – oxidizers, that can fortuitously attack non-growth substrates due to their broad substrate range. Therefore, cometabolic processes require the presence of substrates usually used by the microorganism to support its growth (Fracari et al., 2015; Stroo & Ward, 2010).

Various CAHs, including highly chlorinated ones, can be biodegraded by AC (Fracari et al., 2015). Some examples of CAH that can or cannot be cometabolically biodegraded, and required growth substrates are reported in Fig. 1.5.

Bacteria capable of aerobic cometabolic degradation of CAHs include ethene/ethane-assimilating *Mycobacterium* strains using VC, cDCE and 1,2-DCA as cometabolic substrates (Le & Coleman, 2011); the methane oxidizer *Methylosinus trichosporium*, capable of TCE degradation (Tsien et al., 1989); VC-oxidizing *Pseudomonas* capable of cDCE and tDCE cometabolism (Verge et al., 2002).

Aerobic cometabolism is one of the most exploited processes in *in situ* bioremediation strategies. For bioremediation applications, growth substrates and oxygen can be supplied to degrading microorganisms by several methods such as air sparging and bioventing (Frasconi et al., 2015).

Compound	Cometabolism	Growth substrate
Chloromethane	Yes	Ammonia; Methane
Dichloromethane	Yes	Ammonia; Methane
CF	Yes	Ammonia; Butane; Methane; Propane
Carbon tetrachloride	No	—
Chloroethane	Yes	Ammonia; Methane
1,1-DCA	Yes	Ammonia; Methane; DCA
1,2-DCA	Yes	Ammonia; Chloroethane; Methane; Toluene
1,1,1-TCA	Yes	Ammonia; Methane; DCA; Butane
1,1,2-TCA	Yes	Ammonia; Chloroethane; Propane
1,1,1,2-TeCA	Yes	Ammonia
1,1,2,2-TeCA	Yes	Methane, Propane
PCA	No	—
HCA	No	—
VC	Yes	Ammonia; Butane; Chloroethane; Methane; Propane; Propylene; Ethene
1,1-DCE	Yes	Ammonia; Chloroethane; Methane; Propane; Toluene; VC; DCA; Benzene
cis 1,2-DCE	Yes	Ammonia; Butane; Chloroethane; Methane; Propane; Propylene; Toluene; VC; DCA; Benzene; Chlorobenzene; 1,2-DCB; 1,3-DCB
trans 1,2-DCE	Yes	Ammonia; Chloroethane; Methane; Propylene; Toluene; VC; DCA; Butane
TCE	Yes	Ammonia; Butane; Chloroethane; Butane; 2,4-dichlorophenoxyacetic acid; Isoprene; Methane; Phenol; Propane; Propylene; Toluene DCA; Benzene; Chlorobenzene; 1,2-DCB; 1,3-DCB
PCE	Yes	Toluene; DCA

Figure 1.5. Examples of CAHs susceptible to aerobic cometabolism and relative growth substrates required for cometabolism. Abbreviations used: CF: chloroform; DCA: dichloroethane; DCE: dichloroethene; DCB: dichlorobenzene; HCA: hexachloroethane; PCA: pentachloroethane; PCE: perchloroethylene; TCA: trichloroethane; TCE: trichloroethene; TeCA: 1,1,2,2-tetrachloroethane; VC: vinyl chloride. Modified from (Jesus et al., 2016).

1.4. 1,2-dichloroethane

1,2-dichloroethane (1,2-DCA) is a volatile, synthetic chlorinated alkane in the form of a colorless liquid at room temperature. It is one of the most abundant chlorinated solvents produced industrially, because it serves in the synthesis of vinyl chloride monomer, an intermediate during the manufacture of polyvinyl chloride (PVC) plastic, and in the

production of rubber, cosmetics, drugs, and synthetic textile fibers. 1,2-DCA is also used as a solvent for plastics, resins, and oils, and in cleaning agents and degreasers. Smaller amounts of 1,2-DCA are used in the synthesis of other chlorinated solvents such as chlorinated ethenes (Fracari et al., 2015; Hughes et al., 1998).

1,2-DCA is a widespread contaminant released to the environment during its production and use, and it is defined as a “priority” substance by EU Water Framework Directive. The majority of 1,2-dichloroethane emissions end up into air. If released to soil, it is not expected to adsorb strongly and tends to leach into groundwater, thus becoming one of the most common chloroethane aquifer contaminants (Harper et al., 2022; Hughes et al., 1998) .

1,2-DCA poses serious risks for animal and human health.

Exposure to 1,2-DCA can occur mainly from inhalation, dermal absorption and ingestion of 1,2-dichloroethane in contaminated drinking water or food items. inhalation and dermal contact can also be routes of occupational exposure to 1,2-dichloroethane at workplaces where it is produced or used.

It is classified as a possible human carcinogen (group 2B) by IARC (International Agency Research on Cancer), and a probable human carcinogen (group B2) by US EPA, based mainly on evidence from animal studies. Main targets of mammalian toxicity include liver, kidneys, and neurological, cardiovascular, and immune systems. A limited amount of information about effects in humans is available. Based on reports on people who died after acute exposure to high levels by inhalation or ingestion, symptoms include central nervous system depression, nausea and vomiting, corneal opacity, bronchitis, respiratory distress, lung congestion, myocardial lesions, hemorrhagic gastritis and colitis, hepatocellular damage, renal necrosis, and histopathological changes in brain tissue (Harper et al., 2022). Because of its toxicity and suspected carcinogenicity, maximum allowable concentrations, in Europe are regulated by EU Directives. In Italy, the maximum legal contamination in the environment is 3 µg/L (Legislative Decree 152/2006).

As 1,2-DCA is a xenobiotic, and therefore often recalcitrant to biodegradation, contamination can persist for many years or decades in the environment. For this reason, the interest in the implementation of efficient remediation methods is thriving, in particular for contaminated aquifer, where 1,2-DCA is commonly found. In particular, bioremediation strategies have the potential to be applied for the clean-up of 1,2-DCA contaminated aquifers by exploiting and enhancing the known biodegradation pathways of this contaminant (Fracari et al. 2015; Munro et al., 2013; Wang et al., 2015).

The main 1,2- DCA biodegradation pathways are summarized in Fig. 1.6.

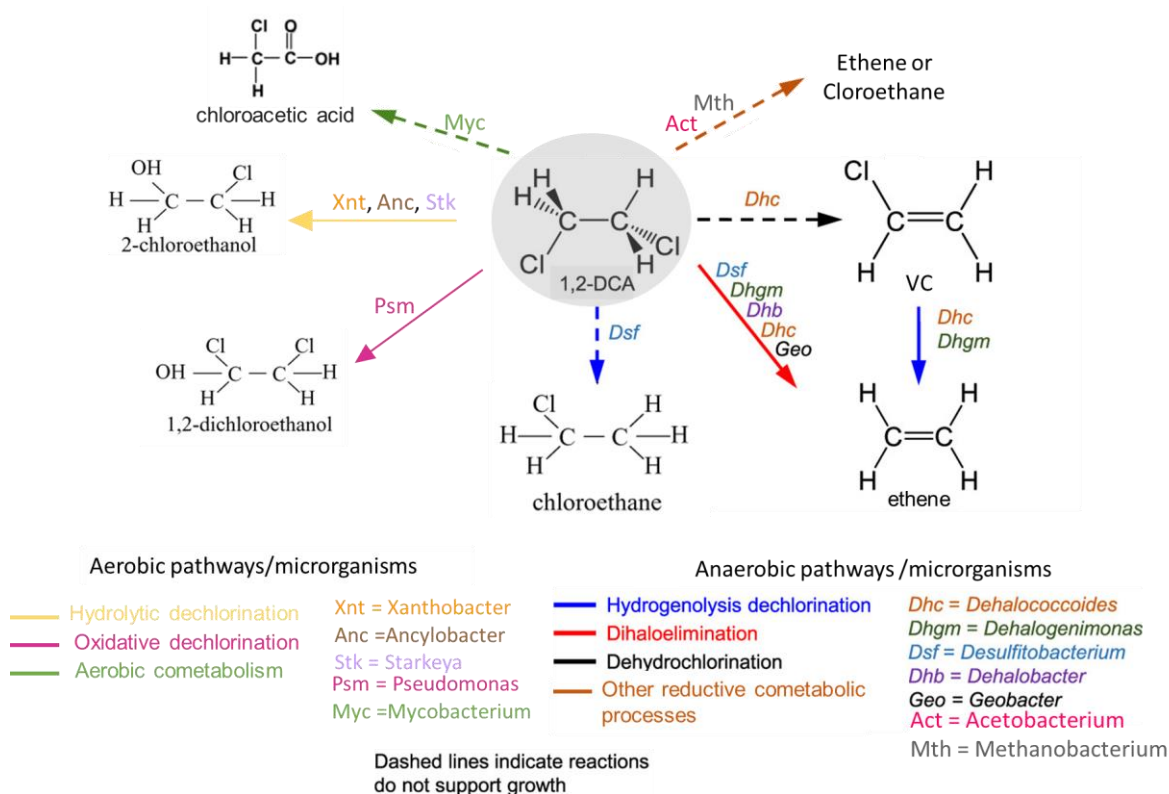


Figure 1.6. Main 1,2-DCA biodegradation pathways and microorganisms involved. Modified from (Jiang et al., 2022)

Aerobic bacteria can grow on 1,2- DCA as a carbon and energy source via either a hydrolytic pathway mediated by a Dh1A enzyme (as in *Xanthobacter* and *Ancylobacter*) or a monooxygenase-mediated pathway (as in *Pseudomonas* and *Polaromonas*) (Hage & Hartmans, 1999; Janssen et al., 1985; Nishin et al., 2013; Van Den Wijngaard et al., 1992). Both pathways lead to the formation of chloroethanol, further oxidation to monochloroacetate and subsequent mineralization. Obligate anaerobic bacteria such as *Dehalococcoides* (Dhc), *Desulfitobacterium* (Dsf), *Dehalobacter* (Dhb), and *Dehalogenimonas* (Dhgm) can grow on 1,2-DCA as an electron acceptor by reductive dehalogenases - mediated pathways that mainly consist in dichloroelimination, yielding ethene (Grostern & Edwards, 2009; Marzorati et al., 2007; Maymò-Gatell et al., 1999). Hydrogen serves as the electron donor for *Dehalococcoides*, *Dehalogenimonas*, *Dehalobacter* and *Desulfitobacterium*. *Desulfitobacterium* also uses lactate and formate as electron donors during the anaerobic DCA biodegradation process (S. Y. Wang et al., 2015). Among genes encoding for reductive dehalogenases active on 1,2-DCA are: *tceA*, *vcrA*, *bvcA* from *Dehalococcoides* strains (Jugder et al., 2016), a (1,2-DCA)-specific *dcaA* from *Desulfitobacterium dichloroeliminans* DCA1 (Marzorati et al., 2007); *rdhA* genes from an

1,2-DCA dechlorinating culture dominated by *Dehalobacter* sp. were found to have high sequence homology with *pceA* of *D. restrictus* and *dcaA* from *D. dichloroeliminans* strain DCA1 (Groster & Edwards, 2009).

Dehydrochlorination is another type of dehalogenation reaction that can occur on 1,2-DCA giving rise to a C=C bond. It is not a reductive reaction therefore not reported as a respiratory process (Adrian & Löffler, 2016).

Finally, cometabolic 1,2-DCA degradation can also occur both under aerobic and anaerobic (sulfate reducing and methanogenic) conditions (S. Y. Wang et al., 2015).

In 1,2-DCA contaminated aquifers, both aerobic and reductive 1,2-DCA biodegradation pathways could be exploited in bioremediation strategies, depending on the characteristics of the site itself. Few recent studies also reported that aerobic and anaerobic 1,2-DCA biodegradation potential can also co-occur (Munro et al., 2017; S. Y. Wang et al., 2015).

1.5. Aim of the project

The aim of the research activities carried out within this PhD project and described in the present thesis was to study the biodegradation potential of the autochthonous microbial communities of an aquifer contaminated by chlorinated solvents and in particular by 1,2-DCA, with the purpose to provide the knowledge required for the eventual future implementation of enhanced bioremediation strategies in the site under study.

The first activities focused on the preliminary microbiological characterization of the aquifer and on the evaluation of the biodegradation potential of the autochthonous microbial communities by a laboratory-scale microcosm study (Chapter II). Different biostimulation treatments were tested to evaluate the possibility to exploit different anaerobic and aerobic 1,2-DCA biodegradation pathways. Contrarily to the commonly used approach, that mainly focuses on the exploitation of reductive processes, the aerobic biodegradation pathways were addressed here with particular interest. In a second phase of the work, the isolation of aerobic 1,2-DCA dechlorinating strains from the previous biostimulation treatments and the evaluation of their biodegradation capabilities were also attempted (Chapter III).

The last phase of the activities focused on the development of innovative biotechnological applications. A pilot test in column was carried out to evaluate the applicability of a permeable reactive barrier (PRB) to 1,2-DCA bioremediation using the enriched consortia obtained in the biostimulation trials. In particular the technology here proposed consists in a

polyhydroxybutyrate-based PRB, where the biodegradable polymer polyhydroxybutyrate is supposed to act as a slow-releasing electron donor source promoting reductive biodegradation processes (Chapter IV).

Finally, innovative biodegradable scaffolds made up of biopolymers were tested at laboratory scale and in a preliminary field experiment to evaluate their suitability as carriers for the immobilization of autochthonous bacteria to be eventually exploited in enhanced bioremediation technologies, such as bioaugmentation approaches (Chapter V).

The activities here described were carried out at the Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF) of the University of Palermo, in the Environmental Microbiology and Microbial Ecology Laboratory (EMME-Lab), under the scientific supervision of the tutor Prof. Paola Quatrini. Chemical analyses were carried out at the Chemistry Laboratory with the collaboration of Prof. Andrea Pace. Column studies and tests on biopolymeric devices were carried out with the collaboration of Dr. Daniele Di Trapani (Environmental and Sanitary Engineering) and with Prof. Roberto Scaffaro (Science and Technology of Materials) of the Engineering Department of the University of Palermo.

CHAPTER II

Evaluation of the catabolic potential of a 1,2-dichloroethane contaminated aquifer

2.1. Introduction

2.1.1. Methods to study the catabolic potential

The implementation of bioremediation strategies finalized to the clean-up of a contaminated site usually implies some preliminary steps to verify its suitability. Fundamental steps to evaluate the potential for the application of a bioremediation technology are the characterization of the contaminant(s) to remediate, the characterization of the site itself in terms of environmental conditions and hydrogeochemical factors in case of an aquifer, and the characterization of the microbial communities (Stroo & Ward, 2010).

For biodegradation-based remedies, the microbiological aspect is particularly important. Studying the intrinsic catabolic potential of the site means to assess if autochthonous contaminant-degrading microbial communities are present and if biodegradation processes are occurring in the site. The assessment of identity and properties of the microorganisms involved and the identification of the conditions that could promote the bioremediation of the area can drive the selection of the appropriate bioremediation strategy to apply, such as monitored natural attenuation, biostimulation or bioaugmentation (Stroo & Ward, 2010).

Methods to study the microbial populations to verify the suitability of a site for biostimulation or bioaugmentation techniques can be divided into cultivation-based methods and cultivation-independent methods (Bombach et al., 2010).

With reference to cultivation-based methods, laboratory microcosms set up using site matrices as inoculum, either water, soil or sediments, and amended with the contaminants of interest, can be periodically monitored by chemical analysis to assess the occurrence of a degradation process at a field site. Microcosm studies allow to distinguish biotic from abiotic chemical reactions, to study single processes and possible toxic effects and to test bioaugmentation cultures when such kind of intervention is needed. Moreover, microcosms can be used to test multiple substrate types, by evaluating the impact of amendments such as nutrients, vitamins, electron acceptors or donors on biodegradation processes and microbial growth rates when investigating on the applicability of biostimulation strategies.

Furthermore, microorganisms enriched in the microcosms can also be isolated by appropriated cultivation techniques, thus allowing a linkage between the microbial identity and the observed biodegradative process (Bombach et al., 2010).

The drawbacks of laboratory experiments based on cultivation-based approaches are due to the difficulties associated with cultivation of microorganisms, to possible changes of microbial community composition during incubation under controlled environmental variables, and difficulties in reproducing the complexity of environmental habitats. Other pitfalls include the chance that the inoculum used for the microcosms may not be representative of the entire contaminated site, and that generally less than 1% of bacteria from environmental samples are cultivable (Bombach et al., 2010). In addition to microcosm studies, column studies can be carried out to provide information for example on hydraulic factors, if required, and test materials for permeable barriers or bioreactors. A further step to determine engineering design aspects and to assess the feasibility of biostimulation is represented by field-scale pilot tests (Stroo & Ward, 2010). These methods are usually combined with analytical methods to characterize degradation processes and to evaluate microbial activity in an integrated approach.

Cultivation based methods can be integrated with cultivation-independent methods, that include molecular tools targeting biomarkers, such as specific nucleic acids, lipids, and proteins, to provide evidence on the composition and activity of microbial communities.

The so called ‘omics’ techniques, such as genomics, proteomics and transcriptomics, have contributed to reach a better understanding of microbial phylogenetic and functional diversity and catabolic pathways. Techniques that allow to unravel structure and function of a microbial communities and the impact they might have on bioremediation processes include metagenomic sequencing of PCR-amplified bacterial 16S rRNA genes and molecular fingerprinting techniques such as DGGE (*Denaturing Gradient Gel Electrophoresis*), T-RFLP (*Terminal Restriction Fragment Length Polymorphism*), ARISA (*Automated Ribosomal Intergenic Spacer Analysis*), ARDRA (*Amplified Ribosomal DNA Restriction Analysis*), RAPD (*Randomly Amplified Polymorphic DNA analysis*) (Maphosa et al., 2012). In particular, metagenomic approaches, that allow to study complex environmental samples harboring microbial communities, can provide insights into identity and potential metabolic capabilities of community members.

2.1.2. Methods to study CAHs dechlorination potential

2.1.2.1. Metagenomic approach and other molecular tools

With reference to dechlorinating microbial systems, the metagenomic approach is particularly important, considering that microbial communities exploited for bioremediation purposes often rely on complex multispecies interactive networks. Therefore, community profiling and functional assessment play a key role when evaluating the potential success or failure of bioremediation approaches (Maphosa et al., 2012). As an example, organohalide respiring bacteria (OHRBs) have proven difficult to obtain in pure culture and are often found to thrive in consortia and mixed communities, where they may also make up only a small percentage of the total microbial community. Interaction between community members include syntrophic relationships between hydrogen producers (such as fermenters and homoacetogens) and hydrogen consumers (such as reductive dechlorinators and methanogens), and the dependence of some OHRBs on microorganisms producing cobalamin, an important cofactor for reductive dechlorination (Maphosa et al., 2012). Common fermenters found in OHRB communities belong to Firmicutes, Bacteroidetes, and Proteobacteria; some examples are genera such as *Pelobacter* and *Desulfovibrio* (He et al., 2007; Mao et al., 2017) that can produce acetate or H₂ from substrates like lactate. Homoacetogens, such as the commonly found genus *Acetobacterium*, generate acetate or H₂ from substrates such as methanol and one-carbon compound (Groster & Edwards, 2009).

Understanding microbial community dynamics and interrelationships, including nutrient exchange and supply between different community members, in addition to the roles of specific OHRBs involved in CAHs degradation, could enable a better manipulation of the site conditions toward the optimal exploitation of such consortia in bioremediation.

Another common use of molecular tools consists in the search for targeted microbial genera and species and functional genes involved in catabolic processes. In the case of chlorinated solvents, as the most common bioremediation approaches are based on reductive dechlorination processes, the 16S rRNA gene sequences of *Dehalococcoides*-related species are the usual target (Stroo & Ward, 2010).

Beside 16S rRNA genes of known dechlorinating species, functional genes encoding key enzymes of degradation pathways constitute useful biomarkers for field diagnostic purposes for bioremediation of CAHs contaminated sites. In particular, genes encoding for four reductive dehalogenases with experimentally determined dechlorination functions (*pceA*,

tceA, *vcrA*, and *bvcA*) are the usual target. As different reductive dehalogenases target specific compounds, searching for specific genes provides more precise information on potential processes occurring in a contaminated site than searching for *Dehalococcoides* 16S rRNA gene. In fact, different strains, indistinguishable on the basis of 16S rRNA genes, have demonstrably different degradation capabilities depending on the repertoire of specific reductive dehalogenases they carry (Maphosa et al., 2012). Nevertheless, this approach requires the knowledge of the degradation pathway of the contaminant and the genes encoding involved enzymes. A challenge of the functional gene approach is the design of primer sets that can encompass the natural sequence diversity of the target genes (Bombach et al., 2010). This is especially relevant in the case of reductive dehalogenase genes, characterized by a notable diversity (Hug & Edwards, 2013). The search for functional genes is therefore often addressed by PCR probing metagenomic DNAs with degenerate primers covering, to a certain extent, the genes diversity. This approach may also lead to the identification of novel genes. As an example, a novel reductive dehalogenase specifically acting on 1,2-DCA was recently obtained and sequenced from the metagenome of a 1,2-DCA-degrading mixed culture (named 6VS) containing *Desulfitobacterium* and *Dehalobacter* spp. (Marzorati et al., 2007). The obtained gene cluster, including five open reading frames (ORF) named *dcaA*, *dcaB*, *dcaC*, *dcaT* (*dcaABCT*), shared 98% identity with a *pceABCT* gene cluster of *Dehalobacter restrictus* strain DSMZ 9455T, coding for an RD active on PCE. Later, a *rdh* sequence was obtained from a pure culture of *Desulfitobacterium dichloroeliminans* DCA1, showing high similarity (98% at the amino acid level) to the 6VS culture, indicating that the 6VS putative 1,2-DCA *rdh* was most likely from *Desulfitobacterium* (Marzorati et al., 2007). Other examples are the identification of three novel *rdh* sequences (WL *rdhA1*, WL *rdhA2* and WL *rdhA3*) from a *Dehalobacter* /*Acetobacterium* co-culture (WL DCA/H₂) enriched on 1,2-DCA and H₂/CO₂. WL *rdhA1* was found to encode for an aminoacidic sequence sharing 95% similarity to PceA of *Dehalobacter restrictus* (Groster & Edwards, 2009).

A drawback in the detection of functional genes is that it does not provide information on the metabolic activity of the microorganisms. Therefore, the application of RNA-based analysis, that allow the assessment of the expression of specific genes, such as the functional ones, could be a further step to overcome this limitation, when genes involved in degradation processes are known.

2.1.2.2. Biostimulation treatment tests in microcosm

Biostimulation treatments as strategy for CAHs bioremediation, as anticipated (Chapter I), consist in the addition of substrates and chemicals to support the nutritional needs of specialized indigenous microorganisms, with the aim to create a suitable environment for dechlorination processes.

Reductive or aerobic cometabolic pathways are usually the most exploited processes in CAHs bioremediation, as they allow the biodegradation of some pollutants of interest, such as PCE and TCE (Stroo & Ward, 2010), that are not prone to be degraded by other processes, such as direct aerobic oxidation. Conversely, direct aerobic pathways are not often exploited in CAHs bioremediation: in most cases, complex mixtures of CAHs, including compounds recalcitrant to aerobic degradation, are found in contaminated sites, thus hampering the applicability of direct aerobic processes.

Both reductive dehalogenation and aerobic cometabolic dehalogenation require the addition of co-substrates to allow degradation to occur. In reductive dechlorination processes, chemicals required by OHRBs include vitamin B₁₂, an important cofactor in reductive dehalogenases, and an electron donor source, as the chlorinated molecules are used as electron acceptors. Depending on the OHRB, the electron donor sustaining the dechlorination is H₂ or, less frequently, acetate. These products can be the result of fermentation processes by non-dechlorinating members of the microbial communities. Several methods of delivering and several electron donor sources have been developed and used with success so far, including gases, solids, and water-soluble donors. Although hydrogen could be directly sparged into the subsurface, this has proven not to be the most suitable solution. Therefore, other substrates have been exploited, such as acetate, propionate, butyrate, lactate, ethanol or slow-release H₂ sources such as molasses, chitin, polymerized lactic acid (Stroo & Ward, 2010). Recently, low-cost slow-release substrates such as poly- β -hydroxybutyrate (Corsino et al., 2022) were also successfully applied in CAHs bioremediation (Matturro et al., 2018; De Marines et al., under revision)

In aerobic cometabolic processes, that consist in the oxidation of CAHs as non-growth substrates accidentally uptaken by nonspecific enzymes, oxygen and appropriate growth substrates (co-substrates) must be supplied. Examples of growth substrates stimulating the production of oxygenase enzymes by appropriate microorganisms include methane, ethane, propane, butane, ethene, propene, isoprene, phenol, toluene, ammonia. Direct injection of

air and gaseous cometabolites, such as methane or butane, is only an example, but several methods are possible (Frascari et al., 2015).

Finally, direct aerobic processes do not require the addition of substrates beside the CAH used by microorganisms as growth and energy source, but they require the supply of oxygen (Xing et al., 2022).

2.1.3. Aim of the work

The selection and set up of bioremediation strategies to be applied for the clean-up of a specific site require prior studies on the intrinsic biodegradation potential of the site and on the feasibility of enhanced bioremediation interventions.

The aim of this work was to investigate the intrinsic biodegradation potential of a CAHs contaminated aquifer, where 1,2-dichloroethane (1,2-DCA) constitutes the main contaminant, and to evaluate the response of the autochthonous microbial communities to biostimulation treatments in microcosm, under anaerobic and aerobic conditions.

In particular, the first aim was the characterization of the microbial communities of groundwater samples collected from selected sampling points within the contaminated area of the site, to individuate, on a taxonomic basis, known or potential dechlorinating taxa and to drive the subsequent set up of laboratory scale studies.

The second aim was to investigate on the best biostimulation treatments in terms of efficiency of 1,2-DCA biodegradation by autochthonous bacteria from the contaminated aquifer, by microcosm studies.

Microcosms under different aerobic and anaerobic conditions were set from contaminated groundwater with appropriate mineral media amended, if required, with growth substrates, to investigate all possible 1,2-DCA biodegradation pathways, although the chemical physical characterization of the aquifer suggested an anaerobic, low redox environment. 1,2-DCA removal was periodically monitored by Gas Chromatography-Mass Spectrometry analysis; dechlorinating microbial communities, biostimulated under various conditions, were analyzed by 16S rRNA metagenomic sequencing, to individuate the enriched dechlorinating taxa involved; the detection of functional genes from the metagenome of dechlorinating communities was performed by PCR probing with appropriate primer set to confirm the biodegradation potential of identified taxa.

2.2. Materials and Methods

2.2.1. Groundwater samples

The area of interest is an aquifer contaminated by CAHs, mainly represented by 1,2-DCA, and other minor pollutants including hydrocarbons and metals. The groundwater (GW) samples used to characterize the autochthonous microbial communities of the aquifer and to set up microcosms were collected from two sampling sites, selected among the several present in the area of interest. Within the area, a highly contaminated zone and a less contaminated zone can be distinguished. In particular, the sites (here referred to as S1 and S2) selected for the activities here described are located within the highly contaminated zone. Groundwater samples from sites S1 and S2 (named GW-S1 and GW-S2 respectively) were collected at different times during the whole timeframe of the experimental activities; four sampling campaigns, carried out in April 2019, September 2020, May 2021 and May 2022, provided material for this study.

Groundwater samples collected in April 2019 (GW-S1-Apr19 and GW-S2-Apr19) were used in the preliminary characterization of the autochthonous microbial communities. Samples collected in September 2020 and May 2021, designated respectively with the labels Sep20 and May21 in the name, were used for microcosms setup. Samples collected in May 2022 (labeled as May22) were used in experimental activities with biopolymeric devices (Chapter V).

GW samples from both sites are characterized by contamination from several CAHs, including PCE, TCE, VC, 1,1-DCE and, mostly, 1,2-DCA. The range of contaminant concentrations (total CAHs and in particular 1,2-DCA) and the average values of main physical-chemical parameters of the samples across the entire duration of the experimental activities (2019-2022) are reported in Tab. 2.1.

GW-S1 was always the most contaminated sample. GW-S2 showed lower contamination values; nevertheless, even the lowest 1,2-DCA concentration value reported (1.5 mg/l) is notably higher than the legal concentration threshold in Italy (Legislative Decree 152/2006, CSC=3 µg/l). Negative redox potentials (Eh) and low oxygen levels (always <0.5 mg/l) indicate an anaerobic groundwater environment. Anaerobic conditions were ensured during the sampling. All samples were processed within 48 h from their arrival into the laboratory.

No further information about the sampling procedures and the site can be provided for confidentiality reasons.

	GW-S1	GW-S2
Total CAHs concentration (mg/l)	78 - 330 mg/L	2 - 27 mg/l
of which 1,2-DCA (mg/l)	77 - 320 mg/L	1.5 - 25 mg/l
Redox potential (mV)	-212 ± 19 mV	-427 ± 42 mV
Dissolved oxygen (mg/l)	0.295 ± 0.025 mg/l	0.09 ± 0.01 mg/l
Temperature (°C)	22.6 ± 0.69 °C	21.7 ± 0.3 °C
pH	6.4 ± 0.5	6.8 ± 0.5
Electric conductivity (µS/cm)	19645 ± 2208 µS/cm	9592 ± 615 µS/cm

Table 2.1. Ranges of contaminants' concentrations and average values of main physical-chemical parameters of groundwater samples GW-S1 and GW-S2 across the period 2019-2022.

2.2.2. Groundwater filtration and DNA extraction

In order to collect the biomass for subsequent microbial characterization, 1 liter of each groundwater sample was filtered through a filtering complex equipped with Millipore 0.22 µm filters connected to a vacuum pump (Fig. 2.1). After filtration, the filters were removed from the complex and stored at -20°C for future analysis.



Figure 2.1. Groundwater filtration through a filtering complex connected to a vacuum pump

Total DNA was extracted from filters using the commercial kit QIAamp Fast DNA™ Stool Mini Kit (QIAGEN) according to manufacturer's instructions, except for a modification applied in the first step. Briefly, the filter was scraped with a sterile scalpel, cut into pieces

and transferred into a conical tube with ASL buffer (2 mL) prior to mixing by vortex to optimize the recovery of biomass. The supernatant was collected, and the subsequent steps were performed according to the manufacturer's recommendations. The resulting genomic DNA was eluted in 50 µL of ultraPURE™ DNase/RNase-free distilled water (GIBCO®); purity and concentration of the extracted DNA were assessed using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The DNA samples were stored at -20°C for subsequent analyses.

2.2.3. 16S rRNA metagenomic sequencing

Community composition of groundwater samples was analyzed by 16S rRNA Illumina sequencing. Bacterial and archaeal V3-V4 hypervariable region of the 16S rRNA gene were amplified using extracted DNA as template with primers Pro341F/ Pro805R (Takahashi et al., 2014) (Attached A).

PCR reactions were performed in a total volume of 25 µl containing 1 x High Fidelity Buffer (Termofisher), 2mM MgSO₄ 0.2 mM dNTPs (Invitrogen), 0.4 µM of forward and reverse primers, 0.04 U/µl of Platinum Taq DNA Polymerase High Fidelity (Termofisher), and 15-50 ng of template DNA.

The following PCR conditions were used: an initial denaturation at 94 °C for 1 min; [94 °C for 30 s, 55 °C for 30 s, 68 °C for 45 s] X 25 cycles; a final extension at 68 °C for 7 min.

PCR products were purified with AMPure XP beads and indexed using the Nextera XT Index Kit in a second PCR step and sequenced on an Illumina MiSeq Sequencer at BMR Genomics (Padova, Italy) with 300 bp paired-end reads. The quality of sequences was checked using the FASTQC software. Qiime2 tools version 2019.4 was used for reads denoising and feature filtering (0.005%). Taxonomy was assigned using trained Operational Taxonomic Units (OTUs) at 99% from GreenGenes database version 13–8.

2.2.4. Microcosms setup

GW samples from sampling sites S1 and S2 collected in September 2020 and May 2021 were used to set up microcosms under different anaerobic and aerobic conditions, in order to assess the presence of a biodegradation activity by stimulating and enriching the dechlorinating component of the autochthonous bacterial communities of the study area. Microcosms are also suitable to individuate the best growth conditions promoting 1,2-DCA biodegradation for the specific site of interest.

As the site had never been characterized before, protocols for microcosms setup and suitable culturing media were established according to the literature and partially to the preliminary microbiological analysis of the site carried out in this thesis.

Microcosms were prepared in 20 mL screw caps serum bottles with 50% (vol/vol) groundwater and culture medium (unless stated otherwise) to a final volume of 12 ml (headspace: 8 ml of air), in four replicates for each condition.

Anaerobic microcosms were set up twice: once using GW samples collected in September 2020 and a second time using GW samples collected in May 2021.

The first series of anaerobic microcosms (September 2020) was set up as a preliminary test in order to refine the technique and subsequent analysis methods and to gain preliminary information about bacterial growth conditions and 1,2-DCA biodegradation. The second series of anaerobic microcosms (May 2021) was set up with an optimized method.

Aerobic microcosms were set up only once using GW samples collected in September 2020.

2.2.4.1. Anaerobic microcosms setup and enrichment cultures

Two series of anaerobic microcosms were set up in September 2020 and in May 2021.

To ensure anaerobic conditions, the microcosms were set up in an anaerobic glove box under an atmosphere saturated with filter sterilized N₂ and the culture medium and other solutions used were previously bubbled with filter sterilized N₂. The last step of glove box conditioning was performed using a filter sterilized gas mixture N₂/CO₂/H₂ (80%/15%/5%) (Marzorati et al., 2007) to provide an initial H₂ supply to dechlorinating microorganisms.

A culture medium, referred to as BTZ, was adapted from the literature (Marzorati et al., 2006). Medium composition was the following: a salt solution containing final concentrations of 0.043 g/l NH₄Cl, 0.5 g/l KH₂PO₄, 0.2 g/l MgCl₂ x 6H₂O, 0.01 g/l CaCl₂ x 2H₂O; 1 mM cysteine (to help maintaining anaerobic conditions); 1:200 of a Trace Elements Solution (Tab. 2.2); 1:1000 of a Seven Vitamins Solution (Tab. 2.3) containing vitamin B₁₂, a basic cofactor for some dehalogenating microorganisms; 0.5 mM Hepes/NaOH pH7 [4- (2-hydroxyethyl) piperazine-1-ethanesulfonic acid solution] to keep the pH in a range of 6.8–8.2; yeast extract 0.05% (w/v).

1,2-DCA 100 ppm was added to the BTZ medium to act as electron acceptor.

Trace Elements Solution		Seven Vitamins Solution	
Nitrilotriacetic acid (NTA)	12.8 g /l	Vitamine B ₁₂	100 mg/l
FeCl ₃ x 6 H ₂ O	1.35 g/l	p-Aminobenzoate	80 mg/l
MnCl ₂ x 4 H ₂ O	0.1 g/l	D(+)-Biotin	20 mg/l
CoCl ₂ x 6 H ₂ O	0.024 g/l	Nicotinic acid	200 mg/l
CaCl ₂ x 2 H ₂ O	0.1 g/l	Calcium pantothenate	100 mg/l
ZnCl ₂	0.1 g/l	Pyridoxine hydrochloride	300 mg/l
CuCl ₂ x 2 H ₂ O	0.025 g/l	Thiamine HCl x 2H ₂ O	200 mg/l
H ₃ BO ₃	0.01 g/l	H ₂ Od	1 l
Na ₂ MoO ₄ x 2 H ₂ O	0.024 g/l		
NaCl	1.0 g/l		
NiCl ₂ x 6 H ₂ O	0.12 g/l		
Na ₂ SeO ₃ x 5 H ₂ O	0.026 g/l		
H ₂ Od	1 l		

Table 2.2 Trace Elements Solution

Table 2.3 Seven Vitamins Solution

The experimental design for both September 2020 and May 2021 anaerobic microcosms included microcosms amended and not amended with an electron donor. In particular, the selected fermentable substrate was lactate, whose efficacy in sustaining reductive dechlorination has been widely proven (Aulenta et al., 2006b; Carpani et al., 2021; Marzorati et al., 2006).

For the first series of anaerobic microcosms (September 2020), the following conditions were realized (Fig. 2.2a):

- microcosms containing medium, GW and lactate amendment (*lac*)
- microcosms containing medium and GW with no additive amendment (*na*)
- microcosms containing raw GW, without any medium or addition (*tq*)
- abiotic control microcosms containing medium and filter sterilized GW (*k-*).

In *lac* and *k-* microcosms, sodium-lactate 5 mM was added to the medium as electron donor (Marzorati et al., 2006). Abiotic control microcosms (*k-*) were obtained with filter sterilized GW. In order to visually assess the establishment and maintenance of anaerobic conditions, resazurin 0.1% w/v was added to the culture medium as a redox indicator (Matteucci et al.,

2015). The redox potential inside the microcosms was routinely monitored by checking the resulting color change of the resazurin (resazurin oxidized form has a blu/purple color; the irreversibly reduced form, resorufin, shows a pinkish color; the reversible, more reduced form, dihydroresorufin, is colorless). The condition *tq*, made up of GW only, without any medium or amendment, was intended as a further control to compare non biostimulated 1,2-DCA biodegradation by autochthonous microbial communities to biostimulation treatments.

The second series of anaerobic microcosms (May 2021) included the same conditions, except for *tq*. Only *lac*, *na* and *k-* microcosms were set up (Fig 2.2b).

Culture conditions and methods were optimized on the basis of the results obtained from the first microcosms series. Microcosms were set up as previously described, except for some modifications. The ratio GW: culture medium was increased to 5:1 (vol/vol), instead of 1:1 (vol/vol). Sodium-lactate amendment in *lac* and *k-* microcosms was decreased to 1 mM (Carpani et al., 2021). GW for abiotic control microcosms was previously treated with 1% sodium azide (NaN_3) as bacteriostatic (Carpani et al., 2021). The redox indicator resazurine was not added. Anaerobic microcosms were incubated statically in the dark, at room temperature until analysis.

Microcosms were analyzed at the moment of the set up and then periodically monitored. Four incubation times (time 0, 15, 30, 45 days from the setup) were planned for September 2020 anaerobic microcosms; three incubation times (time 0, 7, 15 days from the setup) were planned for May 2021 anaerobic microcosms. At the end of each incubation time, microcosms were sacrificed in triplicate to monitor biodegradation by chemical analysis (see paragraph 2.2.5).

A set of *lac* and *na* anaerobic microcosms prepared from GW-S1 sample in May 2021 (*S1lacMay21* and *S1naMay21*) was maintained over the last incubation time to 30 days and used to set up subsequent batches of enrichment cultures. Enrichment cultures were periodically refreshed every 30 days by transferring an inoculum 10% (vol/vol) from the previous culture into fresh 1,2-DCA amended BTZ medium (with or without lactate 1mM) into 160 ml crimp-sealed glass serum bottles with butyl rubber stoppers to a final volume of 96 ml (respecting the liquid:headspace ratio adopted in microcosms), in duplicate. Abiotic controls were set up in correspondence of every transfer with uninoculated 1,2-DCA amended medium. At each transfer, a part of every enriched culture was cryopreserved in glycerol 20% and stored at -80°C . Cultures were incubated statically in the dark, at room

temperature. Biodegradation monitoring in all batches of enrichment cultures was performed at 0, 4, 7, 15 days from the setup.

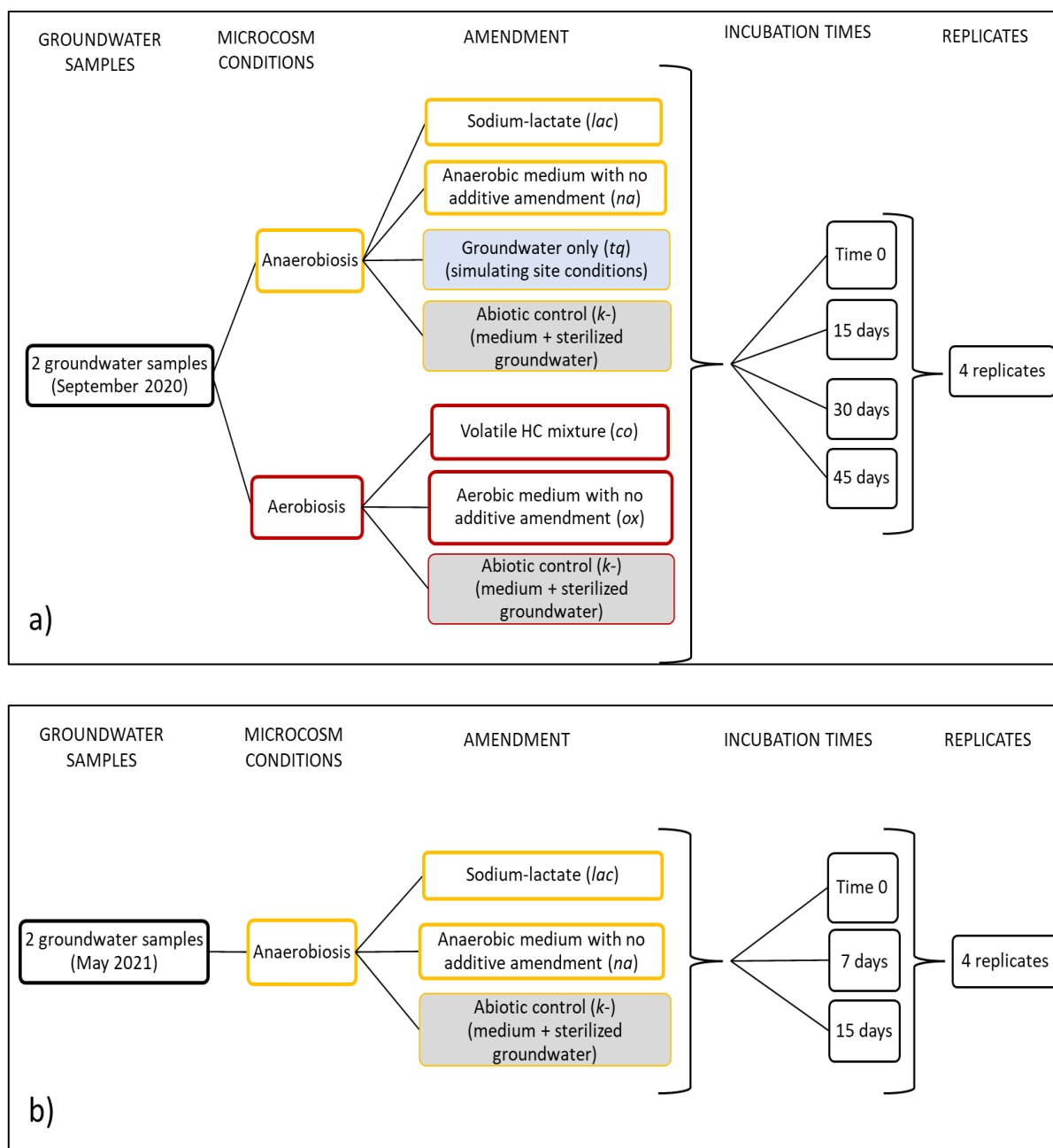


Figure 2.2. Experimental design of a) anaerobic and aerobic microcosms set up in September 2020; b) anaerobic microcosms set up in May 2021.

2.2.4.2. Aerobic microcosms setup and enrichment cultures

Aerobic microcosms were set up from groundwater samples collected in September 2020. A carbon-free mineral salts medium (MSM) for enrichment of specific nutritional types of microbes was adapted from (Coleman et al., 2002). MSM was composed as follows: a salt

solution containing final concentrations of 2.97 g/l $\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$, 0.95 g/l KH_2PO_4 , 0.67 g/l $(\text{NH}_4)_2\text{SO}_4$; 1:2000 of a Seven Vitamins Solution (the same as anaerobic microcosms, Tab. 2.3); 1:500 of a Metals Solution (Tab. 2.4). 1,2-DCA was added to the medium to a final concentration of 100 ppm as carbon and energy source.

Metals Solution

$\text{Na}_2\text{EDTA} \times 2\text{H}_2\text{O}$	6.37 g/l
$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$:	1.0 g/l
$\text{CaCl}_2 \times 2\text{H}_2\text{O}$:	0.5 g/l
$\text{FeSO}_4 \times 7\text{H}_2\text{O}$:	2.5 g/l
$\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$:	0.1 g/l
CuSO_4	0.064 g/l
$\text{CoCl}_2 \times 6\text{H}_2\text{O}$:	0.2 g/l
$\text{MnSO}_4 \times \text{H}_2\text{O}$:	0.52 g/l
$\text{MgSO}_4 \times 7\text{H}_2\text{O}$:	60.0 g/l
H_2O	1 l

Table 2.4. Metals solution for MSM medium

The following conditions were realized (Fig 2.2a):

- microcosms containing medium, GW with no additive amendment (*ox*)
- microcosms containing medium, GW and a co-substrate (*co*)
- abiotic control microcosms containing medium and filter sterilized GW (*k-*).

In *ox* microcosms, set up to biostimulate direct aerobic 1,2-DCA biodegradation processes, 1,2-DCA was the sole carbon and energy source. To determine whether a growth substrate was simultaneously required for 1,2-DCA degradation, as in aerobic cometabolic processes, a volatile hydrocarbons mixture (1% ethane, 1% methane, 0,01% $\text{C}_4\text{-C}_6$) was syringe-injected into sealed *co* and *k-* (abiotic control) microcosms as a co-substrate, in addition to 1,2-DCA 100 ppm. Filter sterilized GW was used in abiotic control microcosms.

Aerobic microcosms were incubated on a rotary shaker at 150 rpm at room temperature in the dark until analysis.

Microcosms were analyzed at the moment of the set up and then periodically monitored. As for September 2020 anaerobic microcosms, four incubation times (time 0, 15, 30, 45 days from the setup) were planned; at the end of each incubation time, microcosms were sacrificed in triplicate to monitor biodegradation.

A set of *ox* and *co* microcosms set up from GW-S1 and GW-S2 samples (named e.g S1*ox*Sep20, S1*co*Sep20, and so on) was maintained until about 90 days to set up subsequent batches of enrichment cultures. Enrichment cultures were obtained by transferring an inoculum 10% (vol/vol) from the previous culture into fresh culture medium (with or without co-substrate) amended with 1,2-DCA 100 ppm into 120 ml crimp-sealed glass serum bottles with butyl rubber stoppers to a final volume of 72 ml, in duplicate. Each transfer was performed after about 90 days of incubation. In correspondence of each transfer, an abiotic control was set up with uninoculated medium. Aliquots of the cultures were cryopreserved in glycerol 20% and stored at -80°C at each transfer. Cultures were incubated as described and periodically monitored for 1,2-DCA biodegradation (at 0, 15, 25 days from the setup).

2.2.5. Chemical monitoring

At the end of every planned incubation time, chemical monitoring of microcosms and subsequent enrichment cultures was performed by Gas Chromatography - Mass spectrometry analysis and/or chloride release assay to assess the removal of 1,2- DCA.

2.2.5.1. Headspace Sampling Gas Chromatography - Mass spectrometry (HS-GC/MS) analysis

Chemical monitoring of 1,2-DCA removal in all microcosms and in aerobic enrichment cultures was performed by Gas Chromatography - Mass spectrometry (GC/MS) by headspace sampling after setting up calibration curves with known concentration solutions.

For September 2020 microcosms, the Gas Chromatograph-Mass Spectrometer GCMS-QP2010 Plus equipped with a Supelcowax 10 column was used. Column features, carrier gas, injection temperature and mode, and the program of column oven temperature are shown in Fig. 2.3.

Gaseous samples (1 ml) were manually withdrawn from the headspace of three replicates of microcosms for each condition using gas-tight syringes (Hamilton) and injected into the column. The areas of the peaks corresponding to 1,2-DCA in the resulting chromatograms were calculated and referred to a calibration curve to obtain 1,2-DCA concentration.

Column Name: Supelcowax 10
Length: 30 m
Thickness: 0.25 μm
Diameter: 0.25 mm

Carrier gas: He
Injection temperature: 240°C
Injection mode: splitless

<u>Program Column Oven Temperature</u>			
	<i>Rate</i>	<i>Final Temp.</i>	<i>Hold time</i>
0	-	40°C	2 min
1	25°C/min	200°C	0

Figure 2.3. Column features, carrier gas, injection temperature and mode, and program of column oven temperature used for September 2020 microcosms chemical monitoring using a Gas Chromatograph-Mass Spectrometer GCMS-QP2010 Plus equipped with a Supelcowax 10 column.

For enrichment cultures from September 2020 microcosms and for May 2021 microcosms, a 7000C GC/MS/MS Triple Quad (Agilent Technologies) GC/MS with an Agilent 19091S-433UI column and equipped with an autosampler was used. Column features, carrier gas, injection temperature and mode, and the program of column oven temperature are shown in Fig. 2.4.

Incubation Temperature: 30°C
Incubation time: 10 min
Column Name: Agilent 19091S-433UI
Length: 30 m
Thickness: 0.25 μm
Diameter: 0.25 mm

Carrier gas: He
Injection temperature: 270°C
Injection volume: 250 μl
Injection mode: split ratio 5:1

<u>Program Column Oven Temperature</u>			
	<i>Rate</i>	<i>Final Temp.</i>	<i>Hold time</i>
0	-	40°C	0,2 min
1	10°C/min	0°C	0

Figure 2.4. Column features, carrier gas, injection temperature and mode, and program of column oven temperature used for chemical monitoring of enrichment cultures from September 2020 microcosms and May 2021 microcosms using a Gas Chromatograph-Mass Spectrometer 7000C GC/MS/MS Triple Quad equipped with an Agilent 19091S-433UI column.

Prior to the headspace sampling, microcosms bottles were incubated 10 min at 30°C with shaking. After incubation, 250 µl of headspace were withdrawn and injected by the autosampler. 1,2-DCA concentration was calculated as already described using the Agilent MassHunter Qualitative Data Analysis software.

2.2.5.2. Chloride release assay

Both in aerobic and anaerobic dechlorination processes, a halogen - carbon bond is broken, and the halogen atom is liberated as a halide. Chloride release is supposed to be proportional to the amount of chlorinated substrate degraded. The assessment of inorganic chloride released from aerobic and anaerobic biodegradation processes was performed by a colorimetric method through the QuantiChrom™ Chloride Assay Kit (BioAssay System). The reagent mercuric 2,4,6-tripyridyl-s-triazine forms a colored complex specifically with chloride. The intensity of the color, measured at 610nm, is directly proportional to the chloride concentration in the sample. Chloride concentration was calculated using a standard curve based on solutions with known chloride concentrations as a reference.

2.2.6. Analysis of the bacterial communities

Based on the results of chemical analysis, microcosms and enrichment cultures whose dechlorinating abilities were confirmed, were further analyzed. At the end of the last planned incubation time for microcosms or enrichment cultures, filtration and DNA extraction were carried out on microcosms (four replicates) and on enrichment cultures (one replicate) as previously described for groundwater samples. Biological monitoring was performed on extracted DNAs by community fingerprinting analysis, 16S rRNA gene metagenomic sequencing and PCR-probing for functional dehalogenation genes.

2.2.6.1. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The microbial communities of the different anaerobic (*lac*, *na tq*) and aerobic (only *ox*) microcosms set up in September 2020 were compared in terms of structure and diversity through ARISA (*Automated Ribosomal Intergenic Spacer Analysis*). ARISA is a fingerprinting molecular method applied to the length polymorphisms of the prokaryotic 16S- 23S rRNA intergenic spacers (ITS) (Danovaro et al., 2006). Bacterial ITS was PCR amplified using total DNA from microcosms as template with the ITSf/ITSReub-Hex primer set (Cardinale et al., 2004) (Attached A).

PCR conditions used were as follows: an initial denaturation at 94°C for 30 s; [94°C for 30 s, 55°C for 1 min, 68°C for 5 min] X 30 cycles, final extension at 68°C for 5 min. PCR products were visualized by electrophoresis on 2% agarose gel stained with GelRed in TAE 1X buffer [40 mM Tris, 20 mM acetic acid, and 1 M EDTA (pH 8.0)] at 80 V for 20 min and later sent to BMR Genomic srl (Padova) to perform ARISA.

Data were processed using the Peak Scanner v1.0 software to obtain the microbial community profiles associated to each microcosm condition. The number of peaks in each community profile represents the number of OTUs (Operational Taxonomic Units) in each sample and is used to estimate species richness. A cluster analysis was performed to assess the level of similarity among the microbial communities by processing data through the PAST v2.17c software.

2.2.6.2. 16S rRNA metagenomic sequencing of microcosms

Community composition of microcosm conditions proving efficient in 1,2-DCA removal according to chemical analysis was analyzed by 16S rRNA Illumina sequencing as described for groundwater samples. Diversity indices based on 16S rRNA metabarcoding were calculated by processing data through the PAST v2.17c software. In particular the following indices were calculated (Marzorati et al., 2010): the species *richness*, the number of species (or OTUs) identified in a sample; the dominance index that quantifies the dominance of one or more taxa within a sample; the *Simpson's index*, the probability that two clones randomly taken from a sample will be the same species; the *Shannon-Wiener index* (H') that takes into account the number of species and their relative importance within the assemblage and reflects the evenness of taxa distribution.

2.2.6.3. Search for known OHRB genera with specific primer sets

The presence of known dechlorinating OHRB genera in microcosms showing dechlorinating activities and in groundwater was confirmed by PCR probing extracted DNA with specific primers targeting OHRB 16S rRNA gene (Attached A) using a nested PCR approach. The 16S rRNA gene was first amplified with universal primer set 27F/1492R (Frank et al., 2008). The PCR reaction was performed in a total volume of 30 µl containing 0.025 U/µl of DreamTaq DNA Polymerase (Thermo Fisher Scientific), 1 x DreamTaq Buffer, 0.2 mM dNTPs (Invitrogen), 0.2 µM of forward and reverse primers and 1-10ng of template DNA.

PCR conditions used were the following: an initial denaturation at 95 °C for 3 min; [95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min] X 30 cycles; a final extension at 72 °C for 15 min.

PCR products were used as template in nested PCR using specific OHRB 16S rRNA gene primer sets. PCR reaction with OHRB primers were conducted in a total volume of 20 μ l containing 0.025 U/ μ l of DreamTaq DNA Polymerase (Thermo Fisher Scientific), 1 x DreamTaq Buffer, 0.2 mM dNTPs (Invitrogen), 0.2 to 0.5 μ M of forward and reverse primers, and 1-10ng of template DNA. PCR conditions used were the following: an initial denaturation at 95 °C for 3 min; [95 °C for 30 s, annealing (Attached A) for 30 s, 72 °C for 1 min] X 30 cycles; a final extension at 72 °C for 15 min.

PCR products were visualized by electrophoresis on a 1% agarose gel stained with GelRed Nucleic Acid Stain (Biotium) in TAE 1X buffer at 80V for 40 min. 1 kb DNA ladder (NEW ENGLANDS BioLabs) was used to quantify and estimate products sizes. Gel images were acquired by ChemiDoc Imaging System (Bio-Rad).

2.2.7. Search for catabolic genes in microcosms and enrichment cultures

PCR amplification with degenerated and specific primers designed on the sequence of known dechlorination genes was performed on DNA extracted from anaerobic and aerobic microcosms to detect the genes potentially involved in biodegradation processes.

2.2.7.1. Search for reductive dehalogenase genes in anaerobic microcosms

Degenerated and specific primers (Attached A) designed on the sequences of known reductive dehalogenase genes were used to search for *rdh* genes in metagenomic DNAs extracted from *lac* and *na* microcosms. PCR reactions were performed in a total volume of 20 μ l containing 0.025 U/ μ l of DreamTaq DNA Polymerase (Thermo Fisher Scientific), 1 x DreamTaq Buffer, 0.2 mM dNTPs (Invitrogen), 0.2 to 0.6 μ M of forward and reverse primers and 1-10ng of template DNA. PCR conditions used were the following: an initial denaturation at 95 °C for 3 min; [95 °C for 30 s, annealing (Attached A) for 30 s, 72 °C for 1 min] X 30 cycles; a final extension at 72 °C for 15 min. Electrophoresis was performed as described for OHRB16S rRNA gene PCR products, except a 100 bp DNA ladder (NEW ENGLANDS BioLabs) was used.

A nested PCR was done with the Rdh552F/Rdh625R primer set, using PCR products obtained with the DHL-F1/DHL-R1 primer set. PCR reactions were performed in a total volume of 30 μ l containing 0.025 U/ μ l of OneTaq DNA Polymerase (NEB), 1 x OneTaq Standard Buffer, 0.2 mM dNTPs (Invitrogen), 0.3 μ M of forward and reverse primers and 1-10ng of template DNA.

The following PCR conditions were used: an initial denaturation at 94 °C for 30 s; [94 °C for 30 s, 52 °C for 30 s, 68 °C for 30 s] X 25 cycles; a final extension at 68 °C for 7 min. PCR products were visualized by electrophoresis as already described.

Nested-PCR products were cloned in pCR 2.1- TOPO vector and *Escherichia coli* Oneshot TOP10 chemically competent cells were transformed with the TOPO® TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Recombinant plasmids from clones were extracted using the GeneElute™ Plasmid Miniprep Kit (SIGMA-ALDRICH®). Inserts were amplified with M13r/M13f primer set and sequenced by the sequencing services provider "Macrogen Europe" (Amsterdam, The Netherlands). Nucleotide sequences obtained were analyzed using the NCBI GenBank database by the nucleotide-BLAST program, MEGABLAST ("Highly similar sequences").

2.2.7.2. Search for haloalkane dehalogenase genes in aerobic enrichment cultures

PCR amplification of aerobic dechlorination genes was performed with degenerate or specific primers (Attached A) designed on the sequences of the *dhlA* gene coding for a 1,2-DCA haloalkane dehalogenase using DNA extracted from aerobic enrichment cultures as template.

PCR reactions with DHMf/DHMr and DHLA 380F/548R primer sets were performed in a total volume of 20 µl containing 0.025 U/ µl of OneTaq DNA Polymerase (NEB), 1 x OneTaq Standard Buffer (NEB), 0.2 mM dNTPs (Invitrogen), 2 µM of DHMf/DHMr primers or 0.5 µM of DHLA 380F/548R primers and 1-10 ng of template DNA. The following PCR conditions were used: an initial denaturation at 94 °C for 30 s; [94 °C for 30 s, 60 °C for 1 min, 68 °C for 30 s] X 30 cycles; a final extension at 68 °C for 5 min.

PCR protocol was optimized for PCR reactions with DHLA 319F/603R primer set: reactions were performed in a total volume of 20 µl containing 0.025 U/ µl of OneTaq DNA Polymerase (NEB), 1 x OneTaq GC Buffer, 0.2 mM dNTPs (Invitrogen), 0.5 µM of forward and reverse primers and 1-10 ng of template DNA. Optimized PCR conditions were: an initial denaturation at 94 °C for 30 s; [94 °C for 15 s, 65 °C for 15 s, 68 °C for 20 s] X 25 cycles; [94 °C for 15 s, 60 °C for 20 s, 68 °C for 20 s] X 7 cycles; a final extension at 68 °C for 5 min.

PCR products were sequenced by the company Macrogen Europe and nucleotide sequences were analyzed as already described.

2.3. Results and discussions

2.3.1. Groundwater microbial communities

In order to gain a preliminary insight into the intrinsic bioremediation potential of the site and to drive the setup of the most suitable conditions for the subsequent biostimulation treatment tests in microcosms, a first sampling was conducted in April 2019, and microbial communities of groundwater samples collected from two sampling points within the most contaminated zone of the study area (GW-S1-Apr19 and GW-S2-Apr19) were analyzed by metagenomic 16S rRNA Illumina sequencing. Final reads of GW-S1-Apr19 and GW-S2-Apr19 samples were 51,563 and 46,058 respectively. Both S1 and S2 groundwater communities were characterized by similar alpha diversity, counting 811 and 759 OTUs (Operational Taxonomic Units at 99%) respectively.

At phylum level (Fig. 2.5a), GW-S1-Apr19 and GW-S2-Apr19 communities mainly differed in the relative abundances of the identified taxa. Both samples were dominated by the phylum Proteobacteria (18% and 41% over the total number of reads in GW-S1-Apr19 and GW-S2-Apr19 respectively). In particular, Deltaproteobacteria were the dominant class within Proteobacteria in both samples (16% and 27% in GW-S1-Apr19 and GW-S2-Apr19 respectively).

GW-S1-Apr19 was also characterized by high relative abundances of the division GN02 (15.5%), Bacteroidetes (10.4%) and Firmicutes (9.4%). Other phyla, such as the archaeal Parvarchaeota, Tenericutes, Chloroflexi and several candidate divisions (SAR406, OD1, WS6, TM7, OP11 among the most abundant) were less represented.

In GW-S2-Apr19, beside Deltaproteobacteria, also Gammaproteobacteria (7%) and Epsilonproteobacteria (6%) were well represented; Parvarchaeota (12.5%), Bacteroidetes (7%), Chloroflexi (3.5%) and candidate divisions OD1 (4.4%), TM7 (4.6%) and OP11 (4%) were among the most abundant groups.

Notably high percentages of unclassified taxa were reported at the family (Fig. 2.5b) and especially genus level. The predominant metabolisms retrieved on the basis of the identified genera within microbial communities of the aquifer were related to the sulfur cycle. Sulfur-oxidizing genera such as *Sulfurimonas* (Inagaki et al., 2003), *Sulfuricurvum* (Kodama & Watanabe, 2004), *Halothiobacillus*, *Thiobacillus*, *Thiomonas*, *Thiotrix* (Pokorna & Zabranska, 2015) were retrieved; sulfate- or thiosulfate-reducing bacteria identified included *Desulfovibrio* (Sasi Jyothsna et al., 2008), *Desulfarculus* (Sun et al., 2010), *Desulfobacca*,

Desulfobulbus, *Desulfocapsa*, *Desulfococcus*, *Desulfomonile*, *Desulfosporosinus* (Castro et al., 2006), *Syntrophobacter* (Boone & Bryant, 1980), *Dethiosulfatibacter* (Takii et al., 2007) and *Fusibacter* (Ravot et al., 1999). Some anaerobic and aerobic dechlorinating taxa could be detected at low abundance; the dechlorinating genera detected in the samples and other non-dechlorinating genera identified within the same family are reported in Tab. 2.5.

At the family level, in sample GW-S1-Apr19 Deltaproteobacteria were mainly made up of the families Desulfuromonadaceae (6.4%) and Pelobacteraceae (3.7%). Desulfuromonadaceae include PCE-respiring strains within the facultative OHRB genus *Desulfuromonas* (Türkowsky et al., 2018), that was also retrieved in GW-S1-Apr19 (0.23%). Within Firmicutes, Eubacteriaceae (4.5%) and Peptococcaceae (2%) were the most represented families. Eubacteriaceae and Pelobacteraceae both include members, *Acetobacterium* and *Pelobacter* respectively, found in consortia or co-culture with dechlorinating genera *Dehalococcoides* or *Dehalobacter*, likely playing a role in production H₂ or acetate, used as electron donors by OHRBs (Grostern & Edwards, 2009; Mao et al., 2017). Within the homoacetogen genus *Acetobacterium*, strains capable of cometabolic reductive dechlorination of 1,2-DCA and direct anaerobic oxidation of chloromethane (Atashgahi et al., 2018; De Wildeman et al., 2003) were also reported. The family Peptococcaceae includes the obligate OHRB genus *Dehalobacter*, known for the chlororespiration of different chlorinated ethanes and ethenes, including 1,2-DCA by *Dehalobacter* sp. strain WL (Adrian & Löffler, 2016; Grostern & Edwards, 2009). In sample GW-S1-Apr19 Peptococcaceae were almost entirely made up of *Desulfosporosinus*, and extremely low abundance of *Dehalobacter* was detected.

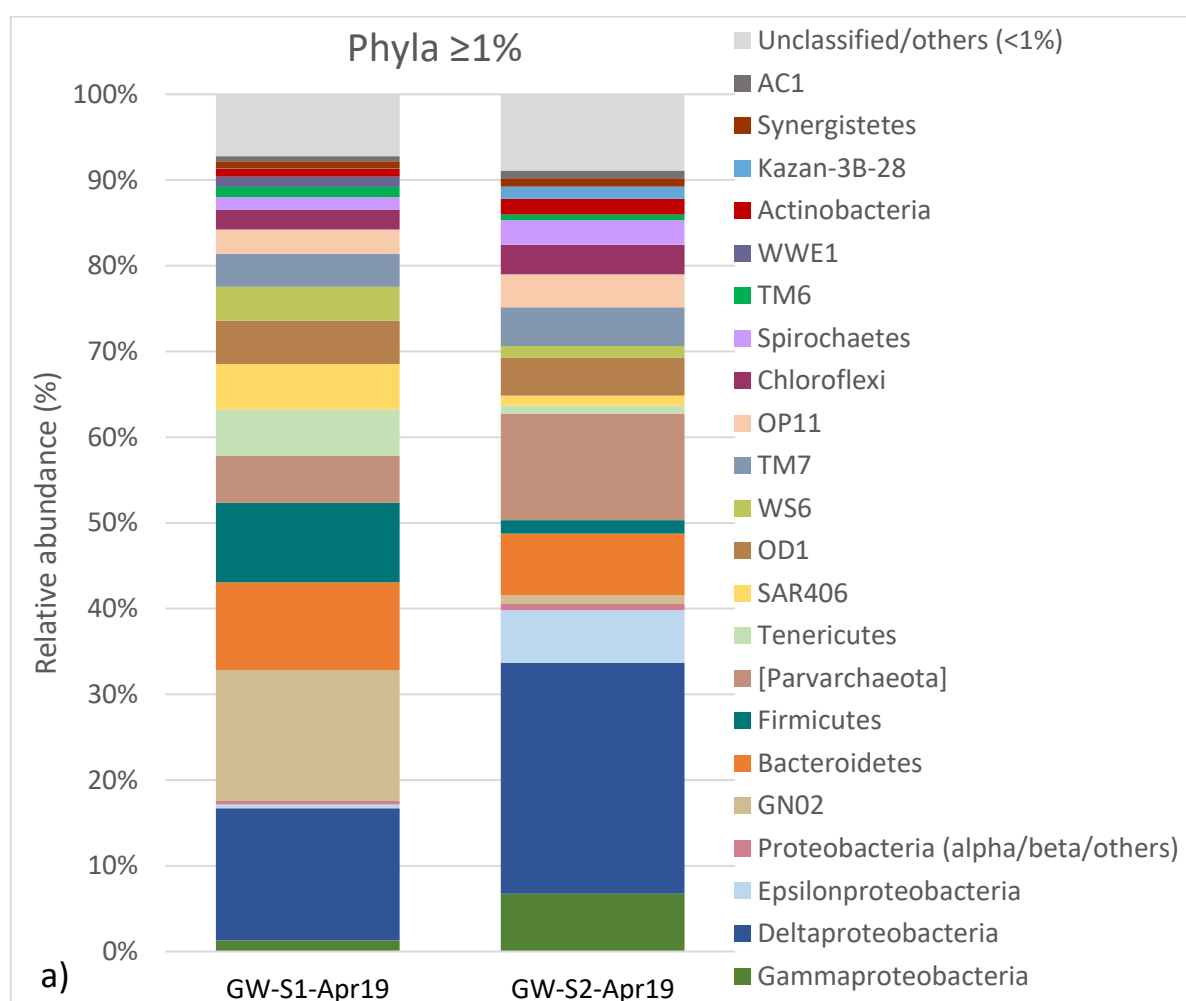
Within the phylum Chloroflexi, in GW-S1-Apr19 sample, the only identified family >1% was Anaerolineaceae (2%). Several members of the family are described as fermenters producing hydrogen and acetate as by-products (McIlroy et al., 2017), thus playing a role in providing electron donors for OHRBs. Dehalococcoidaceae (Chloroflexi), including the obligate OHRB *Dehalogenimonas*, were present but poorly represented (0.4%). The family A714017 (1.5%) might be involved in the reduction of sulfur compounds (Pavlovskaya et al., 2021). Among other identified dechlorinating genera, whose relative abundances are <1%, *Sulfurospirillum* (Campylobacteraceae), *Mycobacterium* (Mycobacteriaceae) and *Stenotrophomonas* (Xanthomonadaceae) were found (Tab. 2.5)

The sample GW-S2-Apr19 was dominated by the strictly anaerobic, fermentative family Syntrophaceae (17%), that includes the facultative OHRB *Desulfomonile* (Adrian & Löffler,

2016) (<0.1% in the sample) and the sulfate reducing genus *Desulfobacca* (16% in the sample). Other abundant families were: Helicobacteraceae (5%) made up of *Sulfuricurvum* and *Sulfurimonas*, Desulfarculaceae (4%) and Syntrophoobacteraceae (2%). All these families were mainly made up of sulfur oxidizing genera (Kodama & Watanabe, 2004; Inagaki et al., 2003). The family Campylobacteraceae (1.3%) included very low abundances of the OHRB genus *Sulfurospirillum* (Goris & Diekert, 2016; Luitjen et al., 2003) and the genus *Arcobacter*, frequently found in various aquatic environments (Ghaju Shrestha et al., 2022). The phylum Chloroflexi in GW-S2-Apr19 included Dehalococcoidaceae (1%, mainly made up of the OHRB *Dehalogenimonas*), and Anaerolineaceae (1.6%).

The dechlorinating genera *Shewanella* and *Desulfovibrio*, capable of reductive dechlorination (Adrian & Löffler, 2016), and genera *Mycobacterium* and *Xanthobacter*, capable of aerobic oxidative or cometabolic dechlorination (Xing et al., 2022) were present but low abundances were reported.

Notably, the method used did not allow the retrieval of the known OHRBs *Dehalococcoides* and *Desulfitobacterium* in any sample analyzed.



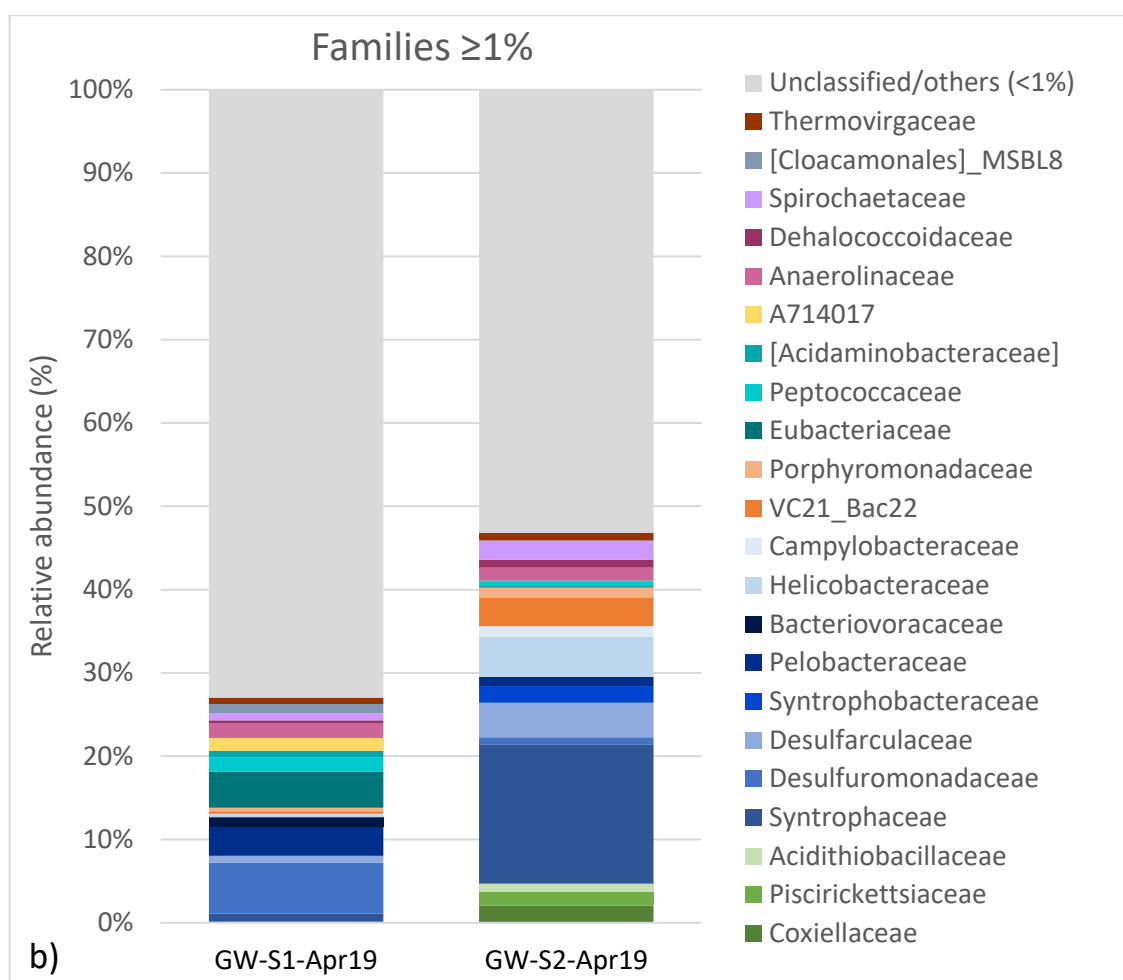


Figure 2.5. Relative abundances of a) phyla (down to class level for Proteobacteria) and b) families of GW-S1-Apr19 and GW-S2-Apr19 samples. Taxa $\geq 1\%$ in at least one sample are shown. Unclassified/others includes unclassified taxa and identified taxa $<1\%$.

Overall, the preliminary characterization of the groundwater samples collected from two highly contaminated sites within the study area, revealed significant differences in community composition, especially at the lower taxonomic levels, with different families being dominant in the samples.

Among the taxonomic groups identified, low relative abundances of known dechlorinating taxa were observed in both groundwater samples, independently from the different CAHs contamination levels (higher in GW-S1-Apr19 than in GW-S2-Apr19), suggesting a low intrinsic dechlorinating potential of the site. Different redox potential of groundwater samples (lower in GW-S2-Apr19 than in GW-S1-Apr19) and dissolved oxygen levels did not affect the bacterial community composition in terms of known aerobic and anaerobic dechlorinating taxa: in both samples, known anaerobic dechlorinating taxa, on which most bioremediation approaches usually rely, showed very low abundances, if present, while aerobic dechlorinating taxa were almost absent.

Dechlorinating metabolism	Phylum	Family	Genus	Relative abundance (%)	
				GW-S1 Apr19	GW-S2 Apr19
Reductive dechlorination	Gammaproteobacteria	Shewanellaceae		0	0.02
			<i>Shewanella</i>		0.02
	Deltaproteobacteria	Desulfovibrionaceae		0.14	0.17
			<i>Desulfovibrio</i>	0.14	0.17
		Desulfuromonadaceae		6.37	0.86
			<i>Desulfuromonas</i>	0.23	
		Syntrophaceae		0.93	17.01
			<i>Desulfobacca</i>	0.08	15.68
			<i>Desulfomonile</i>		0.08
	Epsilonproteobacteria	Campylobacteraceae		0.04	1.28
			<i>Arcobacter</i>	0.02	1.25
			<i>Sulfurospirillum</i>	0.02	0.03
	Chloroflexi	Dehalococcoidaceae		0.4	1
			<i>Dehalogenimonas</i>	0.35	0.87
	Firmicutes	Peptococcaceae		1.80	0.60
			<i>Dehalobacter</i>	0.01	
			<i>Desulfosporosinus</i>	1.76	0.52
			<i>Pelotomaculum</i>	0.01	0.05
Direct aerobic oxidation / aerobic cometabolism	Actinobacteria	Mycobacteriaceae		0.02	0.03
			<i>Mycobacterium</i>	0.02	0.03
	Alphaproteobacteria	Xanthobacteraceae		0	0.02
			<i>Xanthobacter</i>		0.02
Direct aerobic oxidation	Gammaproteobacteria	Xanthomonadaceae		0.09	0.20
			<i>Luteimonas</i>		0.03
			<i>Pseudoxanthomonas</i>		0.01
			<i>Stenotrophomonas</i>	0.09	

Table 2.5. Known dechlorinating genera (in bold character), on a taxonomic basis, detected in the samples and other non-dechlorinating genera identified within the same family. Genera including at least one known dechlorinating strain are taken into account as known dechlorinating genera.

2.3.2. Preliminary monitoring of September 2020 microcosms

The first group of microcosms, set up from GW-S1 and GW-S2 samples collected in September 2020 included preliminary anaerobic microcosms (*lac*, *na*, *tq* and *k*- conditions) and aerobic microcosms (*ox*, *co* and *k*- conditions) (Fig 2.6). All microcosms belonging to this group were chemically monitored by HS-GC/MS at 0, 15, 30, 45 days of incubation from the setup, and total DNA from four replicates of each condition was extracted after 45 days of incubation. Anaerobic microcosms were periodically checked to assess the establishment and maintenance of a reductive environments based on the color changes of the redox indicator resazurin. The redox indicator showed a pinkish color at the moment of the set up and turned clear after 10 days (Fig 2.7), suggesting a small amount of oxygen was present at first and was likely consumed by metabolic processes within few days. Anaerobic conditions were then maintained during the entire experimental activity.

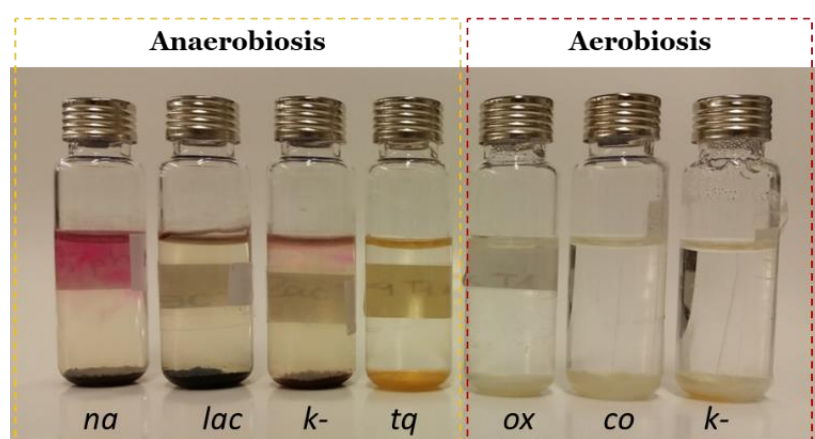


Figure 2.6. Anaerobic and aerobic microcosms (September 2020) from GW-S1, as an example.

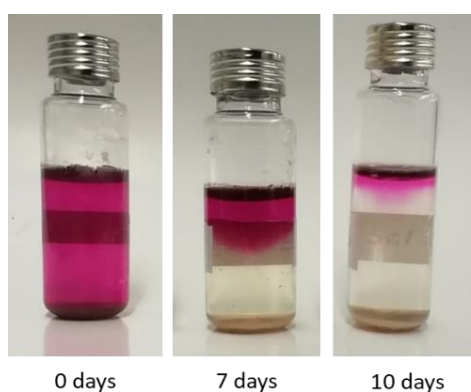


Figure 2.7. The use of resazurin to monitor anaerobic conditions. Example of anaerobic condition establishment and maintenance monitored over time (0, 7 and 10 days) in S1lacSep20 microcosms.

2.3.2.1. 1,2-DCA removal analysis in September 2020 microcosms

In order to assess 1,2-DCA removal in microcosms in respect to the initial concentration and to the abiotic control microcosms, 1,2-DCA concentration was periodically monitored by GC/MS analysis by headspace sampling. Chemical monitoring in this phase did not provide conclusive evidence of 1,2-DCA biodegradation. Indeed, high variability among the replicates was assessed, likely due to the manual headspace sampling and injection method adopted (data not shown). The poor reliability and inconsistency of these results affected the comparison between conditions and incubation times. Nevertheless, it was possible to identify trends in the removal of the contaminant for the different microcosm conditions although they should be considered more as a qualitative rather than a quantitative assessment. Microcosms showing higher 1,2-DCA removal levels (data not shown) than relative abiotic control microcosms were considered dechlorinating. In particular, 1,2-DCA concentration trends highlighted higher 1,2-DCA removal (90-100%) in anaerobic microcosms S1_{lac} and S2_{lac} (September 2020) and aerobic microcosms S1_{ox}, S1_{co} and S2_{co} (September 2020) in respect to the relative abiotic control microcosms after 45 days of incubation (data not shown). Microcosms without additive amendment (*na* condition) and microcosms simulating site conditions (*tq* condition) showed lower removal trends.

2.3.2.2. Biological monitoring of September 2020 microcosms

After 45 days of incubation, total DNA was extracted from microcosms prepared with the groundwater sampled in September 2020. The amount of extracted DNA, referred to filtered volume, was used to estimate the biomass of enriched bacterial communities grown under different conditions. An increase in biomass was recorded for every microcosm condition, particularly for the *lac* microcosms (Fig. 2.8)

Extracted DNAs were used to compare the structures of the bacterial communities of anaerobic (*la*, *na*, *tq* conditions) and aerobic (only *ox* condition) S1 and S2 microcosms by performing an *Automated Ribosomal Intergenic Spacer Analysis* (ARISA).

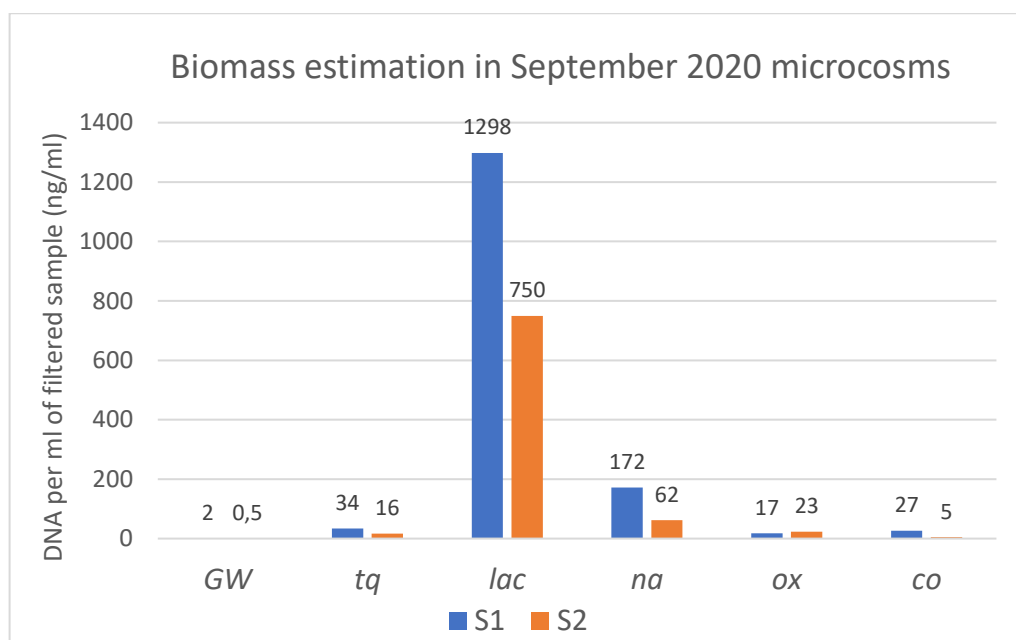


Figure 2.8. Estimation of bacterial growth in September 2020 microcosms and biomass collected from initial groundwater samples (GW) based on the amount of extracted DNA referred to the volume of GW or microcosms filtered (ng/ml).

ARISA profiles were used to build a cladogram, based on the Bray-Curtis similarity coefficient, that takes into account presence/absence and relative abundance of OTUs within each sample. The cladogram (Fig 2.9) shows a clear separation between aerobic and anaerobic microcosms, suggesting, as expected, that different components of the initial bacterial communities were biostimulated on the basis of the presence or absence of oxygen. S1_{lac}, S1_{na} and S2_{na} show relatively high level of similarity, while S2_{lac} is on a separate branch. S1_{tq} and S2_{tq} cluster together and with S1_{ox} microcosm. Likely, aerobic taxa thrived in *tq* microcosms thanks to residual oxygen present in the initial groundwater. Conversely, this residual oxygen may have been completely depleted in *lac* and *na* microcosms thanks to the presence of cysteine as a reducing agent added in the culture medium (and absent in *tq* microcosms made up of GW only). Interestingly, S2_{ox}, the only aerobic condition that does not show degradation according to preliminary chemical monitoring, clusters with the “anaerobic branch” of the cladogram, although showing low similarity.

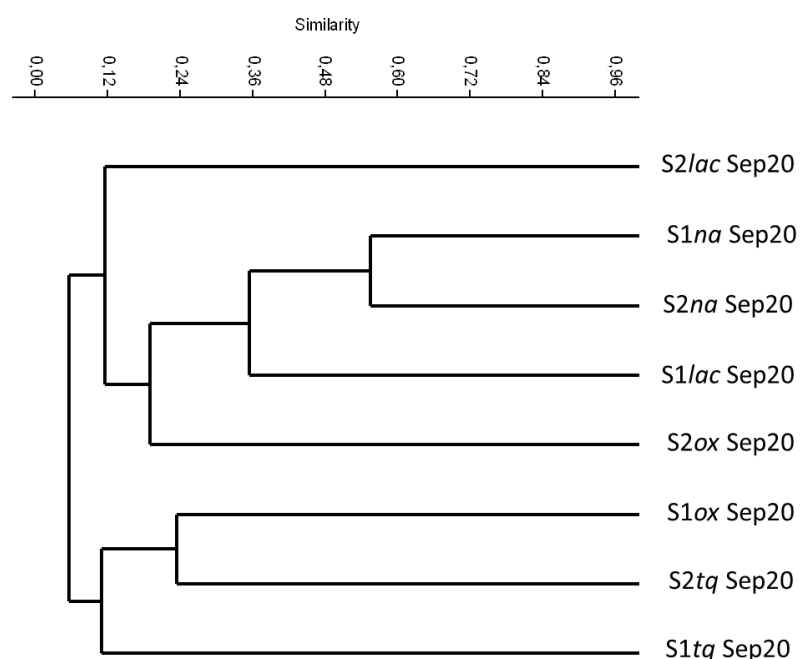
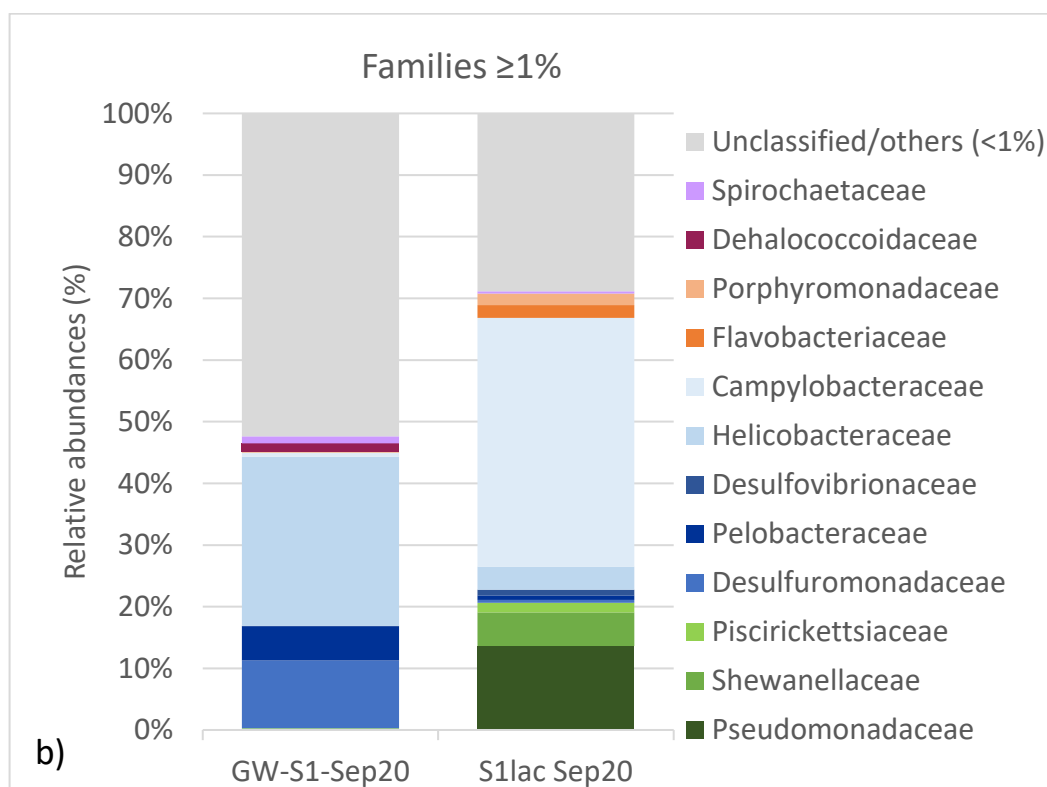
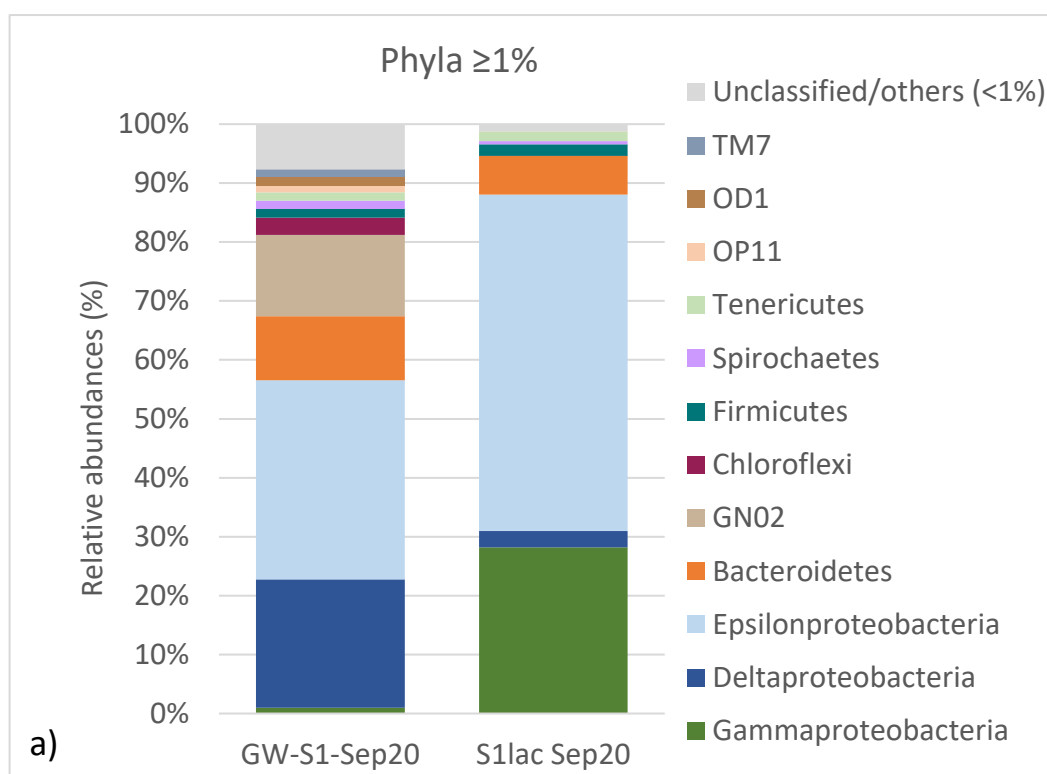


Figure 2.9. Cladogram built on ARISA profiles of microbial communities of microcosm conditions: lactate (lac), no additive amendment (na), only groundwater (tq), direct oxidation (ox) based on Bray-Curtis similarity index.

The composition of the S1lacSep20 microcosm, that showed preliminary evidences of 1,2-DCA removal and the highest values of biomass was analyzed by 16S rRNA Illumina sequencing to investigate the enrichment of dechlorinating microorganisms in respect to the initial GW sample. A total of 60,204 and 45,726 final reads were obtained for GW-S1-Sep20 and S1lacSep20 samples respectively. The reads were assigned to OTUs on a 99% similarity and 401 and 89 OTUs were reported in GW-S1-Sep20 and S1lacSep20 respectively, suggesting a specialization of the enriched community, associated to a reduction in overall diversity in response to anaerobic biostimulating conditions. The comparison between groundwater and S1lac microcosm communities at phylum (Fig. 2.10a), family (Fig. 2.10b) and genus (Fig. 2.10c) level was done taking into account taxa showing $\geq 1\%$ relative abundance. In S1lacSep20 microcosm Gammaproteobacteria, mainly made up of Pseudomonadaceae (18%) and Shewanellaceae (7%), and Epsilonproteobacteria, consisting in Campylobacteraceae (52%) and Helicobacteraceae (5%) completely replaced the dominant taxa in GW-S1-Sep20, represented by Helicobacteraceae (33%), Desulfuromonadaceae (13%) and Pelobacteraceae (7%). *Arcobacter* (Campylobacteraceae), *Pseudomonas* (Pseudomonadaceae) and *Shewanella* (Shewanellaceae) were the most enriched genera in the microcosm. The genus *Arcobacter* is a human and animal pathogen, frequently found in aquatic environments (Ghaju Shrestha et al., 2022). *Arcobacter* is not

known as a dechlorinating genus; its enrichment may have taken advantage of the high lactate concentration.



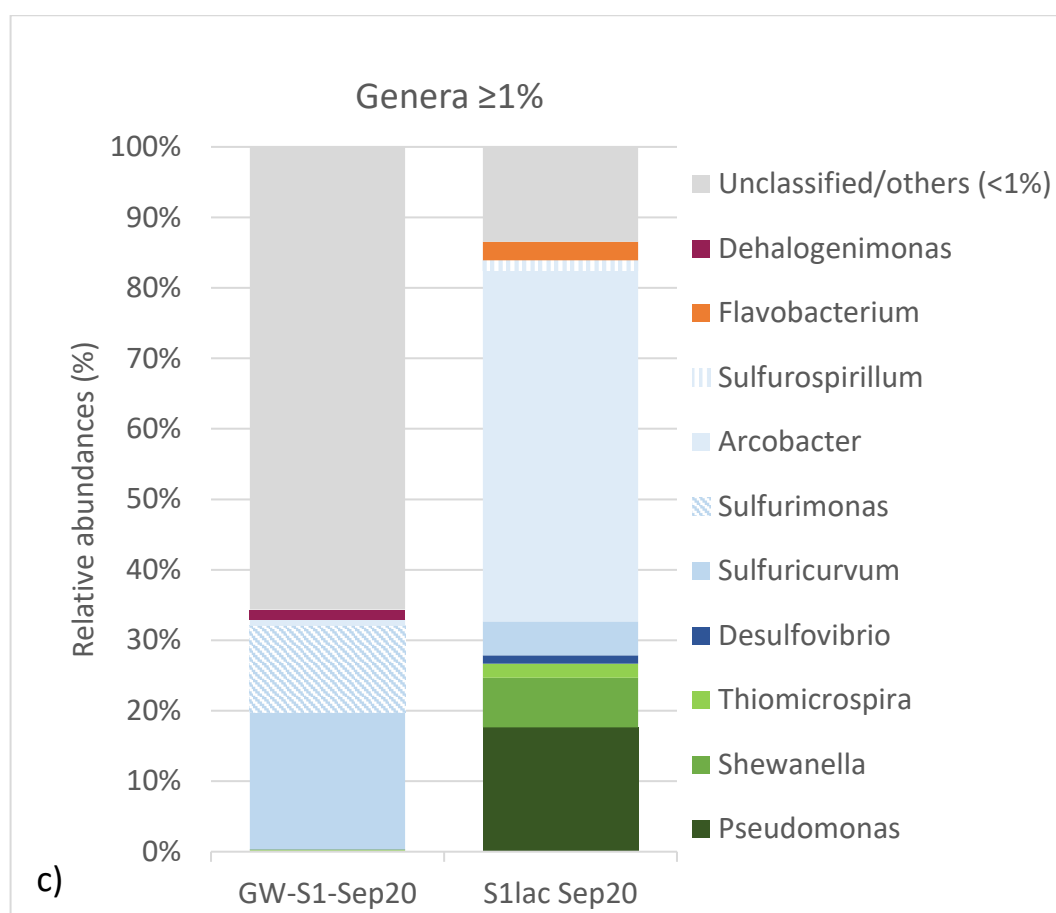


Figure 2.10. Relative abundances of a) phyla (down to class level for Proteobacteria), b) families and c) genera of GW-S1-Sep20 and S1lacSep20 samples. Taxa $\geq 1\%$ in at least one sample are shown. Unclassified/others (<1%) includes unclassified taxa and identified taxa <1%.

The bacterial community of the microcosm showed low relative abundances of potential dechlorinating taxa, and a shift in the presence of OHRBs in groundwater ad in the biostimulated microcosm was observed (Fig. 2.11). Notably, the relative abundances of Dehalococcoidaceae and Peptococcaceae families, that include obligate OHRBs, and Desulfuromonadaceae and Syntrophaceae families, including facultative OHRBs, decreased in the microcosm in respect to the initial groundwater. Conversely, the abundances of Desulfovibrionaceae, Campylobacteraceae (excluding *Arcobacter*) and Shewanellaceae increased. Within these families, the genera *Desulfovibrio*, *Sulfurospirillum* and *Shewanella*, identified in the lac microcosm, include versatile OHRBs (Adrian & Löffler, 2016) that could be the agents responsible of 1,2-DCA removal assessed by chemical monitoring.

The low abundances of dechlorinating taxa in this microcosm were likely due to the high concentration of fermentable substrate used. Fermentative metabolisms may have overcome the dechlorinating metabolism, rather than supplying H_2 for OHRBs activity. Therefore,

another series of anaerobic microcosms with optimized conditions were set up from groundwater samples collected in May 2021.

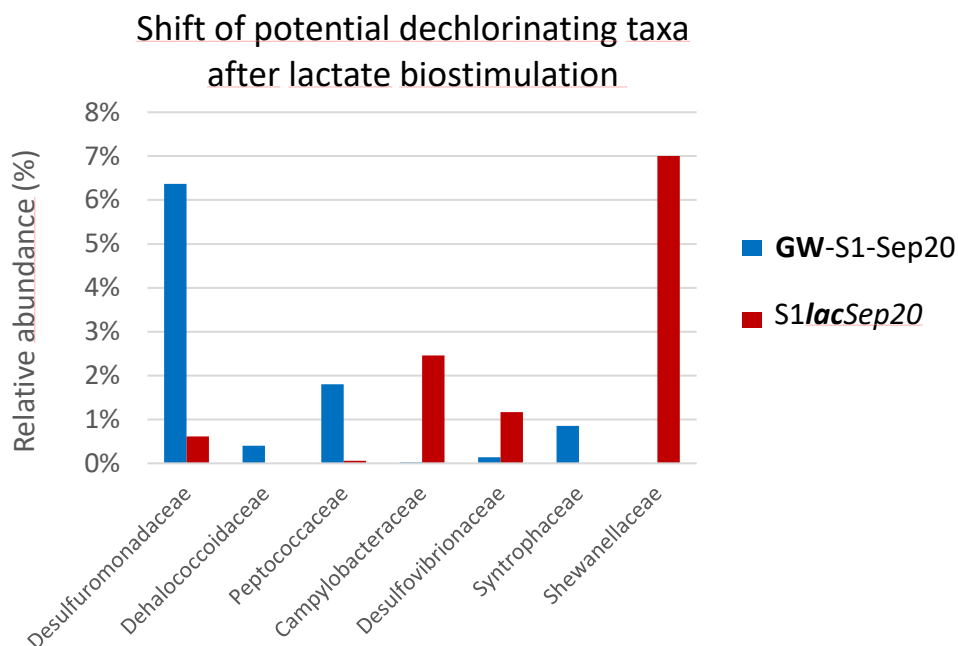


Figure 2.11. Comparison of dechlorinating potential in groundwater GW-S1-Sep20 and lactate microcosm S1lacSep20. Families including at least one known dechlorinating strain are taken into account as potential dechlorinating taxa. Relative abundance of *Arcobacter* (non-dechlorinating) is excluded from Campylobacteraceae abundance.

2.3.3. Anaerobic microcosms (May 2021) and enrichment cultures

A second series of anaerobic microcosms was set up from S1 and S2 groundwater samples in May 2021. 1,2-DCA biodegradation in microcosms was monitored over time by HS-GC/MS with an improved, more reliable method. S1lacMay21 and S1naMay21 were selected for further biological analysis and to set up the subsequent batches of enrichment cultures.

2.3.3.1. 1,2-DCA removal analysis in anaerobic microcosms

Periodical monitoring of anaerobic May 21 microcosms by HS-GC/MS analysis revealed 1,2-DCA removal in respect to its concentration at time 0 (given by the initial contamination levels in groundwater and 1,2-DCA added in BTZ medium) occurred in microcosms set up from both S1 and S2 groundwater samples (Fig. 2.12a, b). 1,2-DCA removal in microcosms set up from S1 groundwater, the more contaminated sample, was faster (7 days of incubation) than in microcosms set up from S2 groundwater (15 days).

Chemical monitoring of *lac* and *na* May 21 microcosms revealed both conditions tested showed a complete removal of 1,2-DCA, while no removal was observed in abiotic control microcosms (Fig. 2.12a, b). This result suggests that the addition of lactate as substrate in *lac* microcosms did not enhance biodegradation if compared with *na* microcosms. Likely other components of the culture medium used, such as yeast extract and vitamin B₁₂ were sufficient to stimulate 1,2-DCA biodegradation. In particular, the presence of vitamin B₁₂ that acts as a cofactor in dehalorespiration and is involved in reductive cometabolic processes (Adrian & Löffler, 2016) could play a key role in 1,2-DCA biological removal in both microcosm conditions.

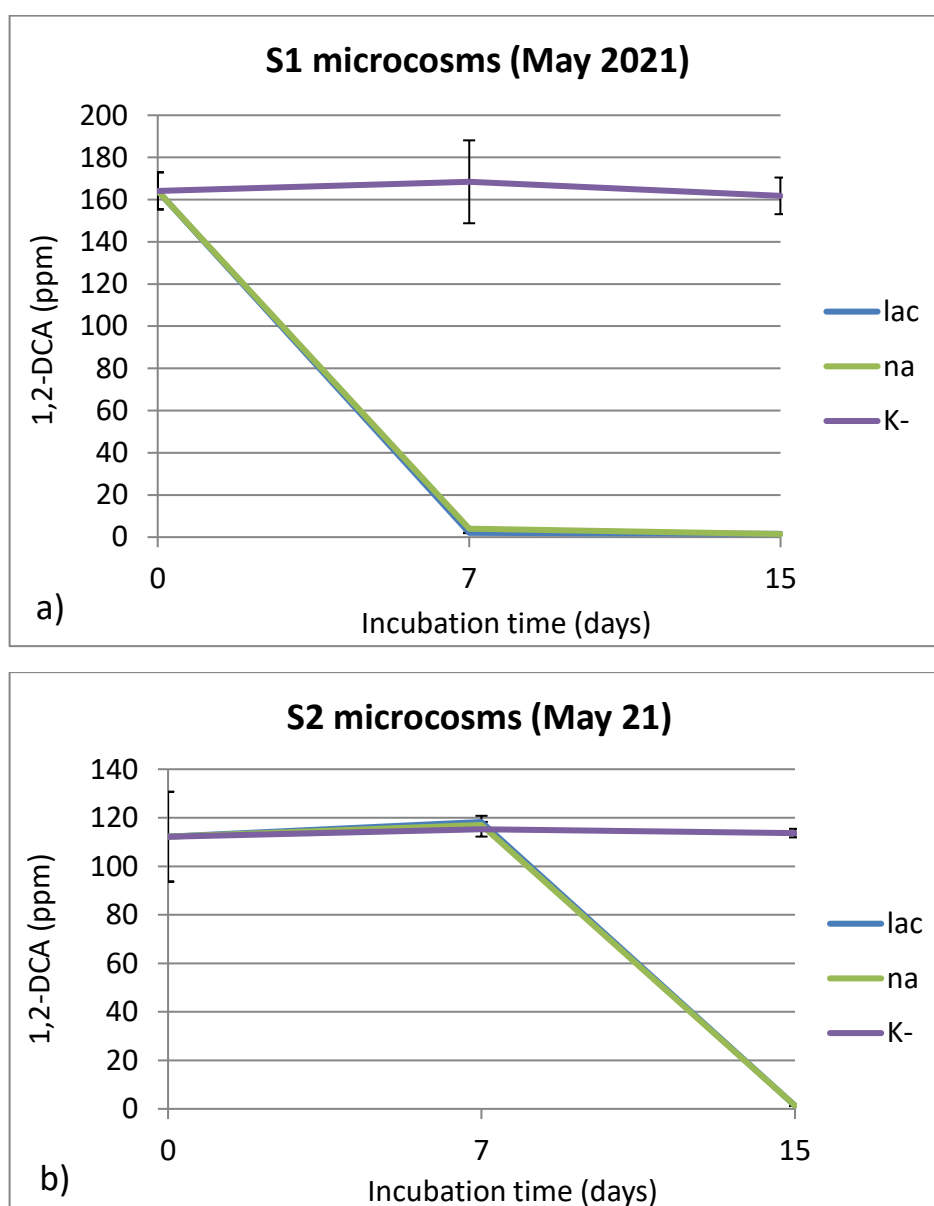


Figure 2.12. Chemical monitoring of 1,2-DCA removal in the second series of anaerobic *lac*, *na* and abiotic control(*k-*) microcosms from groundwater samples S1 (a) and S2 (b).

Microcosms set up from groundwater sample S1 (*S1lac*May21 and *S1na*May21), that were considered the most efficient 1,2-DCA dechlorinating cultures according to the rate of contaminant removal, were transferred after 30 days of incubation by inoculating the cultures into fresh culture medium. Two subsequential batches of enrichment cultures (namely Batch II and Batch III) were obtained. Chemical monitoring of the enrichment cultures, carried out by chloride assay, confirmed the biodegradation abilities of the cultures were successfully maintained after the transfers (Fig 2.13).

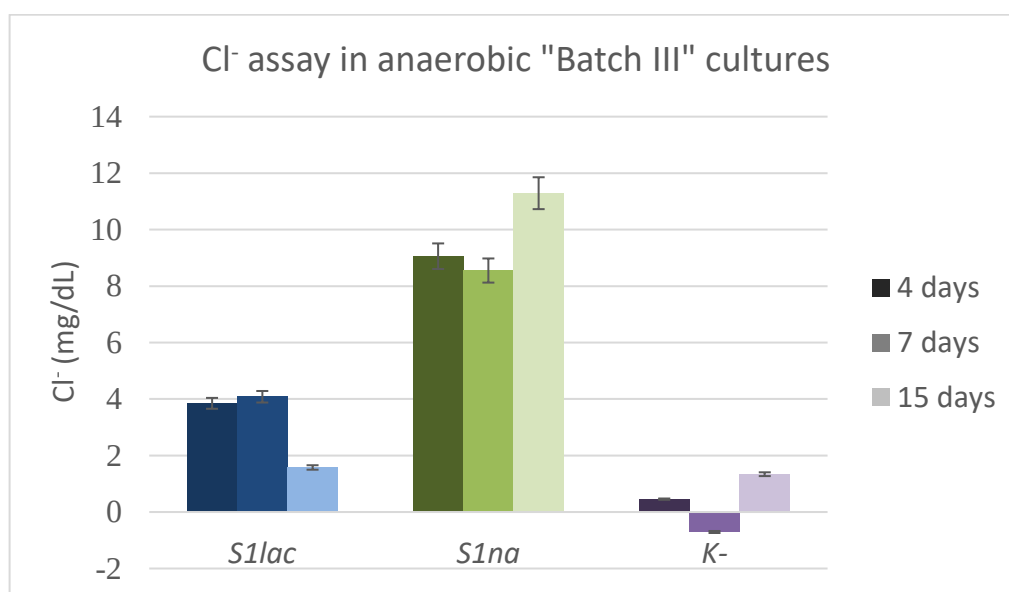


Figure 2.13. Chloride concentration increase in Batch III enrichment cultures obtained from microcosms *S1lac* and *S1na* and in the abiotic control at after 4, 7 and 15 days of incubation in respect to time 0.

2.3.3.2. Anaerobic 1,2-DCA dechlorinating bacterial communities characterization

Microcosms *S1lac*May21 and *S1na*May21 were selected for further biological monitoring. After 30 days of incubation, prior to the transfer into enrichment cultures, bacterial growth was estimated in terms of extracted DNA and compared to the amount of DNA from initial groundwater sample (GW-S1-May21). Extracted DNA was referred to the volume of GW or microcosm filtered (Tab. 2.6). Biomass in microcosms was notably higher than in groundwater, confirming the efficacy of biostimulation treatments.

Community composition of *S1lac*May21 and *S1na*May21 microcosms was analyzed by 16S rRNA Illumina sequencing and compared to GW-S1-May21 microbial community. The final reads obtained (Tab. 2.6) were assigned to OTUs at 99% similarity.

Diversity indices (Tab. 2.6) based on the number of OTUs were calculated to analyze and compare diversity and structure of bacterial communities. Microcosms were characterized by a lower richness if compared to groundwater sample, suggesting a specialization in microbial communities depending on the enrichment conditions imposed. Moreover, taxa in microcosms communities were more evenly distributed than in groundwater communities, that were instead characterized by higher dominance index.

	GW-S1-May21	S1lacMay21	S1naMay21
Total DNA (ng/ml)	3.39	124.54	248.32
Final reads	36,384	27,202	18,516
Richness (N° OTU)	223	79	119
Dominance (D)	0.2007	0.08335	0.04459
Simpson (1-D)	0.7993	0.9166	0.9554
Shannon (H')	2.603	3.027	3.635

Table 2.6. Amount of extracted DNA, 16S rRNA Illumina sequencing reads and diversity indices calculated on Illumina sequencing data of May 2021 groundwater S1 and anaerobic May 2021 microcosms.

Taxonomic composition at phylum, family and genus level of these communities is shown (Fig. 2.14a, b, c) taking into account taxa $\geq 1\%$ in at least one sample. Microcosms S1lacMay21 and S1naMay21 (*lac* and *na* microcosms), independently from presence or absence of lactate, showed similar bacterial compositions in terms of taxa detected, with slight differences in relative abundances, while differed in composition from groundwater GW-S1-May21 bacterial communities.

Epsilonproteobacteria decreased in *lac* and *na* microcosms in respect to the original groundwater (58%) but were still abundant in both conditions (20% and 24% respectively in *lac* and *na* microcosms); in groundwater sample this phylum is mainly made up of the sulfur oxidizing *Sulfuricurvum* (43%) (Kodama & Watanabe, 2004) and *Sulfurimonas* (13%) (Inagaki et al., 2003) genera (Helicobacteraceae family) making up most of the microbial community. Firmicutes, Synergistetes and Bacterioidetes phyla were strongly enriched in both microcosms, while Deltaproteobacteria were only enriched in *na* microcosm.

At the family level, *lac* and *na* microcosms were dominated by Desulfovibrionaceae (Deltaproteobacteria) (17% and 20% respectively), Helicobacteraceae (Epsilonproteobacteria) (17% and 20%) and Dethiosulfovibrionaceae (Synergistetes) (about 14% in both microcosms), while Eubacteriaceae (Firmicutes) were among the most abundant families only in *lac* microcosm (15%) and less represented in *na* microcosm (4%), here entirely represented by the fermenting genus *Acetobacterium*. This may suggest that sulfur

and sulfate metabolisms are comparable in both microcosms, while the role of fermentative metabolism is more important in *lac* microcosm, likely stimulated by lactate addition.

At the genus level, the sulfate- reducing *Desulfovibrio* (17% and 20% in *lac* and *na* microcosm respectively) and the thiosulfate and sulfur- reducing *Dethiosulfovibrio* (13% in both microcosms) (Surkov et al., 2001) were among the most abundant genera in both *lac* and *na* communities. Other, less represented, genera involved in sulfur and sulfate metabolism were also detected. The thiosulfate and sulfur- reducing genus *Dethiosulfatibacter* (2.4% and 3.5%) (Takii et al., 2007) and *Fusibacter* (1.6% and 0.95%), a thiosulfate and sulfur- reducing strictly anaerobic fermentative genus producing acetate, CO₂ and H₂ (Ravot et al., 1999) were detected in both microcosms, while the sulfur oxidizing *Sulfuricurvum* was only abundant in *na* microcosm (7.4%).

Desulfovibrio sp. strains were found able to couple lactate or acetate oxidation to reductive dehalogenation of chlorophenol (Sasi Jyothsna et al., 2008) or bromophenol compounds (Boyle et al., 1999). Moreover, non-dechlorinating *Desulfovibrio sp.* strains may play a role in facilitating organohalide respiration. Syntrophic associations or stable interspecific microbial communities between the sulphate-reducing *Desulfovibrio* and dechlorinating strains, such as a tetrachloroethene respiring *Desulfotobacterium* (Drzyzga et al., 2001) or a polychlorinated biphenyls respiring *Dehalococcoides mccartyi*_strain (Wang et al., 2019) were previously reported, suggesting lactate fermentation and subsequent H₂ production are able to sustain the growth of the dechlorinating strain in co-culture. Nested PCR using OHRB 16SrRNA specific probes allowed the detection of *Dehalococcoides* in *lac* and *na* microcosms and *Desulfotobacterium* in *na* microcosm, although they were not detected by 16S Illumina sequencing approach. Both *Dehalococcoides* (Maymò-Gatell et al., 1999) and *Desulfotobacterium* (Marzorati et al., 2007) include 1,2-DCA strains, and could be therefore responsible of 1,2-DCA biodegradation in microcosms, sustained by lactate fermentation products (in *lac* microcosm) or by H₂ present in the atmosphere since the setup (in both *lac* and *na* conditions).

Acetobacterium (14.4% in *lac* and 4% in *na* microcosm) is a homoacetogenic genus fermenting substrates such as lactate (Balch et al., 1977); this may explain its higher abundance in lactate-amended microcosm. An *Acetobacterium* strain was found able to cometabolically dechlorinate 1,2-dichloroethane (1,2-DCA) to ethene with lactate as substrate thanks to the Co(I) corrinoids (vitamin B₁₂) ability to reduce halogen substituted carbon atoms in halogenated compounds during acetogenesis (De Wildeman et al., 2003).

Among dechlorinating genera, notably the strictly anaerobic sulfur-reducing genus *Desulfuromonas* (Castro et al., 2006) was enriched in microcosms in respect to original groundwater, although the family Desulfuromonadaceae was instead more abundant in original groundwater, but mainly made up of unclassified genera. The presence of *Desulfuromonas* in microcosms and in groundwater was also confirmed by OHRB 16SrRNA specific probes. *Desulfuromonas* strains have been reported to couple PCE reduction to the oxidation of acetate or lactate (Sung et al., 2003) and to cometabolically dechlorinate TCE in sulfate- or sulfur- reducing bacterial communities (Guerrero-Barajas et al., 2011).

Other organohalide-respiring bacteria detected include the genus *Sulfurospirillum* (Campylobacteraceae), a minor component of both *lac* and *na* microcosms (1.4% and 2% respectively). *Sulfurospirillum* is a known facultative OHRB that mainly dechlorinates chlorinated ethenes by using different substrates, including lactate, as electron donor (Goris & Diekert, 2016; Luijten et al., 2003).

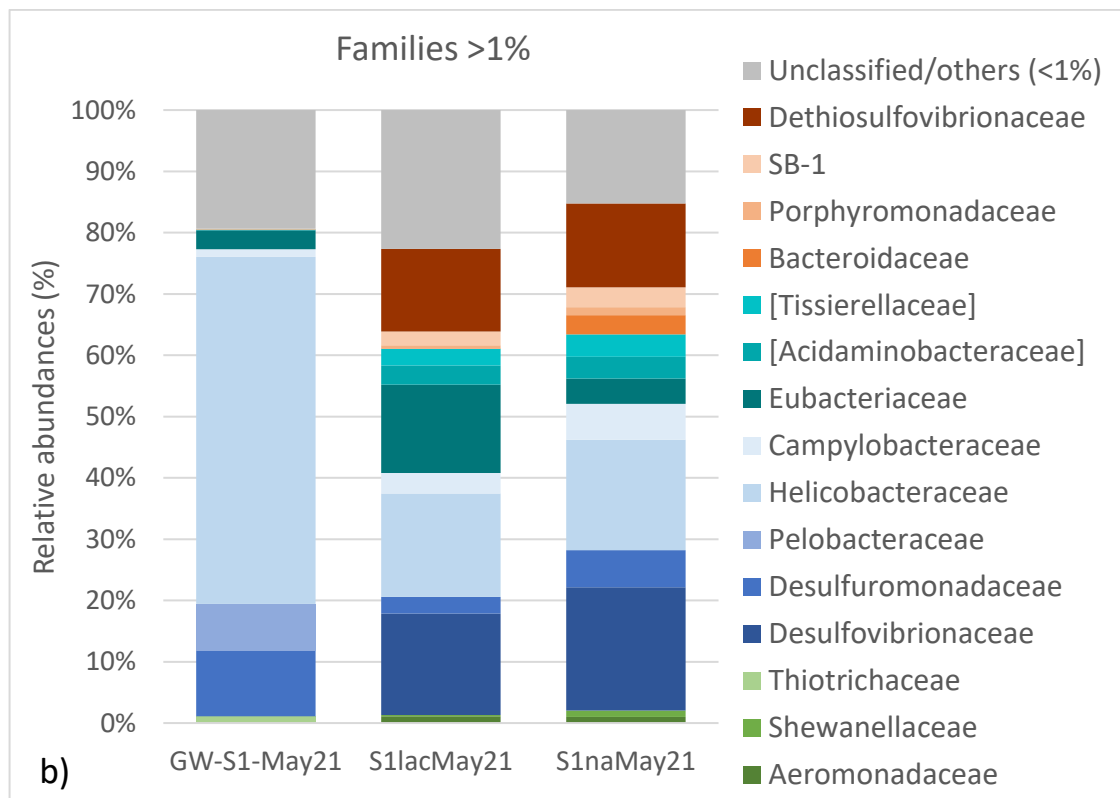
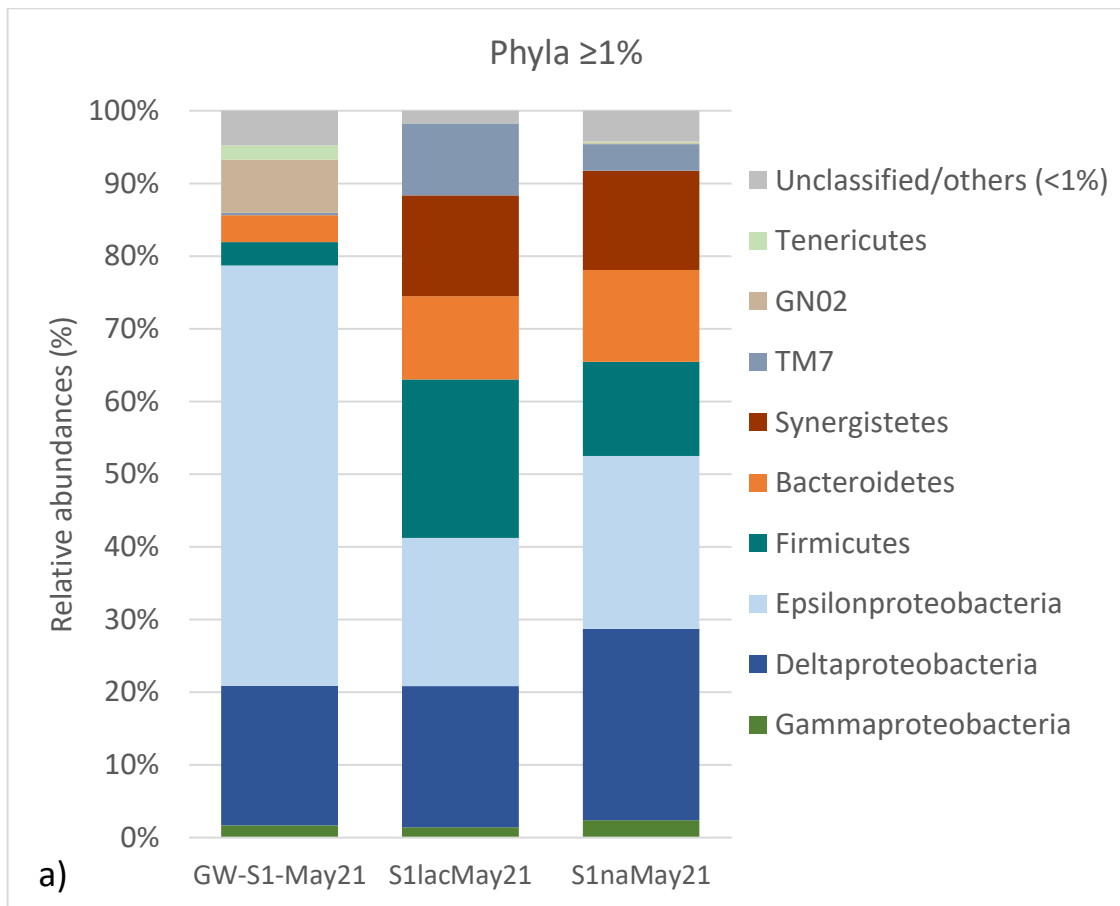
Low abundances of *Shewanella* were also detected (<0.5% in *lac* and 1% in *na* microcosm). *Shewanella* is a versatile OHRB within Shewanellaceae, capable of anaerobic dehalorespiration of halogenated organic compounds such as tetrachloromethane, polychlorinated biphenyls, 1,1,1-trichloroethane (Hau & Gralnick, 2007).

On the other hand, nested PCR with OHRB 16SrRNA specific probes for *Dehalogenimonas* and *Dehalobacter* did not yield expected amplicons.

All genera detected in microcosms are strictly anaerobic, facultatively anaerobic or microaerophilic, thus confirming anaerobic conditions were successfully established.

Moreover results suggest that, although the initial groundwater did not show a significant dechlorination potential considering known dechlorinating microorganisms on a taxonomic basis, the biostimulation treatments allowed to enrich taxa potentially involved in dechlorinating processes, that could explain 1,2-DCA removal assessed via chemical monitoring in both microcosms.

Notably, at family and genera levels, high percentages of unclassified genera (41% and 28% in *lac* and *na* microcosms respectively) were detected that could potentially also include yet unknown dechlorinating taxa.



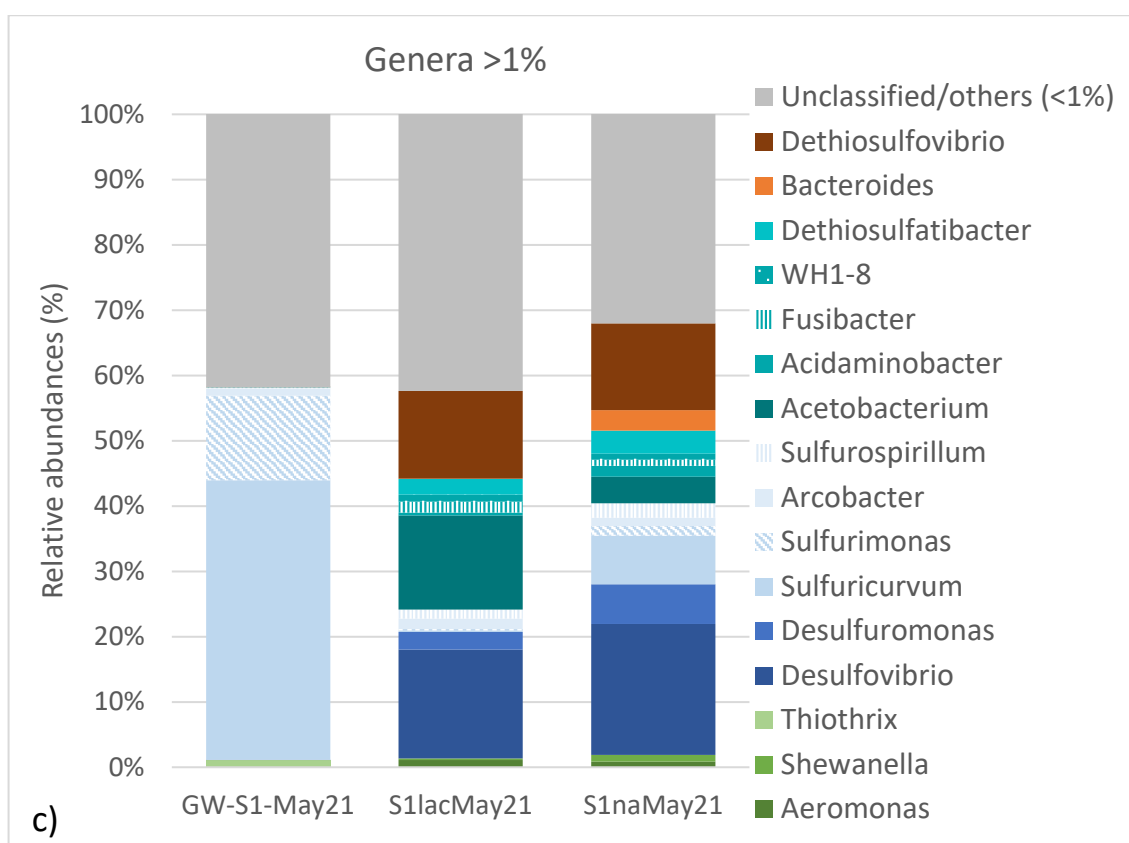


Figure 2.14. Relative abundances of a) phyla (down to class level for Proteobacteria), b) families and c) genera of GW-S1-May21 and S1lacMay21 and S1naMay21 microcosms. Taxa $\geq 1\%$ in at least one sample are shown. Unclassified/others (<1%) includes unclassified taxa and identified taxa <1%.

2.3.3.3. Detection of reductive dehalogenase genes

Catabolic genes involved in reductive dehalogenation were detected in metagenomic DNA from microcosms S1lacMay21 and S1naMay21 by PCR assays with degenerated and specific primers.

Amplicons of 300 bp, shorter than the expected size (400 bp) were obtained from metagenomic DNA of both *lac* and *na* microcosms with the primer set DHL-F1/DHL-R1 targeting a portion of the *dcaA* gene, coding for a reductive dehalogenase specifically involved in 1,2-DCA dichloroelimination (Marzorati et al., 2007). These amplicons were used as template in a nested PCR with Rdh522F/Rdh625R. Expected PCR products (95 bp) were obtained and cloned. 7 clones from S1naMay21 and 7 from S1lacMay21 were selected to be sequenced. The sequences of all 14 inserts had 98-99% identity with reductive dehalogenases gene *pceA* from *Desulfotobacterium* and *Dehalobacter* genera both previously found to be related to 1,2-DCA reductive dechlorination, and with the gene cluster described by (Marzorati et al., 2007), coding for a reductive dehalogenase specifically acting on 1,2-DCA (Tab. 2.7).

BLAST best match (nr/nt database)	Per. Ident.	Accession	Reference
<i>Desulfitobacterium hafniense</i> Y51 orf0, orf1, <i>pceA</i> , <i>pceB</i> genes for transposase, hypothetical protein, PCE dehalogenase, PCE dehalogenase membrane-bound subunit, partial and complete cds	98-99%	AB070709.1	(Suyama et al., 2002)
<i>Dehalobacter restrictus</i> pce operon (<i>pceABCT</i> genes), strain PER-K23	98-99%	AJ439607.2	(Maillard et al., 2003)
Uncultured bacterium dichloroethane reductive dehalogenase gene cluster	98-99%	AM183919.1	(Marzorati et al., 2007)

Table 2.7. Detection of reductive dehalogenase genes in metagenomic DNA of *S1lacMay21* and *S1naMay21* microcosms. BLAST best matches for reductive dehalogenase genes obtained with *Rdh522F/Rdh625R* primer set.

2.3.4. Aerobic microcosms and enrichment cultures

Aerobic microcosms were set up once from groundwater samples S1 and S2 collected in September 2020 in order to enrich aerobic microbial communities using 1,2-DCA as sole carbon source in presence of oxygen only (*ox* microcosms) or dechlorinating 1,2-DCA both directly and cometabolically in presence of a volatile hydrocarbon gaseous mix added as a co-substrate (*co* microcosms). After preliminary 1,2-DCA removal analysis, microcosms were transferred every 90 days into subsequent batches of enrichment cultures (named Batches II and III), that were periodically monitored by HS-GC/MS and Cl^- assay. Selected microcosms or enrichment cultures were further analyzed to characterize microbial communities and detect dehalogenase genes.

2.3.4.1. Aerobic dechlorinating microcosms

As already discussed, September 2020 microcosms were analyzed by HS-GC/MS by manual sampling, producing unreliable results. Nevertheless, based on overall trends of 1,2-DCA removal, microcosm *S1oxSep20*, *S1coSep20* and *S2coSep20* were considered dechlorinating. These preliminary results were confirmed by chemical monitoring of subsequent batches of enrichment cultures, carried out by HS-GC/MS after 15 and 25 days of incubation (Fig. 2.15) and further confirmed by chloride assay on the same cultures (Fig. 2.16). These results suggest dechlorinating communities, intended as cultures showing higher 1,2-DCA removal and Cl^- release than the abiotic control, were established and maintained over time. Here, results relative to the “batch III” of enrichment cultures are shown.

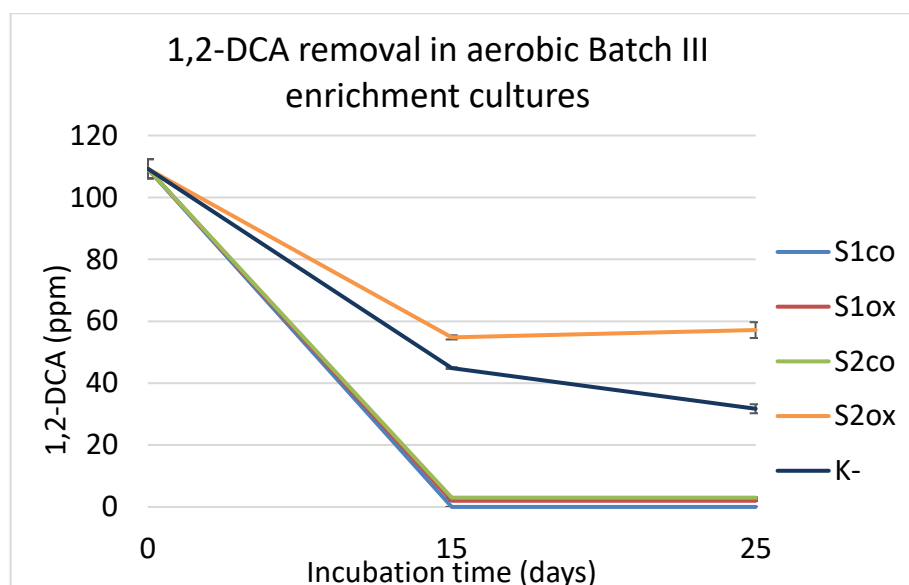


Figure 2.15. Chemical monitoring of BatchIII cultures from ox and co microcosms (S1 and S2) and abiotic control by HS-GC/MS.

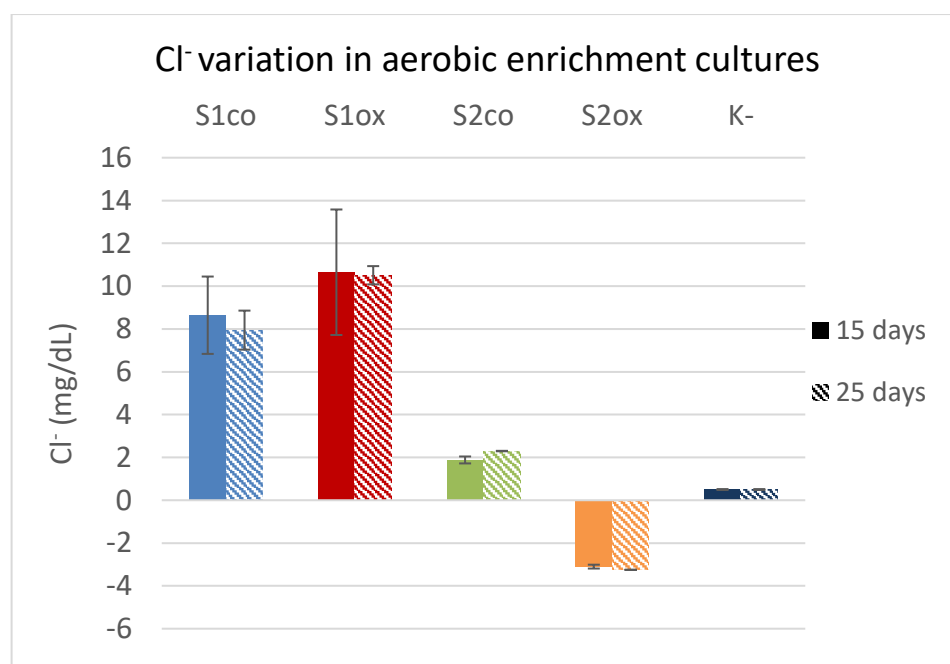


Figure 2.16. Chloride release assay on BatchIII cultures from ox and co microcosms (S1 and S2) and abiotic control(k-) after 15 and 25 days of incubation.

Periodical monitoring revealed both *ox* and *co* cultures from sample S1 were dechlorinating. Nevertheless, in the case of *co* microcosms, where volatile HC mixture was added as a co-substrate, chemical evidences only did not allow to determine if both direct and cometabolic dechlorination processes or only one of them occurred in *co* microcosm, nor if one of the processes was dominant over the other, in the former case. As a consequence, it was not possible to establish if the co-substrate was required for dechlorination or 1,2-DCA is directly degraded.

Evidences relative to S2 microcosms and enrichment cultures revealed only *co* microcosm was dechlorinating, while the corresponding *ox* culture, lacking volatile hydrocarbons as co-substrate, was non dechlorinating. These results suggest microbial communities enriched from S2*co* microcosm depended on volatile hydrocarbons for growth and that cometabolic dechlorination processes likely occurred; indeed the absence of the co-substrate did not allow dechlorination. This was further confirmed by Cl^- assay: lower values of released Cl^- in S2*co* culture, in respect to cultures where direct degradation also took place, suggest 1,2-DCA removal was only due to cometabolic processes. Cometabolic processes likely resulted in chlorinated intermediates, thus explaining why the complete removal of 1,2-DCA in S2*co* microcosm and enrichment culture was associated to low Cl^- release.

A certain amount of 1,2-DCA removal also occurred in the abiotic control, likely due to abiotic transformation processes or volatilization, while no Cl^- release was reported. It can be supposed that abiotic processes also occurred in inoculated microcosms to a certain extent, but the remaining 1,2-DCA removal should be attributed to biological activities.

2.3.4.2. *Aerobic 1,2-DCA dechlorinating bacterial communities characterization*

Bacterial biomass in microcosms was estimated by DNA quantization (Fig. 2.6). Both *co* and *ox* microcosms from GW samples S1 and S2 (September 2020) showed higher biomass values than the original groundwater, although to a lesser extent than anaerobic microcosms.

Microcosms in the conditions *co* and *ox* from groundwater sample S1 (S1*co*Sep20 and S1*ox*Sep20) after 45 days of incubation were chosen for further analysis. The community composition was analyzed by 16S rRNA Illumina sequencing and compared to the community of the original groundwater sample. Furthermore, also the third batch of the enrichment culture (BIIS1*ox*) obtained from S1*ox*Sep20 was analyzed after 100 days of incubation.

Total DNA extracted from the microcosms, *ox* enrichment culture and groundwater (referred to the volume filtered), final reads and diversity indices calculated on metagenomic 16S rRNA sequencing data are reported in Tab. 2.8. Microcosms showed higher biomass and lower richness values than groundwater, suggesting the enrichment in specialized taxa in microcosms thriving under the imposed conditions in spite of others. Moreover, lower Shannon index (H') values and higher dominance (D) values reported for microcosm compared to groundwater confirmed that less taxa were present in microcosms, and some of them, likely playing a major role within the community, were dominant over others.

	GW-S1-Sep20	S1coSep20	S1ox Sep20	BIIS1ox
Total DNA (ng/ml)	1.77	26.59	17.31	22
Final reads	60,204	37,442	27,196	30,526
Richness (N° OTU)	401	155	89	38
Dominance (D)	0.06	0.14	0.20	0.24
Simpson (1-D)	0.94	0.86	0.80	0.76
Shannon (H')	3.65	2.79	2.20	1.98

Table 2.8. Amount of extracted DNA, 16S rRNA Illumina sequencing reads and diversity indices calculated on Illumina sequencing data of September 2020 groundwater S1, aerobic September 2020 S1 microcosms and BIIS1ox enrichment culture.

The comparison of community composition of groundwater, aerobic microcosms and *ox* enrichment culture at phylum (Fig. 2.17a), family (Fig. 2.17b) and genus (Fig 2.17c) level is reported below. Taxa $\geq 1\%$ in at least one sample are reported.

The comparison of bacterial communities from September 2020 groundwater sample S1 (GW-S1-Sep20) and microcosms S1coSep20 and S1oxSep20 showed a total shift in composition. The two microcosms were very similar to each other, but significantly different from groundwater. Epsilonproteobacteria, Deltaproteobacteria and the uncharacterized division GN02, that were dominant in groundwater (34%, 22% and 14% respectively), became notably less abundant in aerobic microcosms, while Alphaproteobacteria, whose abundance in groundwater was $<1\%$, were strongly enriched in both *co* and *ox* microcosm (50% and 62%, respectively), becoming the dominant group. Within Alphaproteobacteria, the Xanthobacteraceae family was dominant in both microcosms (46% and 60%). This family comprises genera known to degrade chlorinated solvents. In particular, the genera *Ancylobacter* and *Starkeya*, both detected and dominant in microcosms, include known 1,2-DCA degraders. *Starkeya* (29% and 34% in *co* and *ox* microcosms respectively) is a methylotroph and facultative chemolithoautotroph sulfur oxidizer (Kappler et al., 2012); a 1,2-DCA degrading *Starkeya novella* strain carrying a plasmid-borne *dhla* gene was recently isolated from a 1,2-DCA polluted aquifer (Munro et al., 2016). Instead, members of the chemoorganotrophic and facultative methylotrophic genus *Ancylobacter* (18% and 26% in *co* and *ox* microcosms) are able to degrade chlorinated compounds such as dichloromethane (Firsova et al., 2009), 2-chloroethanol, chloroacetate, 2-chloropropionate and 1,2-dichloroethane using them as carbon and energy sources (Van den Wijngaard et al., 1992).

Epsilonproteobacteria, the most represented class in groundwater (34%), was also abundant in *co* microcosm (11%). In groundwater, two genera, *Sulfuricurvum* (19%) and *Sulfurimonas* (12%) were present; in *co* microcosm only *Sulfuricurvum* was reported (10%). Both *Sulfuricurvum* (19%) and *Sulfurimonas*, as already stated, are sulfur oxidizing and facultatively anaerobic or microaerophilic genera belonging to the Helicobacteraceae family (Inagaki et al., 2003; Kodama & Watanabe, 2004).

The class Deltaproteobacteria in groundwater (22%) was mainly made up of the families Desulfuromonadaceae (13%) and Pelobacteraceae (7%) both related to anaerobic 1,2-DCA degradation, as they include known OHRBs or genera found in coculture in dechlorinating communities, as previously described. These taxa were almost absent in microcosms communities (<0.5%), where Deltaproteobacteria (12% and 10% in *co* and *ox* microcosms respectively) were mostly made up of Bdellovibrionaceae (about 10% in both microcosms), entirely constituted by *Bdellovibrio*. The role of this genus, an obligate predatory bacterium selectively preying on Gram-negative bacteria (Bratanis et al., 2020) in this microbial community is not clear.

Bacteroidetes were similarly enriched in *co* (19%) and *ox* microcosms (20%) in respect to groundwater (11%). Bacteroidetes in groundwater were mostly unclassified, while the uncharacterized lineages GZKB119 (8% and 11%) and VC21_Bac22 (6% and 4%) were present in *co* and *ox* microcosms and SB-1 (2%) in *co* microcosm. Knowledge on metabolic characteristics and ecological functions of these groups is limited. GZKB119 were originally reported as members of a landfill leachate microbial community (Huang et al., 2005); VC21_Bac22 is an uncultured clade first reported from a hydrothermal vent and then found in samples from sulfide-rich environments (Leng et al., 2022), suggesting a role of this group in sulfur cycling; SB-1 group is related to a clone obtained from a benzene degrading, sulfate-reducing consortium (Phelps et al., 1998).

Actinobacteria, whose abundance in groundwater was <1%, were only enriched in *co* microcosm (2%), mainly made up of the Mycobacteriaceae family (1.4%) and, in particular, of genus *Mycobacterium* (1.4%). A *Mycobacterium* strain was reported to be capable of aerobic cometabolic biodegradation of VC and cDCE when grown on ethene, and of cDCE and 1,2-DCA when grown on ethane (Le & Coleman, 2011). Cometabolic biodegradation of 1,2-DCA in *co* microcosm could be carried out by a *Mycobacterium* strain growing on the ethane provided as co-substrate. Other *Mycobacterium* strains are known to carry the *dhmA* gene, coding for a haloalkane dehalogenase showing broad substrate specificity and also acting on 1,2-dichloroethane (Jesenská et al., 2002).

In the enrichment culture derived from *ox* microcosm, Alphaproteobacteria (83%) were further enriched in respect to *ox* microcosm. Within Alphaproteobacteria, Xanthobacteraceae (49%) made up of genera *Starkeya* (43%) and *Ancylobacter* (5%) were still dominant, but other families, almost absent in *ox* microcosm, were enriched. In particular, Brucellaceae (17%), entirely made up of the genus *Ochrobactrum*, and Erythrobacteraceae (8%), unclassified at genus level, were among the dominant taxa of the

BIIS1_{ox} community. An *Ochrobactrum* strain was found able to use vinyl chloride (VC) as sole carbon and energy source under aerobic conditions by an alkene monooxygenase codified by a plasmid-borne gene (Danko et al., 2004). Minor alphaproteobacterial components in BIIS1_{ox} community included the photosynthetic purple non-sulfur genus *Rhodobacter* (2%, Rhodobacteraceae), the sulfur-oxidizing genus *Bosea* (2%, Bradyrhizobiaceae) and unclassified Phyllobacteriaceae (3%).

Betaproteobacteria, almost absent in *ox* microcosm (<0.5%), were enriched in BIIS1_{ox}, entirely made up of the sulfur-oxidizing genus *Thiobacillus* (Das et al., 1996), in the Hydrogenophilaceae family (2%).

Gammaproteobacteria were enriched in microcosms (from <1% in groundwater to 1.5% in *co* and 4.5% in *ox* microcosm) and especially in the *ox* enrichment culture (13%). Xanthomonadaceae were the main family detected within Gammaproteobacteria both in *ox* microcosm (4%) and in the enrichment culture (10%). Xanthomonadaceae in BIIS1_{ox} were entirely made up of the genus *Dyella*; strains of the genus *Dyella*, usually isolated from sludge or soil, include strains able to dechlorinate the chlorinated aromatic compound triclosan (S. Wang et al., 2018) and biphenyl (Ang et al., 2009). In BIIS1_{ox} also Moraxellaceae, made up the genus *Acinetobacter* (3%), were detected. *Acinetobacter* strains were found able to cometabolically degrade 4- and 3-chlorophenols (CP) in presence of phenol as growth substrate (Kim & Hao, 1999).

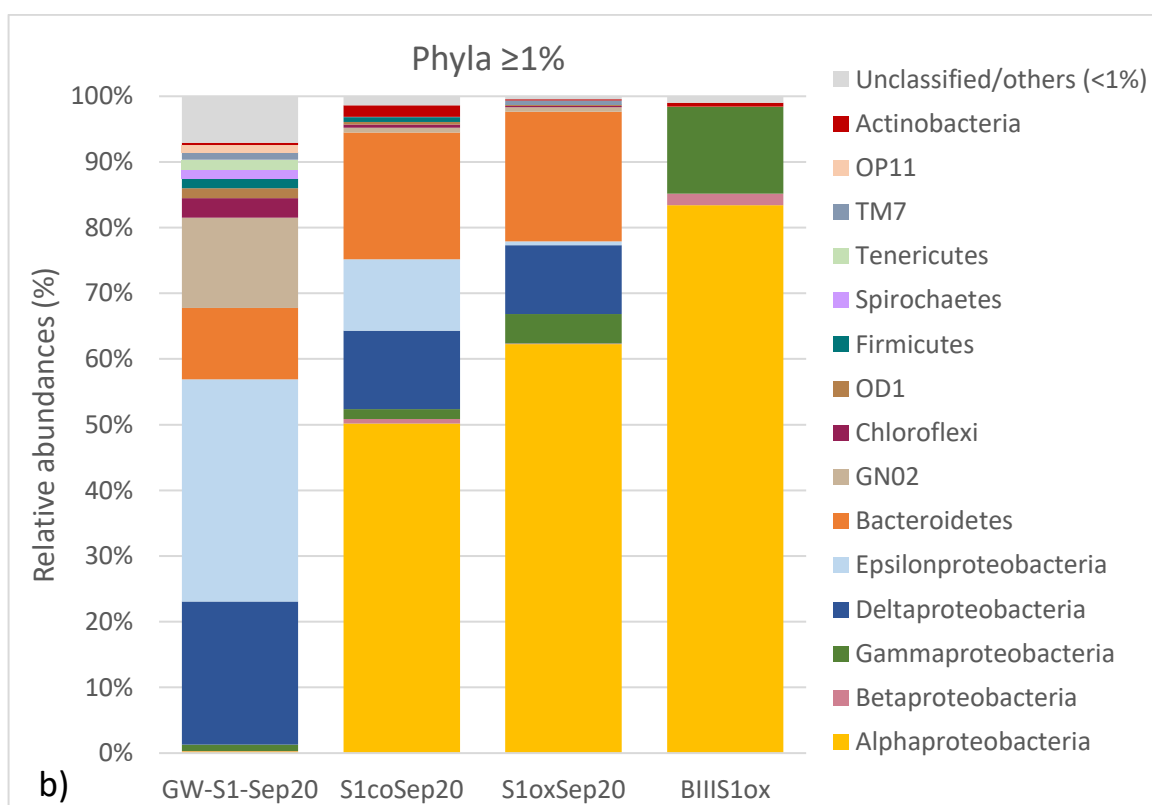
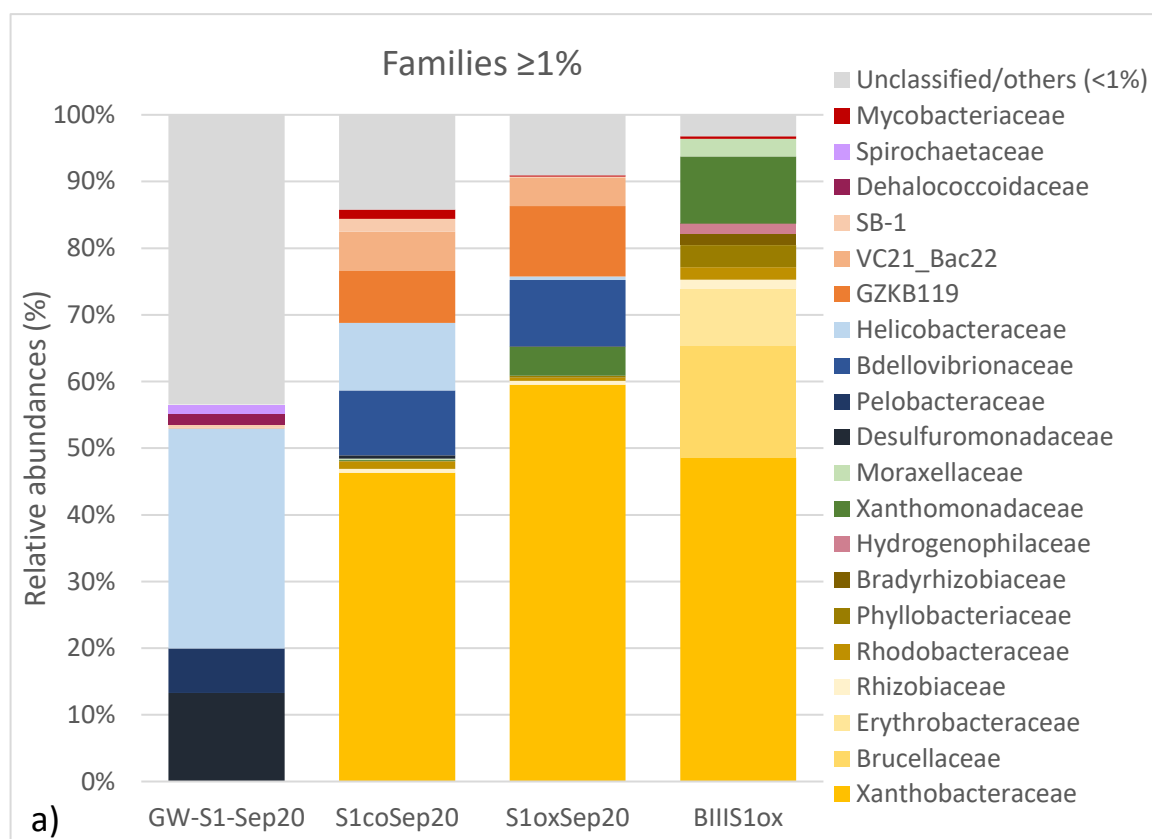
Notably, also in these microcosms, a large percentage of unclassified taxa was observed.

Overall, in response to the aerobic biostimulation, the anaerobic or microaerophilic sulfur oxidizers *Sulfuricurvum* and *Sulfurimonas* constituting most of the identified groundwater community, were replaced by several sulfur oxidizing genera detected in aerobic microcosms and enrichment culture, such as *Starkeya*, *Sulfuricurvum*, *Thiobacillus* and *Bosea*.

Both aerobic microcosms and the subsequent *ox* enrichment culture were dominated by aerobic hydrolytic 1,2-DCA degraders that were below the detection level in groundwater. In particular, *Ancylobacter* and *Starkeya* might play a key role in 1,2-DCA degradation in both *ox* and *co* conditions. Moreover, in *co* microcosm, *Mycobacterium* might also be responsible of cometabolic biodegradation processes sustained by the supply of volatile hydrocarbon mix.

The possibility to biostimulate this aerobic biodegradation potential, in parallel with the biostimulation of reductive pathways obtained in anerobic microcosms, was unexpected, considering the groundwater sample used to set up the microcosms was characterized by a low redox potential. Indeed, only two recent studies investigated both the aerobic and

anaerobic bioremediation potential within 1,2-DCA contaminated aquifers, assessing the coexistence and potential exploitation of aerobic and anaerobic biodegradation pathways (Munro et al., 2017; S. Y. Wang et al., 2015) .



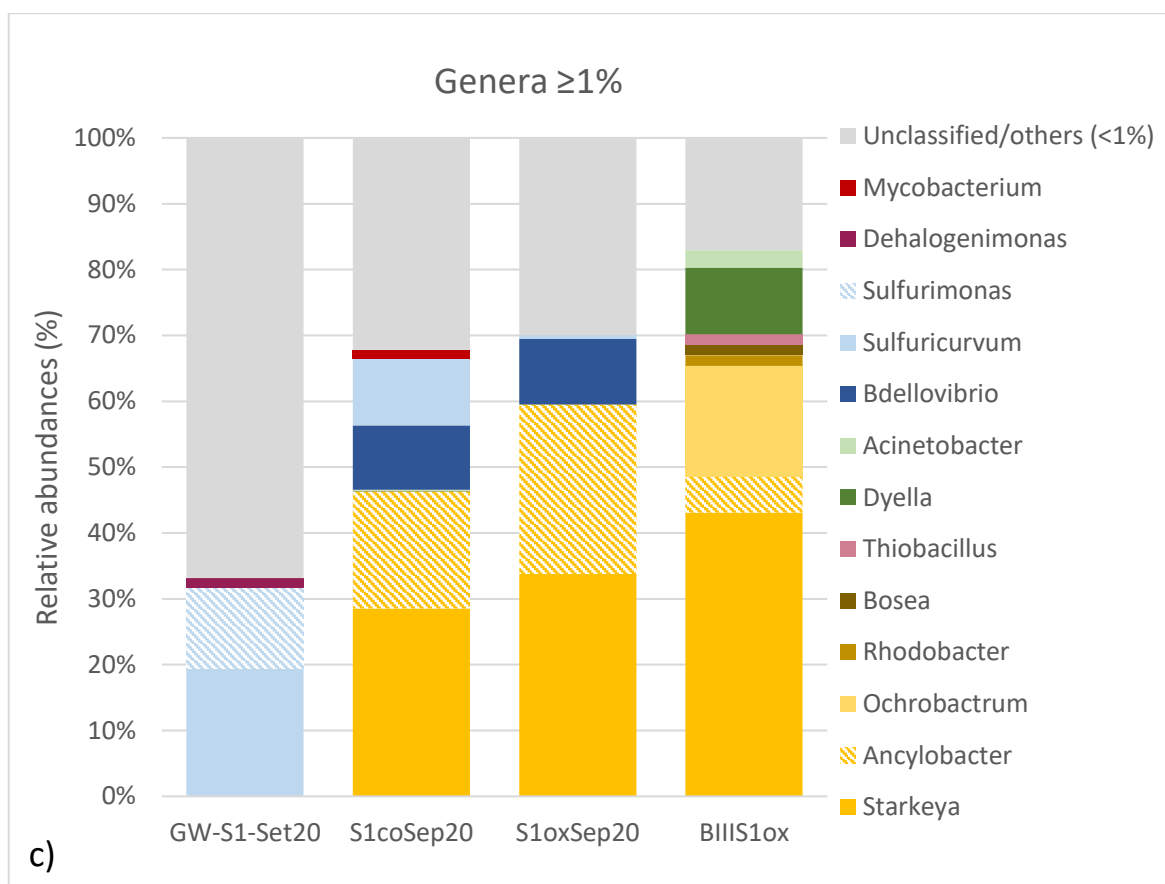


Figure 2.17. Relative abundances of a) phyla (down to class level for Proteobacteria), b) families and c) genera of GW-S1-Sep20, and S1co Sep20 and S1oxSep20 microcosms and BIIIS1ox enrichment culture. Taxa $\geq 1\%$ in at least one sample are shown. Unclassified/others (<1%) includes unclassified taxa and identified taxa <1%.

2.3.4.3. Detection of haloalkane dehalogenase gene *dhlA*

The detection of abundant Xanthobacteraceae in S1ox enrichment cultures, and in particular of genera *Starkeya* and *Ancylobacter*, including known aerobic 1,2-DCA dechlorinating strains, suggested the occurrence of the DhlA-mediated hydrolytic pathway for 1,2-DCA degradation. This hypothesis was confirmed by PCR probing the DNA extracted from S1ox enrichment cultures with degenerated and specific primer sets targeting a portion of the *dhlA* gene, coding for the haloalkane dehalogenase DhlA. Amplicons of the expected size were obtained with all primer sets tested; DHLA 380F/548R primer set yielded amplicons of the expected size and several aspecific products. In particular, a 284 bp fragment of the *dhlA* gene was obtained with the DHLA 319F/603R primer set using as template the DNAs extracted from BII-S1ox and BIII-S1ox cultures after 90 days of incubation. Amplicons obtained from both cultures were sequenced. Sequences (156 and 173 bp in size), compared with those present in the NCBI GeneBank database, showed 100% identity with the plasmid-borne *dhlA* gene from *Xanthobacter* sp., *Ancylobacter* sp. and *Starkeya* sp. (Tab. 2.9).

The detection of genes coding for the DhlA haloalkane dehalogenase confirm that the removal of 1,2-DCA in aerobic enrichment cultures can be attributed to biotic hydrolytic dechlorination processes.

BLAST best match (nr/nt database)	Accession	Per. Ident	Reference
<i>Xanthobacter flavus</i> strain EL8 plasmid pDCA, complete sequence	KU922121.1	100%	(Munro et al., 2016)
<i>Xanthobacter autotrophicus</i> strain EL4 plasmid pDCA	KU922120.1	100%	
<i>Starkeya novella</i> strain EL1 plasmid pDCA, complete sequence	KU922118.1	100%	
<i>Ancylobacter aquaticus</i> strain DH2 haloalkane dehalogenase (dhlA) gene, complete cds	FJ573162.1	100%	(Govender & Pillay, 2011)
<i>Xanthobacter flavus</i> clone UE-15 insertion sequence IS1247 haloalkane dehalogenase (dhlA) and transposase genes, complete cds	AY561847.1	100%	(Song et al., 2004)
<i>X. autotrophicus</i> haloalkane dehalogenase (dhlA) gene, complete cds	M26950.1	100%	(Janssen et al., 1989)

Table 2.9. Detection of haloalkane dehalogenase gene *dhlA* in metagenomic DNA of *S1ox* enrichment cultures. BLAST best matches for haloalkane dehalogenase gene sequences obtained with DHLA 319F/603R primer set.

2.4. Conclusions

The evaluation of the intrinsic dechlorination potential of an aquifer contaminated by chlorinated solvents, mainly represented by 1,2-DCA, was carried out here by a preliminary microbiological characterization of the site, performed by 16S rRNA Illumina sequencing, and a laboratory-scale study based on biostimulated microcosms.

The microbiological characterization of the aquifer, carried out on groundwater samples collected from the two most contaminated sampling sites within the study area, revealed a low intrinsic dechlorination potential, in terms of presence and abundance of known dechlorinating bacteria, on a taxonomic basis, independently from the different CAHs contamination levels and physical-chemical characteristics of the samples. Low abundances of known dechlorinating taxa were confirmed by the microbiological characterization of groundwater samples collected in different time periods to provide material for microcosm set up. Although the composition of the autochthonous microbial communities in groundwater was slightly different over the sampling periods, a limited presence of known or potential OHRBs was reported and, at the same time, the absence of aerobic CAHs degraders. Interestingly, the Dehalococcoidaceae family, one of the most commonly found in CAHs contaminated sites and exploited in enhanced bioremediation strategies, was poorly represented or not detected by 16S rRNA Illumina sequencing, although specific probes allowed its detection. Notably, large percentages of unclassified taxa were reported, that could potentially include yet unknown dechlorinating bacteria, and uncharacterized divisions, highlighting the need to further study yet unexplored environments like the aquifer here considered.

The biostimulation treatments in microcosm allowed the enrichment of 1,2-DCA dechlorinating communities both under the aerobic and anaerobic conditions tested. The efficacy of the biostimulation was evaluated by chemical monitoring (HS-GC/MS and chloride assay) of microcosms over time to assess the removal of 1,2-DCA and by biological monitoring, to study the enriched dechlorinating communities and search for reductive or hydrolytic dehalogenase genes.

Under anaerobic conditions, microcosms containing the BTZ medium with or without addition of lactate as a fermentable substrate showed similar 1,2-DCA removal efficiency and community composition, suggesting the N₂/CO₂/H₂ (80%/15%/5%) atmosphere and vitamins added in the medium (including vitamin B₁₂) were sufficient to sustain dechlorination processes.

In both anaerobic microcosms, biodegradation may be carried out by reductive dechlorination processes mediated by facultative or obligate OHRB genera such as *Desulfitobacterium* and *Dehalococcoides*, and by cometabolic reductive dechlorination mediated by *Acetobacterium*. Other taxa detected, such as *Acetobacterium* and *Desulfovibrio*, could play a key role in sustaining dechlorinating processes. The detection and sequencing of *rdhA* genes in both microcosms confirmed the presence of a dechlorinating potential.

Under aerobic conditions, microcosms containing aerobic medium amended or not with a volatile hydrocarbon mix showed different results in terms of 1,2-DCA removal: with reference to S2 microcosms, only the “co” condition amended with the co-substrate showed 1,2-DCA removal. Nevertheless, the S1 aerobic microcosms, selected for further biological analysis, showed similar results from chemical and biological monitoring. Both conditions were efficient in dechlorination, and both showed a strong enrichment in known hydrolytic 1,2-DCA degraders, although they were not detected in the original groundwater samples, characterized by low redox potential. In S1 microcosm the presence or absence of the co-substrate seems not to affect the dechlorination processes, suggesting they are mainly direct processes. *Ancylobacter* and *Starkeya* may mediate direct aerobic dechlorination, using 1,2-DCA as carbon and energy source. Aerobic cometabolic processes may also occur to a lesser extent in presence of the co-substrate, mediated by *Mycobacterium*. Chemical and biological monitoring carried out on an enrichment culture obtained from *ox* microcosm confirmed this aerobic dechlorinating community was maintained over time. The haloalkane dehalogenase gene *dhlA*, known to be active on 1,2-DCA was detected and sequenced, confirming the occurrence of the hydrolytic dechlorination pathway.

The possibility to stimulate this aerobic dechlorination potential, that could be sustained by oxygen supply only, was unexpected, considered the chemical physical characteristics of groundwater sample used.

The results obtained so far suggest both anaerobic and, surprisingly, aerobic biostimulation treatments could potentially be exploited for bioremediation purposes in the aquifer here studied.

CHAPTER III

Isolation and characterization of aerobic dechlorinating bacteria

3.1 Introduction

3.1.1 Aerobic 1,2-DCA dechlorinating bacteria

Some bacteria are known to carry out direct aerobic biodegradation of 1,2-DCA using this molecule as a carbon and energy source after an initial oxidative or hydrolytic reaction.

The oxidative pathway has been observed in two *Pseudomonas* strains (*Pseudomonas* sp. Strain DCA1 and *Pseudomonas* sp. strain DE2) and the proposed degradation mechanism involves a monooxygenase-mediated initial step (Hage & Hartmans, 1999).

The hydrolytic pathway was observed in *Xanthobacter* sp., *Ancylobacter* sp. and *Starkeya novella* strain EL1 (Janssen et al., 1985; Munro et al., 2016; Van Den Wijngaard et al., 1992), but first proposed in *X. autotrophicus* GJ10 (Janssen et al., 1989) (Fig. 3.1). During 1,2-dichloroethane hydrolysis in the model organism *X. autotrophicus* GJ10, the substrate is taken up into the cytoplasm, where it is dehalogenated to 2-chloroethanol. 2-chloroethanol is subsequently exported to the periplasm and converted by a periplasmic alcohol dehalogenase into chloroacetaldehyde, that is then re-uptaken and further degraded in the cytoplasm (Janssen et al., 1987). This pathway involves the hydrolytic removal of a chlorine atom mediated by a haloalkane dehalogenase (DhlA), yielding 2-chloroethanol; this metabolite is sequentially oxidized by an alcohol dehydrogenase (Mox) and an aldehyde dehydrogenase (AldA) to chloroacetic acid, that is then converted by a hydrolytic haloacid dehalogenase (DhlB) into glycolic acid, then metabolized via the glyoxylate cycle.

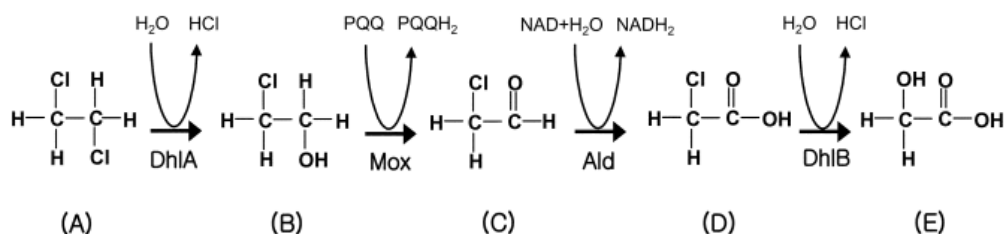


Figure 3.1. Proposed catabolic pathway of 1,2-DCA. (A), 1,2-Dichloroethane (1,2-DCA); (B), 2-Chloroethanol; (C), 2-Chloroacetaldehyde; (D), Monochloroacetic acid (MCA); (E), Glycolic acid; DhlA, haloalkane dehalogenase; Mox, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; DhlB, haloacetate dehalogenase. From (Song et al., 2004).

The key enzymes in the process are the haloalkane dehalogenase (DhlA) and the haloacid dehalogenase (DhlB), that catalyze the release of chlorine atoms from 1,2-DCA. In particular, the first enzyme involved, the haloalkane dehalogenase (DhlA), and the relative encoding gene *dhlA*, were originally discovered in *X. autotrophicus* GJ10. Later, 100% identical DhlA encoding genes have been discovered in several other bacterial strains isolated on 1,2-DCA as carbon source in the Netherlands, Germany, Australia, South Korea, South Africa and affiliated to *Xanthobacter* sp., *Ancylobacter* sp. and *Starkeya novella* strain EL1 (Janssen et al., 1989; Munro et al., 2016; Song et al., 2004; Van Den Wijngaard et al., 1992), suggesting this gene (933 bp) would make a good target for molecular methods when monitoring 1,2-DCA degradation. In fact, DhlA is still the only known hydrolytic haloalkane dehalogenase that operates in 1,2-dichloroethane degrading bacteria and no variants have been described (Munro et al., 2013).

The wide geographical distribution of the isolated strains suggests these dehalogenase genes may be widespread, but also poses questions on the mechanism of distribution and the ancestry of 1,2-DCA-degrading isolates, that is still unknown.

Interestingly, these genes in different isolates are located on different plasmids and associated with insertion sequences and transposase that are identical or closely related among isolates, illustrating the high mobility of the genes (Atashgahi et al., 2018; Munro et al., 2016). As an example, in *X. autotrophicus* GJ10, the *dhlA* and *dhlB* genes are located on a 225 kb linear plasmid and on the chromosome, respectively (Tardif et al., 1991); in other strains of *X. autotrophicus*, *X. flavus* and *Starkeya novella*, *dhlA* and *dhlB* homologs are carried on a variant of a relatively small (37 kb) circular plasmid, named pDCA, that represents a true organochlorine-catabolic plasmid as its only obvious metabolic phenotype seems to be organochlorine dehalogenation (Fig. 3.2). This latter evidence of pDCA variants within different bacterial strains isolated from a contaminated site suggests the 1,2-DCA degradative phenotype can be acquired and passed on by bacteria, although mobilization mechanisms and host range have not been cleared to date (Munro et al., 2016). Since the ability to degrade 2-chloroethanol was also found in other bacteria producing alcohol and aldehyde dehydrogenase enzymes (Dijk et al., 2004) and the function of 2-chloroethanol dehydrogenase can be played by non-specific alcohol dehydrogenase enzymes, such as the methanol dehydrogenases in methylotrophic bacteria (Janssen et al., 1987), likely the acquisition of just DhlA and DhlB would be sufficient to enable many types of bacteria to grow on DCA (Munro et al., 2016).

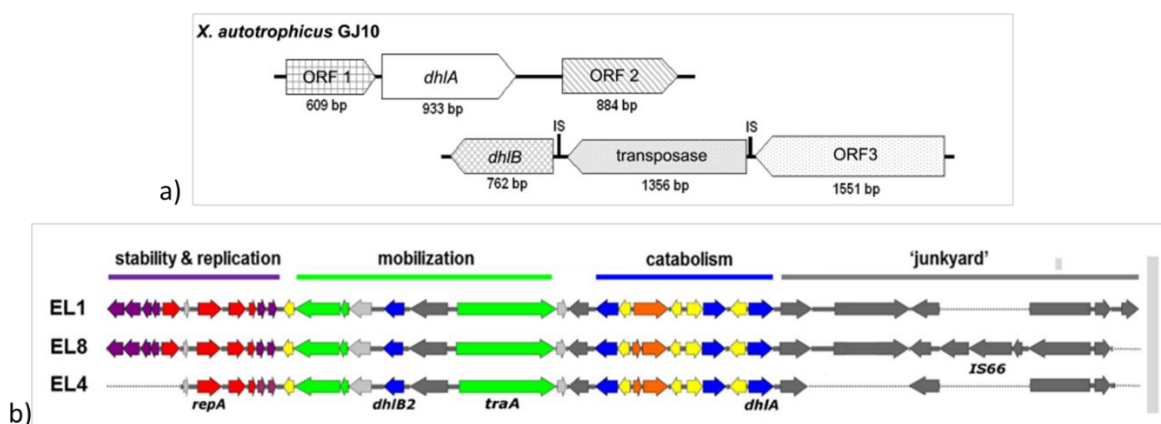


Figure 3.2. Schematic representation of *dhIA* and *dhIB* organization in different 1,2-DCA degraders. a) In *X. autotrophicus* the *dhIA* and the *dhIB* genes are located on a plasmid and on the chromosome, respectively. From Song et al., 2004; b) Alignment of open reading frames in the three variants of pDCA plasmid obtained from Australian isolates *Starkeya* strain EL1, and *Xanthobacter* strains EL4 and EL8. Apart from genes *dhIA* and *dhIB2* (*dhIB* gene homolog), some other features of the plasmid are shown (*repA*, *traA*, IS66). From Munro et al., 2016.

The detection of identical *dhIA* genes in independent environmental samples is usually possible after classical selective enrichment on 1,2-DCA, while it does not occur in (meta)genome sequences from organisms or samples with no history of 1,2-dichloroethane metabolism. This observation suggests that enrichment procedures allow to unravel a part of dehalogenation potential and natural biodiversity that remains undisclosed when environmental samples are only explored by genomic studies (Atashgahi et al., 2018).

All of the aerobic hydrolytic 1,2-DCA degraders isolated so far (*Xanthobacter* sp., *Ancylobacter* sp. and *Starkeya novella* strain EL1) are facultative methylotrophs, affiliated to the Xanthobacteraceae family, belonging to the order Hyphomicrobiales within the Alphaproteobacteria.

3.1.2 Aim of the work

The aims of the activities here described were the isolation, identification and characterization of 1,2-DCA dechlorinating bacteria from the aerobic dechlorinating enrichment cultures obtained from the contaminated aquifer under study described in Chapter II. Although much information can be obtained by omics approaches, the isolation of bacteria endowed with particular environmental and biotechnological properties is always recommended. The dechlorinating bacterial cultures were preliminarily identified and 1,2-DCA biodegradation abilities and the presence of *dhIA* gene were assessed. Selected cultures were analyzed by a Whole Genome Sequencing approach, revealing the presence other genes involved in the hydrolytic process, apart from *dhIA*.

3.2 Materials and methods

3.2.1 Bacterial isolations from the aerobic enrichment cultures

In Chapter II, the enrichment cultures obtained by subsequential transfers from aerobic *ox* and *co* microcosms set up from S1 and S2 groundwater samples were described and the enrichment of dechlorinating communities was confirmed by chemical assessment of 1,2-DCA removal, bacterial community composition analysis and detection of hydrolytic haloalkane dehalogenase genes. These cultures were used as inoculum to enrich and isolate potential dechlorinating strains.

Bacterial isolations were carried out on slope agar MSM medium (Coleman et al., 2002) amended with 1,2-DCA 100 ppm. Agar MSM medium (5 ml) was solidified in 20 ml screw cap glass vials to avoid the contaminant volatilization.

Briefly, 10 µl inoculum from the enrichment cultures were transferred and streaked onto the agar MSM medium amended with 1,2-DCA as sole carbon source and onto medium without the contaminant, as control test. A volatile hydrocarbon mixture (1% ethane, 1% methane, 0.01% C₄-C₆) was also syringe injected into the vials where inocula from *co* enrichment cultures were streaked.

In order to obtain pure cultures, purification steps were performed by colony picking and transferring the inoculum onto fresh agar 1,2-DCA amended-MSM. Further purification was attempted on a low nutrient agar medium (R2A) (Munro et al., 2016; Reasoner & Geldreich, 1985) containing 0.5 g/L yeast extract, 0.5 g/L Difco proteose peptone no.3, 0.5 g/L casamino acids, 0.5 g/L glucose, 0.5 g/L soluble starch, 0.3 g/L sodium pyruvate, K₂HPO₄ 0.3 g/L, 0.05 g/L MgSO₄ x 7H₂O, 15 g/L agar.

Isolates were incubated at room temperature (25°C) in the dark until growth was observed.

Apparent single colony-derived cultures were identified by 16S rRNA sequencing and screened for their 1,2-DCA biodegradation ability by chloride release assay and for the presence of the *dhlA* gene by PCR probing.

3.2.2 Growth in liquid medium and biomass collection

Isolates were grown in liquid medium to test 1,2-DCA biodegradation ability and collect biomass for subsequent DNA extraction. Single colonies were picked and transferred into 5 ml liquid MSM amended with 1,2-DCA (100 ppm) in 20 ml screw cap vials (pre-inoculum). A pre-inoculum (10% v/v) of each culture was transferred into 25 ml fresh MSM medium amended with 1,2-DCA (100 ppm) in 60 ml crimp-sealed glass serum bottles with butyl

rubber stoppers in duplicate. An abiotic control was set up with uninoculated medium. All cultures in liquid MSM were incubated at room temperature (25°C), in darkness, with shaking (150 rpm) for seven days and periodically monitored by chloride assay.

Cultures that could not grow on 1,2-DCA as sole carbon and energy source and release Cl⁻ were grown on R2A liquid medium to collect biomass for DNA extraction. Single colonies were picked from R2A agar medium and inoculated into 100 ml flasks containing 25 ml liquid medium. Cultures in R2A were incubated aerobically at room temperature (25°C), in darkness, with shaking (150 rpm) for 48 h.

Aliquots of each culture were stored in 20% glycerol at -80°C.

Biomass collection from both MSM and R2A cultures was performed by centrifugation at 10000 rpm for 15 minutes. Pellets were stored at -20°C until DNA extraction.

3.2.3 Chloride release assay

All the isolates grown in 1,2-DCA amended MSM were screened by chloride release assay. Before incubation and after 3 and 7 days of incubation, 100 µl of each culture and uninoculated control (prepared as described) were collected, transferred in 2 ml Eppendorf tubes and centrifuged at 14000 rpm for 2 minutes; the supernatant was collected and stored at 4°C until chloride release test was performed with the QuantiChrom™ Chloride Assay Kit (BioAssay System) as previously described for microcosms.

3.2.4 DNA extraction from isolates

Total DNA was extracted from pellets using the commercial kit QIAamp Fast DNA™ Stool Mini Kit (QIAGEN) according to manufacturer's instructions. Purity and concentration of extracted nucleic acids were assessed using the Spectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). The DNA samples were stored at -20°C for subsequent analyses.

Raw DNA extraction from isolates for colony PCR was performed by cell lysis through thermic shock. Briefly, colonies were picked from solid medium and diluted into sterile TE 1x buffer solution (10mM tris and 1mM EDTA). Thermic shock was performed by 5 minutes incubation at 100°C and subsequent 5 minutes incubation on ice. After centrifugation at 14000 rpm for 5 minutes, 1 µl of supernatant was used as template in PCR reactions.

3.2.5 Selection of representative R2A isolates

Prior to identification and chloride assay, isolates obtained on R2A medium showing similar phenotypes were compared based on their 16S- 23S rRNA intergenic spacers (ITS) profiles,

to select representative isolates. Bacterial ITS was PCR amplified using DNA extracted from pellets as template with the ITSf/ ITSReub primer set (Cardinale et al., 2004) (Attached A).

PCR conditions used were as follows: an initial denaturation at 94°C for 30 s; [94°C for 30 s, 55°C for 1 min, 68°C for 5 min] X 30 cycles, final extension at 68°C for 5 min. PCR products were visualized by electrophoresis on 2% agarose gel stained with GelRed in TAE 1X buffer [40 mM Tris, 20 mM acetic acid, and 1 M EDTA (pH 8.0)] at 80 V for 40 min and visualized by UV transillumination by ChemiDoc Imaging System (Bio-Rad).

3.2.6 Identification of isolates

For the taxonomic identification of the isolates, amplification of the 16S rRNA gene was performed using the primer set 27F/1492R (Frank et al., 2008) (Attached A). The PCR reaction was performed in a total volume of 30 µl containing 0.025 U/µl of DreamTaq DNA Polymerase (Thermo Fisher Scientific), 1 x DreamTaq Buffer, 0.2 mM dNTPs (Invitrogen), 0.2 µM of forward and reverse primers and 1-10ng of template DNA. DNA extracted from pellets or colonies was used as template.

PCR conditions used were: an initial denaturation at 95 °C for 3 min; [95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min] X 30 cycles; a final extension at 72 °C for 15 min.

PCR products were visualized by electrophoresis on a 1% agarose gel stained with GelRed Nucleic Acid Stain (Biotium) in TAE 1X buffer at 80V for 40 min. 1 kb DNA ladder (NEW ENGLANDS BioLabs) was used to quantify and estimate products sizes.

Digestion of PCR products with restriction enzyme *Afa* I (*Rsa* I) (Invitrogen) was used to confirm that single sequences of 16S rRNA gene were obtained. Reactions were performed in a final volume of 20 µl containing 0.5 µl *Afa* I, 1x Buffer T, 0.01% BSA and 8 µl of PCR product as substrate and incubated at 37°C for 2 hours. Digestion products were visualized by electrophoresis on a 2% agarose gel stained with GelRed Nucleic Acid Stain (Biotium) in TAE 1X buffer at 80V for 90 minutes. 100 bp DNA ladder (NEW ENGLANDS BioLabs) was used to quantify and estimate products sizes.

Sequencing was performed by the company “Macrogen Europe” (Amsterdam, The Netherlands). The nucleotide sequences obtained were analyzed using the RDP (Ribosomal Database Project) Naive Bayesian rDNA Classifier database (“Confidence threshold” 80%), and the NCBI GenBank database by the nucleotide-BLAST program, MEGABLAST (“Highly similar sequences”).

3.2.7 Search for *dhlA* gene

PCR amplification of the *dhlA* gene coding for a 1,2-DCA haloalkane dehalogenase was performed with degenerate or specific primers (Attached A) as described for aerobic microcosms using DNA extracted from pellets or colonies as template.

PCR products were sequenced by the company “Macrogen Europe” (Amsterdam, The Netherlands) and nucleotide sequences were analyzed using the NCBI GenBank database by the nucleotide-BLAST program, MEGABLAST (“Highly similar sequences”).

3.2.8 Whole genome sequencing

Selected S10x cultures were sequenced using a Whole Genome Sequencing (WGS) approach. Shotgun libraries, sequencing on an Illumina MiSeq platform with 300 bp paired-end reads and bioinformatic analysis were performed by BMR Genomics (Padova, Italy). Briefly, the bioinformatics pipeline included the following steps: residual PCR primer sequences removal and low quality bases (Q <30) and short reads (length <150) filtration by Adapter Trimming and Preprocessing with Fastp v0.23.2; Reads Alignment on the reference genome of the species under analysis with Bowtie 2 v2.4.5; reads mapped are extracted and used in the following steps; Profiling with MetaPhlan v4.0.1 in order to evaluate the sample content, including possible contaminants; Genome Assembly with SPAdes v 3.15.5; Assembly Evaluation with QUAST v 5.2.0 and with BUSCO v 5.4.3; Gene Prediction with Prokka v 1.14.6 and Gene Annotation with eggNOG-mapper v 2.1.7.

In parallel another bioinformatic analysis was conducted. Differences in pipeline steps are the following: residual PCR primer sequences removal and low quality bases (Q <30) and short reads (length <150) filtration was performed by Trimmomatic (v. 0.38.1); Profiling was performed by Kraken2 (v. 2.2.1); Genome Assembly with SPAdes v 3.15.4.

The sequences of annotated genes were compared to those present in the NCBI nr/nt database by the nucleotide-BLAST program.

3.3 Results and discussions

3.3.1 Isolation of 1,2-DCA dechlorinating cultures

The aerobic enrichment cultures obtained from S1 and S2 microcosms in *ox* and *co* conditions (namely BIIS1ox, BIIS1co, BIIS2ox, BIIS2co) were used to further enrich 1,2-DCA dechlorinating strains and attempt their isolation. In particular, among these, cultures BIIS1ox, BIIS1co and BIIS2co were proven to be dechlorinating on the basis of chemical analysis, bacterial community composition analysis and *dhlA* gene detection (Chapter II). A total of 15 isolates or mixed cultures were obtained (Tab. 3.1).

Original culture	N. cultures on 1,2-DCA agar medium
BIIS1ox	9
BIIS1co	1
BIIS2ox	2
BIIS2co	3

Table 3.1. Number of cultures obtained by enrichment on 1,2-DCA agar medium from aerobic BIII enrichment cultures set up from biostimulation treatments.

After the first enrichment step on 1,2-DCA agar medium, the 9 cultures from BIIS1ox were selected for further analysis, while the others were stored for future studies.

The cultures were named after the third enrichment culture they were obtained from: BIIS1ox1a1, BIIS1ox1a2, BIIS1ox1a2p, BIIS1ox2c1, BIIS1ox2c2, BIIS1ox3aN, BIIS1ox3a, BIIS1ox3b, BIIS1ox3bx; in the following paragraphs and in Figures 3.4 and 3.5, the cultures will be briefly referred to as 1a1, 1a2, 1a2p, 2c1, 2c2, 3aN, 3a, 3b, 3bx respectively.

In order to obtain pure cultures, up to five purification steps on 1,2-DCA amended agar medium were performed by periodically picking and transferring single colonies into fresh medium. Six out of nine cultures (1a1, 1a2, 1a2p, 2c2, 3aN, 3bx) were found able to keep growing on 1,2-DCA as sole carbon and energy source, and unable to grow on control medium not amended with the contaminant (Fig 3.3); two cultures (3a, 3b) grew on both 1,2-DCA and control medium; one culture (2c1) stopped growing after the first transfer into fresh 1,2-DCA medium. For every culture, growth on solid mineral medium amended with 1,2-DCA occurred in 3-7 days.

Cultures 1a1, 1a2, 1a2p, 2c2, 3aN, 3bx grew on 1,2-DCA amended medium as small yellow circular colonies; 3a and 3b grew as punctiform clear colonies; 2c1 was characterized by small, pink-pigmented colonies (Fig. 3.4).

Purification of isolates was difficult because of the small dimensions of colonies, and for the presence, in some cases, of several punctiform clear colonies interspersed all over the cultures and also in control media not amended with 1,2-DCA.



Figure 3.3. Growth of a single colony-derived culture streaked on 1,2-DCA MSM agar medium (right); no growth is observed on not amended MSM medium (left).

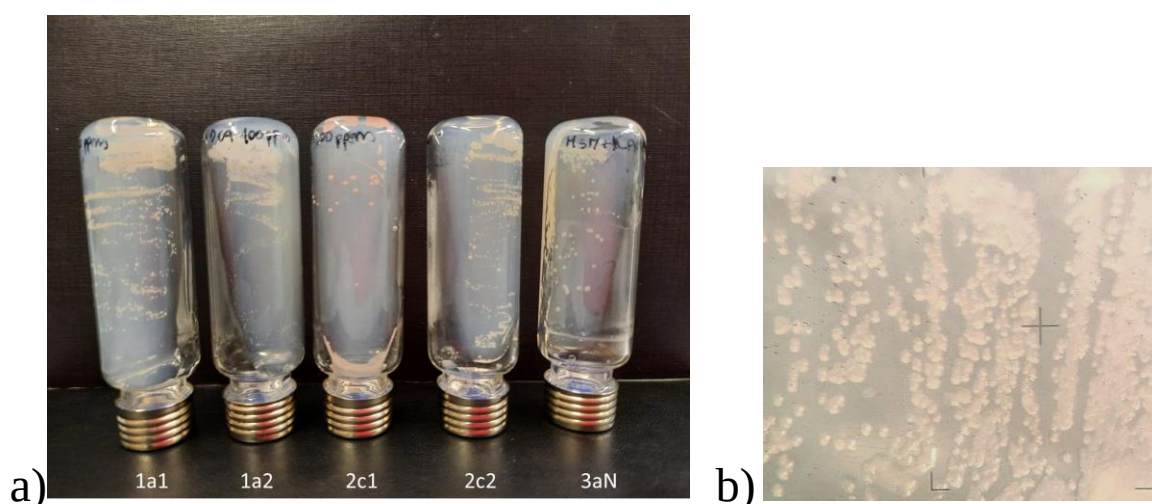


Figure 3.4. a) Some isolates obtained from S10x enrichment culture on mineral medium amended with 1,2-DCA as sole carbon and energy source; b) single colonies of isolate BIIS10x2c2 observed at the stereomicroscope.

3.3.2 Biodegradation abilities of the cultures BIIS10x

The cultures that kept growing on 1,2-DCA agar medium were tested for their biodegradation abilities in liquid 1,2-DCA medium. Growth in liquid mineral medium amended with 1,2-DCA occurred in the form of flocs for some of the cultures or pale turbidity for others. Chloride assay confirmed six cultures (1a1, 1a2, 1a2p, 2c2, 3aN, 3bx) could grow on 1,2-DCA as sole carbon and energy source, showing turbidity and releasing chloride from dechlorination (Fig. 3.5). The remaining cultures (3a, 3b) did not either show

turbidity or Cl^- release and were thus considered not able to grow on 1,2-DCA as sole carbon source. The dechlorinating cultures were pelleted to collect biomass for DNA extraction and subsequent identification and dehalogenase genes detection.

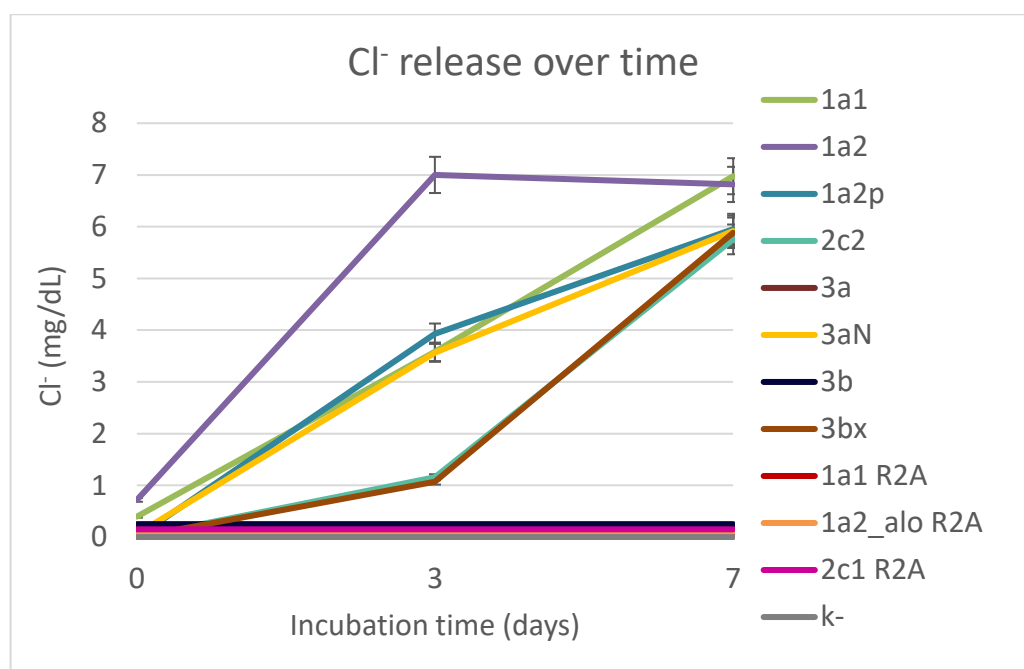


Figure 3.5. Assessment of 1,2-DCA biodegradation abilities of the BIIS1 cultures. Chloride release assay was performed on cultures at 3 and 7 days of incubation. 1a1, 1a2, 1a2p, 2c2, 3a, 3aN, 3b, 3bx: abbreviated names of cultures obtained on 1,2-DCA medium; 1a1 R2A, 1a2_alo R2A, 2c1 R2A: pure cultures obtained on R2A medium (see below).

3.3.3 Detection of the haloalkane dehalogenase gene *dhlA* in dechlorinating cultures

The same primer sets used to PCR probe DNA extracted from S1 ox enrichment cultures (Chapter II) were used to search for the *dhlA* gene in the cultures. The haloalkane dehalogenase gene *dhlA* was searched in DNA extracted from pellets obtained after growth on 1,2-DCA medium. An amplicon of the expected size was obtained for cultures already identified as dechlorinating on the basis of Cl^- assay (1a1, 1a2, 1a2p, 2c2, 3aN, 3bx) both with degenerate primers DHMf/DHMr (Govender et al., 2011) (450 bp) and with specific primers DHLA 319F/603R (Munro et al., 2013) (284 bp) with an optimized protocol; amplicons of expected size were also obtained with DHLA 380F/548R primer set (Munro et al., 2013) (168 bp), but several unspecific products were also amplified.

Fragments obtained with DHLA 319F/603R primer set were selected for sequencing. Sequences obtained from each culture, ranging from 158 to 184 bp, were identical and showed 99-100% identity with *dhlA* gene from *Xanthobacter* sp., *Ancylobacter* sp. and

Starkeya sp. (Tab. 3.2) by comparison with sequences in NCBI GeneBank database, in agreement with results of *dhlA* detection in enrichment cultures (Chapter II).

Culture	Seq lenght (bp)	BLAST best match (nr/nt database) (Accession)	Per. Ident	Reference
1a1	158 - 184	<i>Xanthobacter flavus</i> strain EL8 plasmid pDCA, complete sequence (KU922121.1)	99-100%	(Munro et al., 2016)
1a2		<i>Xanthobacter autotrophicus</i> strain EL4 plasmid pDCA (KU922120.1)	99-100%	
1a2p		<i>Starkeya novella</i> strain EL1 plasmid pDCA, complete sequence (KU922118.1)	99-100%	
2c2		<i>Ancylobacter aquaticus</i> strain DH2 haloalkane dehalogenase (<i>dhlA</i>) gene, complete cds (FJ573162.1)	99-100%	(Govender & Pillay, 2011)
3aN		<i>Xanthobacter flavus</i> clone UE-15 insertion sequence IS1247, haloalkane dehalogenase (<i>dhlA</i>) and transposase genes, complete cds (AY561847.1)	99-100%	(Song et al., 2004)
3bx				
		<i>X. autotrophicus</i> haloalkane dehalogenase (<i>dhlA</i>) gene, complete cds (M26950.1)	99-100%	(Janssen et al., 1989)

Table 3.2. Detection of haloalkane dehalogenase gene (*dhlA*) in DNA extracted from dechlorinating cultures. BLAST best matches for haloalkane dehalogenase gene sequences obtained with DHLA 319F/603R primer set. 1a1, 1a2, 1a2p, 2c2, 3a, 3aN, 3b, 3bx: abbreviated names of cultures obtained on 1,2-DCA medium.

3.3.4 Identification of dechlorinating isolates

Preliminary identification attempts, carried out by 16S rRNA gene colony PCR, led to inconsistent results.

Even when single colonies-derived pure cultures seemed to be achieved on the basis of phenotypes, microscopic observations by stereomicroscope and restriction analysis on 16S rRNA gene amplified by colony PCR, the identification attempts led either to bad quality sequences with low identity or to different identifications of the same culture, suggesting that cultures were not pure.

As colony PCR was not resolutive, bacterial pellets were collected from 1,2-DCA dechlorinating cultures and DNAs extracted from cultures were used for 16S rRNA gene amplification and sequencing. Results of taxonomic identifications are reported in tab 3.3. Within dechlorinating cultures, three sequences (of 1a1, 2c2 and 3aN) were affiliated to *Ochrobactrum anthropi* with 97-99% identity. 16S rRNA sequences from two other

dechlorinating cultures (1a2 and 1a2p) were affiliated to *Pseudomonas khazarica* with 94-97.5% identity. However low identity obtained (94%) was attributed to the bad quality of the sequence, suggesting it was still a mixed amplicon. Mixed sequences were also obtained from 3bx, hampering its identification.

Culture on 1,2-DCA MSM	Seq lenght (bp)	BLAST best match (16S_typesrain database)	Per. Ident
1a1	484	<i>Ochrobactrum anthropi</i> ATCC 49188 (NR_074243.1)	97%
1a2	484	<i>Pseudomonas khazarica</i> strain TBZ2 (NR_169334.1)	94%
1a2p	995	<i>Pseudomonas khazarica</i> strain TBZ2 NR_169334.1	97.5%
2c2	949	<i>Ochrobactrum anthropi</i> ATCC 49188 NR_074243.1	98%
3aN	975	<i>Ochrobactrum anthropi</i> ATCC 49188 NR_074243.1	99%
3bx		/	

Table 3.3. Identification of dechlorinating cultures obtained from BIIS1ox enrichment culture on 1,2-DCA agar medium. BLAST best matches for 16S rRNA sequences. 1a1, 1a2, 1a2p, 2c2, 3a, 3aN, 3b, 3bx: abbreviated names of cultures obtained on 1,2-DCA medium.

3.3.5 Isolation in pure culture on nutrient medium

A further purification step was attempted on a low nutrient medium, R2A. The transfer of single colonies from each of the nine cultures from 1,2-DCA medium to R2A medium resulted in the isolation of a single strain, with the exception of 1a2 and 1a2p, that after growth on R2A resulted in the isolation of colonies showing two different phenotypes, leading to the isolation of 11 pure cultures. Obtaining more than one isolate from the transfer of a single colony further confirmed that some cultures obtained on 1,2-DCA agar medium were probably mixed communities. The pure cultures obtained on R2A were named from the original mixed culture on 1,2-DCA medium labeled with R2A (e.g., 1a1 R2A, 1a2 R2A and so on). R2A isolates that showed similar or identical phenotypes were grouped by comparison of ITS profiles. Based on phenotypes and ITS profiles, some isolates were selected as representatives for chloride release assay and identification by 16S rRNA sequencing. In particular the isolate 1a1 R2A was selected as representative of isolates with an ITS profile shared by 1a1 R2A, 1a2 R2A, 1a2p R2A, 2c2 R2A, 3aN R2A, 3a R2A, 3b R2A, 3bx R2A; the isolate 1a2_alo R2A was selected as representative of isolates with an

ITS profile shared by 1a2_alo R2A and 1a2p_alo R2A; 2c1 R2A showed a distinguishable phenotype. All isolates growing on R2A were found unable to grow on 1,2-DCA as sole carbon and energy source by chloride release assay (Fig. 3.5) and gave negative result when tested for the presence of the *dhlA* gene, as expected.

Isolates obtained on R2A were grown in liquid medium and pellets were used for DNA extraction and identification by 16S rRNA amplification and sequencing. These isolates were affiliated to *Ochrobactrum*, *Pseudomonas* and *Methylobacterium* spp. (Tab. 3.4). The isolation of *Ochrobactrum* and *Pseudomonas* confirmed the preliminary identification carried out on cultures grown on 1,2-DCA MSM and, therefore, the presence of these genera in the dechlorinating mixed cultures.

Isolates on R2A	Seq lenght (bp)	BLAST best match (16S_typesrain database)	Per. Ident
1a1 R2A (Representative of 1a2p R2A, 2c2 R2A, 3aN R2A, 3bx R2A)	1089	<i>Ochrobactrum anthropi</i> ATCC 49188 (NR_074243.1)	98.5%
1a2 R2A	1243	<i>Ochrobactrum anthropi</i> ATCC 49188 (NR_074243.1)	98%
3a R2A	1111	<i>Ochrobactrum anthropi</i> ATCC 49188 (NR_074243.1)	98%
3b R2A	1148	<i>Ochrobactrum anthropi</i> ATCC 49188 (NR_074243.1)	98%
1a2_alo R2A (Representative of 1a2p_alo R2A)	1180	<i>Pseudomonas khazarica</i> strain TBZ2 (NR_169334.1)	99%
2c1 R2A	905	<i>Methylobacterium aminovorans</i> strain JCM 8240	98%

Table 3.4. Identification of isolates obtained on R2A medium. BLAST best matches for 16S rRNA sequences of the isolates.

Ochrobactrum and *Methylobacterium* belong to families Brucellaceae and Methylobacteriaceae, respectively, within the order Hyphomicrobiales (Alphaproteobacteria); *Pseudomonas* belongs to family Pseudomonadaceae within the Gammaproteobacteria. The Brucellaceae were among the most abundant families detected in the BIIS1ox enrichment culture, source of the isolates; conversely, Methylobacteriaceae and Pseudomonadaceae were <1% or not detected in BIIS1ox enrichment culture.

As already reported in Chapter II, an *Ochrobactrum* strain using vinyl chloride (VC) as sole carbon and energy source by a plasmid-borne gene encoded alkene monooxygenase was described (Danko et al., 2004). With reference to *Pseudomonas* sp., some strains are known

to dechlorinate 1,2-DCA by a monooxygenase-mediated oxidative pathway (Hage & Hartmans, 1999); nevertheless, neither the *Ochrobactrum* nor the *Pseudomonas* isolated here can grow on 1,2-DCA as sole carbon source.

Methylobacterium species are pink-pigmented facultative methylotrophs (Green & Ardley, 2018). All of the aerobic hydrolytic DCA degraders isolated so far (*Xanthobacter*, *Ancylobacter* and *Starkeya*) are also facultative methylotrophs, and this seems to be related to the fact that the methanol dehydrogenase Mox involved in methanol degradation, is an effective 2-chloroethanol dehydrogenase (involved in 1,2-DCA hydrolytic pathway) (Munro et al., 2016). Although the *Methylobacterium* sp. isolated did not grow on 1,2-DCA as sole carbon source, its initial growth on the 1,2-DCA medium could be related to the degradation of 2-chloroethanol, an intermediate in 1,2-DCA degradation, likely produced by dechlorinating strain initially present in co-culture.

Based on these results, it was clear that the pseudo-isolates obtained on 1,2-DCA were indeed mixed culture or consortia composed of a few bacterial strains not all involved in direct dechlorination.

It was concluded that the results of isolation and characterization of the isolates led to 6 stable 1,2-DCA dechlorinating oligospecific consortia, suggested that the dechlorinating cultures were actually stable 1,2-DCA dechlorinating consortia, made up of both dechlorinating and non-dechlorinating members.

3.3.6 Whole genome sequencing of selected dechlorinating consortia

Two dechlorinating consortia were selected for further analysis on the basis of the preliminary identification and the quality of DNA extracted. Cultures 2c2 and 1a2p, identified by 16S rRNA gene sequencing as *Ochrobactrum* and *Pseudomonas* respectively (Tab. 3.3) (and here defined as *Ochrobactrum* consortium and *Pseudomonas* consortium) were analyzed by a WGS (Whole Genome Sequencing) approach.

3.3.6.1 Community profiling of the aerobic BIIS1ox dechlorinating consortia

Two consortia, tentatively named *Ochrobactrum* consortium and *Pseudomonas* consortium, were selected for WGS. Bioinformatic analysis on sequences obtained were carried out by using two different tools and results were compared. In particular, Metaphlan, a marker gene-based taxonomic profiler, and Kraken, a multiple genomes-based taxonomic classifier (Breitwieser et al., 2019) were used. Community profiling of both consortia, made by both tools are reported in Tab. 3.5. Kraken resulted in a deeper detection of the components of the consortia and a lower % of unclassified sequences in respect to Metaphlan.

According to the Kraken analysis, both consortia are mainly made up of three more abundant genera (within Alphaproteobacteria or Gammaproteobacteria), accounting for more than 50% of the communities, followed by other minor components.

In particular, the *Pseudomonas* consortium (referred to as culture 1a2p in the previous paragraphs and in table 3.3) was dominated by *Pseudomonas* (22%), followed by *Ancylobacter* (20%) and *Ochrobactrum* (17%) spp. Other less abundant members included *Starkeya* (7%), *Dyella* (2%) spp. and other unclassified or <2% genera, that included *Xanthobacter* sp. (1%). The prevalence of *Pseudomonas* in the culture is consistent with 16S rRNA sequencing results.

In the *Ochrobactrum* consortium (culture 2c2 in the previous paragraphs and table 3.3), *Ancylobacter* sp. was dominant (30%), followed by *Ochrobactrum* (13%) and *Starkeya* (10%) spp. *Rhodopseudomonas* (2%), *Xanthobacter* (2%), *Bradyrhizobium* (2%) spp. and other unclassified or <2% genera were also present. All components of this consortium are Alphaproteobacteria (order Hyphomicrobiales)

Other components of both consortia included unclassified Actinobacteria (2%).

Consortium	16S rRNA identity (Table 3.3)		Community profiling (WGS)		
	BLAST best match (16S_typesrain database) (accession)	Id. (%)	Genera ($\geq 2\%$)	Rel. Abundance (%)	
				MetaPhlAn	Kraken
1a2p	<i>Pseudomonas khazarica</i> strain TBZ2 (NR_169334.1)	97.5	<i>Pseudomonas</i> sp.	24	22
			<i>Ochrobactrum</i> sp.	21	17
			<i>Ancylobacter</i> sp.		20
			<i>Starkeya</i> sp.		7
			<i>Dyella</i> sp.		2
			Unclassified /others <2%	55	32
			(including <i>Xanthobacter</i>)		
2c2	<i>Ochrobactrum anthropi</i> ATCC 49188 (NR_074243.1)	98	<i>Ancylobacter</i> sp.		30
			<i>Ochrobactrum</i> sp.	19	13
			<i>Starkeya</i> sp.		10
			<i>Rhodopseudomonas</i> sp.	2	4
			<i>Xanthobacter</i> sp.		2
			<i>Bradyrhizobium</i> sp.		2
			Unclassified /others <2%	79	39

Table 3.5. Community profiling of two 1,2-DCA dechlorinating consortia obtained from enrichment culture BIIS1ox. Results of the taxonomic identification obtained by 16S sequencing and by Whole Genome Sequencing (WGS) using two bioinformatic tools (MetaPhlAn and Kraken). Genera $\geq 2\%$ are reported.

Considering the role of each member of the consortia, *Ochrobactrum* and *Pseudomonas* isolated from the consortia on R2A medium (whose 16S rRNA sequences were >98% identical to 16S rRNA sequences of *Ochrobactrum* and *Pseudomonas* detected in the consortia) were unable to grow on 1,2-DCA as sole carbon and energy source when in pure culture. 1,2-DCA dechlorination abilities of other genera detected in the consortia, could not be assessed as they were not isolated in pure culture.

Strains of the genera *Ancylobacter*, *Xanthobacter* and *Starkeya*, detected in both the consortia, are known 1,2-DCA hydrolytic degraders (Janssen et al., 1985; Munro et al., 2016; Van Den Wijngaard et al., 1992). These genera could be responsible of 1,2-DCA degradation by the consortia.

The role of the other components in the two consortia is not clear. It could be supposed that their growth is dependent on some intermediates of 1,2-DCA degradation or metabolites produced by other members of the community, such as 2-chloroethanol from 1,2-dichloroethane hydrolysis, that can temporarily accumulate in the medium (Janssen et al., 1987). Different *Pseudomonas* sp. strains, as an example, were found able to degrade 2-chloroethanol thanks to specific or non-specific quinoprotein alcohol dehalogenase enzymes, NAD-dependent aldehyde dehydrogenases and a chloroacetate dehalogenase (Dijk et al., 2004; Strotmann et al., 1990).

Rhodopseudomonas, in particular *Rhodopseudomonas palustris*, the species detected in the *Ochrobactrum* consortium, is a metabolically versatile bacterium capable of four different modes of growth (photoautotrophic, photoheterotrophic, chemoheterotrophic, chemoautotrophic). In absence of light (as the incubation conditions for the dechlorinating consortia) lignin, monomers, thiosulphate, H₂ and other organic or inorganic compounds can supply reductant for both energy generation and carbon dioxide fixation. This bacterium also degrades nitrogen-containing compounds, including amino acids and heterocyclic aromatic compounds, and it dehalogenates chlorinated benzoates and chlorinated fatty acids. Moreover, the genome contains several dehydrogenases, oxidoreductases, genes for carbon degradation pathways enzymes and several mono- or dioxygenase and cytochrome P450 genes (Larimer et al., 2004). Furthermore, *Rhodopseudomonas* sp. was previously found as a member of the bacterial community of a 1,2-DCA-degrading pilot-scale membrane bioreactor (MBR) also including *Ancylobacter* and *Xanthobacter* (Munro et al., 2013).

Dyella, in particular the species *Dyella thiooxydans* detected in the *Pseudomonas* consortium, is a facultatively chemolithotrophic, thiosulfate-oxidizing bacterium

(Anandham et al., 2011). Biphenyl and triclosan degrading *Dyella* sp. strains are known (Ang et al., 2009; S. Wang et al., 2018). Moreover, a chloroacetaldehyde dehydrogenase gene (*aldA*) from the 1,2-DCA degrading bacterium *Ancylobacter aquaticus* shares 87% gene homology with a chloroacetaldehyde dehydrogenase gene from *Dyella thiooxydans* (Kumar et al., 2018).

Bradyrhizobium sp. is nitrogen fixing bacterium. 1,2-dibromoethane- degrading *Bradyrhizobium* sp. strains are known. These bacteria were found to carry a haloalkane dehalogenase mainly active toward 1,2-dibromoethane, that can convert several other halogenated substrates, including 1,2-dichloroethane, to the corresponding alcohols, although subsequent alcohols degradation was not observed (Sato et al., 2005).

3.3.6.2 Detection of catabolic genes in the aerobic dechlorinating consortia

Genes encoding for all the enzymes involved in 1,2-DCA degradation were detected based on PROKKA annotation. The sequences of annotated genes were compared to those present in nr/nt NCBI database.

Sequences of the haloalkane dehalogenase gene *dhla* were detected, as expected, in both consortia. Sequences (933 bp) showed 100% identity with *dhla* gene from *Xanthobacter* (KU922121.1; KU922120.1), *Ancylobacter* (FJ573162.1) and *Starkeya* (KU922118.1) spp. The region upstream (172bp) the *dhla* gene showed 99% identity with a sequence encoding for a TetR family transcriptional regulator found immediately upstream of *dhla* gene in *Xanthobacter* strain GJ10 and in the pDCA catabolic plasmid found in *Xanthobacter* spp. and *Starkeya novella* strains isolated from a groundwater treatment plant in Australia (Munro et al., 2016). Upstream this element, a sequence (127bp) was found, showing 99% identity with an insertion element IS1247 previously found to be located beside the *dhla* gene in *X. autotrophicus* GJ10 (X84038.1) (Van Der Ploeg et al., 1995). This insertion element was also found in the flanking region of the *dhla* gene in the 1,2-DCA degrader *X. flavus* UE15 (Song et al., 2004). In both consortia, *dhla* sequences are located in a contig including sequences for an *AraC* family transcriptional regulator, for transposase Tn3 family proteins and for a toxin-antitoxin gene pair (*relE-relB* family), suggesting it could be located on a plasmid. Similar elements, although in a different order, were also reported on the pDCA catabolic plasmid.

The second step of the hydrolytic 1,2-DCA dechlorination consists in the transformation of 2-chloroethanol into chloroacetaldehyde, mediated by an alcohol dehydrogenase. The methanol dehydrogenases of methylotrophs are effective 2-chloroethanol dehydrogenases

(Munro et al., 2016). In both consortia, sequences showing 85-93% similarity to *Xanthobacter* (CP085618.1), *Ancylobacter* (CP083239.1) and *Starkeya* (CP002026.1) spp. methanol dehydrogenase genes (*mox*) were detected. Other sequences showed 81-85% similarity with zinc-binding alcohol dehydrogenase genes of *Starkeya* and *Ancylobacter* spp. In *Xanthobacter GJ10* a quinoprotein alcohol dehydrogenase is involved in the metabolism of methanol and 2-chloroethanol (Janssen et al., 1987). In the *Pseudomonas* consortium a sequence showing 99% similarity with a quinoprotein ethanol dehydrogenase gene of *Pseudomonas khazarica* was retrieved (CP068551.1). Other sequences showed 100% similarity to alcohol dehydrogenase genes from *Ochrobactrum* sp. (CP061772.1).

Chloroacetaldehyde is degraded by an inducible NAD-dependent chloroacetaldehyde dehydrogenase. Sequences identical or similar to *aldB* aldehyde dehydrogenase from *Ochrobactrum* sp. (100% identity) or *Xanthobacter*, *Ancylobacter* and *Starkeya* spp. (80% similarity) and sequences identical to *Ochrobactrum* sp. acetaldehyde dehydrogenase *acoD* gene were found.

Sequences encoding for the haloacetate dehalogenase *DhlB*, involved in the last step of the hydrolytic pathway, were detected in both consortia, showing 90% identity with the chromosomal *dhlB* gene from *X. autotrophicus GJ10* (M81691.1).

Moreover, sequences of another haloacetate dehalogenase were retrieved in both consortia. These sequences were 100% identical to the haloacetate dehalogenase *dhlB2* found in the variants of the pDCA plasmid (Munro et al., 2016).

Similarly, the strains of *Xanthobacter* and *Starkeya* isolated by Munro et al. and carrying the pDCA plasmid, carried two haloacetate dehalogenase genes: one related to the chromosomal *dhlB* gene from *X. autotrophicus GJ10* and likely chromosomally located, and another one, pDCA plasmid-borne, not closely related to strain GJ10 gene, but capable of chloroacetate dechlorination (Munro et al., 2016).

In both consortia, this haloacetate dehalogenase is located on the same contig of a sequence encoding for a transposase of the insertion sequence ISPme1 found associated to the *dhlB2* gene carried on pDCA plasmid. This transposase is also closely related to that of an insertion sequence previously found upstream of the *dhlB* gene in *Xanthobacter strain GJ10*. Munro et al. (2016) hypothesized that ISPme1 was responsible for the *dhlB2* gene recruitment into the plasmid pDCA and that it may play a role in activating the transcription of *dhlB2*. The strong association of gene *dhlB2* with ISPme1 transposase gene may confirm the role of ISPme1 in *dhlB* genes mobilization and recruitment into plasmids.

On a separate contig, plasmid replication, stabilization and mobilization systems were found. A *repA* gene sequence, encoding for a transcriptional regulator, was found to be identical to the replication initiator gene of pDCA plasmid. This sequence is related to replication initiators of catabolic plasmids from the pTAR plasmid family, that are widespread within the Alphaproteobacteria (Munro et al., 2017). The *repA* sequence also shared 92% identity with a plasmid sequence of an *Ochrobactrum* strain (CP061775.1). The mobilization system consisted of *mobC*, *traA*, and *traG* homologs (encoding for a relaxosome protein, relaxase and a conjugative coupling protein, respectively), identical to those found in pDCA plasmid variants (Munro et al., 2017). Within the same contig, other element of the pDCA plasmids were found, including a RelE / RelB toxin-antitoxin system, a HigB-like toxin killer system, a DNA methylase gene and a plasmid resolvase gene. This may suggest the presence of some variants of the pDCA plasmid or similar plasmids of the pTAR family in the two consortia.

3.4 Conclusions

The aerobic biostimulation treatment of a 1,2-DCA contaminated groundwater sample led to stable dechlorinating aerobic enrichment cultures that were used to isolate 1,2-DCA aerobic dechlorinating bacteria. Six cultures able to grow on 1,2-DCA as sole carbon and energy source, releasing Cl^- were positive to the *dhlA* gene detection, suggesting that 1,2-DCA dechlorination occurred via the hydrolytic pathway. Based on a preliminary identification of the dechlorinating colonies by 16S rRNA gene sequencing, the genera *Ochrobactrum* and *Pseudomonas* were detected; further purification step on the nutrient R2A medium revealed that the dechlorinating cultures were not pure cultures as they included also non-dechlorinating bacteria. A Whole Genome Sequencing (WGS) analysis carried out on two selected cultures, confirmed that single colony-derived bacterial cultures were actually stable oligospecific dechlorinating consortia. The consortia could only grow on 1,2-DCA amended mineral medium, and the transfer into R2A nutrient medium led to the loss of the dechlorinating members of the community. The WGS approach revealed a different bacterial composition between the two analyzed consortia. Both consortia were mainly made up of Alphaproteobacteria, including the known dechlorinating genera *Ancylobacter*, *Starkeya* and *Xanthobacter* in different relative abundances. Other members of the consortia included *Ochrobactrum*, *Pseudomonas*, *Rhodopseudomonas* and *Dyella* among the most abundant ($\geq 2\%$). These genera are not known as 1,2-DCA degrading bacteria. Moreover, *Ochrobactrum* and *Pseudomonas*, isolated from the consortia, were not able to grow on 1,2-DCA as sole carbon and energy source when in pure culture. Nevertheless, these genera could grow on intermediates of 1,2-DCA degradation likely carried out by *Ancylobacter*, *Starkeya* and *Xanthobacter*, such as 2-chloroethanol. Genes encoding for the enzymes involved in 1,2-DCA hydrolytic dechlorination steps were featured by PROKKA annotation in the two consortia. *dhlA* genes identical to the *dhlA* gene obtained so far from the known 1,2-DCA hydrolytic degraders were detected in both consortia. Two haloacetate dehalogenase homologs genes were detected, one similar to the chromosomal located *dhlB* gene from *X. autotrophicus* GJ10 and one related to a plasmid-borne homolog *dhlB2* gene from 1,2-DCA dechlorinating strains of *Xanthobacter* and *Starkeya* recently isolated in Australia. The presence of other elements related to the catabolic plasmid pDCA (carrying the *dhlA* and *dhlB2* genes) from these isolates suggests a variant of this plasmid or a similar one could carry these genes in the *Ancylobacter*, *Starkeya* and *Xanthobacter* strains member of the dechlorinating consortia here described. This work confirms 1,2-DCA hydrolytic degradation is associated to the presence of a *dhlA* gene

identical to the *dhlA* gene of all the other 1,2-DCA degrading bacterial strains isolated so far in the Netherlands, Germany, Australia, South Korea, South Africa and affiliated to genera *Xanthobacter*, *Ancylobacter* and *Starkeya*. Moreover, this work confirms and expands the already wide geographical distribution of the *dhlA* gene. Further studies are required to confirm the chromosomal or plasmid localization of these genes and the eventual assignation of the plasmid to one or more of the members within the consortia. Further investigation could also provide information on the mechanisms of plasmid or dehalogenase genes mobilization to better understand if and how the 1,2-DCA degradation abilities can spread among bacteria in presence of the contaminant. The role of the other members of the consortia is also worth to be further investigated.

CHAPTER IV

Poly- β -hydroxybutyrate as electron donor: a pilot study in column

After assessing the possibility to biostimulate reductive and aerobic 1,2-DCA dechlorination processes in CAHs contaminated groundwater by microcosms studies, a pilot study in column was carried out to test the efficacy of two simulated 1,2-DCA bioremediation approaches. In particular, 1,2- DCA removal was evaluated in a column simulation of a poly- β -hydroxybutyrate-based permeable reactive barrier (PRB) and of an aerobic approach.

The permeable reactive barrier (PRB) technology is a commonly accepted standard practice for groundwater remediation. PRB can be defined as an *in situ* treatment zone positioned in a contaminated aquifer with the aim to passively capture a contaminant plume and remove or degrade the contaminants, letting uncontaminated water flow out of it (Stroo & Ward, 2010). PRB-mediated remediation is an advantageous approach because the reactions can proceed *in situ* under ambient groundwater conditions. This technology is usually used in combination with metallic iron, exploiting its properties of chlorinated organic compounds degradation (Stroo & Ward, 2010). In this study, this technology is used in combination with the biopolymer poly- β -hydroxybutyrate (PHB).

PHB is a completely biodegradable polymer whose production, for several commercial application, can rely on the use of both pure and mixed bacterial cultures from several sources, including low-cost feedstock such as wastewater (Corsino et al., 2022). Poly- β -hydroxybutyrate, here used in powder form to fill the PRB, has already been proved to be effective in sustaining reductive dechlorination processes (Aulenta et al., 2008; Baric et al., 2014). PHB, indeed, is a long-lasting fermentable substrate, acting as a slow-release electron donor source, whose fermentation products, acetate and H_2 , can be used as electron donors by Organohalide Respiring Bacteria.

The efficacy of this PHB based-PRB technology in supporting 1,2-DCA biodegradation is tested in this study by using a continuous flow apparatus to feed the columns with a real CAHs contaminated groundwater sample (collected from site S2 of the aquifer under study) and by periodically monitoring 1,2-DCA concentration and acetate production from PHB fermentation.

The columns simulating the PHB based-PRB and the aerobic bioremediation approach, were inoculated with anaerobic and aerobic dechlorinating consortia, respectively, previously microcosm-enriched under appropriate conditions and tested for dechlorinating abilities. At

the end of the experimental activities, microbial communities within the columns were analyzed by 16S rRNA Illumina sequencing to identify the agents responsible for 1,2-DCA biodegradation.

This activity was carried out in collaboration with Dr. Ilenia Cruciata (Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo) during her PhD project, and Dr. Daniele Di Trapani (Department of Engineering, University of Palermo).

The experimental plant was set up at the Department of Engineering. The microbiological and molecular activities were carried out at EMME-Lab, at the Department of Biological, Chemical and Pharmaceutical Sciences and Technologies.

The manuscript describing these activities and results obtained, currently submitted for publication, is reported here.

Degradation of 1,2-dichloroethane in real polluted groundwater by using enriched bacterial consortia in aerobic and anaerobic laboratory-scale conditions

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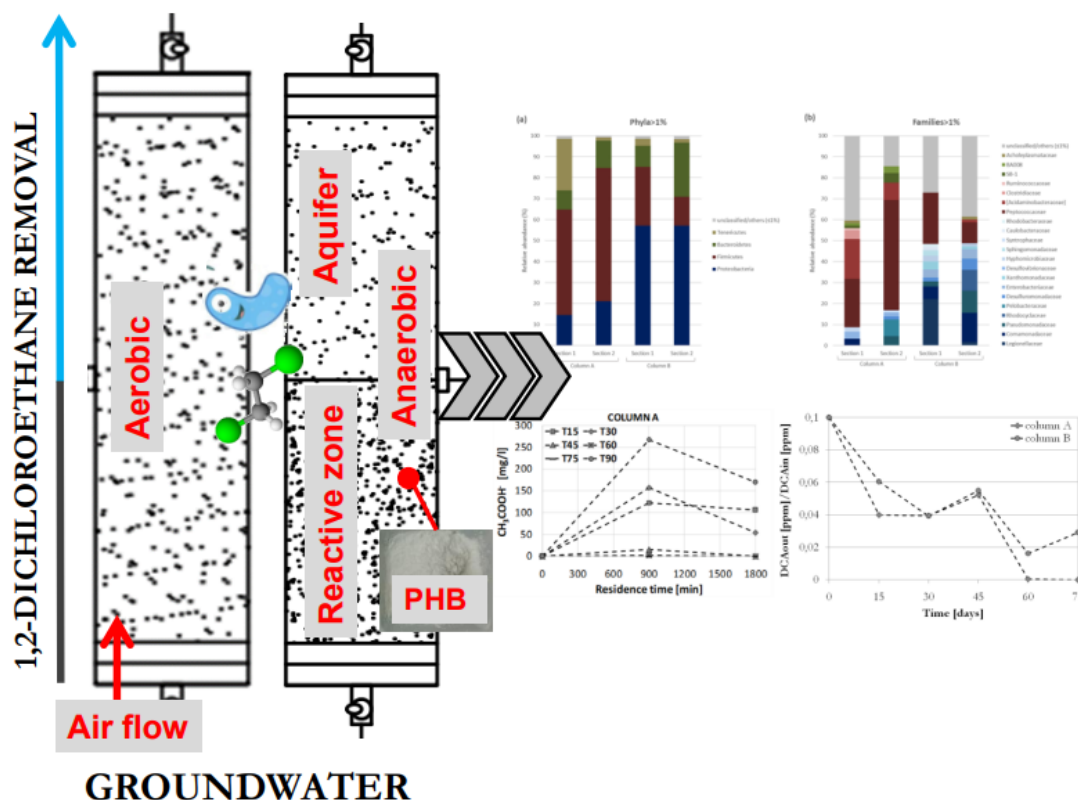
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Abstract

The aim of this work was to gain insights about the feasibility of chlorinated solvents removal through an anaerobic biological process in a laboratory-scale permeable reactive barrier (PRB), based on the use of poly- β -hydroxybutyrate (PHB) as electron donor, and comparing the results with those achieved under aerobic conditions. The experimental plant consisted of two Plexiglas cylindrical columns filled with silica sand and fed with groundwater contaminated by chlorinated solvents (mainly 1,2-dichloroethane, 1,2-DCA); column A simulated a PRB containing PHB powder and worked under anaerobic conditions, in column B an inlet air flow rate ensured aerobic conditions. Both columns were inoculated with dechlorinating bacterial consortia obtained by enrichment cultures from the same contaminated groundwater. Results from column A showed that PHB can be fermented and used as a slow-releasing carbon source for sustaining reductive dechlorination, as revealed by acetate production and complete 1,2-DCA removal. The microbial community detected in column A at the end of the experimental period was mainly enriched in sulfate reducing bacteria that could act as both fermenting and dechlorinating agents. Column B showed a slight lower 1,2-DCA removal efficiency (98%) likely related to the establishment of aerobic (co)metabolic processes.

Keywords: Chlorinated solvents; Permeable reactive barrier; Poly- β -hydroxybutyrate; Biological reductive dechlorination; Direct or cometabolic aerobic oxidation.

Graphical Abstract



1. Introduction

Chlorinated solvents are organic compounds belonging to the class of chlorinated aliphatic hydrocarbons and they are a subgroup of organ-halogen compounds. These compounds have been widely used in various industrial sectors (Anam et al., 2019), as degreasers of mechanical and electronic parts, dry cleaning of metals and clothes, as solvents and raw materials in the chemical-pharmaceutical industry (Chang et al., 2017). The major issues related to these compounds are represented by high toxicity and carcinogenicity; furthermore, they are poorly soluble and degradable and very often, because of improper handling and disposal methods, they can be released into the environment. For this reason they can be found in the environmental matrices, promoting in particular aquifer contamination (APAT and IRSA-CNR, 2003; Bradley, 2003; Anam et al., 2019; Aulenta et al., 2019). To date, in fact, several cases of soils and groundwater contamination by

chlorinated solvents have been reported in technical literature (Majone et al., 2015; Pierro et al., 2016, 2017; Aulenta et al., 2019).

Chlorinated solvents commonly found in the environment belong to the class of chlorinated methanes, ethanes and ethenes, such as 1,2-dichloroethane (1,2-DCA), 1,1,1-trichloroethane (1,1,1-TCA), carbon tetrachloride, methylene chloride, chloroform, tetrachloroethylene (PCE) and trichloroethylene (TCE). Most of them have some peculiar chemical-physical characteristics that describe their behavior in soils and groundwater, including a high vapor pressure, a greater density than water and a very limited water solubility (Ajo-Franklin et al., 2006). Consequently, most of the chlorinated compounds, if released in high quantities, are prone to form dense non-aqueous phase liquids (DNAPLs). Moreover, the high density favors their rapid percolation through the unsaturated soil, reaching the aquifer, where these compounds settle down to the impermeable bottom and are then dispersed along the water flow direction (Sleep and Sykes, 1989; Cohen and Mercer, 1993; Pierro et al., 2016).

Conventionally applied remediation processes, such as Pump & Treat (P&T), are ineffective to solve DNAPLs contamination; these treatments, in fact, rarely allow reaching the low standard limits imposed by Regulations for water reinjection into groundwater; therefore, the final destiny is often the discharge into surface water bodies. Moreover, this treatment is based on contaminant phase transfer, regardless its effective degradation (Starr and Cherry, 1994; Beretta, 2000; Majone, 2009).

This led to the interest in the development of new in situ remediation technologies, including permeable reactive barriers (PRBs) based on physical-chemical and biological processes (Majone et al., 2015; Fan et al., 2017; Cameselle and Gouveia, 2018; Li et al., 2019). PRBs present potential advantages over the P&T in terms of lower management costs, lower energy demand, absence of external discharges as well as less site disturbance issues. Furthermore, this technology promotes the contaminant degradation, rather than its phase transfer.

Concerning PRB based on physical-chemical processes, zero-valent iron (ZVI), activated carbon and natural and synthetic zeolites, have been widely applied (Accardi et al., 2012; Baric et al., 2012; Piscitello et al., 2020; Rossi et al., 2020). ZVI is the most frequently employed reactive media, especially for the treatment of chlorinated hydrocarbon plumes, referring in particular to chlorinated ethenes and ethanes (Odziemkowski et al., 1998; Furukawa et al., 2002). It has been demonstrated that ZVI can effectively degrade these pollutants to nontoxic compounds, such as ethene and ethane, through a series of dechlorination reactions (Baric et al., 2012). However, PRB-ZVI technology is affected by some operational issues, the main

one likely being the long-term performance decrease due to loss of ZVI reactivity and/or porosity reduction (Song and Carraway, 2005; Henderson and Demond, 2007). Indeed, the alkaline pH (8–9) and the presence of ferrous and ferric ions within the barrier, due to ZVI corrosion, can lead to the precipitation of minerals, which can form a passive layer over the iron surface. This aspect might hamper the electron transfer process, thus reducing the iron reactivity (Odziemkowski et al., 1998; Furukawa et al., 2002). Additionally, iron precipitation could cause pore occlusion, resulting in a greater flow velocity, thus reducing the treatment effectiveness due to the decrease of the residence time within the PRB (Morrison and Bartlett, 2009; Baric et al., 2012). However, some compounds, such as chloromethane, dichloromethane and 1,2-DCA, do not undergo chemical reduction in the presence of ZVI (Song and Carraway, 2005; Baric et al., 2012; Song et al., 2023).

In addition of physical-chemical processes, most of chlorinated solvents can be effectively degraded by biological processes carried out by bacteria. The biodegradation of chlorinated compounds can occur through an oxidative (aerobic) or a reductive (anaerobic) reaction, via a direct or cometabolic pathway (Frasconi et al., 2015). In direct aerobic oxidation, the chlorinated compound acts as electron donor and growth substrate for microorganisms, while oxygen is the electron acceptor. Conversely, in aerobic cometabolism, oxygenase enzymes, synthesized by microorganisms for the uptake of growth substrates, fortuitously catalyze the oxidation of the chlorinated compound. Oxidative processes require NADH as source of reducing energy, which is regenerated only following the oxidation of the growth substrate. In aerobic cometabolism of chlorinated compounds, NADH cannot be regenerated, and the biodegradation can continue until the stocks of reducing energy are exhausted (Van Hylckama Vlieg et al., 1996). Furthermore, cometabolism of chlorinated compounds can produce intermediate products very toxic for microorganisms themselves. Figure S1 shows a schematic representation of oxidation process of the growth and cometabolic substrate mediated by monooxygenase enzyme. Generally, low-chlorinated compounds are degraded through an oxidative direct pathway; while polychlorinated compounds can be effectively degraded via reductive dechlorination (Furukawa et al., 2002). Biological reductive dechlorination (BRD) is an anaerobic multi-step reaction carried out by specialized microorganisms present in anoxic environments, such as groundwater ecosystems, in which highly chlorinated compounds are converted to less chlorinated by-products and finally to non-toxic end products, e.g., ethene and ethane (Xiao et al., 2020; Lin et al., 2021; Amanat et al., 2022).

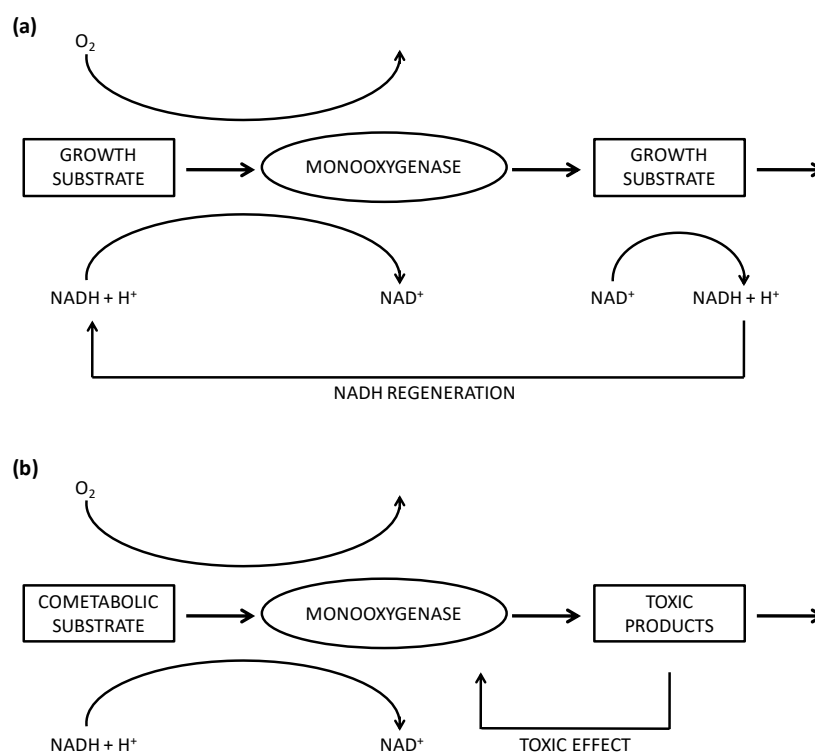


Figure S1. Schematic representation of oxidation process of the growth substrate (a) and the cometabolic substrate (b) mediated by monooxygenase enzyme.

BRD is an anaerobic respiratory process, called dehalorespiration, and microorganisms able to respire chlorinated compounds, utilizing them as terminal electron acceptors, are called organohalide respiring bacteria (OHRBs) (Hug et al., 2013; Song et al., 2023). The reduction mechanism takes place in two stages (Figure S2). The first stage consists in the transfer of a single electron from an electron donor to the chlorinated aliphatic compound with the removal of a chlorine atom and the consequent formation of an alkyl radical. In most cases, the alkyl radical formation is the rate-limiting process in the overall reaction. The second stage consists in a hydrogenolysis reaction, in which the alkyl radical binds a hydrogen ion to itself. BRD is an electron consuming process and is often limited by the lack of suitable electron donors resulting in an incomplete degradation and the accumulation of undesired by-products (Li et al., 2021; Yan et al., 2021; Amanat et al., 2022). Therefore, BRD can be sustained and enhanced through the addition of fermentable substrates (e.g., hydrogen, butyrate, lactate), available as source of electron donors for microorganisms. The additional source of carbon and energy as a co-substrate supports bacterial growth and metabolism, and biodegradation of the target chlorinated compound is carried out by the biochemical capability of microorganisms (Gao and Gu, 2021).

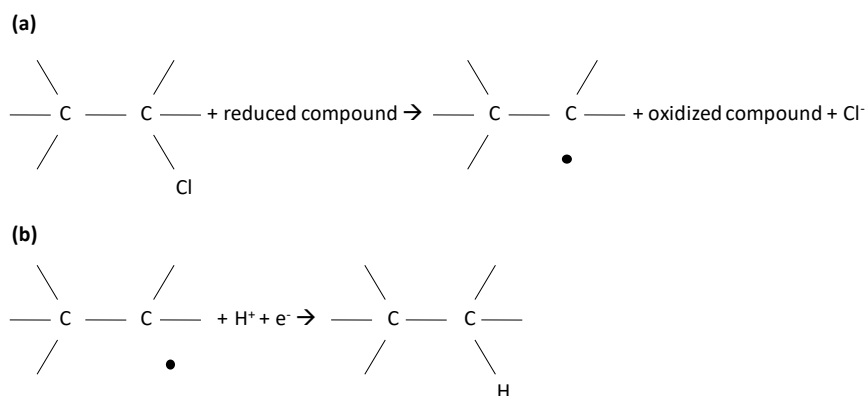


Figure S2. Stage 1 (a) and 2 (b) of the reduction mechanism of a chlorinated compound.

To date, poly-β-hydroxybutyrate (PHB), derived from both pure and mixed microbial culture, has proven to be effective as slow-release electron donor for the BRD process (Aulenta et al., 2008; Pierro et al., 2017; Amanat et al., 2021a, 2021b, 2022). Moreover, various studies confirmed the feasibility to use PHB as reactive medium in PRBs (Baric et al., 2012, 2014; Matturro et al., 2018; Amanat et al., 2021a, 2022). In this light, a bio-PRB represents an environmental biotechnology to use in detoxifying and eliminating pollutants, and remediate contaminated aquifers (Gu, 2020). Furthermore, reactive materials in bio-PRBs provide a carbon supply for the attachment and growth of potentially biodegrading microorganisms transported in the soil water (Freidman et al., 2017). Heretofore, the most common way to produce PHB is to use pure bacterial cultures and selected substrates; however, the main drawback is the high cost associated with fermentative production (Raza et al., 2018). Costs of production are affected by type of carbon source, running cost of fermentation, process productivity, yields on the selected carbon sources and downstream processing (Raza et al., 2018), thus making the production process economically unsustainable (Ivanov et al., 2014; Villano et al., 2014). For these reasons, in recent years, more sustainable processes, based on the use of mixed microbial cultures (MMC) (Valentino et al., 2019; Amanat et al., 2021a) and low-cost feedstocks (such as wastewaters) (Morgan-Sagastume, 2016; Kourmentza et al., 2017; Corsino et al., 2022), have been extensively studied. This represents an economically and environmentally sustainable solution, according to principles of circular bio-economy. To date, there are few experimental studies in which PHB has been used as fermentable substrate to support BRD (Aulenta et al., 2005a, 2005b; Baric et al., 2012, 2014; Matturro et al., 2018; Amanat et al., 2021a, 2021b, 2022); these studies have been conducted on microcosm- or laboratory-scale, ensuring anaerobic conditions within the system, and used synthetic waters contaminated with chlorinated

compounds (PCE, TCE, 1,2-DCA). This aspect, if on one hand simplified the understanding of mechanisms occurring within the system, on the other hand, did not allow to appreciate the possible issues related to the treatment of a real contaminated water, in which there are many other compounds and factors that could cause interferences.

In this context, the aim of the present study was to evaluate the feasibility of a bioremediation approach for a real groundwater contaminated by chlorinated solvents, based on a bio-PRB, using PHB as fermentable substrate and slow-release electron donor to sustain microbial growth and BRD process. An experimental bench-scale apparatus was realized, consisting in two parallel Plexiglas cylindrical columns (column A and column B) filled with silica sand. Column A, simulating a PRB, was additioned with PHB powder and run under anaerobic conditions; column B was filled only with silica sand and operated under aerobic conditions. Both columns were fed with a real groundwater contaminated by chlorinated solvents (mainly 1,2-DCA). At the beginning of the experimentation, both columns were inoculated with two different enrichment cultures obtained from the same contaminated groundwater under anaerobic PHB conditions and under aerobic conditions.

1,2-DCA, a carcinogenic compound, is of particular concern because it is one of the most widespread groundwater chlorinated contaminants (De Wildeman et al., 2003). The novelty of this study relies in the use of the groundwater collected from an aquifer mainly contaminated by 1,2-DCA. Our results can provide useful preliminary indications towards the evaluation of 1,2-DCA biodegradation pathways, both anaerobic and aerobic, when treating real contaminated groundwater.

2. Materials and Methods

The experimental campaign had an overall duration of 180 days. During the first 90 days, enrichment cultures were set up from the groundwater contaminated by chlorinated solvents, in order to favor the autochthonous dechlorinating bacterial population. A dechlorinating bacterial consortium was enriched in presence of PHB powder, as electron donor for the BRD process, under anaerobic conditions. In parallel, an enrichment culture on 1,2-DCA was carried out from the same groundwater under aerobic conditions, in order to biostimulate oxidative processes. During the subsequent 90 days, a pilot study was carried out on a laboratory-scale plant fed with the real contaminated groundwater, inoculated with the dechlorinating bacterial consortia mentioned above.

2.1. Contaminated groundwater characterization

Before being used for experimentation, the real groundwater sample was stored at 4 °C in a container hermetically sealed with no headspace, in order to limit the exposure to atmospheric oxygen, maintain anaerobic conditions and limit contaminants volatilization. Groundwater sample was contaminated by a variety of chlorinated solvents, and 1,2-dichloroethane (1,2-DCA) represented the major contaminant with a concentration of 20 mg l⁻¹. It was also characterized by a negative redox potential, a neutral pH, high values of electrical conductivity and total dissolved solids. Table S1 shows the main physical-chemical characteristics of groundwater sample used in this study.

2.2. Set up of enrichment cultures in microcosm

Enrichment cultures in microcosm were set up using the real groundwater sample from the contaminated aquifer in order to enrich the autochthonous 1,2-DCA dechlorinating bacterial community. Anaerobic enrichment cultures were set up with groundwater and culture medium (Marzorati et al., 2006) (50% vol/vol) and biostimulated with PHB powder 3.4 mM to a final volume of 12 ml into 20 ml screw cap vials in an anaerobic box under an atmosphere of filter-sterilized N₂. The anaerobic cultures were incubated statically in the dark at room temperature. In parallel, aerobic enrichment cultures were set up with groundwater and culture medium (Coleman et al., 2002) and were amended with 1,2-DCA 100 ppm and a mixture of volatile hydrocarbons (1% methane, 1% ethane, 0.01% C₄-C₆) in a final volume of 12 ml into 20 ml screw cap vials. The aerobic cultures were incubated in the dark at room temperature on a rotary shaker. In order to confirm that 1,2-DCA degradation was carried out by microorganisms, in both anaerobic and aerobic conditions, abiotic controls were set up in parallel to the biologically active treatments (Gu, 2016) using filter-sterilized groundwater. All microcosms were prepared in triplicate. At the end of four established incubation times (0, 15, 30 and 45 days), a set of microcosms of each condition was analyzed by gas-chromatography as described in 2.4. section. After 45 days of incubation, the obtained anaerobic and aerobic dechlorinating cultures were further enriched by transferring an inoculum (10% vol/vol) into fresh culture medium for 45 more days. These consortia were used as inoculum into the column systems as described below.

Table S1. Chemical-physical characteristics of groundwater and analytical methods for their determination.

PARAMETER	ANALYTICAL METHOD	UNIT OF MEASURE	CSC D.LGS. 152/06	MEASURED CONCENTRATION
REDOX POTENTIAL	ASTM D1498-14 (III)	mV	-	-450.00
ELECTRIC CONDUCTIBILITY	APAT CNR IRSA 2030 Man 29 2003	$\mu\text{S cm}^{-1}$	-	9,400.00
pH	APAT CNR IRSA 2060 Man 29 2003	pH	-	7.30
TOTAL DISSOLVED SOLIDS	SM 2540 C 2017	mg l^{-1}	-	7,000.00
CHLORIDE	EPA 9056A 2007	mg l^{-1} (as Cl^-)	-	1,800.00
NITRATES	EPA 9056A 2007 (PRI)	mg l^{-1} (as NO_3)	-	< 0.88
SULPHATES	EPA 9056A 2007	mg l^{-1} (as SO_4)	250	1,900.00
TOTAL CYANIDE	EPA 9014A 2014 T	$\mu\text{g l}^{-1}$ (as CN)	-	< 5
METHYL-ter-BUTYLETHER	EPA 8260D 2018	$\mu\text{g l}^{-1}$	-	< 7.6
AMMONIUM	APAT CNR IRSA 4030 A2 Man 29 2003	mg l^{-1} (as NH_4)	-	2.00
HEXAVALENT CHROMIUM	APAT CNR IRSA 3150 C Man 29 2003	$\mu\text{g l}^{-1}$ (as Cr)	5.00	< 0.84
TOTAL CHROMIUM	EPA 6020B 2014	$\mu\text{g l}^{-1}$ (as Cr)	50.00	< 0.92
IRON	EPA 6020B 2014	$\mu\text{g l}^{-1}$ (as Fe)	200.00	25,000.00
MANGANESE	EPA 6020B 2014	$\mu\text{g l}^{-1}$ (as Mn)	50.00	1,700.00
CHLOROMETHANE	EPA 8260D 2018	$\mu\text{g l}^{-1}$	1.50	< 7.5
CHLOROFORM	EPA 8260D 2018	$\mu\text{g l}^{-1}$	0.15	< 1.3
VINYL CHLORIDE	EPA 8260D 2018	$\mu\text{g l}^{-1}$	0.50	520.00
1,2-DCA	EPA 8260D 2019	$\mu\text{g l}^{-1}$	3.00	20,000.00
1,1-DCE	EPA 8260D 2020	$\mu\text{g l}^{-1}$	0.05	18.00
TCE	EPA 8260D 2021	$\mu\text{g l}^{-1}$	1.50	< 7
PCE	EPA 8260D 2022	$\mu\text{g l}^{-1}$	1.10	< 6.9

2.3. Continuous-flow experimental apparatus

Two parallel Plexiglas columns (length: 44 cm, inner diameter: 2.8 cm), namely column A and column B, were realized and continuously fed upward with the same contaminated groundwater sample. Column A was conceived to simulate a first PRB portion (from 0 to 22 cm) and a down-gradient aquifer (from 22 to 44 cm): PRB portion (section 1) was filled with a mixture of silica sand and PHB (Sigma-Aldrich, CAS 29435-48-1, purity 99.9%) at 5% on a weight basis; aquifer portion (section 2) was filled with silica sand only. Before the start-up of the experimental activity, column A was preliminarily flushed with nitrogen in order to eliminate oxygen and establish anaerobic conditions. Column B was entirely filled with silica sand and was equipped with an aeration system, placed at the flow inlet, in order to ensure aerobic conditions. A 2 cm sand and coarse gravel layer was placed at the bottom of each column to homogenize the distribution of the water flow. Both columns were equipped with a sampling port placed at the middle of the column; in addition, another sampling point has been realized at the outlet of each column.

Both columns were inoculated with a dechlorinating microbial consortium previously obtained and enriched from the same 1,2-DCA contaminated groundwater. In particular, column A was inoculated with the microbial community enriched on PHB under anoxic conditions, with the specific aim of creating a biological reactive zone where inoculated microorganisms could use PHB fermentation products as suitable electron donors for BRD. Instead, column B was inoculated with the microbial consortium enriched on 1,2-DCA in presence of O₂, with the aim of promoting bacterial aerobic oxidative (co)metabolism. In each column, 70 ml of groundwater were inoculated with 10% (vol/vol) bacterial culture at OD₆₀₀ = 0.1 and left in batch conditions for one week, before starting the continuous groundwater feeding.

During the experimentation, both columns were covered and kept in the dark in order to simulate the aquifer real conditions and avoid algae formation. Throughout the entire experimental activity (90 days), groundwater was continuously pumped into the columns by two peristaltic pumps at a flow rate of 0.1 ml min⁻¹, which corresponded to 30 hours of residence time or 1.3 cm h⁻¹ of pore water velocity. The feed water was stored in sealed bags with a capacity of about 2 l, which were periodically refilled (autonomy of about 14 days). For column A, the bags were previously flushed with nitrogen gas to eliminate oxygen ensuring anaerobic conditions. Furthermore, in order to remove any residual dissolved oxygen in the feeding water for column A, a deoxygenation procedure with sodium sulphite and cobalt chloride, was performed in accordance with (Capodici et al., 2019). Before each

bags refill, groundwater was enriched in nutrients (urea - $\text{CH}_4\text{N}_2\text{O}$: 7.5 ml in 1 liter of water and potassium dihydrogen phosphate - K_2HPO_4 : 8.6 ml in 1 liter of water) in order to avoid limitations for microbial growth; nutrients were dosed according to (Baric et al., 2014).

Figure S3 shows a schematic layout (a) and a panoramic view (b) of both laboratory-scale column systems.

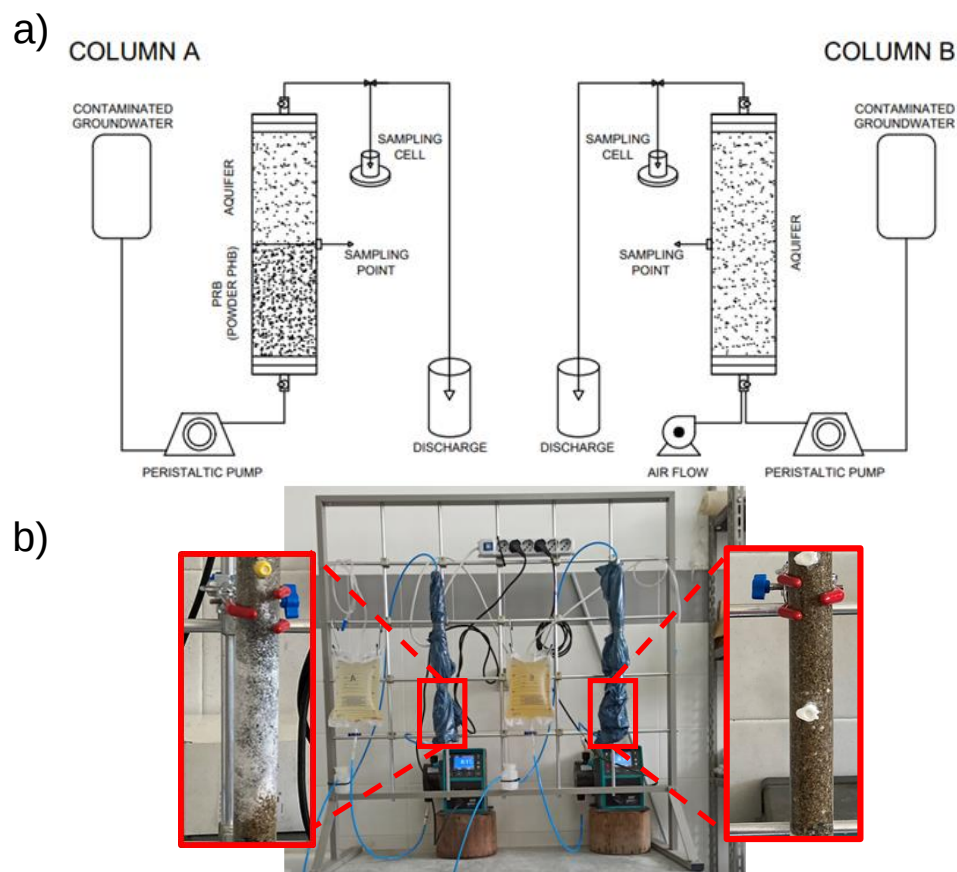


Figure S3. Schematic (a) and panoramic overview (b) of both laboratory-scale columns.

2.2. Analytical methods

During the experimental activity, the main operational parameters, i.e. dissolved oxygen (DO), redox potential (ORP), electric conductivity (EC) and pH have been regularly measured in the sampling cell located at the outlet of both columns, by means of dedicated probes connected to a multimeter (WTW 3340).

Liquid samples were periodically (every 14 days) collected at the inlet, at the middle (corresponding to a retention time of 900 min) and at the outlet of each column (corresponding to a retention time of 1800 min). In column A, where the middle point of sampling corresponded with the outlet of PRB, the aim was to monitor influent water physical-chemical parameters, PHB fermentation in terms of acetate production, and BRD

progress in terms of 1,2-DCA removal. In column B, the aim was to monitor oxidative degradative reactions in terms of 1,2-DCA removal. In detail, 1,2-DCA concentration in both enrichment cultures in microcosm and in column systems was measured by headspace-gaschromatography/mass spectrometry using the 7000C GC/MS Triple Quad GC/MS System, equipped with the column Agilent 19091S-433UI; helium was used as carrier gas, oven temperature was set at 240 °C and injection temperature was 270 °C. In column A, Volatile Fatty Acids (VFAs) were analyzed by ion chromatography (DIONEX ICS-1100) according to APAT IRSA CNR 4020 method.

2.5. Characterization of microbial communities by Illumina Miseq16S rRNA gene sequencing

The microbial communities of two portions (section 1 and 2 respectively, in column A corresponding to PRB and aquifer) of both columns were characterized using high throughput 16S rRNA gene amplicons sequencing. At the end of the experimental period, samples of sand were recovered from both sections of columns A and B. Metagenomic DNA was extracted from 0.5 g of each sample using the Fast DNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following manufacturer's instructions. Purity and concentration of extracted DNA were assessed using a NanoDROP ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). As reported in (Corsino et al., 2022), metagenomic DNA obtained from each sample was used as template to amplify bacterial and archaeal V3-V4 hypervariable region of the 16S rRNA gene; the amplicons sequencing was carried out using Illumina-MiSeq® platform; reads filtering and denoising were performed using Qiime2 tools version 2019.4 and taxonomy was assigned using trained Operational Taxonomic Units (OTUs) at 99% from Green Genes database version 13–8. 16S rRNA gene sequences obtained in this study were deposited in GenBank (NCBI) within the PRJNA939953 Bioproject (<https://www.ncbi.nlm.nih.gov/sra/PRJNA939953>).

3. Results

3.1. Enrichment cultures in microcosm

Gas-chromatographic analysis conducted on anaerobic and aerobic enrichment cultures after 45 days incubation during the preliminary experimental study revealed a removal of 1,2-DCA of about 90% in the PHB- biostimulated anaerobic microcosms, and of 100% in the aerobic microcosms; a decrease of 1,2-DCA concentration was also observed in anaerobic/aerobic abiotic control microcosms, but to a lesser extent (30% and 70%, respectively) (Figure S4), thus highlighting that a dechlorinating community was enriched

in microcosms in both conditions. The obtained dechlorinating consortia were further enriched and used to inoculate the two columns of the continuous-flow experimental apparatus.

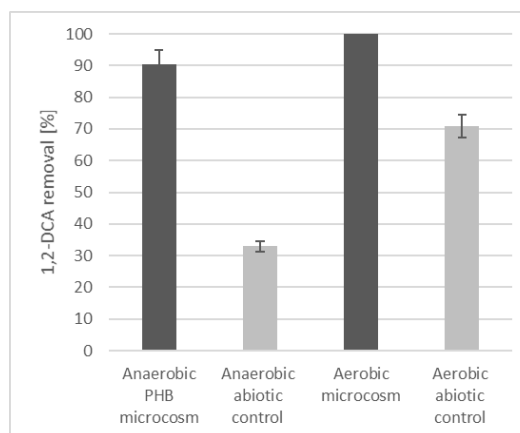


Figure S4. 1,2-DCA removal in PHB anaerobic microcosm, in aerobic microcosm and in their relative abiotic controls after 45 days of incubation.

3.2 Performance of continuous-flow experiments

3.2.1. Physical-chemical parameters monitoring

As expected, the two columns showed a different behavior throughout experiments. Figure 1 shows physical-chemical parameters variation during the continuous-flow experimental campaign within both columns. Figure 1a reports the trend profile of DO concentration. In column A, DO concentration was always equal to zero confirming the establishment of anaerobic conditions into the column, excepting a short period (from day 60 to day 78) when a sudden increase of the DO concentration was observed. This increase could be likely due to an improper water-bag storage. In contrast, in column B, DO concentration measured at the outlet of the system, was significantly high, thus confirming aerobic conditions into the column. Interestingly, a decreasing trend of DO concentration in column B during experiments was observed, probably due to biotic oxygen consumption. DO behavior within the two columns was confirmed by ORP values measured throughout experiments and shown in Figure 1c. In particular, in column A, ORP always showed negative values, excepting the short period corresponding to the increase of DO concentration. Also, ORP negative values confirmed anaerobic conditions within column A. Conversely, in column B, ORP always showed positive values, thus indicating aerobic conditions within the system. EC remained almost constant over time in both columns, with higher values observed in column A compared to column B (Figure 1b). Regarding to pH (Figure 1d), it showed an increasing trend in column A. This trend could be explained by a balance between two opposite phenomena occurring simultaneously into column A: on one hand, PHB hydrolysis

produced acetate, leading to a pH decrease, while on the other hand, acetate consumption by microorganisms caused an increase in alkalinity. It is probable that, from a kinetic point of view, acetate consumption overwhelmed its production, thus determining a pH increasing trend. In column B, pH remained constant around 7.6.

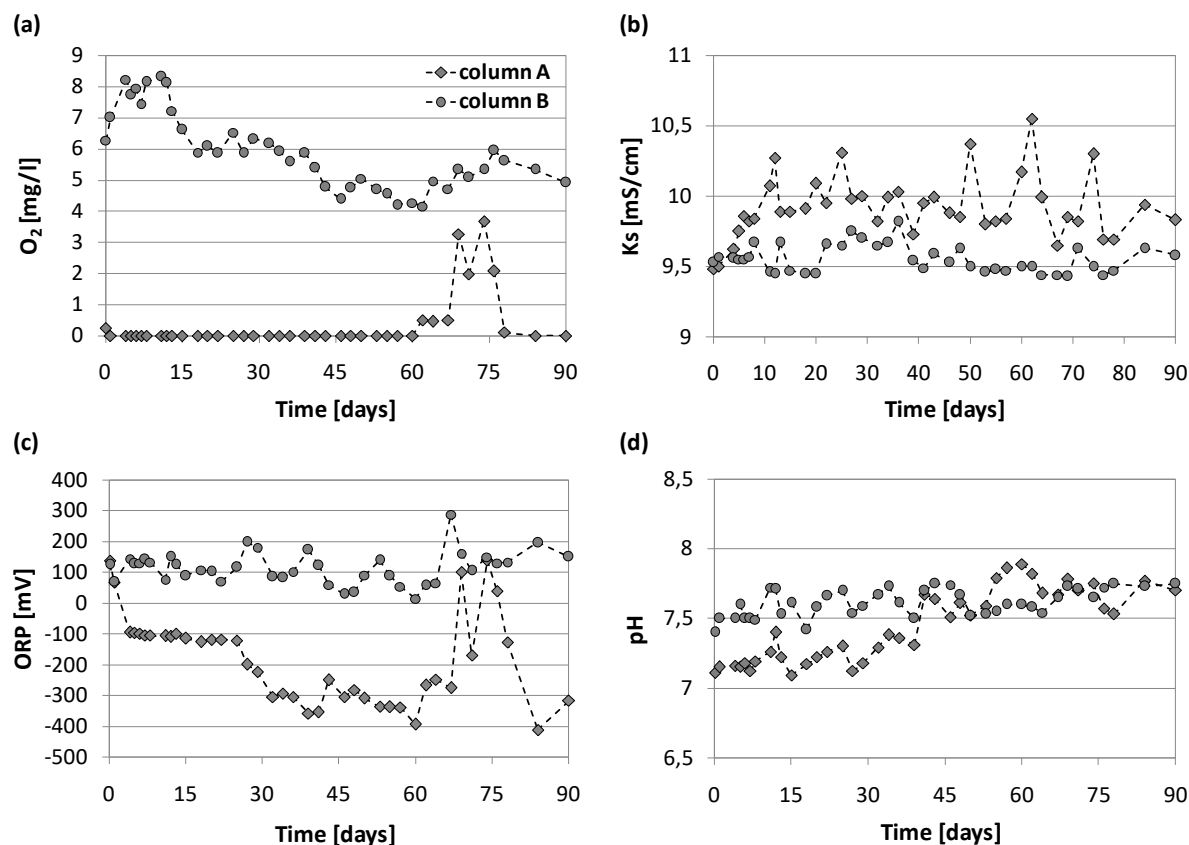


Figure 1. DO concentration (a), electric conductivity (b), redox potential (c), and pH (d) over time for column A and B

3.2.2. PHB fermentation and acetate production in column A

PHB fermentation results in the production of acetate. In column A an increase in acetate concentration over incubation time was observed, demonstrating that, under anaerobic conditions, bacterial PHB fermentation was enhanced (Figure 2). This increased trend of acetate concentration suggests a high rate of microbial activity inside column A, stimulated by the high specific surface area of PHB powder, that can explain the rapid biopolymer degradation (Baric et al., 2012).

Furthermore, it was observed that during the first 15 days the acetate production was higher than the consumption rate, denoting a greater fermentation activity compared to acetate consumption; consequently, no significant increase in pH was observed at this stage (Figure 3).

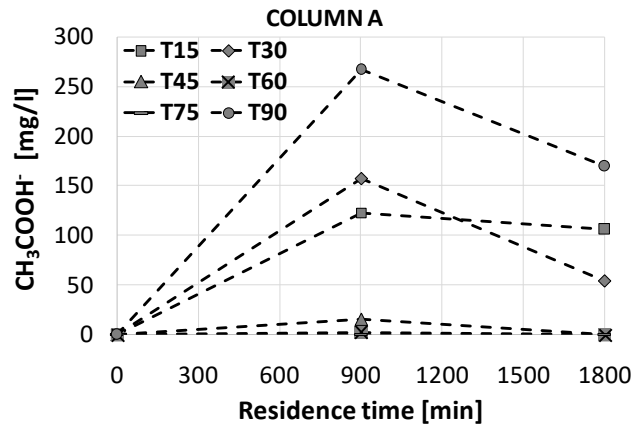


Figure 2. Acetate concentration trend over time along the column A at day 15, 30, 45, 60, 75 and 90.

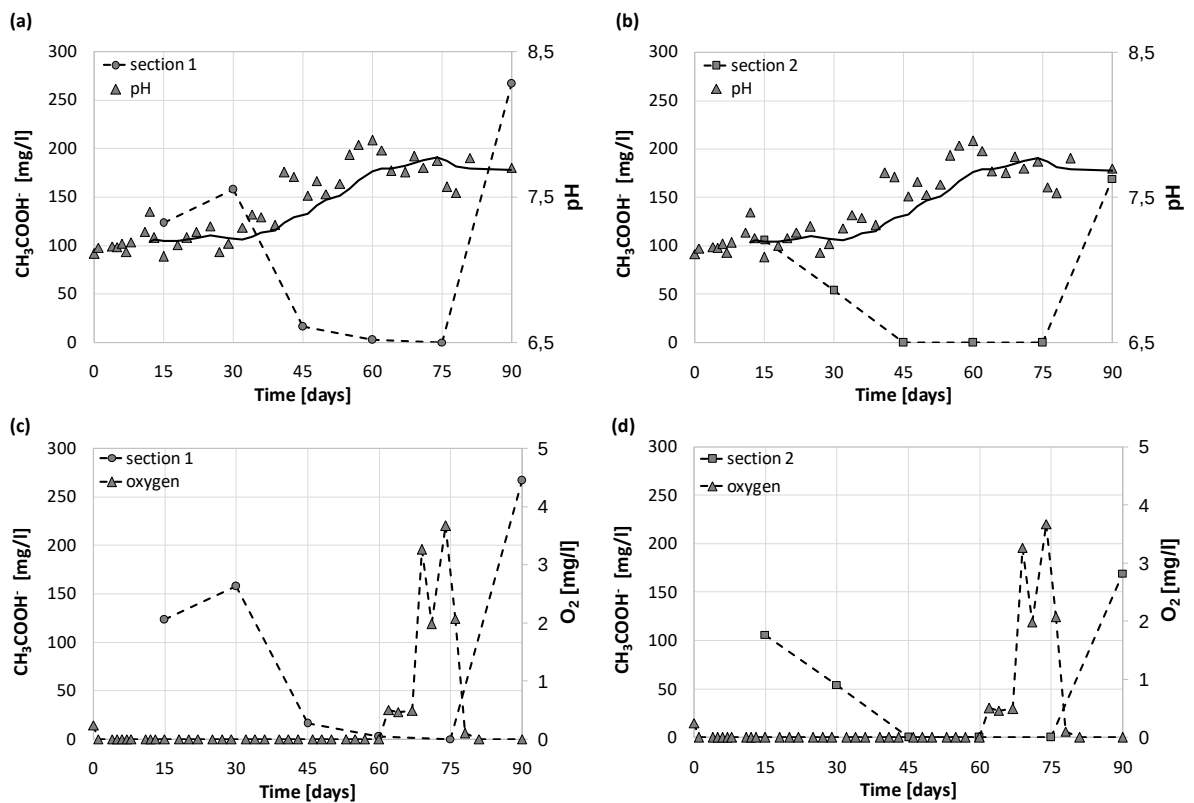


Figure 3. Correlation between pH and acetate in section 1 (a) and 2 (b) and DO and acetate in section 1 (c) and 2 (d) in column A.

Subsequently, from 30th to 60th day, acetate consumption kinetics increased resulting in pH increase. Indeed, acetate concentration at day 45 decreased significantly in section 1 and was close to zero in section 2; this behavior was observed also at day 60, with acetate concentration close to zero in both sections. Thereafter, up to day 75, acetate concentration remained close to zero in both sections, but in this case for a zeroing of the fermentation kinetics which caused a reduction in the pH growth trend; this could be due to the inhibition of fermentation process caused by the unfavorable aerobic conditions established in that

period inside the column. Finally, at experimental day 90, when optimal conditions for biopolymer fermentation were recovered within the system, a significant increase of acetate production was observed, reaching the maximum concentration of the entire experimental campaign, close to 267 mg l⁻¹.

3.2.3. 1,2-DCA concentration

Referring to column A, 1,2-DCA was degraded until reaching a concentration close to the standard limit imposed by Legislative Decree No 152/06 (1,2-DCA = 6 µg l⁻¹; CSC - contamination threshold concentration = 3 µg l⁻¹) on the 60th experimental day, and it was below the instrument detection limit after 75 days of operation. On the contrary, in column B, the degradation rate was lower and 1,2-DCA concentration was significantly higher than CSC (1,2-DCA = 324 µg l⁻¹). However, in column B it was observed a significant 1,2-DCA removal. Figure 4 shows 1,2-DCA concentration trend along the two columns. Furthermore, Figure S5 shows the normalized trend of the effluent 1,2-DCA for the two columns during the experimental period. In both columns very low values of normalized 1,2-DCA were found, with a general decreasing trend, confirming a high 1,2-DCA degradation, with removal efficiencies always higher than 90%. In column A, after 75 experimental days, an almost complete removal of the 1,2-DCA was obtained, with an efficiency close to 100%. The maximum removal efficiency observed in column B, on the other hand, was approximately 98%.

Acetate concentration trend over time along the column A at day 15, 30, 45, 60, 75 and 90.

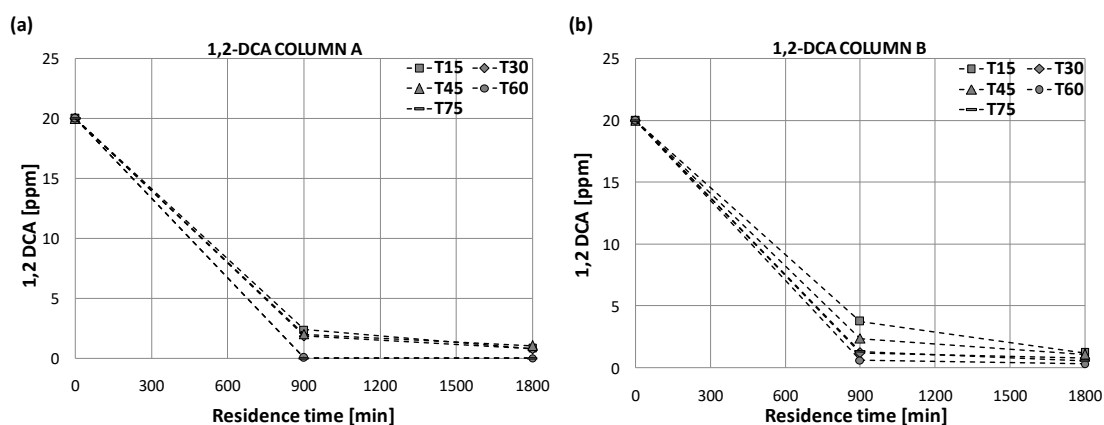


Figure 4. 1,2-DCA concentration trend along column A (a) and B (b).

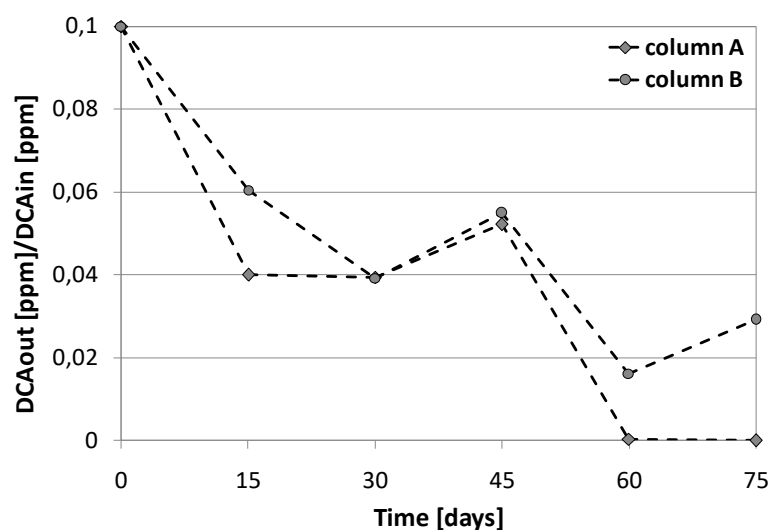


Figure S5. Normalized 1,2-DCA concentration trend over time for column A and B.

3.3. Microbial consortia in the columns

Microbial biomass within both columns was estimated as total double strand extracted DNA (dsDNA) (Catania et al., 2022). Columns A and B had a similar biomass content that was higher in section 1 in respect to section 2 for both columns (Table 1). Illumina MiSeq 16S rRNA gene sequencing was performed on the metagenomic DNA extracted from the silica sand samples from columns A and B in order to detect and identify the bacterial communities and to verify the presence of microbes involved in BRD and direct or cometabolic oxidation of chlorinated solvents.

The summary of Illumina sequencing output is reported in Table 1. Clustering of 16S rRNA gene sequences at 99% similarity, led to a similar number of operational taxonomic units (OTUs) in the two sections from column A (143 for section 1 and 131 for section 2), and a slightly higher number of OTUs in column B. The clusters were taxonomically identified using GreenGenes database version 13–8.

The histograms in Figure 5 show the composition of the two microbial consortia at phylum (Figure 5a), family (Figure 5b) and genus (Figure 5c) taxonomic level. Taxonomic groups with relative abundance greater than 1% were taken into consideration. The most represented phyla in both columns were Firmicutes and Proteobacteria, followed by Bacteroidetes and Tenericutes. Column A was dominated by Firmicutes (50% and 64%, in section 1 and 2 respectively) followed by Tenericutes (25% and 1%). Conversely, column B was dominated by Proteobacteria (57% in both sections), followed by Firmicutes (28% and 14%).

Table 1. Microbial biomass of columns and summary of 16S Illumina sequencing output.

Column	Section	Microbial biomass (dsDNA) ($\mu\text{g g}^{-1}$ column content)	non-chimeric reads (n)	Bacterial diversity observed OTUs (99% id.)
A	1	2.3	31640	143
A	2	1.6	29555	131
B	1	2.0	42446	214
B	2	1.8	32309	203

At family level, column A was mainly composed of Peptococcaceae (23% and 53%, in section 1 and 2 respectively) and Acidaminobacteraceae (19% and 8%), and, to a lesser extent, Pelobacteraceae (8% in section 2), Desulfovibrionaceae (3% and 2%) and Syntrophaceae (2% and 1%). Members of these families are almost exclusively obligate anaerobes, confirming that anaerobic conditions were established throughout column A (Figure 1). Peptococcaceae were represented mainly by *Desulfosporosinus* (3% and 15%, in section 1 and 2 respectively), while Acidaminobacteraceae were totally represented by *Fusibacter*, which was the most abundant identified genus in section 1. In at least one section of column A the families Pseudomonadaceae (4.5% in section 2), Comamonadaceae (3% in section 1) and Desulfuromonadaceae (1.5% in section 2) were also identified. Five out of the total detected families, namely Peptococcaceae, Desulfovibrionaceae, Desulfuromonadaceae, Syntrophaceae and Comamonadaceae, include known OHRB genera (Molenda et al., 2020; Türkowsky et al., 2018).

In column B, Proteobacteria were mainly made up of families Legionellaceae (22%) and Comamonadaceae (6%) in section 1, Comamonadaceae (14%), Pseudomonadaceae (11%), Rhodocyclaceae (10%) and Desulfuromonadaceae (5%) in section 2, and Enterobacteriaceae (4%) in both sections. Firmicutes were mainly made up of Peptococcaceae (including the unidentified phylotype EtOH8) in both sections (24.5% in section 1 and 10% in section). Legionellaceae are aerobic Gram-negative bacilli that are found mainly in aqueous environments (Edelstein and Lück, 2015); they were probably present in the groundwater used to feed the columns and enriched in column B taking advantage of oxygen. Beyond aerobic Legionellaceae, Comamonadaceae and Pseudomonadaceae, column B hosts also facultative and anaerobic bacteria (i.e., Enterobacteriaceae, Peptococcaceae and Desulfuromonadaceae). The presence of facultative and anaerobic bacteria suggests the formation of micro-oxygen depletion zones inside column B, probably due to aerobic microbial consumption, in accordance to the detected decrease of DO (Figure 1). Anaerobic Peptococcaceae were mainly represented by *Desulfosporosinus* (19% and 9%, in section 1 and 2 respectively). The genera identified within Comamonadaceae were *Delftia* (just over 4% in both sections) and *Hydrogenophaga* (1% in section 1 and 5% in section 2).

Pseudomonadaceae were totally represented by the genus *Pseudomonas* (2.5% and 10.5%, in section 1 and 2 respectively), that is the most abundant genus in section 2. Among families hosted in column B, Comamonadaceae, Pseudomonadaceae, Peptococcaceae and Desulfuromonadaceae, include known degraders of chlorinated solvents (Bertolini et al., 2021; Dolinová et al., 2017).

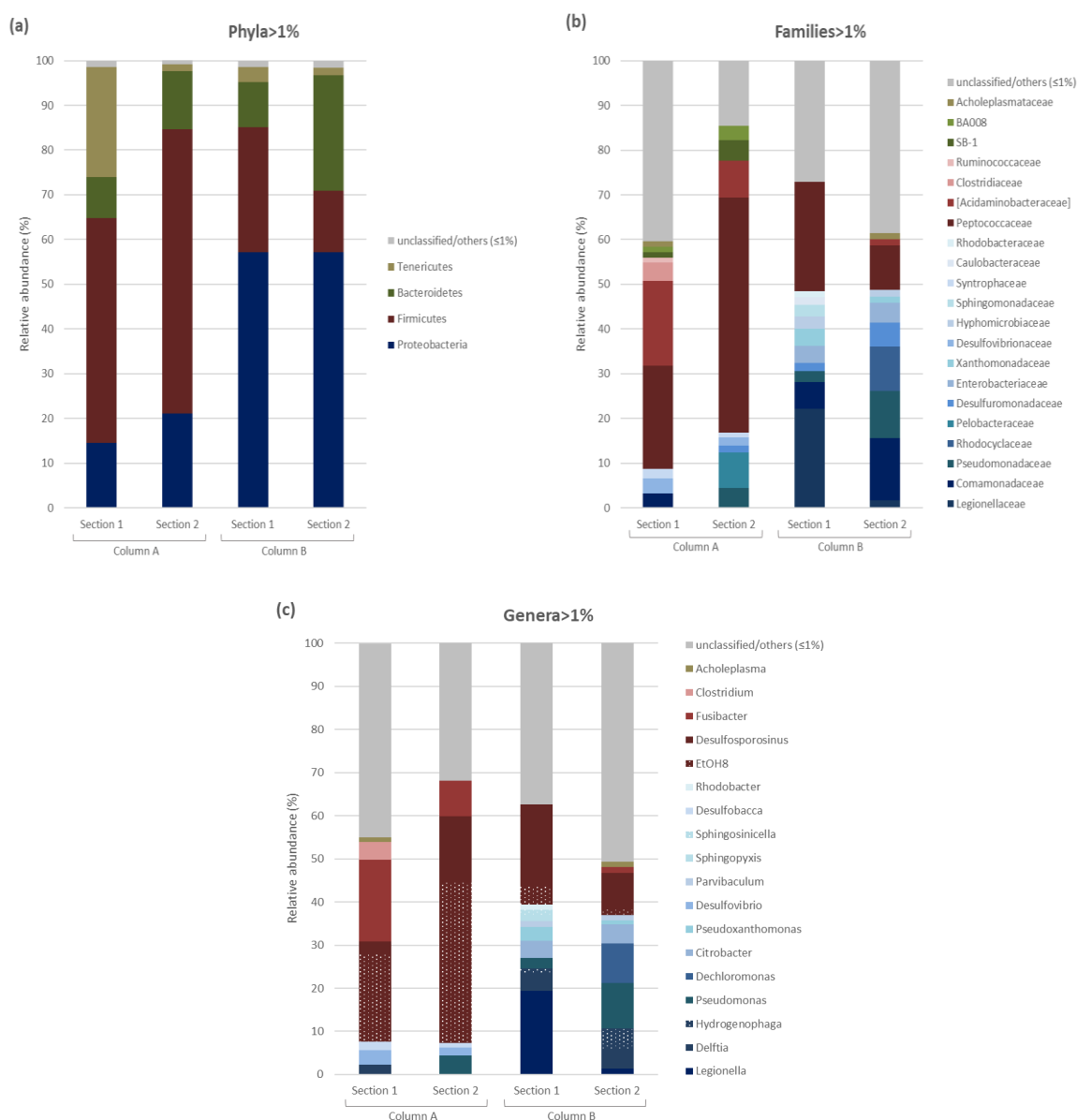


Figure 5. Relative abundances (%) of bacterial phyla (a), families (b) and genera (c) in section 1 and section 2 of columns A and B. Only taxa with relative abundance >1% in at least one sample are represented.

2. Discussion

The very high 1,2-DCA removal achieved in the preliminary microcosm test, under anaerobic and aerobic conditions, highlighted that a dechlorinating microbial community

was enriched in both conditions, thus confirming that both biological pathways (anaerobic BRD and aerobic direct/cometabolic oxidation) are feasible alternatives for 1,2-DCA removal from contaminated groundwater (Frasconi et al., 2015; Amanat et al., 2022).

Results from column A showed that PHB powder was effectively hydrolyzed and used as substrate by autochthonous groundwater microorganisms that converted it into acetate (which was accumulated up to 267 mg l⁻¹ after 90 experimentation days). The rapid fermentation was determined not only by the favorable conditions established into the system, but also by the high specific surface of the biopolymer itself. In particular, along column A, the highest acetate concentration was observed at the end of PRB section, rather than at the exit of the column. This is probably due because PHB hydrolysis took place in the reactive portion of the column, resulting in the production of acetate, while in the aquifer section acetate was consumed and used as growth substrate by microorganisms. However, the amount of acetate deriving from PHB fermentation was sufficient to sustain an efficient and prolonged reductive dechlorination activity in the column, as demonstrated by the completely 1,2-DCA removal. So PHB, ensuring electron donor slow-release in the form of acetate, can be effectively used to support a long-term reductive dechlorination microbial metabolism, allowing the removal of the chlorinated contaminant and confirming the effectiveness of anaerobic BRD pathway for 1,2-DCA biodegradation (Baric et al., 2014).

Column B showed a slightly lower 1,2-DCA removal efficiency (98%). In this case, the biodegradation of the chlorinated compound was probably mediated by the establishment of both aerobic and anaerobic dechlorination pathways, as suggested by DO and redox potential values achieved during the experimental campaign. Aerobic degradation of 1,2-DCA has been observed in previous studies, in which it has been shown that 1,2-DCA can be degraded both by direct oxidation and by oxidative cometabolism (Munro et al., 2017). However, the slightly lower removal of 1,2-DCA observed in column B suggested that anaerobic BRD enhanced by PHB in column A was more effective in 1,2-DCA biodegradation, compared to aerobic metabolisms, at least under the operational conditions under study. The microbial consortia established in columns A and B, analyzed at the end of experimental period, derived from the autochthonous microbial community of the groundwater used to feed both columns and from the two 1,2-DCA dechlorinating consortia previously enriched in microcosm from the same groundwater and used as inoculum in both columns. In particular, column A inoculum was made up of a microbial consortium enriched on PHB under anaerobic conditions, while column B inoculum was made up of a microbial consortium enriched on 1,2-DCA under aerobic conditions. The microbial communities of columns A

and B showed quite similar profiles at phylum level, although the relative abundances of the identified taxa varied between the two columns and between sections within each column. Since the columns were fed with the same groundwater sample, the difference in the composition of the two final communities could depend on the presence of PHB in column A, on the different physical-chemical conditions in the two columns, referring in particular to DO concentrations, as well as on the different microbial inocula used for the two columns.

In column A, the degradation of PHB powder was evidenced by acetate formation and by microbial biomass proliferation. Anaerobic conditions in the presence of PHB favored the enrichment of fermentative/sulfate reducing bacteria. Indeed, the most abundant genera, *Desulfosporosinus* and *Fusibacter*, are both sulfate and thiosulfate reducers and they have also a fermentative metabolism that could be responsible for acetate production from PHB (Hippe and Stackebrandt, 2015; Ravot et al., 2015). In particular, *Desulfosporosinus*, a strictly anaerobic spore forming sulfate-reducing bacterium, can produce acetate as fermentation end product (Hippe and Stackebrandt, 2015). Moreover, sulfate reducing bacteria (SRB) are known to be involved also in biodegradation of aromatic and chlorinated hydrocarbons (Dutta et al., 2022) and *Desulfosporosinus*, in particular, includes PCE-degrading species (Spring and Rosenzweig, 2006), such as *D. meridiei* (Robertson et al., 2001). *Desulfosporosinus* was recently detected as the most abundant active genus, resident in an organohalide polluted groundwater underneath a landfill stimulated with molasses (Bertolini et al., 2021). Also, the genus *Fusibacter* could have a role as both fermenting and OHRB agent responsible for both acetate production from the degradation of PHB and dehalogenation of 1,2 DCA (Ravot et al., 1999). Although both genera have been described as OHRB, the specific role of *Desulfosporosinus* and *Fusibacter* in 1,2-DCA dehalogenation is observed here for the first time; further investigations are needed to confirm it. Beyond the aforementioned genera, other members of Peptococcaceae and members of Desulfovibrionaceae, Desulfuromonadaceae, Syntrophaceae and Comamonadaceae, detected in column A at lower abundance, include dehalogenating species (Türkowsky et al., 2018; Molenda et al., 2020), which could be involved in 1,2-DCA biodegradation in our experimental system. Surprisingly the most well-known OHRB genera (i.e., *Dehalococcoides*, *Dehalogenimonas*, *Dehalobacter*, *Desulfitobacterium*) were not detected in column A, but it is worth to note that a large part of the reads could not be assigned at genus level suggesting that other dehalogenating taxa could be present. According to microbial community composition, the most probable mechanism for 1,2-DCA

biodegradation in column A is reductive dechlorination carried out by SRB and enhanced by fermentation products from PHB degradation.

Unlike column A, alternate aerobic/anoxic microsites in column B enriched the consortium with a less abundant and more diverse microbial community, composed mainly of aerobic chemoorganotrophs and anaerobic fermentative/sulfate reducing bacteria. Among identified families, Pseudomonadaceae, Comamonadaceae, Peptococcaceae and Desulfuromonadaceae include known degraders of chlorinated solvents (Dolinová et al., 2017; Bertolini et al., 2021). One of the most abundant genus in section 1 was *Desulfosporosinus*, already described referring to column A. While the most abundant genus in section 2 was *Pseudomonas* that includes known hydrocarbon degraders and species able to carry out (co)metabolic oxidation of chlorinated solvents (Alvarez-Cohen and Speitel, 2001). *Pseudomonas sp.* strain DCA1, for example, can utilize 1,2-DCA as sole carbon and energy source (Hage et al., 2001), suggesting that members of this genus could have a pivotal role in 1,2-DCA degradation in column B by both direct and cometabolic oxidation. Also, the genera *Delftia* and *Hydrogenophaga*, detected in column B at lower abundance, could be responsible of 1,2-DCA biodegradation. It has been reported that many species of *Delftia* live in anoxic-aerobic environments, and they could dehalogenate and degrade halogenated aromatic compounds, such as diuron, 2,4-dichlorophenoxyacetate, chlorophenoxy herbicides, trifluoroacetate and 3,5-dibromo-4-hydroxybenzoate (Zhang et al., 2019). *Hydrogenophaga* includes aerobic chemorganotrophic or chemolithoautotrophic bacteria and some strains have demonstrated the ability to aerobically degrade chlorinated compounds, such as polychlorinated biphenyls (Lambo and Patel, 2007). However further investigations should assess the ability of these genera to dehalogenate also 1,2-DCA. According to column B microbial composition, 1,2-DCA biodegradation in the imposed aerobic experimental system could mostly occur by an aerobic oxidative pathway, both direct and cometabolic, and could be supported by anaerobic reductive dechlorination processes.

3. Conclusions

In the present study, aerobic and anaerobic biostimulation and bioaugmentation treatments were investigated for the bioremediation of real groundwater mainly polluted by 1,2-DCA. Anaerobic 1,2-DCA degradation was carried out mainly by fermentative/sulfate reducing bacteria stimulated by acetate derived from PHB fermentation in column A. Conversely, mainly aerobic (co)metabolic oxidation by aerobic chemoorganotrophs occurred in column B. Our results highlight that a variety of biological processes can be effectively stimulated

for the remediation of 1,2-DCA contaminated aquifer, promoting the application of more environmentally sustainable remediation approaches.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this paper.

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CHAPTER V

Biopolymeric scaffolds for enhanced bioremediation

5.1. Introduction

5.1.1. Carriers in bioremediation and sampling

In the context of the strategies applied to enhance bioremediation efficiency, cell immobilization on supports is an emerging and promising technique. Immobilization, that consists in limiting the mobility of the microbial cells, but simultaneously preserving their viability and metabolic functions, can be achieved by exploiting the natural ability of microorganisms in the environment to form biofilms on various surfaces (Dzionic et al., 2016). Immobilized cell systems have been used in several applications such as in pharmaceutical and food industries, process engineering and biosensor applications (Mehrotra et al., 2021).

In bioremediation, immobilized cells were successfully exploited in wastewater treatment and biodegradation of heavy metals, hydrocarbons, and chlorinated solvents, among others (Mehrotra et al., 2021). Biofilm-mediated bioremediation has some advantages over the use of free cells, making it a low-cost and effective strategy. In fact, cell immobilization preserves cells viability by providing favorable environmental conditions, such as possibility for nutrient, pH, temperature; it provides resistance to the surrounding environment, tolerance to high concentrations of pollutants and toxic chemicals and protection from shear damage; it prevents washout problems and dispersion of cells in the environment, allowing a better retention of biodegrading biomass, and therefore the bioaugmentation of highly efficient bacteria, in the contaminated sites even after a prolonged period (Bayat et al., 2015; Scaffaro et al., 2017a).

Fundamental aspects to take into account when setting up an immobilization system are the selection of appropriate degrading microorganisms, microbial consortia or enzymes, the immobilization method used and the selection of the carrier.

About immobilization methods (Fig. 5.1), some of the most used include: adsorption, consisting in a reversible physical interaction between microorganisms and the surface of the matrix, based on weak forces; covalent binding, based on the presence of a binding agent; entrapment, that consists in capturing cells within porous structures of the matrix; encapsulation, that is the irreversible embedding of cells within semi permeable membranes (Bayat et al., 2015).

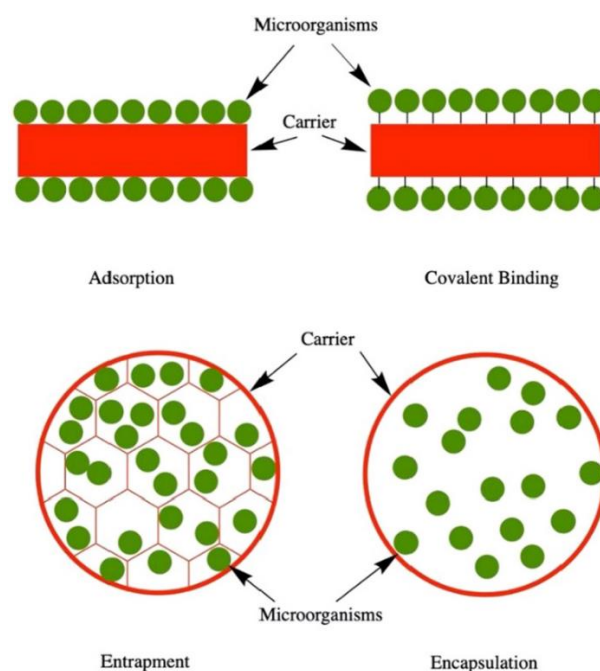


Figure 5.1. Methods of immobilization of microorganisms on carriers for bioremediation. From (Valdivia-Rivera et al., 2021)

The other critical aspect concerns the selection of the carrier type and material for cell immobilization, as it should be cost effective, non-polluting, non-toxic, insoluble in aqueous medium, available in large quantities, and chemically and physically suitable for proper adhesion of specific microorganisms. So far, both inorganic and (natural or synthetic) organic materials have been exploited (Mehrotra et al., 2021).

Among organic carriers, that are preferably used over inorganic ones for cell immobilization, synthetic polymers such as polyethylene (PE), polyurethane (PU), polypropylene (PP) and polyvinyl chloride (PVC) are commonly used, as they show higher mechanical strength and provide a more stable biofilm formation than natural polymers (Mehrotra et al., 2021), but they are non-biodegradable and polluting. On the other hand, using natural carriers such as plant fibers and agro-industrial residues would represent an eco-friendly, inexpensive and low-impact alternative, but with the drawback of low mechanical and chemical stability (Dzionic et al., 2016).

Over the last years, interest in biodegradable polymers as alternative to hydrocarbon derived materials has raised. Among synthetic biopolymers, polylactic acid (PLA) and polycaprolactone (PCL) received a great attention and have been widely used for several applications, including biomedical applications, such as tissue engineering (Scaffaro et al., 2016a) and in improving bioproductivity by immobilized cells (Scaffaro, et al., 2016b), thanks to the peculiar properties of these materials such as biodegradation, biocompatibility, mechanical properties, low costs and ease of production.

Recently, biopolymeric carriers have also been successfully applied to hydrocarbon (HC) bioremediation. In particular, PLA and PCL tridimensional porous structures, in a sponge-like or membrane form, were reported to have a high selective oil uptake potential and suitability for bacterial colonization; moreover, evidence of enhanced biodegradation activity by selected HC-degrading immobilized bacteria was observed (Scaffaro et al., 2017a; Catania et al., 2020).

Under another, recent and still unexplored, perspective, carriers with similar features to the ones applied in cell immobilization have also been tested for potential application in the very first steps of planning bioremediation strategies, that consist in investigating the bacterial communities of the site of interest. Electrospinning polyurethane nanofibers were found to be efficient as biomass sampling tools from contaminated sites for molecular analysis (Nechanická et al., 2021). Biomass sampling from contaminated sites and microbial community characterization are crucial steps in controlling and planning (bio)remediation interventions, but soil or groundwater samplings are time-consuming and costly and they often provide low biomass yield for subsequent analysis, thus requiring filtration or centrifugation of the samples to increase the biomass concentration. Sampling biomass in the form of a biofilm that colonizes easy-to-collect carriers could therefore overcome sampling drawbacks (Nechanická et al., 2021).

In conclusion, immobilization-based bioremediation and carrier-based sampling constitute emerging, alternative and efficient technologies for environmental remediation. Nevertheless, further effort is required in the development of cost-effective, eco-friendly, easily available and stable appropriate systems to extend the commercial and field adaptations of this technologies to diverse environments and against different pollutant.

5.1.2. Aim of the work

On the basis of the intriguing applicative potential of biopolymeric carriers combined with cells immobilization, different types of scaffolds were produced and tested for their suitability as bacterial carriers for the enhanced bioremediation of chlorinated solvents, for example as immobilization-based bioaugmentation technology. Porous biopolymeric scaffolds were realized in polylactic acid (PLA) and polycaprolactone (PCL) and preliminary tests were conducted to assess: i) scaffolds uptake potential toward the contaminant of interest in this study, 1,2-dichloroethane (1,2-DCA); ii) scaffolds biocompatibility and suitability for the immobilization of a bacterial biofilm; iii) scaffolds suitability colonization by a complex biofilm from a contaminated environmental water sample; iv) the possible enrichment in biodegrading taxa of the scaffold-colonizing biofilm.

5.2. Materials and methods

5.2.1. Production and characterization of scaffolds

Biopolymeric scaffolds were provided by Prof. Ing. Roberto Scaffaro, Dept. of Engineering, University of Palermo. Two production techniques were used, melt mixing and electrospinning, and two biopolymeric materials, PLA (polylactic acid) and PCL (polycaprolactone).

Materials used were as described in (Scaffaro et al., 2017a) and (Catania et al., 2020): PCL ($M_w = 80$ kDa, $\rho = 1.145$ g/cm³), polyethylene glycol (PEG) ($M_w = 2000$ Da, $\rho = 1.12$ g/cm³), and Sodium Chloride (NaCl) (purity > 99%, $\rho = 2.17$ g/cm³), commercial grade of amorphous PLA (PLA 2002D, Natureworks® LLC, Minnetonka, MN 55345, USA).

PLA and PCL melt mixing scaffolds, called “M-M scaffolds” (Fig 5.2a), were prepared as described in (Scaffaro et al., 2017a) by melt mixing PCL or PLA, PEG and NaCl (11/7/82%wt) in an internal batch mixer (Brabender PLE-330, $T = 100$ °C, rotor speed = 64 rpm) and processing until a constant value of torque was achieved. The blends were then cooled in liquid nitrogen and compression molded in a Carver laboratory press in cylindrical molds with a diameter of 15 mm and a height of 10 mm. NaCl and PEG, constituting the porogen part of the blends, were removed by leaching in distilled water for 3 h at 50 °C. Pores dimension range was 50-100 μ m. The resulting structures were then left to dry for 12 h at room temperature.

PLA and PCL electrospinning scaffolds, called “ES scaffolds” (Fig 5.2b), were prepared by electrospinning as described in (Catania et al., 2020). Briefly, a PLA or PCL solution was prepared as reported in (Scaffaro et al., 2017b) and fed in a 10 mL glass syringe fitted with a 19-gauge stainless steel needle. An electrospinning equipment (Linari Engineering-Biomedical Division, Pisa, Italy) was used to prepare the nanofiber mats. Nanofiber average diameter was 1.4 μ m. Constant parameters were used: distance between the needle tips and the collector, 15 cm; supplied high voltage, +15 kV.

Prior to any experiment, all scaffolds were weighted to refer any measure to the initial weight.

Scaffolds were washed three times with distilled water to remove residual salts and dried at 37° until constant weight before assessing their initial dried weight.

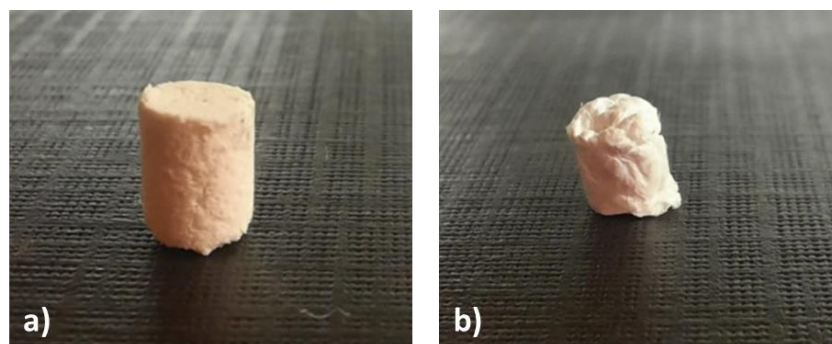


Figure 5.2. a) a melt-mixing scaffold and b) an electrospinning scaffold.

5.2.2. 1,2-DCA uptake test

To determine whether the scaffolds can uptake 1,2-DCA from a water solution, thus working as an enrichment environment for dechlorinating biofilm-forming microorganisms, an absorption test was carried out by incubating pre-weighted ES and M-M scaffolds in 20 ml screw caps vials containing 12 ml of 1,2-DCA 10 ppm solution (Sigma-Aldrich). A negative control without any carrier was set up for comparison. After 48 hours of static incubation in dark at room temperature, the removal of 1,2-DCA from the aqueous medium was assessed by Gas Chromatographic-Mass Spectrometry Analysis by head space sampling (HS-GC/MS). A Gas Chromatography-Mass Spectrometry GCMS-QP2010 Plus equipped with a Supelcowax 10 column was used. Gaseous samples (1 ml) were manually withdrawn from the headspace of the vial, using gas-tight syringes (Hamilton) and injected in the column. The amount of contaminant trapped by every scaffold was obtained by subtracting the residual 1,2-DCA from the initial solution and referred to initial dried weight of the scaffold.

5.2.3. Sterilization of biopolymeric scaffolds

The scaffolds used in adhesion and colonization tests were sterilized before immersion in inoculated medium or groundwater samples in microcosms. The sterilization protocol was modified from (Scaffaro et al., 2017a). Briefly, the scaffolds were washed in EtOH 70% for 30 minutes, rinsed in sterile distilled H₂O for 30 minutes twice and air dried overnight under a laminar flow hood.

5.2.4. Adhesion tests with a model biofilm-forming microorganism

A marine biofilm-forming microorganism, *Pseudoalteromonas* sp. (Iijima et al., 2009) was chosen as a model microorganism to assess the suitability of scaffolds to work as bacterial biofilm carriers. *Pseudoalteromonas* sp. strain used in this test was previously isolated from a PLA film exposure experiment in marine environment (unpublished) and available in the

laboratory's strain library; the microorganism, stored in glycerol 20% at -80°C, was streaked on a Marine agar (Condalab) plate and incubated overnight at 30°C. A single colony was reinoculated into 3 ml marine broth and incubated for 5 days at 30°C in a rotary shaker (150 rpm). Growth was assessed by Optical Density Measurement (OD₆₀₀).

Weighted and sterilized PLA and PCL scaffolds were incubated in presence of a pure culture of *Pseudoalteromonas sp.* (OD₆₀₀ 0.2) in triplicate in 3 ml marine broth for 48 and 96 hours at 30°C with shaking (150 rpm). Uninoculated controls were prepared and incubated under the same conditions. At the end of every incubation period, three scaffolds of each kind were collected, air dried and weighted until constant weight. Viability of adhered cells was checked at the end of every incubation time by quickly touching a marine agar plate with the carrier before drying it. The adhered biomass was calculated as dried weight increase (%) in reference to the initial weight of the pristine scaffold.

5.2.5. Groundwater samples collection and filtration

As immobilization of pure culture bacteria is generally easier and more efficient if compared to immobilizing a heterogeneous naturally growing microbial population from a complex contaminated environment because of inter-dependent growth patterns and synergistic/antagonistic (Mehrotra et al., 2021), the colonization of scaffolds by a complex microbial community was also tested in this study. Therefore, after testing pure culture biofilm formation on biopolymeric scaffolds, selected scaffolds were incubated in the presence of real contaminated groundwater samples. Two groundwater samples were used: one was collected during the third sampling (the same used to set up anaerobic microcosms, GW-S1-May21, described in Chapter II); another sample was collected later on purpose from site 2 (GW-S2-May22).

The microbial communities from groundwater used to inoculate the scaffolds, were analyzed as previously described (Chapter II). Briefly, groundwater samples were filtered using Millipore 0.22 µm filters to collect microbial biomass and filters were used for DNA extraction as described below.

5.2.6. Microbial adhesion test in 1,2-DCA contaminated groundwater in microcosms

Microbial adhesion tests with contaminated groundwater in microcosm were performed using the PLA electrospinning scaffolds. Six sterilized scaffolds (Fig. 5.2b) were incubated in 60 ml crimp-sealed glass serum bottles with butyl rubber stoppers containing 36 ml of groundwater (Fig. 5.3). Two microcosms were set up. A first microcosm was set up in an anaerobic glove box under an atmosphere saturated with filter sterilized N₂/CO₂/H₂

(80%/15%/5%) gas mix (Marzorati et al., 2007) from sample GW-S1-May21 and incubated statically in the dark at room temperature (25°C) for 30 days. Another microcosm was set up under aerobic conditions from sample GW-S2-May22 and incubated in the dark at room temperature (25°C) with shaking (150 x g) for 60 days. At the end of established incubation times, scaffolds were retrieved from microcosms. Colonizing microbial communities were analyzed by 16S rRNA Illumina sequencing. Microbial adhesion and proliferation on the scaffolds were evaluated by scanning electron microscope (SEM) observations. The scaffolds destined to DNA extraction were retrieved and stored at -20° until further analysis; those destined to SEM observations were processed as described below.



Figure 5.3. Microcosm containing groundwater and PLA ES scaffolds (right) and control microcosm with groundwater only.

5.2.7. Field exposure experiment

PLA electrospinning scaffolds were used to set up a preliminary in field exposure experiment in the aquifer under study. Scaffolds were sunk at 20 m depth in groundwater through a monitoring well in the site S2 of the study area. The placement was carried out in May 2022, at the same time of the sampling for microcosm experiment with GW-S2-May22; scaffolds were retrieved after 60 days of exposure. Scaffolds were put into perforated plastic containers (Fig. 5.4) attached to a lead weight to soak them in groundwater and lowered into the monitoring well by a nylon thread. In total 34 PLA ES scaffolds divided into two containers were put in field. After the retrieval, the scaffolds were transported to the laboratory immersed in groundwater and processed within 24h. Total DNA was extracted from 15 scaffolds and the composition of the microbial community recruited on the scaffolds in the form of a biofilm was analyzed by 16S rRNA Illumina sequencing. Other scaffolds were stored at -20°C for further analysis.



Figure 5.4. Plastic containers used to sink the scaffolds in groundwater

5.2.8. Scanning electron microscopy

The scaffolds before and after colonization were processed for Scanning Electron Microscopy (SEM, Phenom ProX, PhenomWorld) observation as described in (Scaffaro et al., 2017a). The carriers were washed 3 times with PBS 1 × for 3 min; adhered cells were fixed with glutaraldehyde 4% for 30 min at 4 °C. The samples were dehydrated with increasing ethanol solutions (15, 30, 50, 75, 100%); each wash was performed for 3 min.

5.2.9. DNA extraction

Total DNA extraction was performed using the commercial kit QIAamp Fast DNA™ Stool Mini Kit (QIAGEN) as already described. For DNA extraction from scaffolds, the first step was adapted from the manufacturer's instructions. The scaffolds were soaked in 2 ml InhibitEx Buffer and subjected to 3 min ultrasonic bath and 3 min vortex to maximize biofilm detachment and retrieval.

Purity and concentration of the extracted DNA were assessed using the Spectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). The DNA samples were stored at -20°C for subsequent analyses.

5.2.10. 16S rRNA metagenomic sequencing

Groundwater and carrier-colonizing microbial communities were analyzed by 16S rRNA Illumina sequencing. Bacterial and archaeal V3-V4 hypervariable region of the 16S rRNA gene were amplified using extracted DNA as template with primers Pro341F/Pro805R (Takahashi et al., 2014) as already described (Chapter II).

5.3. Results and discussions

5.3.1. 1,2-DCA uptake by biopolymeric scaffolds

PLA and PCL (ES and M-M) scaffolds were tested for the capacity to absorb 1,2-DCA from an aqueous solution. After 48 hours incubation of scaffolds with a 1,2-DCA aqueous solution, residual 1,2-DCA concentration was assessed by HS-GC/MS. Percentage of 1,2-DCA absorbed was reported to the weight of the scaffold. Preliminary results of the test revealed PLA ES scaffolds could entrap up to 12% of 1,2-DCA per mg of scaffold (Fig. 5.5). PLA M-M scaffolds were also good in adsorbing the contaminant, while the PCL scaffold resulted less suitable to adsorption. The presence of entrapped 1,2-DCA within the scaffold could favor the enrichment of a dechlorinating bacterial community colonizing the scaffold.

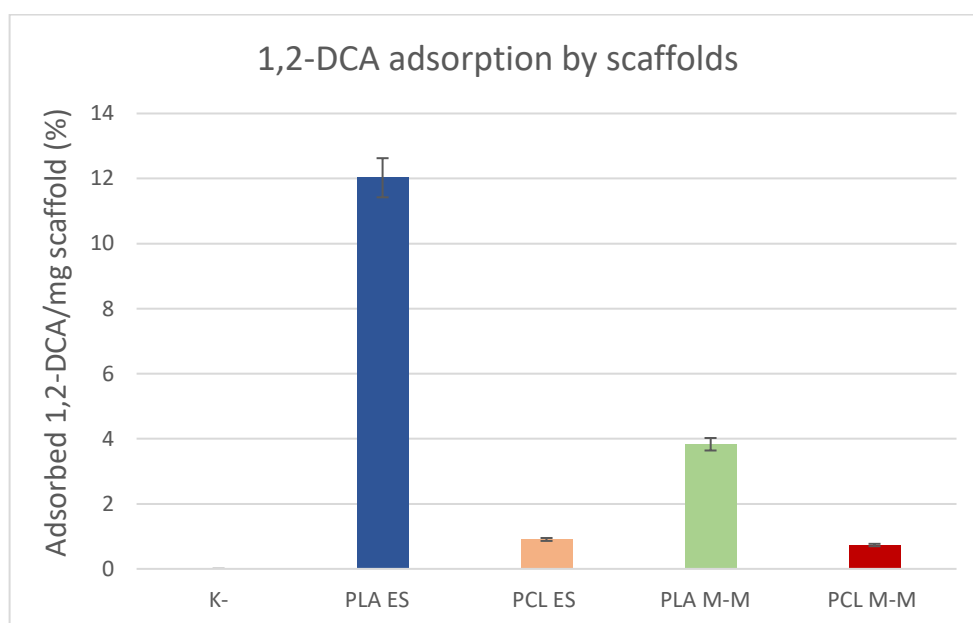


Figure 5.5. 1,2-DCA uptake test on scaffolds. Percentage of 1,2-DCA uptaken by scaffolds reported to the weight of each scaffold after 48h incubation. K-: negative control system without scaffold; PLA: polylactic acid; PCL: polycaprolactone; ES: electrospinning scaffold; M-M: melt-mixing scaffold.

5.3.2. Bacterial biofilm formation on biopolymeric scaffolds

To test if the scaffolds can host a bacterial biofilm, PLA and PCL (ES and M-M) scaffolds were incubated with the marine biofilm-forming bacterium *Pseudoalteromonas sp.*

After 48 and 96 hours incubation, scaffolds were retrieved, air dried and weighted. Results show all scaffolds could host a microbial biofilm to a different extent even after 48 h, and biofilm amount increases over time (Fig. 5.6). The best performing scaffolds were PLA and PCL M-M. Viability of attached microorganisms was confirmed by growth on marine agar at the end of every incubation time.

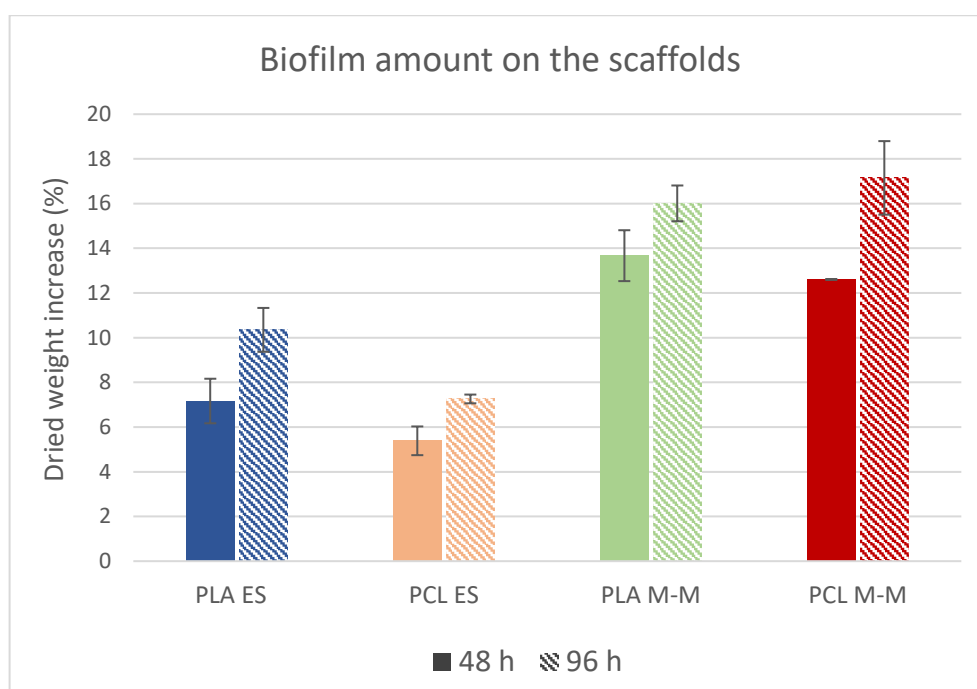


Figure 5.6. Bacterial adhesion test on scaffolds. Amount of biomass on scaffolds after two incubation times, calculated as dried weight increase in respect to initial dried weight. PLA: polylactic acid; PCL: polycaprolactone; ES: electrospinning scaffold; M-M: melt-mixing scaffold.

5.3.3. Scaffolds colonization by groundwater microbial communities

PLA ES scaffolds were chosen for further studies in order to assess whether scaffolds can function as traps by recruiting complex microbial communities from an environmental sample. Therefore, the scaffolds were exposed to contaminated groundwater both by a microcosm-scale study and by a preliminary *in situ* experiment. With reference to microcosms studies, PLA ES scaffolds were incubated in raw groundwater in microcosms set up under a $N_2/CO_2/H_2$ (80%/15%/5%) atmosphere or under aerobic conditions and scaffolds colonization was assessed by SEM observations. SEM images confirmed bacterial colonization within the tridimensional structure of the scaffolds showing a significant presence of cell aggregates along the fibers under both aerobic and anaerobic conditions, with thicker colonization of aerobic biofilms (Fig. 5.7).

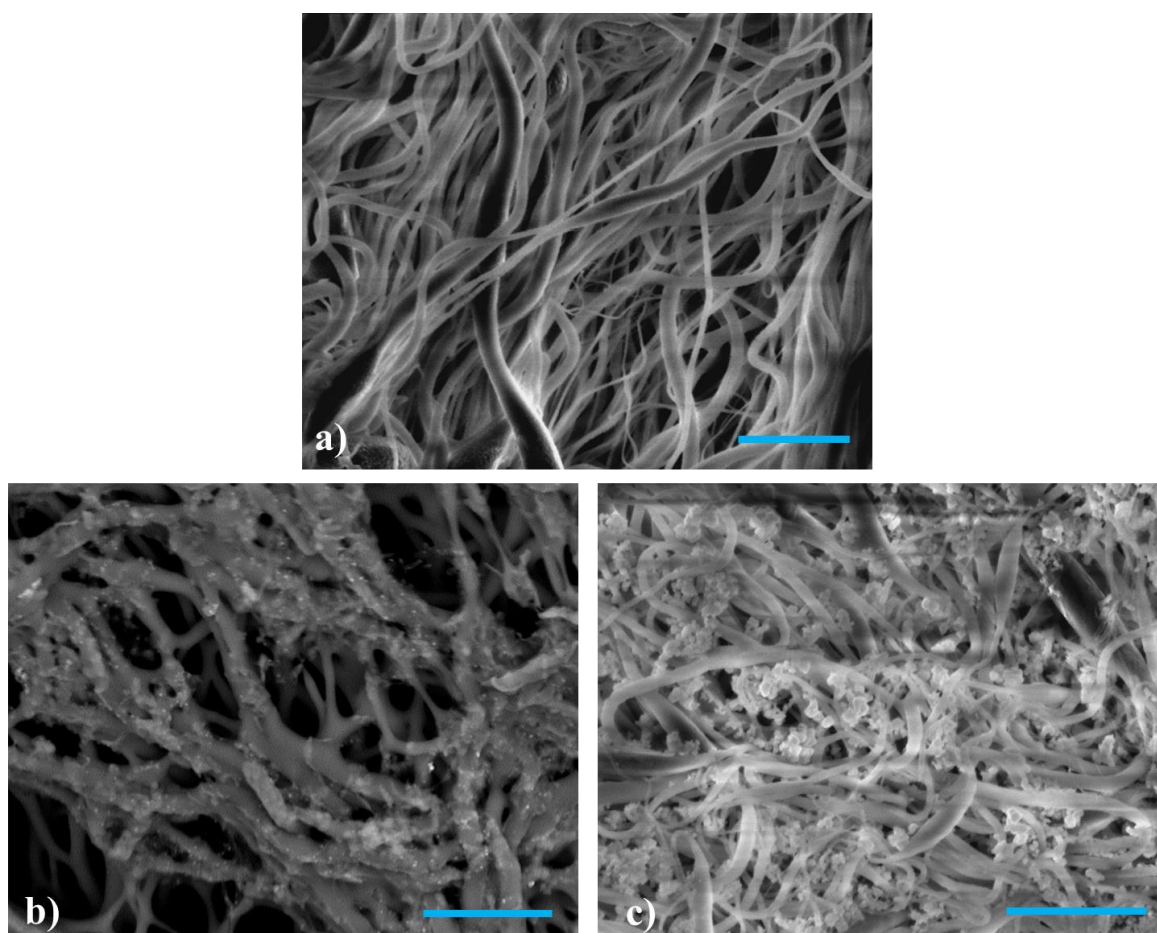


Figure 5.7. SEM images of a) uncolonized fibers of PLA ES scaffold, b) biofilm formed on PLA ES scaffold under anaerobic conditions and c) biofilm formed on PLA ES scaffold under aerobic conditions. Blue bars are 10 μ m.

After exposure to groundwater in microcosm or in the aquifer, the microbial biofilm was retrieved from the scaffolds and total DNA was extracted. In order to estimate bacterial biomass in groundwater samples and biomass adhered to the scaffolds, DNA amount was referred to the volume of groundwater filtered or to the single unit of scaffolds retrieved after the incubation. Both groundwater samples (GW-S1-May21 and GW-S2-May22) are characterized by low biomass. About 60 ng of DNA per scaffold and 26 ng of DNA per scaffold were extracted respectively from the ES scaffolds in the anaerobic microcosm (ES-S1) and from the ES scaffolds in aerobic microcosm (ES-S2). Both values are notably higher than DNA extracted from scaffolds incubated in the aquifer (ES-aquifer). Microbial communities from groundwater at the moment of the sampling (GW-S1-may21 and GW-S2-May22) and biofilm communities from scaffolds were analyzed by metagenomic 16S Illumina sequencing. Reads number and OTUs for each sample are reported in Tab. 5.1.

	GW-S1-May 21	ES-S1	GW-S2-May22	ES-S2	ES-aquifer
Biomass (Extracted DNA)	3.4 ng/ml	60 ng/ scaffold	6.1 ng/ml	26 ng/ scaffold	6.4 ng/ scaffold
Final Reads	36,384	18,725	62,453	41,833	61,372
OTUs	223	93	323	99	290

Table 5.1. Microbial biomass, reads number and OTUs obtained from 16S Illumina sequencing of groundwater samples (GW-S1-May21 and GWS2-May22) and biofilm colonizing scaffolds (ES-S1 and ES- S2).

5.3.3.1. Scaffold-colonizing groundwater microbial communities in microcosm

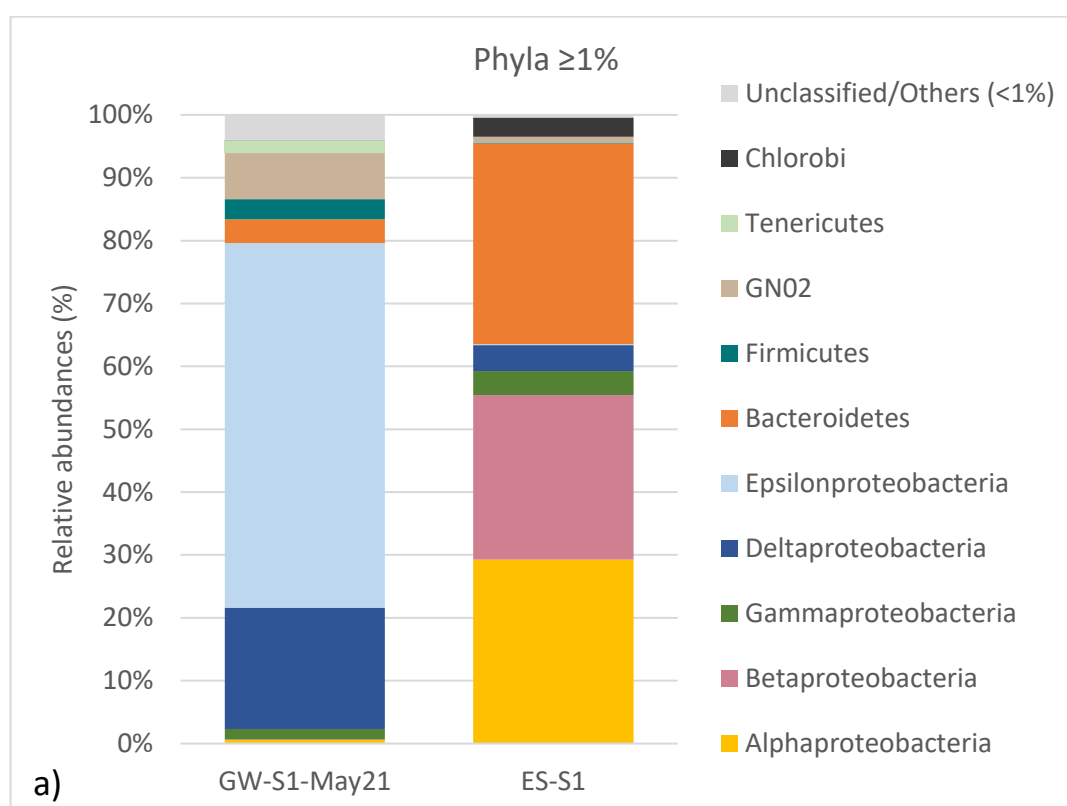
In the experiment of scaffolds colonization set up under N₂/CO₂/H₂ (80%/15%/5%) atmosphere, the same groundwater sample (GW-S1-May21) used to set up anaerobic *lac* and *na* microcosms (Chapter II) was used. The scaffolds were retrieved and analyzed after 30 days of incubation (Fig. 5.8).

The number of OTUs in the biofilms retrieved from the ES scaffolds after exposure to GW in microcosm systems was significantly lower than the number of OTUs in groundwater (Tab 5.1).

The microbial community retrieved from the scaffold was significantly different in composition from original planktonic communities in groundwater at the beginning of the incubation. GW-S1-May21 was dominated by family *Helicobacteraceae* (57%) within *Epsilonproteobacteria*, in particular by sulfur-oxidizing genera *Sulfuricurvum* (43%) and *Sulfurimonas* (13%), followed by the family *Desulfuromonadaceae* (11%), comprising known anaerobic dechlorinating genera (Löffler et al., 2000), within *Deltaproteobacteria*. Concerning the scaffolds, *Deltaproteobacteria* were poorly represented in the biofilm, but the *Desulfuromonadaceae* family, potentially including *OHRB* genera, was still present (1.2%). *Alphaproteobacteria*, *Betaproteobacteria* and *Bacteroidetes* were the dominant taxa in the scaffold biofilm. Within *Alphaproteobacteria*, *Xanthobacteraceae* (11%) and *Rhodobacteraceae* (10.5%) were the most abundant families. In particular, the known aerobic 1,2-DCA dechlorinating genus *Ancylobacter* (*Xanthobacteraceae*) was strongly represented (8.4%)(Van Den Wijngaard et al., 1992). *Rhodobacteraceae* were mainly made up of the genus *Paracoccus* (5.6%), while *Rhodobacter* (2.2%) was a minor component in the biofilm. *Paracoccus* is considered a metabolically versatile genus: members of this genus have been reported to grow by aerobic or anaerobic respiration on a variety of carbon sources, to be methylotrophs and to be involved in the biodegradation of many environmental contaminants, including chlorinated hydrocarbons (Puri et al., 2022).

The most abundant identified family in the biofilm community was *Comamonadaceae* (*Betaproteobacteria*), entirely made up by the genus *Hydrogenophaga* (24.7%) in the

sample. Members of this genus are aerobic chemoorganotrophic or chemolithoautotrophic bacteria and they can use the oxidation of H₂ as an energy source (Willems et al., 1989). Both *Hydrogenophaga* and *Rhodobacter* have been reported to be associated to aerobic cometabolism of TCE (Liang et al., 2011). Flavobacteriaceae (Bacteroidetes), the other major component of scaffold-colonizing community (about 16%) include *Flavobacterium* (10%) and *Muricauda* (5%). *Flavobacterium* is an aerobic chemoorganotrophic genus with a respiratory type of metabolism. In some species, anaerobic growth can occur fermentatively or by respiration reducing nitrate (Bernardet & Bowman, 2006) and aerobic, chlorophenol-utilizing *Flavobacterium* sp. have been reported (Orser & Lange, 1994).



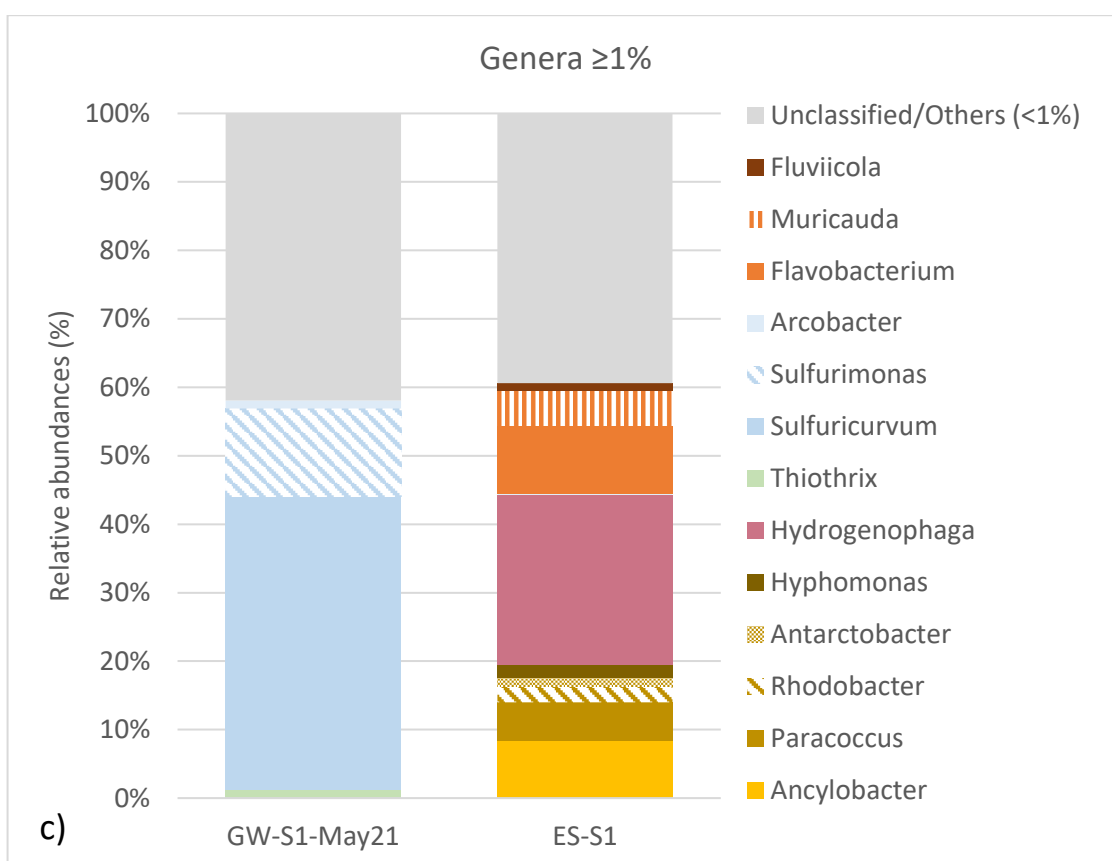
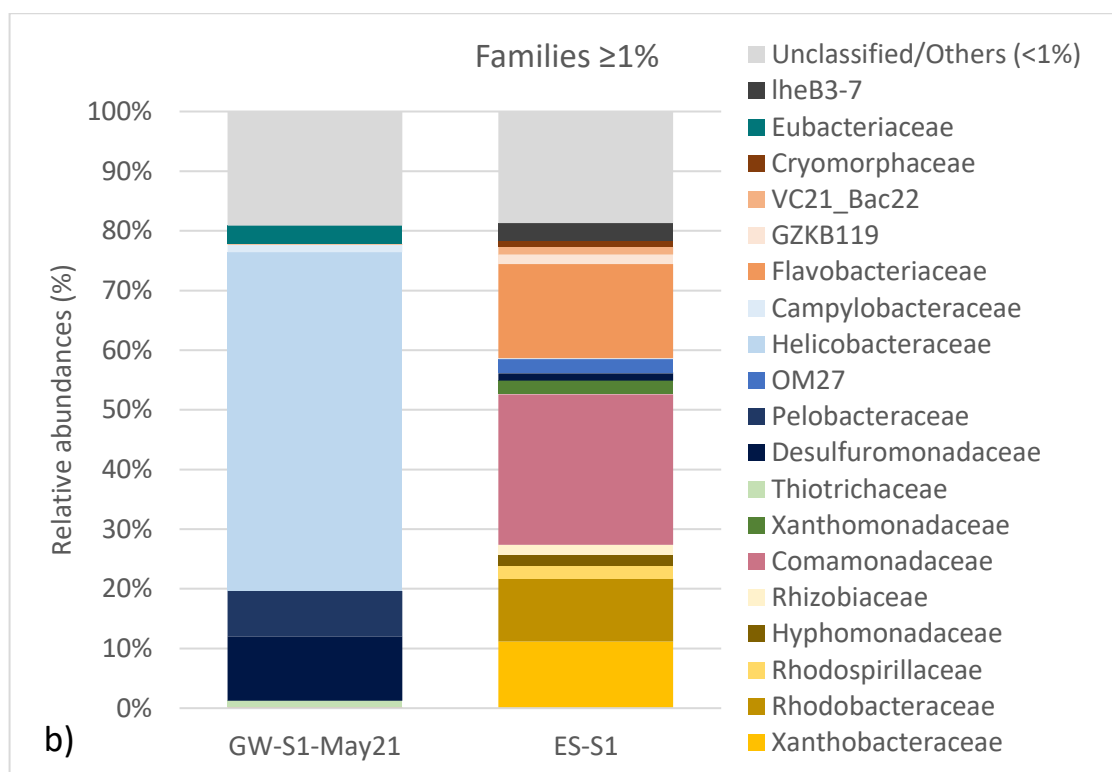


Figure 5.8. Relative abundances (%) of bacterial a) phyla (down to class level for *Proteobacteria*), b) families and c) genera in 1,2-DCA contaminated groundwater sample GW-S1-May21 and biofilm formed on the scaffold ES-S1. Taxa with relative abundance $\geq 1\%$ in at least one sample are represented. Others/unclassified includes unclassified and $<1\%$ taxa.

The unexpected enrichment of aerobic genera in a microcosm prepared under anaerobic atmosphere with low redox groundwater, can be explained by the presence of a certain amount of dissolved oxygen in the groundwater sample. While in *lac* and *na* microcosms (described in Chapter II), set up under the same conditions, the presence of a reducing agent in culture medium (cysteine) probably allowed to establish true anaerobic conditions, oxygen in untreated groundwater was likely enough to sustain the growth of aerobic taxa. At the same time the presence of H₂ in the atmosphere of the glove box where the microcosm was set up, allowed the enrichment of *Hydrogenophaga*. Nevertheless, dissolved oxygen allowed the enrichment of the aerobic 1,2-DCA dechlorinating genus *Ancylobacter*.

Encouraged by the enrichment and immobilization of aerobic dechlorinating genera as scaffold-colonizing microorganisms, another experiment of scaffold exposure to groundwater in microcosm was attempted by setting up the microcosm under aerobic conditions. Another groundwater sample (GW-S2-May22) was collected from Site 2 within the study area. The scaffolds were soaked in groundwater and retrieved after 60 days incubation (Fig. 5.9). Microbial community composition in GW-S2-May22 was similar to the groundwater sample used in the previous experiment, with Epsilonproteobacteria being dominant and made up of the Helicobacteraceae (70.6%) and Campylobacteraceae (4%) families. *Sulfuricurvum* was the most abundant identified genus, suggesting again the most relevant microbial activity in untreated groundwater is sulfur oxidation, while *Sulfurospirillum* (Campylobacteraceae), a genus including organohalide respiring species, may play a role in dechlorination or contribute to sulfur compounds oxidation (Goris & Diekert, 2016).

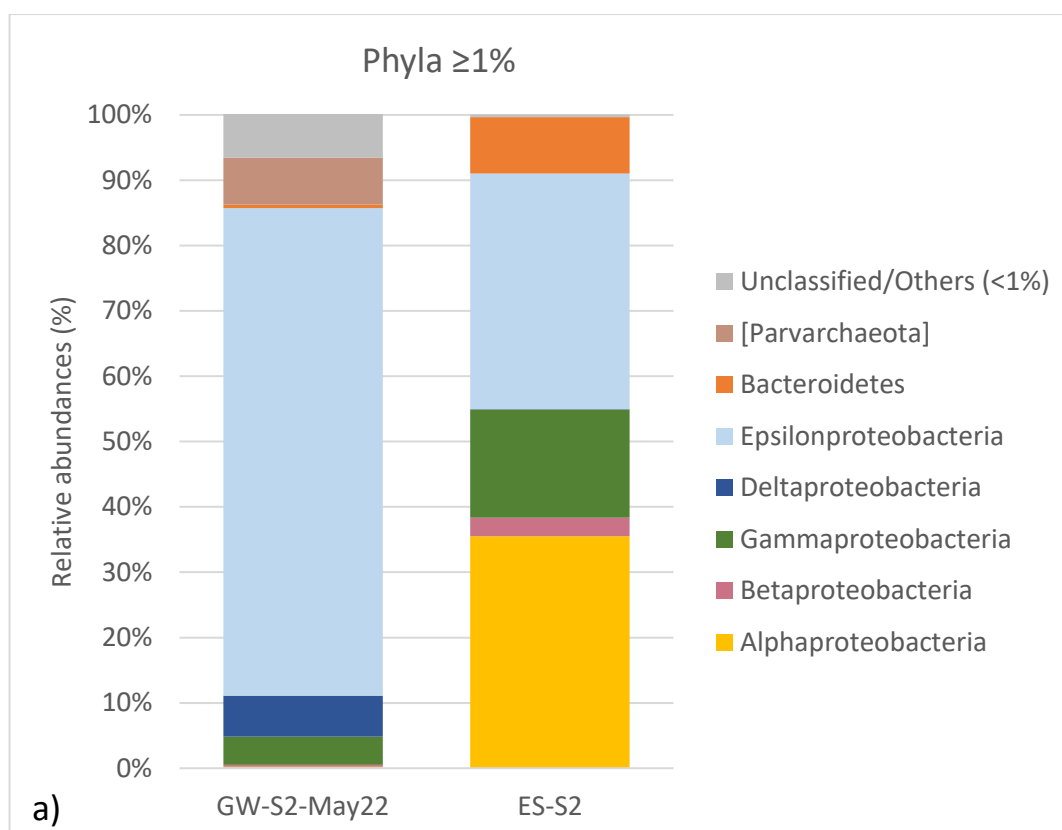
Both genera *Sulfuricurvum* and *Sulfurospirillum*, were still present as components of the scaffold colonizing community (respectively 31% and 4.5% of the biofilm community). Alphaproteobacteria (35.5%) and Gammaproteobacteria (16.5%) were also abundant within the scaffold-colonizing community. A major component within Alphaproteobacteria was the genus *Paracoccus* (about 14%) (Rhodobacteraceae) that might play a role in oxidation of sulfur compounds (Puri et al., 2022) as well as *Sulfuricurvum*. Rhodospirillaceae (8%) and Erytrobacteraceae (9.5%) were mostly unidentified, except for the genus *Phaeospirillum* (2%). Members of this genus are anaerobic and anoxygenic phototrophic bacteria, but chemotrophic growth is possible under dark and micro-oxic conditions (Imhoff et al., 1998).

Shewanella was the most represented genus (7.6%) among identified Gammaproteobacteria in the biofilm. Members of this genus are facultative aerobic, but mainly found in anaerobic and microaerobic conditions (Lemaire et al., 2021). As already reported, reductive dehalogenation in *Shewanella* spp. is known during anaerobic respiration (Hau & Gralnick, 2007).

The genus *Pseudomonas* was detected in the biofilm (1.7%), but not in groundwater (GW-S2-May22). The isolation of a 1,2-DCA dechlorinating strain of *Pseudomonas* from a DCA-degrading biofilm is described in (Hage & Hartmans, 1999). This strain requires oxygen to use 1,2- DCA as sole carbon and energy source by a monooxygenase-mediated oxidation.

The TCE cometabolic aerobic degraders *Rhodobacter* (not shown, 0.9%) and *Hydrogenophaga* (1.5%), already described as biofilm components also in the microcosm set up under anaerobic conditions, were detected.

The setup of this microcosm was carried out under aerobic condition and favoring aeration by incubation in a rotary shaker. The enrichment of potential aerobic dechlorinating strains was obtained together with microaerobic and facultative anaerobic taxa, suggesting that low oxygen micro-niches could be formed in the biofilm.



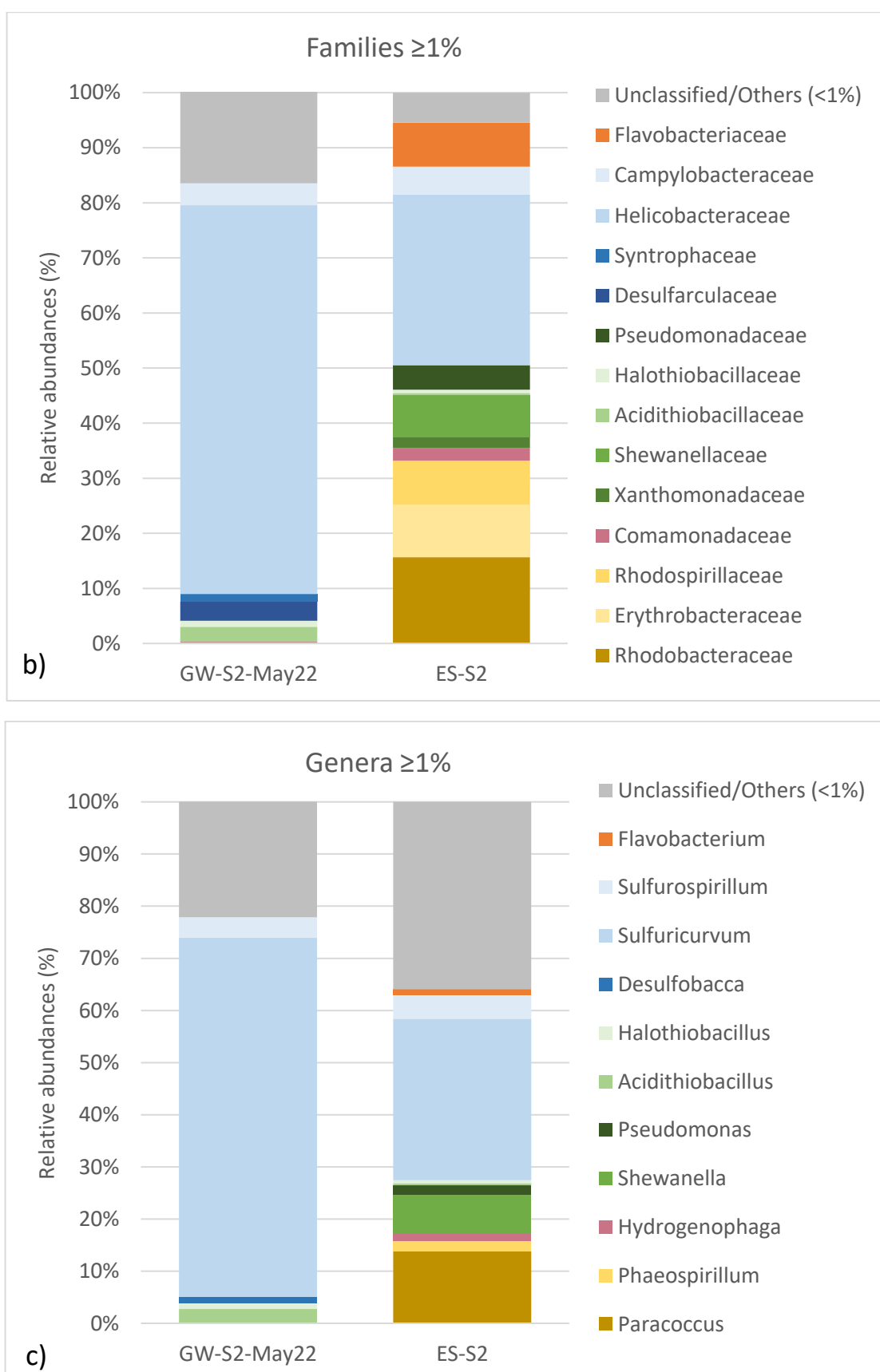


Figure 5.9. Relative abundances (%) of bacterial a) phyla (down to class level for *Proteobacteria*), b) families and c) genera in 1,2-DCA contaminated groundwater sample GW-S2-May22 and biofilm formed on the scaffold ES-S2. Taxa with relative abundance $\geq 1\%$ in at least one sample are represented. Others/unclassified includes unclassified and $<1\%$ taxa.

5.3.3.2. Scaffold-colonizing microbial communities in the aquifer

In the perspective of possible *in situ* bioaugmentation purposes, a further step in testing the scaffolds suitability as bacterial carriers for the immobilization of bioremediation agents consisted in exposing the scaffolds in a real contaminated aquifer.

The preliminary *in situ* experiment was carried out in parallel with the aerobic microcosms set up. The scaffolds were immersed in the aquifer and retrieved after 60 days of incubation. When retrieved, scaffolds were covered in a dark layer of viscous material (Fig 5.10). The amount of biomass collected per scaffold (ES-aquifer), estimated in terms of extracted DNA (Tab. 5.1), is lower than biomass colonizing scaffolds in microcosms.



Figure 5.10. PLA ES scaffolds retrieved after 60 days of incubation at 20 m depth in the aquifer, site S2.

The composition of the microbial community recruited from the aquifer was analyzed by 16S rRNA Illumina sequencing. The number of OTUs in the community retrieved from the scaffolds was similar to the number of OTUs found in groundwater (Tab 5.1).

The composition of groundwater (GW-S2-May22) and biofilm (ES-aquifer) microbial communities was significantly different. In the biofilm Firmicutes (27%), Bacteroidetes (17%) and Actinobacteria (9%), almost absent in groundwater (<1%), were the dominant groups, while the most abundant GW groups Epsilonproteobacteria (77%) and Parvarchaeota (7%) were less represented on the scaffold (4 and 1.5%, respectively). Other relatively abundant taxa on the scaffold were Gammaproteobacteria (8%), Spirochaetes (7.5%), Deltaproteobacteria (6%) and Alphaproteobacteria (3%).

At a lower taxonomic level, the most abundant group in biofilm community was represented by Staphylococcaceae, entirely made up of *Staphylococcus* (15%), a pathogenic bacterium whose presence was assessed in diverse aquatic environments (Silva et al., 2020). Other Firmicutes included Clostridiaceae (the genus *Clostridium*, in particular, 5.5%) and

Acidaminobacteraceae (5%) mainly made up of *Fusibacter* (2%), an anaerobic sulfate-reducing and fermentative bacterium (Ravot et al., 1999), and *WH1-8* (3%). Both genera *Fusibacter* and *WH1-8* have been reported to increase in abundance in microcosms systems treated with oil; *Fusibacter*, in particular, has also been reported to degrade crude oil (Koo et al., 2015). *Clostridium* sp. strains have been reported to degrade cDCE and VC by organohalide respiration (Dolinová et al., 2017).

Within Bacteroidetes, the uncharacterized family SB-1, related to a benzene degrading consortium dependent on sulfate-reduction (Phelps et al., 1998), was particularly abundant (8.3%) as component of the scaffold colonizing biofilm. Bacteroidetes also included the family Rikenellaceae (3%), whose members are described as anaerobic, carbohydrate-fermenting bacteria. Genera of this family, commonly found in wastewater or freshwater environments, include hydrogen and acetate producing bacteria (Su et al., 2014).

Actinobacteria, only detected as biofilm components, were made up of *Corynebacterium* (4%, Corynebacteriaceae), and *Propionibacterium* (4%, Propionibacteriaceae). *Corynebacterium* strains able to grow aerobically on 1,2-DCA (Olaniran et al., 2004) or anaerobically 1-chlorobutan (Yokota et al., 1987) were isolated; two *Propionibacterium* sp. strains were found able to anaerobically dechlorinate PCE and 1,2-DCE (Chang et al., 2011).

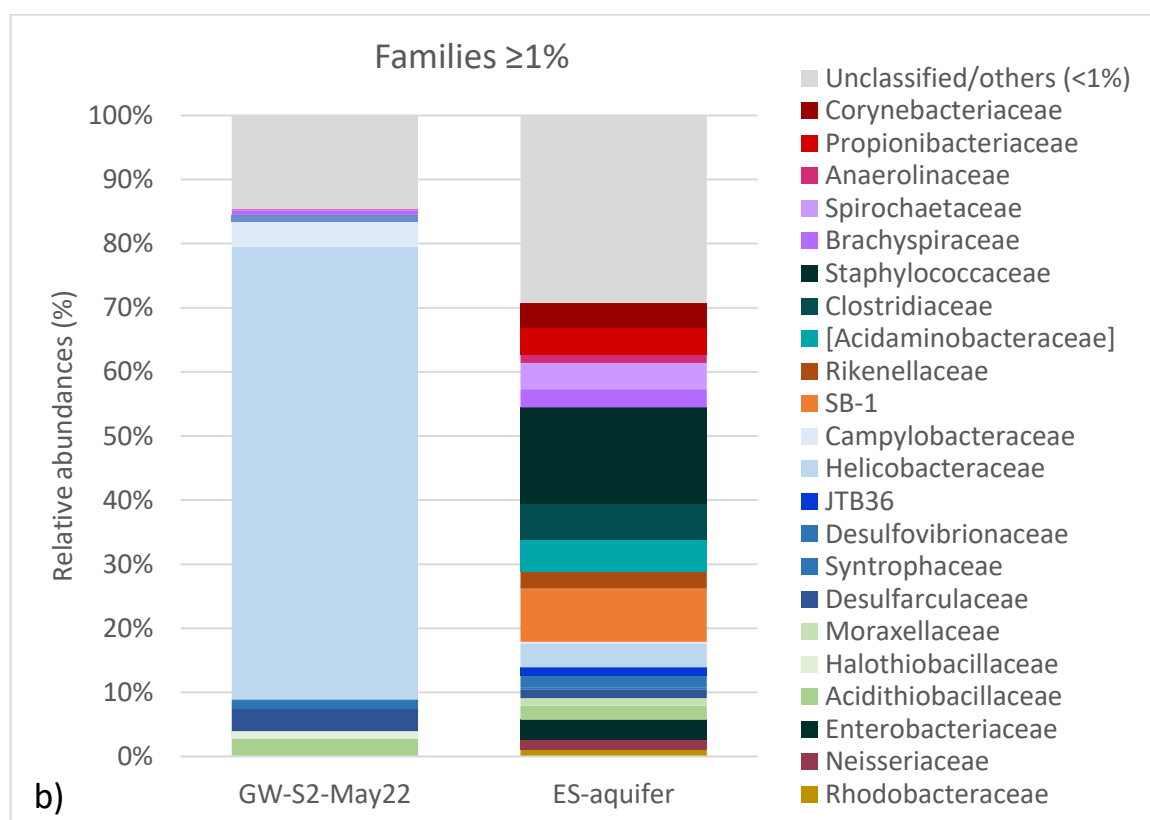
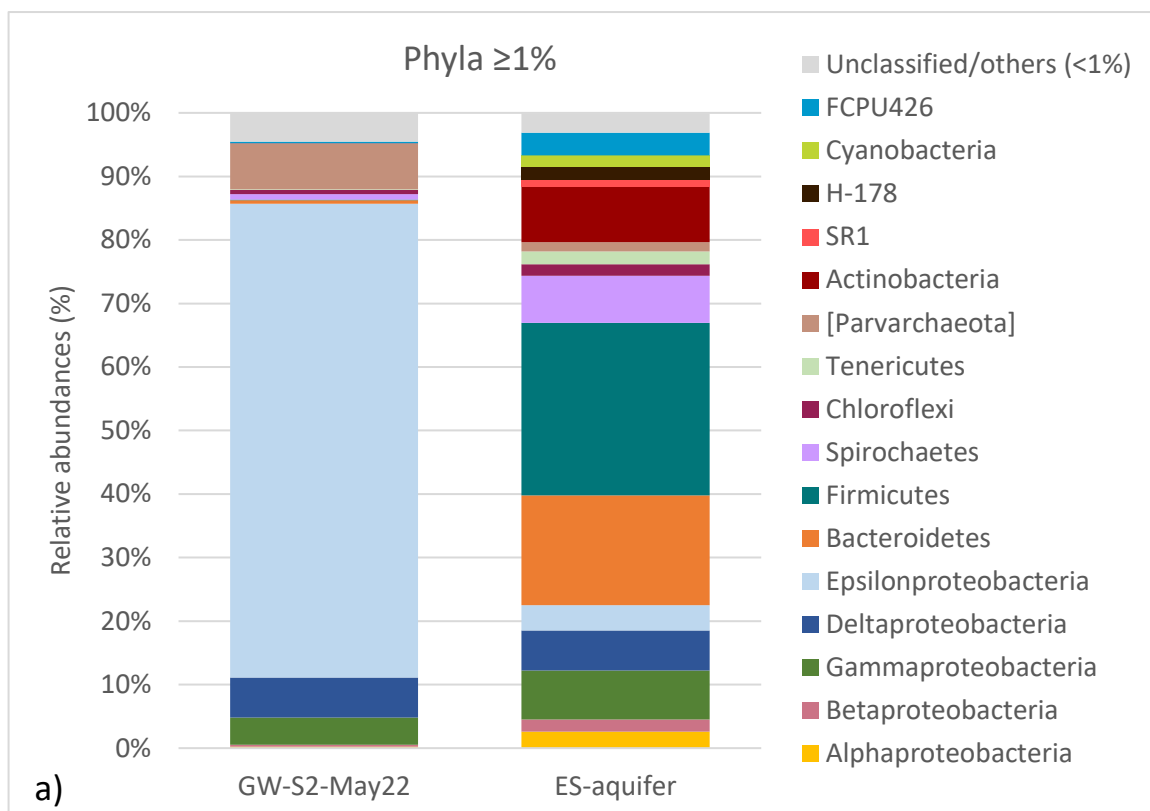
Sulfur-oxidizing genera, *Sulfuricurvum* (Epsilonproteobacteria, Helicobacteraceae) and *Acidithiobacillus* (Gammaproteobacteria, Acidithiobacillaceae) (Pokorna & Zabranska, 2015) were detected in the scaffold-colonizing community (3% and 2% respectively), but they were notably less abundant than in groundwater (69% and 3% respectively).

Within Gammaproteobacteria, the Enterobacteriaceae family was detected in the biofilm (3%); this family includes members able to respire the chloroethenes PCE and TCE (Dolinová et al., 2017).

Other genera detected in the biofilm to a lesser extent included the potential chlorinated hydrocarbons degrader *Paracoccus* (1%, Rhodobacteraceae) (Puri et al., 2022), also found as a biofilm component on scaffolds in microcosms, and the facultative OHRB *Desulfovibrio* (2%, Desulfovibrionaceae) (Türkowsky et al., 2018).

Overall, the sulfur-oxidizing taxa, accounting for most of the groundwater community, notably decreased in the biofilm forming microbial community recruited on the scaffold, while some known dechlorinating taxa were enriched. Although most taxa detected are not

known as 1,2-DCA degraders, they could act on other chlorinated contaminants present in the aquifer, such as PCE, TCE, 1,2-DCE and VC.



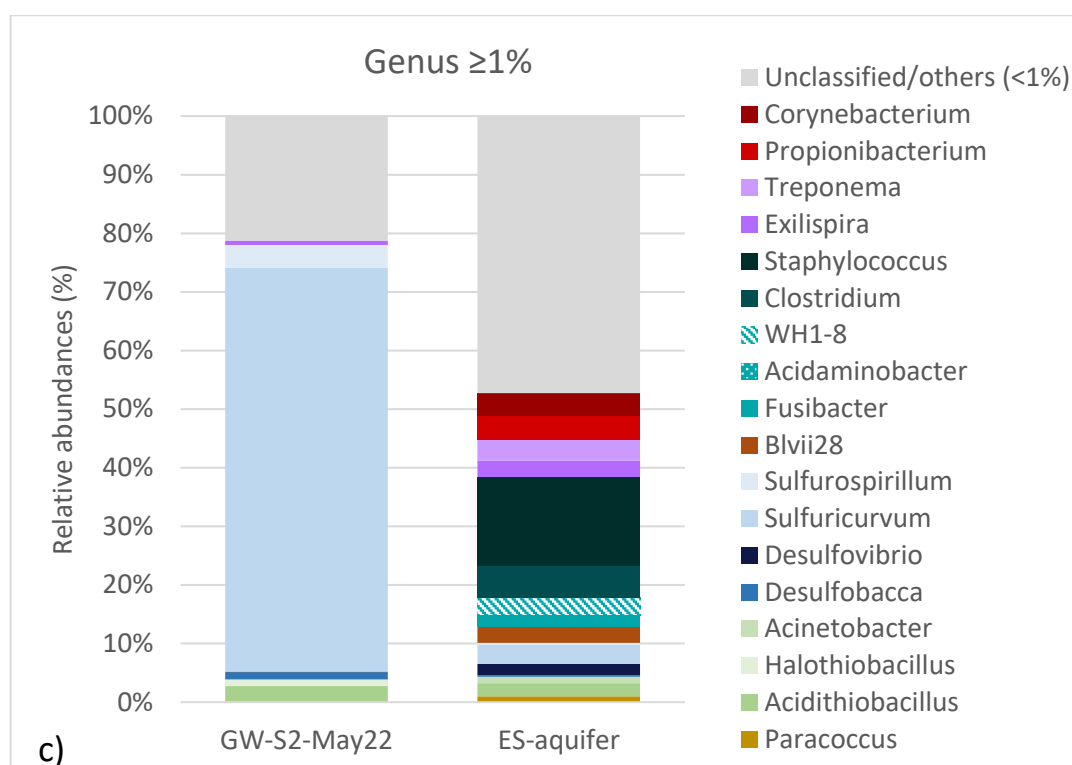


Figure 5.11. Relative abundances (%) of bacterial a) phyla (down to class level for *Proteobacteria*), b) families and c) genera in 1,2-DCA contaminated groundwater sample GW-S2-May22 and biofilm formed on the scaffold. Taxa with relative abundance $\geq 1\%$ in at least one sample are represented. Others/unclassified includes unclassified and $<1\%$ taxa.

5.4. Conclusions

The biopolymeric PLA and PCL scaffolds tested in this study were found able to entrap 1,2-DCA from an aqueous solution and to host a bacterial biofilm.

Based on preliminary tests to assess contaminant uptake and biofilm formation, PLA scaffolds produced by electrospinning were selected for further studies. A microcosm-scale study with real groundwater samples was set up to evaluate if the autochthonous microbial community can be immobilized on scaffolds and if dechlorinating taxa can be enriched within the immobilized biofilm. The scaffolds were successfully colonized by microorganisms from real groundwater samples, confirming they can host a complex biofilm, as revealed by SEM observation and 16S Illumina sequencing. Moreover, within immobilized bacterial communities, taxa including aerobic and anaerobic dechlorinating strains were detected, such as the Desulfuromondaceae family, and the genera *Ancylobacter*, *Hydrogenophaga*, *Rhodobacter* and *Pseudomonas*. A preliminary *in situ* exposure experiment, set up to investigate whether these scaffolds can favor the colonization by autochthonous dechlorinating communities (if present in the site) resulted in the detection of taxa potentially involved in the dechlorination of various CAHs as components of the biofilm formed on the scaffold. These results suggest that the scaffolds could be used to host a dechlorinating biofilm.

Further studies are necessary to test if these scaffolds can potentially be applied into *in situ* or *ex situ* enhanced bioremediation strategies based on immobilized selected dechlorinating biofilm. Chemical monitoring of microcosm systems containing pre-colonized scaffolds using selected dechlorinating taxa or communities should be performed to assess if their immobilization within a biofilm leads to the enhancement of biodegradation activity. Immobilization tests and biodegradation monitoring could also be carried out with 1,2-DCA dechlorinating isolates obtained in this study.

Final conclusions

The research activities carried out in the context of this PhD fall in the framework of bioremediation of chlorinated solvents contaminated sites. Chlorinated solvents are widespread, recalcitrant and toxic environmental pollutants, and bioremediation has recently emerged as an effective and eco-friendly solution. As the implementation of bioremediation strategies is usually site-specific, the evaluation of the catabolic potential of a 1,2-dichloroethane (1,2-DCA) contaminated aquifer was carried out with the aim to provide information in the perspective of eventual *in situ* bioremediation interventions.

The activities here described led to the first bacterial characterization of the 1,2-dichloroethane contaminated aquifer under study, yet unexplored under the microbiological aspect, revealing a significant diversity among the different sampling sites of the aquifer and a low intrinsic bioremediation potential. Notably, on the basis of the taxonomic analysis of the aquifer bacterial communities, the known and most common dechlorinating taxa, usually exploited in bioremediation interventions, were scarce or absent. Nevertheless, both anaerobic and aerobic dechlorinating taxa were successfully biostimulated in laboratory scale microcosm studies, suggesting the autochthonous bacterial communities could be potentially exploited for 1,2-DCA removal, if properly biostimulated. Under anaerobic conditions, the enrichment in Organohalide Respiring Bacteria and the detection of reductive dehalogenase genes suggested reductive dechlorination was the main catabolic pathway in anaerobic microcosms; cometabolic reductive dechlorination processes mediated by *Acetobacterium* also occurred. Under aerobic conditions, known aerobic hydrolytic 1,2-DCA degraders were enriched and the haloalkane dehalogenase gene was detected, suggesting 1,2-DCA was degraded by an aerobic hydrolytic pathway. Although the anaerobic process of reductive dechlorination is the most common pathway exploited in aquifer bioremediation strategies, this study provided evidence that relying on the aerobic processes could be a promising approach for site-specific clean-up interventions.

Moreover, stable aerobic 1,2-DCA dechlorinating consortia were obtained. These consortia included genera *Xanthobacter*, *Ancylobacter* and *Starkeya* spp., known for the hydrolytic 1,2-DCA dechlorination abilities. The detection of the haloalkane dehalogenase gene *dhlA*, identical to the ones so far detected in aerobic 1,2-DCA degraders isolated in other distant geographic locations, further confirms its strong association with 1,2-DCA hydrolytic degradation and expands its geographical distribution. The gene repertoire also suggests

these genes could be located on a catabolic plasmid, similar to one recently found in other isolates.

Innovative biotechnological applications were tested, yielding promising results.

The enriched dechlorinating cultures obtained from the anaerobic and aerobic biostimulation treatments were successfully applied in a pilot test in column simulating two bioremediation approaches using a continuous flow system fed with real contaminated groundwater. A polyhydroxybutyrate-based permeable reactive barrier set up under anaerobic condition and an aerobic approach were both proven to be suitable as bioremediation systems, leading to the removal of 1,2-DCA by anaerobic and aerobic biodegradation processes. The possibility to exploit polyhydroxybutyrate, a completely biodegradable biopolymer that can be produced from low-cost sources such as wastewater, would be particularly interesting.

Moreover, innovative biopolymeric tridimensional scaffolds were proven to be suitable as carriers for bacterial immobilization and potential application in bioaugmentation approaches. Laboratory scale studies and a preliminary field experiment revealed the scaffolds can uptake 1,2-DCA and host bacterial biofilms that include anaerobic or aerobic dechlorinating taxa, suggesting a potential application in enhanced CAHs bioremediation based on immobilized dechlorinating biofilms.

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Attached A

Primer sets used in this study.

Abbreviations of degenerate nucleotides: k = g/t; m = a/c; r = a/g; s = c/g; w = a/t; y = c/t

Target gene	Primer set	Sequence (5'->3')	Annealing temp (°C)	PCR product (bp)	Ref.
Primer sets targeting 16S rRNA gene					
V3-V4 region (16S rRNA gene)	Pro341F	TCGTCGGCAGCGTCAGATGTGTATAAGAGAC	55	460	1
	Pro805R	AGCCTACGGGNBGCASCAG GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGGACTACNVGGGTATCTAATCC			
16S rRNA gene (universal primers)	27F	AGAGTTTGATCMTGGCTCAG	50	1500	2
	1492R	TACGGYTACCTTGTACGACTT			
Primer sets targeting 16S- 23S rRNA intergenic spacers (ITS)					
16S- 23S rRNA intergenic spacer (ITS)	ITSf	GTCGTAACAAGGTAGCCGTA	55	-	3
	ITSReub-Hex (ITSReub)	GCCAAGGCATCCACC			
Primer sets targeting OHRB 16S rRNA gene tested in anaerobic microcosms DNA					
<i>Dehalococcoides</i> 16S rRNA gene	<i>Dhc1f</i> <i>Dhc264r</i>	GATGAACGCTAGCGGCG CCTCTCAGACCAGCTACCGATCGAA	59	264	4
<i>Dehalogenimonas</i> 16S rRNA gene	<i>BL-DC-142f</i> <i>BL-DC-1351r</i>	GTGGGGGATAACACTTCGAAAGAAGTGC AAC GCG CTA TGC TGA CAC GCG T	63	1199	5
<i>Dehalobacter</i> 16S rRNA gene	<i>Dhb477f</i> <i>Dhb647r</i>	GATTGACGGTACCTAACGAGG TACAGTTTCCAATGCTTTACGG	63	170	6
<i>Desulfitobacterium</i> 16S rRNA gene	<i>Desulfo16sF3</i> <i>Desulfo16sR5</i>	TTAAGTAGATGGATCCGCGTCTG TTCCGATGCAGTCCCAGG	51	500	7
<i>Desulfuromonas</i> (PCE dechlorinating species) 16S rRNA gene	<i>dsm16sF</i> <i>dsm16sR</i>	AACCTTCGGGTCCTACTGTC GCCGAAGTACCCCTATGTT	58	835	8

Primer sets targeting reductive dehalogenase genes tested on DNA extracted from anaerobic microcosms					
<i>dcaA</i> gene (1,2-DCA specific <i>rdh</i>)	DHL-F1	GGACCTCGTTGGACTCC	54	400	9
	DHL-R1	GGCAAATCCCATGGCATT			
	DHL-F2	GTAAAAAAGGCAGCCTGTT	54	326	
	DHL-R2	GTAAACTTTCCCGTCGC			10
	PceAFor1	ACGTGCAATTATTATTAAGG	54	1944	
	DcaBRev	TGGTATTCACGCTCCGA			
	Rdh552F	ATGACCAATGAAATAGCTAATGA	52	95	
<i>rdh</i> gene	Rdh625R	TGTTGGCCTTGTTGTCGCACT			
	ceRD2L	GCAGCACGCCTTTTTGGIGCIKMIYTNGTIGG	47	700-900	11
	RD7	AA NGG RCA IAC IGC IWC RCA			
	ceRD2S	GCAGCACGCCTTTTTGGIGCIKMIWSIGTIGG	47	700-900	6; 12
	RD7	AA NGG RCA IAC IGC IWC RCA			
	RRF2	SHMGBMGWGATTTYATGAARR	48	900	
<i>Sulfurospirillum</i> spp. <i>rdh</i> gene	RD7r	AA NGG RCA IAC IGC IWC RCA			
	RDH_Group20F	GCCTGAACTCTCAAGAAGAG	50	1470	13
	RDH_Group20R	GCTTACAGTGTCTCTAAAATGGTC			
<i>Shewanella</i> spp. <i>rdh</i> gene	Sul-rdhA-f	TTRGTRGGTRTTGCAAGATT	53	394	14
	Sul-rdhA-r	CTTGTCTTAAACCTGCTTC			
	RDH_group31BF	TGGATAAAACAMARAGTYGAAGG	50	960	13
	RDH_group31R	GRCAWGMHTCRGCRC			
Primer sets targeting haloalkane dehalogenase genes tested on DNA extracted from aerobic microcosms					
<i>Mycobacterium dhmA</i> gene; <i>Xanthobacter dhIA</i> gene	DHMf	GGCGAGCCCACCTGGAGYTAC	60	450	15
	DHMr	GWMKYGTCRGGGAARGGCGC			
<i>Xanthobacter dhIA</i> gene	DHLA 380F	GATTTTTGGGGCTGACCTTA	60	168	16
	DHLA 548R	GATGGCGTAACCAGATCGTA			
	DHLA 319F	CTTGCACTAATCGAACGGCTTG	65	284	
	DHLA 603R	AGCTTCGGTCAGTGTGGGCG			

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