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Enrichment and characterization of marine organohalide respiring bacteria and of their dehalogenating enzymes

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Abstract

Environmental pollution from dangerous and persistent man-made organohalide compounds have been going on for most of the 20th century. These compounds accumulated in freshwater and, ultimately, in marine sediments. Biological cycles of matter bring to the accumulation and concentration of these substances in living beings, inevitably reaching humans. Once in the human organism, this family of compounds cause a very wide spectrum of toxic effects, ranging from immune system disruption and cancerogenicity to hormonal or endocrine disorders and skin diseases, varying based on the specific substance and amount. Traditional strategies for remediation of contaminated sediments involve environmental balance-compromising techniques. In freshwater and marine sediments a natural anaerobic microbiological process called reductive dehalogenation (RD) takes place. The reaction involves the removal of the halogen atoms from organohalide compounds as these are used a terminal electron acceptor in respiration by some microbial species called organohalide-respiring bacteria (OHRB). The effect of microbial reductive dehalogenation (MRD) is the reduction of the halogenation level of organohalide substances, which makes them less toxic and more susceptible to further biodegradation. For these reasons, MRD is an interesting tool in the remediation of contaminated soils and sediments. Identification of OHRB and the study of their biochemistry made it possible to harness their biocatalytic power and stimulate their activity in organohalide-contaminated soils. The key enzymes responsible for the biocatalysis are called reductive dehalogenases, membrane-bound oxidoreductases which transfer electrons from the respiratory chain to organohalide compounds. MRD was initially discovered in freshwater soils and sediments and that is where most of the discoveries were done in terms of species and reductive dehalogenases involved. Nevertheless, MRD was subsequently observed in marine sediments too and a great diversity of reductive dehalogenases homologous (rdh) genes was detected, suggesting that the marine environment could be a source of powerful biocatalysts. The present study tackled MRD in the marine environment of two sites in the Adriatic Sea (Italy), the Venice Lagoon (VL) and Ravenna Harbor (RH). In a microcosm study, primary sediment from the two sites and from an OHRB-enriched PCB-dechlorinating slurry culture originally derived from a Venice lagoon sediment were spiked with different organochloride compounds: hexachlorobenzene (HCBe), 1,2,3,5-tetrachlorobenzene (TeCBe), pentachlorophenol (PCP), 2,3,5-trichlorophenol (TCP) and trichloroethylene (TCE), 1,2,3,4-tetrachlorodibenzo-p-dioxin (TeCDD) and a commercial mixture of PCBs (Aroclor© 1254). While all compounds were dechlorinated to a certain extent in OHRB-enriched cultures, primary sediments showed MRD of HCBe, TeCBe, TCP, TCE but not of PCP, and TeCDD, while PCBs dechlorination in VL sediments was not observed in RH but only in VL in previous studies. Microbial communities from the two primary sediments showed the same dechlorination capabilities but different specificities when it came to the type of chlorine substituents removed from the spiked compounds. Microbial community analysis revealed the specific enrichment of bacteria from the Dehalococcoidia class where dechlorination was taking place in most primary cultures. In enriched cultures the increase of previously identified specific phylotypes from the same taxon, VLD-1 and VLD-2, correlated with dechlorination. A sediment free TCE-dechlorinating consortium with only VLD-1 (17% of the total microbial population) was established first in synthetic marine water and then in a defined mineral medium with lactate and CO_2 as carbon sources and hydrogen as electron donor. Ampicillin and vancomycin proved efficient in positively selecting VLD-1 even further (69% of the total microbial population). The rdh diversity in the TCE-dechlorinating consortium was explored with an approach involving PCR degenerate primer pairs and Next Generation Sequencing of amplicons, which yielded 81 novel

rdh genes sequences. RNA expression studies of the 81 genes carried out on the TCE-dechlorinating cultures to link gene to function highlighted the expression of only a sub-set of them and in particular the specific overexpression of a cluster of three rdh genes. RNA expression information was backed by proteomic mass spectrometry analyses, which also identified three proteins, specifically the same enzymes whose overexpression was measured in RNA expression analyses, hinting at a TCE-dechlorinating activity of one or more of them.

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1 Introduction

1.1 Main organohalide pollutants: chemical features, environmental distribution, toxicity

Organohalide compounds are chemicals in which one or more carbon atoms are linked by covalent bonds with one or more halogen atoms (fluorine, chlorine, bromine or iodine from group 17 of the periodic table) resulting in the formation of organofluorine, organochlorine, organobromine, and organoiodine compounds. Chlorine halocarbons are the most common. Organohalide chemical families are: haloalkanes, haloalkenese, haloaromatics. These compounds represent one of the step of what is called the "halogen cycle" (Fig. 1), which consists of the various chemical, physical and biological processes which collectively move halogen atoms and rearrange them chemically in the world.

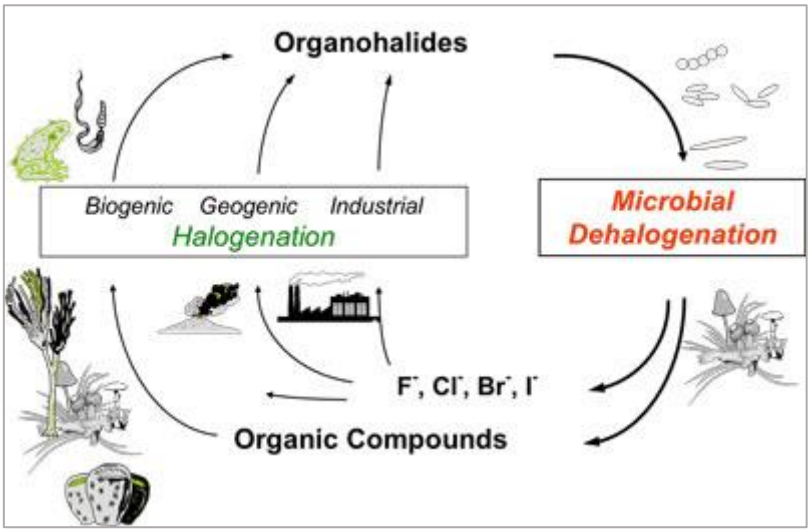


Figure 1 Schematic representation of the cycle of halogen atoms in nature, involving the inclusion of them in organic molecules and the following release by biological processes.

More than 5000 organohalogen compounds are produced by living organisms or are formed during natural abiogenic processes such as volcanoes, forest fires and other thermic processes which cause incomplete combustion reactions[1,2,3]. The halogens fluorine, chlorine, bromine and iodine (astatine is scarcely present on Earth) are strongly electronegative and form stable bonds with carbon atoms, with strength decreasing with increasing molecular weight. The carbon-fluoride bond is thus the strongest bond and very few organofluorines occur naturally, but living organisms should be involved in this element cycle since evidences of anaerobic defluorination have recently been found[4]. However, most naturally occurring organohalogens are brominated or chlorinated compounds and the oceans are the single largest source of biogenic organohalogens, mostly biosynthesized by seaweeds, sponges, corals, tunicates, bacteria and other marine life[5]. Although there are many natural sources of organohalides, most of the ones released in the environment are manmade because many of them proved to have extremely valuable physiochemical properties. For their many different properties, they are used as solvents, pesticides, refrigerants, fire-resistant oils, ingredients of elastomers, adhesives and sealants, electrically insulating coatings, plasticizers and plastics. One halocarbon, sucralose, is a sweetener. For their intrinsic use, they are easily accidentally or deliberately released in the environment. Organochloride pesticides include the infamous DDT,

methoxychlor, dieldrin, chlordane, toxaphene, mirex, kepone and lindane, dicofol, mirex, kepone and pentachlorophenol, they persist in the environment and in the bodies of humans and other animals long after exposure, and are largely banned in many countries, even if they have been used extensively during the 20th century throughout the world. Polychlorobiphenyls, dibenzo-p-dioxins, chlorophenols and chlorobenzenes are among these toxic compounds and have been regulated by the Stockholm Convention since 2011, regarded as Persistent Organic Pollutants (POPs)[6], i.e. as chemicals that are resistant to degradation processes, travel over long distances, bioaccumulate in ecosystems and are toxic for the humans and the environment. In general, physio-chemical characteristics of these compounds are closely linked to the degree of chlorination of their structures, but they all are slightly volatile, slightly soluble in water and soluble in lipids, i.e. they have high octanol/water and organic carbon/water partition coefficient. As semivolatile compounds, after being released or revolatilized, they partition between the gas and aerosol phases and are subject to long-range atmospheric transport. They can deposit in water bodies by dry deposition of particulate-bound pollutants, diffusive gas exchange between the lower atmospheric layer and the water surface or by the scavenging effect of rain[7]. Air-water exchange by diffusion is the most common mechanism, but for high chlorinated compounds, particle-phase sorption is more favourable and wet and dry particle deposition can also occur[8]. Because of their very low solubility, these pollutants become strongly bound to particulate material in water and accumulate in sediments, from which they are separated by biota and enter the marine food web, biomagnifying at high trophic level (i.e. increasing their concentration in tissues of organisms not capable to degrade them) (Fig. 2).

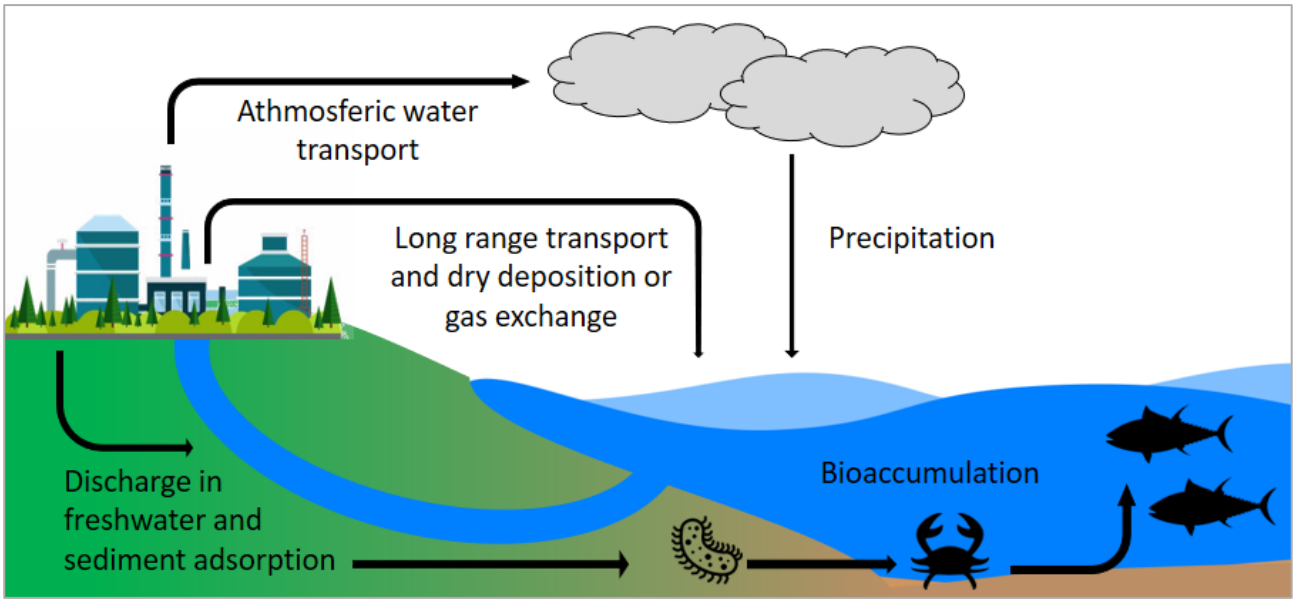


Figure 2 Environmental transport schematics of anthropogenic organohalide pollutants.

Sediments are thus sinks and source of contaminants in the aquatic environment, therefore food from such environments becomes a dominant path of human exposure to most of these compounds[9,10]. With the realization of the threat posed by these compounds, assessing the concentration in aquatic environments has become of great concern in recent years. This has been addressed measuring their concentration either through lipid-containing semipermeable membrane devices (SPMDs)[11] in sediments or in benthic organisms and fish fatty tissues, taking into account species-specificity of bioaccumulation (due to diverse metabolism of different congeners and feeding habits), organisms fat content and migration patterns[12]. The stability of

organohalides brought to the belief that they were mostly harmless, until in the mid-1920s physicians reported workers in polychlorinated naphthalene (PCN) manufacturing suffering from chloracne[13]. By the late 1930s it was known that workers exposed to PCNs could die from liver disease[14] and that DDT would kill mosquitos and other insects. By the 1950s, there had been several reports and investigations of workplace hazards. In 1956, for example, after testing hydraulic oils containing polychlorobyphenyls (PCBs), the U.S. Navy found that skin contact caused fatal liver disease in animals and rejected them[15]. Here are presented classes of organohalides relevant in this particular study.

1.1.1 Poychlorobiphenyls (PCBs)

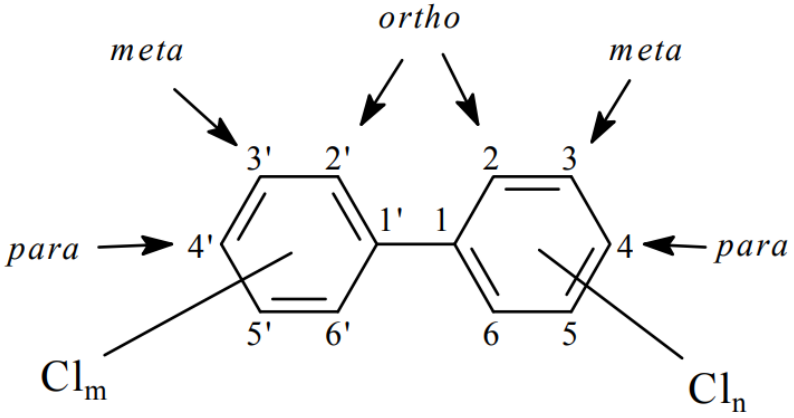


Figure 3 Chemical structure of polychlorobiphenyls. Substituent position name and number of chlorine substituents are shown.

PCBs are a family of chlorinated non-polar hydrocarbons composed of a biphenyl molecule, which may carry from 1 to 10 chlorine substitutions (Fig. 3). Given different combination of chlorine substituent, 209 congeners are possible and are named according to a numeric sequence from 1 to 209, as pioneered by Ballschmiter and Zell[16]. PCBs were first discovered and produced in Germany from 1881 to 1914, then commercial production was taken over by the Monsanto Chemical Company in the U.S. in 1929 from Swann Chemical Company. Commercial production of PCBs involved the batch chlorination of biphenyl with chlorine gas in the presence of a catalyst, resulting in the formation of complex mixtures containing a range of PCBs rather than just individual congeners. Studies on the structure-activity relationship suggest that non-ortho chlorine substituted PCBs, especially, 3,3',4,4'-tetrachlorobiphenyl, 3,3',4,4',5-pentachlorobiphenyl, and 3,3',4,4',5,5'-hexachlorobiphenyl, are able to adopt a planar configuration which makes them behave in a similar fashion as the highly toxic 2,3,7,8-tetrachlorodibenzo-p-dioxin. A very effective cooling and insulating fluid for transformers and capacitors, as well as stabilizing, flame-resistant additives of PVC electrical coatings, PCBs were sold as complex mixtures carrying 60 to 90 congeners[17] with commercial brands known as Aroclor® in the US and the UK (Monsanto company), Clophen® in Germany (Bayer), Kanechlor® or Santotherm® in Japan (Kanegafuchi and Mitsubishi, respectively), Fenclor® in Italy (Caffaro/Monsanto). More than half of all PCB uses were coolants and insulants fluids for transformers and capacitors. Moreover they were used as plasticizers in paints and cements, stabilizing additives in PVC coatings of electrical components and wiring, pesticide extenders, cutting oils, lubricating oils, flame retardants, hydraulic fluids, sealants, adhesives, wood finishes, de-dusting agents, vacuum pump fluids, fixatives for microscopy, surgical implants and carbonless copy paper (UNEP, 1999). The cumulative production until 1993 is estimated to be around 1.3 billion tons of PCBs, with a peak production in the 1970s[18]. PCBs were mainly emitted in the atmosphere

during the utilization and production process, but other sources of emission include volatilization from reservoirs, from combustion and disposal of materials containing PCBs, as well as incorrect operations[18,19].

Volatile low chlorinated PCBs can be present in the city's air and in air of buildings which were constructed using PCBs in sealants, caulking and other building materials[20]. On account of their low water solubility and high lipophilicity, the major source of PCBs for the population is food intake, since they tend to adsorb to particles in the sediments of oceans, sea and lakes, being released from the sediment water interface under certain conditions and then ingested by benthic organisms and further accumulated in the food chain[21]. PCBs have been quantified in the Black sea, Bulgaria[22], along the Red Sea coast, Egypt[23], in Bering Sea, Chuckchi Sea and Canada Basin[24], in Marseille Bay[25], in Trinidad[26]. The aforementioned tendency of POPs to bioaccumulate and biomagnify bringing them in close range of humans is particularly true for PCBs. After the discovery of their toxicity, they were detected in a great range of aquatic animals[27,28] and, from there, also in mammals and humans[29]. The first mechanism of toxicity in animals relies on the ability of PCBs to bind to the Aryl hydrocarbon receptor AhR[30] which activates the detoxification cytochrome system and acts as a transcription factor activating the expression of heat-shock proteins and triggering, this activates cytotoxic responses pathways, including lymphocyte suppression and hormonal interference[31]. The second toxicity mechanism involves Cytochrome P450 proteins, which participate in the metabolism of these compounds hydroxylating them, generating reactive epoxides from para and meta substituted congeners, which in turn form adducts with biomacromolecules and are longer retained in the blood[32]. This is why the non-ortho congeners exhibit the highest toxicity, followed by the moderately toxic mono-ortho and the less toxic di-ortho- substituted PCBs. Risk assessment can thus be evaluated either as relative toxicity in comparison to 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (i.e. toxic equivalency factors, TEQs), as determined by in vitro and in vivo studies[33], or as extension of induction of cytochrome P-450 enzyme activity, especially in marine organisms[34].Even though PCBs can be toxic by themselves, recent studies provide also convincing evidence that specific PCB congeners can be also biotransformed to genotoxic metabolites[35]. Moreover, P450- and P480-catalyzed reactions can bring to reductive dechlorination or hydroxylation of PCBs. Hydroxilated PCBs inhibit the catabolic pathway[36] and might bind to a thyroid hormone transporter[37]. PCBs proved to have carcinogenetic effects on laboratory animals and there is also evidence for immunotoxic effects in guinea pig and Sprague-Dawley male rats[38,39]. In humans, PCBs were correlated with toxic effects including: hormonal interference and thus sexual and reproductive disorders[40,41], immune system deficiencies[42], diabetes[43], neurological disorders, cognitive problems during development[44,45], chloracne[46], metabolic issues[47], non-Hodgkin lymphoma and breast cancer[48,49]. In particular, carcinogenicity studies on humans revealed an increment in prostate cancer incidence[50], and a comprehensive cohort study found a correlation between PCB exposure and cancer[51]. Although PCB production is banned worldwide and not significant releases of newly manufactured or imported materials to the environment occur, exposure to these substances can still happen in several ways: incineration, leakage from older electrical equipment or uncontrolled landfills and hazardous waste sites, improper disposal of old PCB-containing liquids, leachate from contaminated sewage sludge, dispersion from contaminated soils and vehicular emissions (Agency for Toxic Substances and Disease Registry (US Department of Health and Human Services), 2000).

1.1.2 Chlorobenzenes

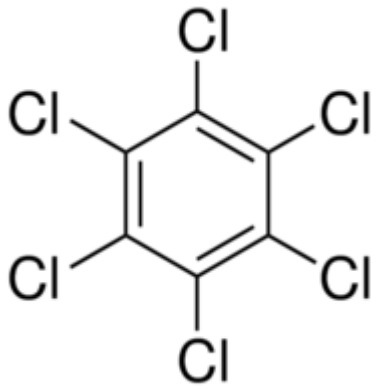


Figure 4 Chemical structure of hexachlorobenzene.

Chlorinated benzenes are a group of cyclic aromatic compounds in which one or more hydrogen atoms of the benzene ring have been replaced by a chlorine atom (Fig. 4). Historically, hexachlorobenzene (HCB) had many uses in industry and agriculture. The major agricultural application for HCB used to be as a seed dressing for crops such as wheat, barley, oats and rye to prevent growth of fungi. The use of HCB in such applications was discontinued in many countries in the 1970s owing to concerns about adverse effects on the environment and human health. Other uses included were also in the production of rubber, aluminum, and dyes and in wood preservation. In industry, HCB has been used directly in the manufacture of pyrotechnics, tracer bullets and as a fluxing agent in the manufacture of aluminum, as a wood-preserving agent, a porosity-control agent in the manufacture of graphite anodes, and as a peptizing agent in the production of nitroso and styrene rubber for tyres[52]. The main origin of HCB today is to be found in the production of chlorinated solvents and pesticides, where it is a reaction by-product of thermal chlorination, oxychlorination, and pyrolysis operations (mainly to produce carbon tetrachloride, trichloroethylene and tetrachloroethylene). Other chlorobenzenes are used mainly as intermediates in the synthesis of pesticides and other chemicals: 1,4-DCB is used in space deodorants and as a moth repellent, while the higher chlorinated benzenes (trichlorobenzenes, 1,2,3,4-TeCB, and PeCB) have been used as components of dielectric fluids. There is no information about symptoms and signs of acute poisoning by HCB in humans. However, there are data on health effects of chronic intoxication with this compound. HCB toxicity main targets are liver, thyroid gland, reproductive end points, developmental end points, and carcinogenesis. Chronic (long-term) oral exposure to hexachlorobenzene in humans results in a liver disease with associated skin lesions. Chronic administration of HCB to experimental animals produces numerous effects, including increased synthesis of liver microsomal enzymes, triggering of hepatic porphyria, hypothyroxinemia and thyroid adenomas. HCB is also known to be carcinogenic in animals[53]. As far as humans are concerned, in Anatolia (Turkey), where it was used to dress seeds as a fungicide, over 3000 people developed porphyria cutanea tarda and mixed porphyria lasting several years due to HCB ingestion. In the same region almost all children born to mothers who consumed HCB-contaminated bread during pregnancy died within one year, after exhibiting weakness, convulsions, and skin lesions.[54,55] In a group of 341 men, the association between HCB exposure and thyroid hormones was examined. Studying workers in chlorinated solvents production facilities, an inverse relationship between total triiodothyronine (T3) and serum HCB levels was found. In another study conducted in pregnant women, a 10-fold increase in HCB concentration was associated with an 8% decrease in free thyroxin (fT4) and a 51% decrease in total T4.[56,57] In a large case-control study including 422 cases and

460 controls, an increased risk of non-Hodgkin's lymphona was found, also among some of the subtypes. Moreover, a significant positive trend for the relationship between HCB plasma level and non-Hodgkin's lymphoma incidence was noted. [58] Epidemiologic studies of persons orally exposed to hexachlorobenzene have not shown an increased cancer incidence. However, based on animal studies that have reported cancer of the liver, thyroid, and kidney from oral exposure to hexachlorobenzene, EPA has classified hexachlorobenzene as a probable human carcinogen (Group B2). Less chlorinated chlorobenzenes can be considered volatile organic compounds and do not have toxic effects, unless at very high concentration: German Ministry of Labor and Social Affairs (Arbeitsplatzgrenzwert, AGW) and the American Conference of Industrial Hygienists (Threshold Limit Value, TLV) adopted a limit of 10 ppm for the occupational exposure to CBs[59]. Above this concentration chlorobenzene can cause irritation of the mucosa in nose and throat as well as headaches and dizziness.

1.1.3 Chlorophenols

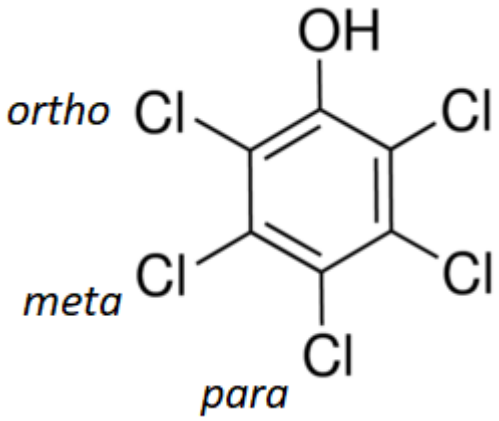


Figure 5 Chemical structure of pentachlorophenol. names of chlorine substituents are indicated.

Chlorophenols are a group of 19 chemicals in which from one to five chlorines can be present on a phenol. Chlorinated phenols are intermediates of industrial production of pesticides, herbicides, dyes, pigments, phenolic resins and paper, but can also be used as flea repellents, fungicides, wood preservatives, fungus inhibitors, antiseptics, disinfectants and antigumming agents for gasoline[60]. In particular, pentachlorophenol (PCP) (Fig. 5) has been used as an herbicide, insecticide, fungicide, algaecide, and disinfectant and as an ingredient in anti-fouling paint. Some applications were in agricultural seeds (for nonfood uses), leather, masonry, wood preservation, cooling tower water, rope, and paper mills. Its use has declined due to its high toxicity and slow biodegradation. The release in the environment is anyway still happening because of accidental spills, waste disposal sites, storage tanks, or municipal landfills. Chlorophenols can also origin from processes of biodegradation of pesticides and herbicides. Microbial degradation of herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), yields numerous chlorophenols as intermediate metabolites of their decomposition[61]. The herbicides 2,4-dichlorophenoxyacetic acids (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) often used on food crop can be broken down to 2,4dichlorophenols and 2,4,5-tricholorphenols (ATSDR, 2007). During the combustion of organic matters chlorophenolic compounds are formed[62]. Findings have shown that chlorophenols are formed during the incineration of municipal waste and, moreover, they can react to form polychlorinated

dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF), which are organic compounds listed as Persistent Organic Pollutants (POP) by the Stockholm Convention and are more toxic that their phenolic precursors.[63] Chlorophenols are found in wastewater, sludge products, surface waters, and groundwater.[64–66] Chlorophenols are of serious environmental concern because of their widespread occurrence throughout the environment. Pentachlorophenol has been evaluated by the International Agency for Research on Cancer (IARC) as a 2B carcinogen in 1991, for inadequate evidence but sufficient data on animals. Since its use has been forbidden after 1970s, PCP levels have diminished. However, they are still high in Chinese surface/water sediments because since 1960 sodium-pentachlorophenate has been extensively sprayed to control the spread of snailborne schistosomiasis: studying the impact of PCP exposure on the popolution living in those areas has associated it to leukemia, lymphoma, esophageal and nasopharyngeal cancer[67]. Other studies have found a correlation between occupational exposure to chlorophenols and non-Hodgkin's lymphoma, multiple myeloma, and kidney cancer[68], but also hematopoietic cancer[69].

1.1.4 Trichloroethylene

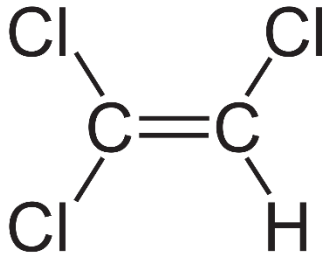


Figure 6 Chemical structure of trichloroethylene.

First synthesized by Fischer in 1864, trichloroethylene (TCE) is a haloalkene with the raw formula C_2HCl_3(Fig. 6). When it was first widely produced in the 1920s, trichloroethylene's major use was to extract vegetable oils from plant materials such as soy, coconut, and palm. Other uses in the food industry included coffee decaffeination and the preparation of flavoring extracts from hops and spices. It has also been used for evaporating the last bit of water for production of 100% ethanol. Between the 1930s and the 1970s, both in Europe and in North America, trichloroethylene was used as a volatile anesthetic almost invariably administered with nitrous oxide. Marketed in the UK by ICI under the trade name Trilene it was coloured blue (with a dye called waxoline blue) to avoid confusion with the similar smelling chloroform. TCE replaced earlier anesthetics chloroform and ether in the 1940s, but was itself replaced in the 1960s in developed countries with the introduction of halothane, which allowed much faster induction and recovery times. Trilene was also used as a potent inhaled analgesic, mainly during childbirth. It has been used as a dry cleaning solvent worldwide, although replaced in the 1950s by tetrachloroethylene (also known as perchloroethylene), because of lower toxicity and higher stability, except for spot cleaning where it was used until the year 2000. The U.S. EPA proposed a ban on the commercial use of trichloroethylene as an aerosol degreasing agent or spot cleaner by dry cleaners in December 2016; final decision on this rule is pending. Trichloroethylene use was banned by the European Union in April of 2016. Beside the dry cleaning facilities, the places that mostly released TCE in the environment were industries with machinery, where TCE was used as an efficient de-greaser. In such environments intentional and unintentional spills, along with occupational exposure is where the first signs of toxicity were observed. TCE has many negative effects on human health. It has the potential

to cause cardiac defects in humans when exposure occurs at sufficient doses during a sensitive window of fetal development[70]. It has the ability to affect the central nervous system[71], liver[72], kidney[73], immune system[74], and reproductive systems and developing embryo/fetus (NRC 2006). effects on kidney are generally associated with metabolites resulting from GSH conjugation[73]. The identity of TCE metabolites involved in the induction of other health effects of TCE is less clear, although similarities have been observed between TCE and its oxidative metabolites in the respiratory tract[75] and developmental toxicity[76]. TCE is metabolized in humans and experimental animal species by both oxidation and glutathione (GSH)-conjugation metabolic pathways, with subsequent production of numerous toxicologically active compounds[77,78]. These include the oxidative metabolites chloral hydrate, trichloroacetic acid (TCA), and dichloroacetic acid, and the GSH conjugation metabolites dichlorovinyl glutathione and dichlorovinyl cysteine. This complex assortment of metabolic compounds is generated from and transported across multiple tissues, making evaluation of mechanistic data especially challenging[76]. Liver effects of TCE are thought to result from oxidative metabolites[72,79], The cancer hazard of TCE was evaluated by IARC (2014), the US EPA (2011), and NTP (2015). The conclusion of all three assessments is that there is sufficient evidence that TCE is a human carcinogen, with the kidney being the target tissue[80]. Limited evidence, on the basis of positive associations observed with TCE exposure, was found for non-Hodgkin lymphoma (NHL) and related cancers (including Chronic Lymphocytic Leukemia, Multiple Myeloma, Hairy Cell Leukemia) and liver cancer.

1.1.5 Polychlorinated Dibenzo-*p*-Dioxins

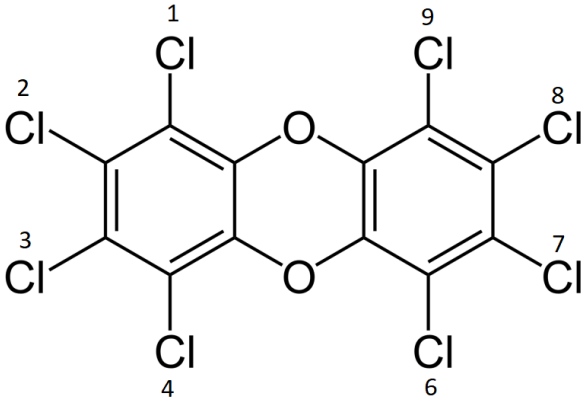


Figure 7 Chemical structure of 1,2,3,4-tetrachloro dibenzo-para-dioxin. Number of substituent positions are indicated.

Polychlorinated dibenzo-p-dioxins (PCDD) are polycyclic aromatic compounds with two oxygen atoms, they are listed as Persistent Organic Pollutants (POP) by the Stockholm Convention. According to position and number of chlorine atoms, 75 congeners can be distinguished (Fig. 7). Among these, 17 congeners are particularly harmful to humans and one, 2,3,7,8-TeCDD is a group 1 carcinogen. In contrast to other chlorinated pollutants, PCDDs have never been produced intentionally for any industrial use, but they occur as unwanted trace contaminants in many industrial and thermal processes. PCDD and polychlorinated dibenzofurans (PCDF) are formed together by three chemical pathways: high temperature homogeneous synthesis, precursor synthesis, and de novo synthesis, the latter two combined in heterogeneous synthesis. Homogeneous synthesis is based on gas phase reactions proceeding at high temperature (>500 C). These include a wide variety of pyrolysis and partial oxidation reactions, accompanied by the cyclisation of reactive intermediates to aromatic structures, self-condensation of precursors, and both chlorination and

dechlorination reactions. These pathways occur in combustion of municipal waste, wood and industrial waste, coal boiler, iron ore sinter strands, electric arc furnaces using recycled steel, metal industries, landfill fires and auto exhausts mainly (but not only) by precursors such as chlorophenols and chlorobenzenes and catalyzed by metal ions[81],[82]. Moreover, dioxins can be released from so-called secondary sources such as landfills and contaminated areas or re-enter the environment via the application of sewage sludge, compost or liquid manure[83]. Other sources of PCDDs are pulp paper manufacture (when bleaching is carried out with chlorine) and by-products in the manufacture of some pesticides. During the last 20 years, different kinds of improvements have been introduced in the processes responsible for the generation of PCDDs and in the technologies for air pollution control, so that for instance the impact of municipal solid waste incineration has considerably been reduced[84]. However, other sectors have become important sources of PCDDs, such as residential wood burning or mechanical-biological treatments, such as composting, biostabilization and biodrying[85]. Dioxins enter the general population almost exclusively from ingestion of food, specifically through the consumption of fish, meat, and dairy products since dioxins are fat-soluble and readily climb the food chain[86]. Dioxins accumulate in food chains in a fashion similar to other chlorinated compounds (bioaccumulation). This means that even small concentrations in contaminated water can be concentrated up a food chain to dangerous levels because of the long biological half life and low water solubility of dioxins. The AhR receptor is recognized as the culprit for most toxic responses observed after exposure to dioxins and related compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)[87]. Toxic effect of PCDD involve many different pathologies: developmental abnormalities in the enamel of children's teeth[88,89], central and peripheral nervous system pathology[90], thyroid disorders[91], damage to the immune system[92], endometriosis[93], diabetes[94].

1.2 Marine sediments as final sink for organohalide accumulation: remediation strategies

All pollutants mentioned above, once diffused into the environment, will diffuse in the soil and, depending on the hydrophobicity, will be transported by water to ultimately accumulating in freshwater (river or lakes) and then marine sediments. Sediments act therefore as the ultimate receptors of chemical and biological contaminants resulting from agricultural, urban, industrial, and recreational activities, making it the place where to apply possible remediation strategies. Characterizing and remediating contaminated sediments is complex since underwater conditions, sensitive habitats, water currents, and access difficulties must be taken into account. Moreover, it is often difficult to establish appropriate site-specific cleanup goals, i.e. which areas to remediate, to what extent and depth, for what ultimate purpose[95]. Sediment risk management strategies are based on three main principles: i) minimize contaminant risk to human health and the environment, ii) minimize cost, iii) minimize the risks associated with the response action itself, such as habitat destruction and/or modification, as well as injury to workers or the public during remediation. Sediment remediation techniques are commonly classified as in situ, i.e. without sediment dredging, and ex situ, i.e. including sediment dredging or resuspension phenomena to some extent. Dredging consists in the physical removal of the contaminated sediment layers, basically shifting the contamination problem to another place. The United States National Research Council[96], for example, has evaluated 26 dredging projects and observed systematic difficulties in achieving target cleanup thresholds, in addition to

impairment of sediment-associated benthic ecosystem. Dredging has some inherent disadvantages: the dredge may miss contaminated areas and residual contaminated sediment may mix with underlying or surrounding sediment. Moreover resuspended material can migrate downstream and affect ecosystems there as well; dredging may lead to long- or short-term increases of contaminant bioavailability, facilitating pollutant entrance into food-chains. Most importantly, dredging very likely leads to the alteration or destruction of the benthic community; finally the presence of boulders and debris may limit the effectiveness and impede the achievement of low contaminant cleanup levels[97]. Dewatering the water body before dredging (dry excavation)is, when possible, a good alternative, access to the contaminated areas is enhanced and contaminant migration downstream during excavation minimized, but the process is very expensive and removed sediment requires subsequent treatment anyway. Capping is an in situ treatment, which consists of covering the contaminated sediment surface with clean material, isolating the sediment. Sand is the most commonly used material for capping, but also other natural or synthetic materials of varying sizes are available[98]. Jacobs and Foerstner[99] suggest the use of zeolites as capping material, as they are a natural sorptive agent easily available and environmentally compatible. During the placement of capping material, contaminated sediments can be mixed with the clean material or resuspended. A related strategy to capping involves the mixing of the capping material with contaminated sediment. The mixing can be carried out with natural substrates or other inert materials which bind to hydrophobic organic contaminants (HOCs) reducing their bioavailability and therefore their accumulation in the aquatic food web. This "natural" contaminant sequestration (named active mixing) can be greatly enhanced by the addition of clean, manufactured carbonaceous materials into sediments, such as activated carbon, which modify the sediment geochemistry actively increasing contaminant binding and stability and reducing the overall cap thickness required. In time, the amended layer will be covered with clean new sediment deposit and continue to serve as a barrier to the release of legacy contaminants to surficial sediments and water column. Diverse and numerous amendments have been developed and some patented commercial products are already available to be applied at full-scale. The problems, though, are that the effectiveness of the treatment vary for different compounds and site conditions[100] and that data on amendments possible toxicity are still scarce. Solidification/stabilization (S/S) is another in situ treatment which consists in the addition of chemicals and/or cements to encapsulate contaminated sediment and/or convert pollutants into less soluble, less mobile or less toxic forms. As in thin capping, in situ conditions (pH, mixing, water/binder ratio) strongly influence the efficacy of the process. Moreover, site characteristics can be significantly modified: volume can increase up to two times the original one and it is usually necessary to reestablish benthic invertebrate community[101]. In situ sediment flushing treatment removes harmful chemicals by injecting water or chemicals into the sediment, washing them out and conveying hydrophilic contaminants toward the extraction wells. Elutriates (what is flushed away from sediment) are pumped and treated in on-site wastewater treatment plants. Sediment flushing is often difficult to apply, since environmentally sustainable surfactants can be used to increase the solubility of organic compounds, but the flushing solution could significantly alter the physico-chemical properties of sediment or it could be ineffective in presence of heterogeneous mixtures of contaminants, because no universal flushing solution is available[97]. Electrokinetics remediation, which has been successfully applied in batch to remove PCBs from soil, can be coupled with sediment flushing[102]. Under the provided electrical current, the contaminants can be removed from the soil via electromigration, electroosmosis and electrophoresis. For organic pollutants like PCBs, the electroosmosis is the main transport mechanism, but soil flushing can help overcome organic pollutants scarce solubility. Advantages of electrokinetics are close

control over the direction of movement of water and dissolved contaminants, retention of contaminants within a confined zone, low power consumption[103]. Another remediation strategy employed require a non-action approach and it is called monitored natural recovery (MNR). It involves leaving the contaminated sediments in place and allowing the ongoing aquatic processes to contain, destroy, or otherwise reduce the bioavailability of the contaminants. Scientific evidence in support of the effectiveness of this approach typically focus on demonstrating contaminant burial and surface sediment recovery, reduced contaminant mobility, chemical and biological transformation, and sediment (hence, contaminant) stability[104]. The biological processes involved in the degradation of contaminants in water and soil are the basis for the remediation strategies collectively called bioremediation. Bioremediation is a process used to treat contaminated media, including water, soil and subsurface material, by altering environmental conditions to stimulate growth of microorganisms and degrade the target pollutants. Even though it requires a thorough understanding of the geochemistry, hydrogeology, microbiology and ecology of contaminated soils, groundwater and sediments, in many cases, bioremediation is, although slower, less expensive and more sustainable than other remediation alternatives as it does not require land modification[105]. Anaerobic bioremediation can be employed to treat a broad range of contaminants including halogenated compounds, which are subject to microbial reductive dehalogenation, a biological process which brings to the substitution of halogen atoms with hydrogen in organohalide compounds, generally making them less toxic, more bioavailable, less stable and more prone to biodegradation[106].

1.3 Microbial reductive dehalogenation importance in the fate of organohalide pollutants: species involved in freshwater and marine environments, their biochemistry and approaches in their study

Microbial reductive dehalogenation is the biological process carried out by some species of anaerobic bacteria which reduced the halogenation level of organohalide compounds in nature. Because of the role it plays in the biochemistry of microbes which carry out such process, microbial reductive dehalogenation can be referred to as organohalide respiration, which has been restricted to the bacterial domain of life. As a unique trait, organohalide-respiring bacteria (OHRB) benefit from reductive dehalogenase enzymes enabling them to use different organohalides as terminal electron acceptors[107]. Known OHRB are spread among several phyla comprising both Gram-positive and Gram-negative bacteria (Fig. 9). These microbes have greatly contributed to global cycling of halogens by breathing (rather) toxic organohalides and preventing their accumulation in the environment employing reductive dehalogenases (RDase in case of functionally characterized enzymes and RdhA for yet uncharacterized reductive dehalogenases predicted from genomes and molecular surveys). Since the description of *Desulfomonile tiedjei* as the first isolated OHRB[108], numerous bacterial strains capable of OHR have been obtained in axenic culture, providing indispensable insights into their phylogenetic, physiological and biochemical traits. Members of the genus *Dehalococcoides* comprise the biggest groups of isolates to date (19 isolates) followed by strains of *Desulfitobacterium* (17 isolates). The known species can be divided into facultative and obligate groups based on whether OHR is their only energy-gaining metabolism[109]. The members of the facultative OHRB are characterized by a more versatile metabolism, in general have the ability to grow on a wide range of electron acceptors, and include proteobacterial OHRB such as *Geobacter, Desulfuromonas, Anaeromyxobacter, Desulfomonile, Desulfovibrio,*

Desulfoluna, Sulfurospirillum, Comamonas, Shewanella as well as *Desulfitobacterium* from the phylum *Firmicutes*. The fact that some facultative OHRB such as *Comamonas, Geobacter* and *Shewanella* belong to phylogenetic groups that mostly comprise non-OHRB points towards horizontal acquisition of reductive dehalogenase genes.

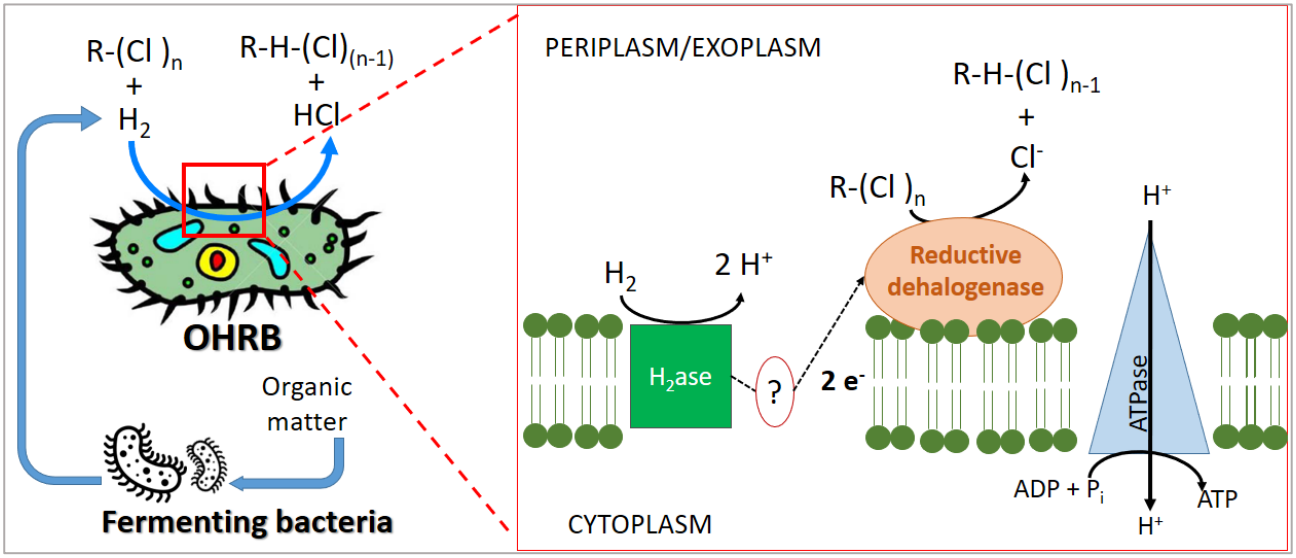


Figure 8 Schematics of microbial reductive dehalogenation by organohalide respiring bacteria (OHRB).

The obligate OHRB on the other hand are restricted to OHR for energy conservation and growth and include *Dehalobacter* (phylum *Firmicutes*) and the OHRB belonging to the *Dehalococcoidia* class (phylum *Chloroflexi*) including strains of *Dehalococcoides mccartyi*, *Dehalogenimonas* spp. and the single isolate '*Dehalobium chlorocoercia*' DF-1[109,110,111]. Recent studies showed fermentative growth of *Dehalobacter* spp. on chloromethane[112,113] without reductive dechlorination in a mixed culture, suggesting that at least some of the isolates previously considered obligate OHRB might harbor additional modes of metabolism beyond the canonical OHR. It is also remarkable that recent single-cell genomic studies of marine *Dehalococcoidia* did not reveal any evidence for catabolic reductive dehalogenation, indicating that microorganisms closely related to known obligate OHRB do not rely on OHR for energy conservation, but rather utilize organic matter degradation pathways[114,115]. With the exception of *Comamonas* sp. 7D-2 as a strict aerobic bacterium capable of OHR[116] and *A. dehalogenans* as a facultative anaerobe[117], the currently known OHRB are generally strictly anaerobic bacteria and sensitive to oxygen exposure. This is of high importance for the more delicate OHRB such as *Dehalococcoides* and *Dehalobacter* spp. that require strict anoxic techniques and presence of reducing chemicals in laboratory cultures. Lacking the genes necessary for direct utilization of oxygen, these OHRB are highly dependent on an array of natural shields in the environment. Recent findings showed that in spite of stringent requirement for anoxic conditions in axenic cultures, members of *Dehalococcoides* might be well protected in their natural habitats such as riverbed sediments[118]. The organic rich matrix of riverbed sediments limits oxygen penetration to the surficial sediment layers. Moreover, sediment aggregates can host anoxic microniches inside sediment-associated biofilms. Presence of facultative aerobic bacteria colonizing the outer layers of sediment biofilms, which rapidly consume oxygen, can protect the strict anaerobes such as OHRB in core microniches[118]. In line with this, a former study on distribution and localization of *D. mccartyi 195* and methanogenic populations in an enrichment culture using fluorescence in situ hybridization (FISH) showed that methanogens reside in the exterior layer of bioflocs, whereas *D.*

mccartyi 195 cells were more evenly distributed within bioflocs as well as between biofloc and planktonic phases[119]. A comparative metagenomic study on three different *Dehalococcoides*-containing dechlorinating microbial consortia indicated the presence of several genes related to oxygen tolerance and scavenging from acetogens, fermenters and methanogens commonly found to co-exist with OHRB in enrichment cultures and natural environments[120]. Activity of such genes in non-dehalogenating community members would enforce oxygen scavenging mechanisms, collaterally protecting the OHRB along-side. In their microbial community, OHRB depend on other non-dehalogenating community members for the supply of electron donors and growth factors such as vitamins. Fermentation intermediates such as acetate, formate and H_2 are common products of anaerobic hydrocarbon degradation[121] (Fig. 8) and are used by other community members such as iron reducers, sulphates reducing bacteria (SRB), OHRB and methanogens. This overlapping nutritional niche caused controversial discussions in the field of OHR. Early reports considered hydrogenotrophic SRB and methanogens as competitors of OHRB for reducing equivalents[122,123], however, the predicted supply of nutrients by SRB and methanogens[120] points towards interactive networks between these microbial guilds rather than pure competition for the shared resources. Indeed, the importance of symbiotic interactions between SRB and OHRB for effective OHR in their co-cultures was previously reported and was attributed to interspecies H_2 transfer[124,125] or supply of unknown growth factors by the SRB partner[126]. Recent dedicated studies showed that supply of corrinoid cofactors by SRB and methanogens sustained OHR by *D. mccartyi 195*, although corrinoid modification and remodelling by strain 195 was essential to add 5',6'-dimethylbenzimidazole (DMB) as the lower ligand to cobamide precursors and produce cobalamin as the preferred corrinoid cofactor[127–129]. Similarly, *Geobacter lovleyi* strain SZ was shown to fulfil *Dehalococcoides'* corrinoid requirements by the exchange of DMB[127]. Given the fact that all *D. mccartyi* strains for which genome sequences are available lack the capability of de novo cobalamin synthesis[130], and all share the same putative genes involved in corrinoid remodelling pathways[128], it is tempting to assume that OHRB exploited the biotopes shared with non-dechlorinating community members and have lost the costly but still key metabolic trait of de novo cobalamin synthesis. In line with this, recent studies on corrinoid-auxotrophy of *Dehalobacter restrictus* showed that in spite of presence of both anaerobic cobalamin biosynthesis and cobinamide-salvaging pathways, one important protein, CbiH (precorrin-3B C17 methyltransferase), was missing from the upper cobalamin biosynthesis pathway, which was believed to be due to frame-shift mutations[131]. Supply of the OHRB with corrinoids in their natural environment or even during long-term enrichments in the lab may enforce such a genetic trade-off that disables the unused trait. This may explain why OHRB and in particular those who rely on OHR as the sole mode of metabolism have shared/overlapping niches with nondechlorinators that are capable of de novo corrinoid biosynthesis. Experimental evidence also suggest that OHRB are mostly enriched in methanogenic and sulphatereducing conditions. For example, a recent batch-scale biostimulation of TCE dechlorination showed incomplete TCE dechlorination in microcosms where methanogens were absent[132]. In another study, inhibition of methanogenesis negatively impacted community members such as Desulfovibrio[133] that are known to synthesize corrinoids de novo. In natural habitats such as cell aggregates and biofilms where cells form close physical association, OHRB and methanogens may play complementary roles in alleviating thermodynamic constraints for other syntrophic microorganisms. The degradation of fatty acids such as propionate and butyrate only takes place in a thermodynamically interdependent lifestyle where degradation intermediates, usually acetate, formate and H_2, are maintained at very low concentrations[121]. Such metabolic/physical networks may also explain why most fastidious OHRB like *D. mccartyi* strains do not submit readily to growth in the laboratory as they need

metabolites, signals and structure provided by the community. Further, the structured associations would be more successful in retaining metabolites such as H_2 that would otherwise be lost by diffusion. However, in spite of the central role of microbe-microbe interactions, this subject has received surprisingly little attention in studies involved in OHR. Such information would highly assist in understanding the ecophysiology of OHRB and facilitate design of effective bioremediation strategies.

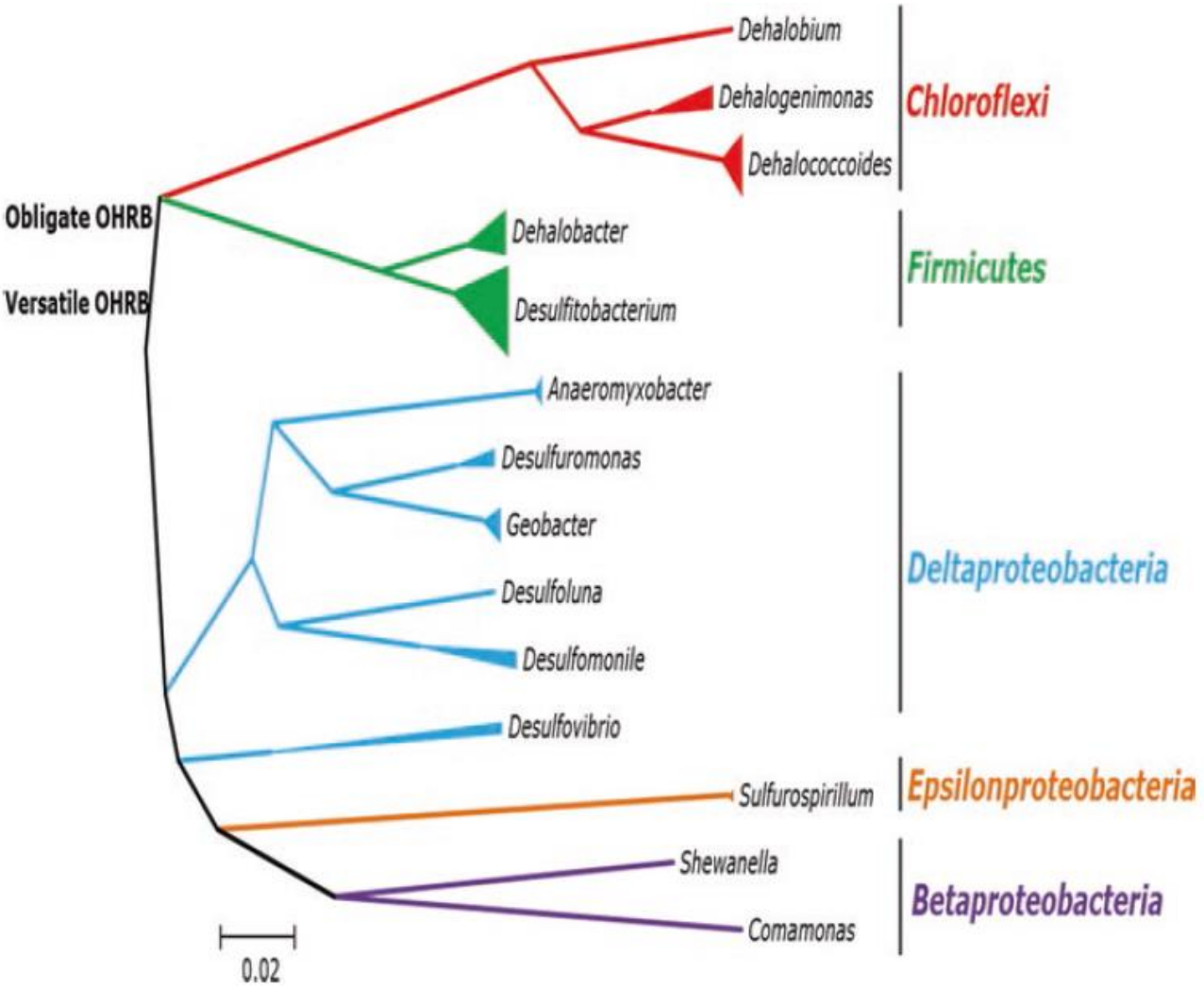


Figure 9 Phylogeny of OHRB species distribution in bacterial phyla.

With few exceptions, OHRB from different phyla, i.e. members of the *Firmicutes*, *Chloroflexi* and different classes of *Proteobacteria*, benefit from similar enzymes/pathways for OHR indicating that these genes could be acquired from common ancestors via transposon mediated dissemination. Anyways, little to no correlation could be found between the type of organohalides used as electron acceptor and the phylogenetic affiliation of OHRB, and haloaliphatic and haloaromatic compounds can be dehalogenated by isolates of taxonomically different genera[134]. Furthermore, isolates with similar phylogeny and physiology have been obtained from very different environments. For example, *Desulfuromonas michiganensis* strain BB1 was isolated from pristine river sediment while the closely related strain BRS1 was obtained from chloroethene-contaminated aquifer material[135]. Similarly, the closely related uncultured *Chloroflexi* Lahn and Tidal Flat Clusters were both capable of dechlorination of perchloroethene (PCE) to transDCE while being enriched from sediments from river and marine environments, respectively[136,137]. Another example is the

case of *D. mccartyi* strains KS and RC. Both bacteria grow with 1,2-dichloropropane (1,2-D) as an electron acceptor in enrichment cultures while being derived from hydrocarbon-contaminated and pristine river sediments, respectively[138,139]. Our current understanding of the environmental distribution of OHRB has to some extent been obtained by domestication of microbial communities capable of OHR in the form of laboratory culturing (microcosm studies). Nevertheless, laboratory culturing shed light on the biochemistry of OHRB in a way not possible in field studies, leading to the comprehension of their metabolism and how to boost it to employ them in bioremediation. Moreover, it allowed scientists to develop a suite of molecular techniques targeting both phylogenetic and functional gene markers such as 16S rRNA and rdhA genes to monitor the presence of OHRB in the environment. Studying the abundance and environmental distribution of OHRB was propelled by application of DNA/RNA-based quantitative PCR methods of recent development[140–142]. Using these methods, the 16S rRNA and tceA, bvcA and vcrA genes of D. mccartyi were detected in groundwater samples collected from a contaminated site near Montague, MI, USA and a large number of unidentified *D. mccartyi* rdhA genes were discovered[141]. Analysis of 45 groundwater samples taken from Rugårdsvej 234, Odense, Denmark for *D. mccartyi* 16S rRNA and vcrA genes indicated the presence of indigenous biomarkers at concentrations below detection limit of qPCR[143]. Increases in ethene concentrations after lactate addition were accompanied by a 3-4 order of magnitude increase in concentrations of vcrA gene copies throughout the groundwater monitoring network[143]. Cultivation independent approaches extended to subsurface aquifers further enhanced our understanding of metabolic plasticity of *Dehalococcoidia*-like bacteria. A recent metagenomic analysis from terrestrial aquifer sediment retrieved two *Dehalococcoidia* related pan-genomes ('RBG-2' and 'RBG-1351')[144], evidences of a versatile heterotrophic life styles were found. These findings indicate that OHR should not be considered as sole mode of metabolism of *Dehalococcoidia*-like bacteria. Marine and estuarine environments are repositories of numerous chlorinated, brominated and iodinated organic compounds that have been produced and released both from natural and anthropogenic sources. The study of King was the first report on OHR of 2,4-dibromophenol in coastal sediments[145]. Since then, numerous studies reported OHR as an essential step in the marine and estuarine biodegradation of halophenols[146], halogenated ethenes[147,148], halobenzoates[149], lindane[150] and polychlorinated biphenyls[151,152,111]. Of all known OHRB, the organohalide-respiring *Chloroflexi* seem to play the most prominent role in OHR in marine, estuarine and tidal flat sediments. The ortho-PCB-dechlorinating bacterium o-17 was isolated from a PCB dechlorinating culture enriched from estuarine sediments of Baltimore Harbour (USA)[153]. Similarly, The PCB-dechlorinating *D. chlorocoercia* DF-1 is indigenous to estuarine sediment and was isolated from Charleston harbour in South Carolina[154]. An active role of these isolates and other *Chloroflexi* members that are distinct from *D. mccartyi* were shown in PCB dechlorination in sediment microcosms from Baltimore Harbour, USA[155]. The Tidal Flat Cluster, a deep clade in the phylum Chloroflexi, was characterized from North Sea tidal flat sediment of the German Wadden Sea and was found to be involved in the dechlorination of PCE to trans-DCE as the predominant end product[137]. Molecular analysis of the bacterial community in enrichment cultures obtained from estuarine sediments of the San Diego Bay, USA, indicated involvement of *Chloroflexi*-like microorganisms related to *Dehalococcoides* in OHR of the model dioxin, 1,2,3,4-tetrachlorodibenzo-p-dioxin (TeCDD)[156]. In addition to former studies where detection of organohalide-respiring *Chloroflexi* was coupled to physiological/biochemical demonstration of OHR potential, there are other reports on abundant presence of bacteria affiliated with the *Dehalococcoidia* in marine sediments[157–159]. Wasmund and colleagues performed a comprehensive study on distribution and diversity of *Dehalococcoidia*-affiliated bacteria along vertical profiles of various marine

sediment cores obtained from Baffin Bay (Greenland), Aarhus Bay (Denmark), and tidal flat sediments of the Wadden Sea (Germany)[115]. qPCR analysis showed that *Dehalococcoidia* affiliated bacteria constituted only low proportions of the total bacterial in shallow sediment and generally became more prevalent in deeper sediments. Pyrosequencing of 16S rRNA genes of different clades revealed diverse and divergent co-occurring *Dehalococcoidia* populations within single biogeochemical zones. The varying local biogeochemistry was proposed as the driving force for such abrupt shifts. Hence different subgroups of *Dehalococcoidia*-affiliated bacteria with different metabolic properties likely occupy a wide range of ecological niches over sediment depth. A number of reports exist on distribution and diversity of rdhA genes in marine subsurface sediments. Using an array of degenerate primer sets, 32 putative rdhA sequences were detected from marine subsurface sediments collected from the southeast Pacific off the Peruvian coast, the eastern equatorial Pacific, the Juan de Fuca Ridge flank off the coast of Oregon, and the northwest Pacific off Japan down to a depth of 358 m below the seafloor[160]. OHR was noted using 2,4,6-tribromophenol and trichloroethene in sediment slurry collected from the Nankai Trough. During another study on sediments of the northwest Pacific off the Kii Peninsula of Japan, OHR was restricted to 4.7 meters below the seafloor, despite detection of rdhA genes in deeper sediments[161]. A recent metagenomic survey on deep sub-seafloor further broadened the diversity of currently known rdhA genes[162]. These results are in contrast to single-cell genomic studies of marine *Dehalococcoidia* where no evidence for OHR were found[114,163]. These studies indicated that microorganisms closely related to known obligate OHRB do not rely on OHR for energy conservation, but rather utilize organic matter degradation pathways which are in line with a similar study from aquifer materials[164]. Marine and estuarine environments have been an important source for the isolation of OHRB given their likely exposure to abundant organohalides and remarkable distribution of rdhA genes. As far as the Mediterranean region is concerned, a notable body of research has been carried out on reductive dehalogenation in groundwater and marine sediments in Italy. *Chloroflexi* phylotypes were reported for PCB dechlorination in enrichments from contaminated marine sediment of the Venice lagoon, Italy[165,166]. In the southern part of the Italian coast several papers identified the classes of bacteria involved in RD and the reductive dehalogenases involved. Dhc strains carrying the reductive dehalogenase genes tceA and vcrA were detected in the marine sediment near Sarno river mouth and their number increased during the treatment with tetrachloroethylene (PCE)[147]. Another study explored the composition and the dynamics of the microbial communities of a marine sediment collected from one of the largest Sites of National Interest (SIN) in Italy (Mar Piccolo, Taranto)[167] Among other bacteria from the Dehalococcoidia class, *Dehalococcoides mccartyi* was enriched during the treatment of sediments with PCBs and the screening of the specific reductive dehalogenase genes revealed the occurrence of undescribed strains. Considering the vastness of the marine environment and the complexity of microbial communities, much is yet to be investigated about the players in the catabolism of halogenated compounds.

1.4 Reductive dehalogenases: diversity study and features

Reductive dehalogenases, the active enzymes in organohalide respiration, are a diverse protein family with low sequence similarity and a punctuated distribution across the tree of life. The first reductive dehalogenase was purified in 1995[168], with the first gene sequence determined in 1998 from *Dehalospirillum multivorans*[169]. Since these initial studies, the number, sequence diversity, and taxonomic distribution of reductive

dehalogenase homologous sequences (rdhs) have increased greatly. The more reductive dehalogenase homologous (rdh) genes sequences were identified, the easier it became to create molecular tools for their study, like degenerate or specific PCR primers or probes. Reductive dehalogenase genes comprise an operon containing rdhA and rdhB, the former being the gene for the enzyme, while the latter is a gene encoding a predicted membrane-integral protein putatively anchoring the RdhA subunit to the membrane, and other associated genes. The rdhB genes encode small proteins of ~90 amino acids with predicted trans-membrane regions, and can be located up or downstream of the rdhA gene. In an analysis of 337 RdhB sequences identified by keyword search in the NCBI database, the sequence identity for the full alignment was 29.8 %, ranging from 3.2 to 100 % pairwise identity. The level of sequence conservation across the RdhA and RdhB families is low, but is in line with other described protein families. Very few molecular structures of reductive dehalogenases have been described to date: one from the species *Nitratireductor pacificus pht-3B*[170] and several structures of the same tetrachloroethene reductive dehalogenase (PceA) from S. multivorans in complex with different organohalides[171]. PceA's main features include a unique base-off conformation in which the adenine lower ligand has been "curled" away from the Co(II) ion to generate an apparent four-coordinate corrinoid in complex with the substrate. Additionally, two cubane Fe-S centers are present in each monomer, and are positioned within a favorable range to transfer electrons to the corrin ring. RdhA from *Nitratireductor pacificus* revealed a similar conformation for the bound cobalamin cofactor, as well as two [4Fe-4S] clusters positioned favorably for reduction of cobalamin. Because of the lack of knowledge of the features of the reductive dehalogenases' protein structure, the RdhA family is recognized by the HMM-based databases of homologous proteins PFAM and TIGRFAM (PF13486, TIGR02486), but is not present in PROSITE or SMART, which require structural information. The PFAM and TIGRFAM models are similar in length, spanning 308 and 314 amino acids respectively of what is typically a ~500 amino acid protein. The PFAM model gives a relatively complete picture of the sequence diversity within this family. It was built with a seed alignment of 89 RdhAs, with a full dataset of 620 protein sequences (accessed pfam.sanger.ac.uk/family/PF13486). The average identity for the full alignment is 27 % at the amino acid level, with an average 31.77 % coverage of a given protein sequence by this model. Both the PFAM and TIGRFAM models cover only the N-terminal domain of RdhAs, and do not include C-terminal 4Fe–4S cluster binding domains or TAT signal sequences, features typically associated with RdhAs (Fig. 10) The C-terminal portion of the RdhA proteins is typically more highly conserved, containing a relatively tightly constrained 4Fe–4S double cluster binding domain (PF13484, average identity of 42 %). While the discovery of rdh genes from cultures and metagenomes proceeds faster and faster, biochemical characterization lags behind mainly because of a lack of sufficient protein yield and homogeneity. RdhA sequence similarity does not always correspond to shared substrate specificities and vice versa. As an instance, the two closely related *Dehalobacter* strains CF and DCA encode the cfrA and dcrA genes respectively. cfrA and dcrA are syntenic within the two genomes, share 95 % sequence identity at the amino acid level, and are tightly grouped within a global reductive dehalogenase tree[172,173]. Despite these strong relationships, the CfrA and DcrA proteins have been biochemically proven to dechlorinate chloroform/1,1,1trichloroethane and 1,1-dichloroethane respectively; nonoverlapping substrate ranges[173]. Similarly, the *Desulfitobacterium dichloroeliminans* dichloroethane-dehalogenating enzyme DcaA shares 90 % amino acid identity with the *Dehalobacter restrictus* strain PER-K23 tetrachloroethene (PCE)-dechlorinating enzyme PceA, a relatively high level of conservation within this protein family. Despite this, the two enzymes do not share a common substrate[174]. As an instance of the opposite situation, the five proteins biochemically proven to share specificity for

tetrachloroethene come from different genera (*Shewanella, Dehalococcoides, Dehalobacter, Sulfurospirillum,* and *Desulfitobacterium*)[147,175–178], are broadly distributed on an evolutionary tree of reductive dehalogenases, and share at maximum 29.8 % pairwise identity (range 20.9–29.8 %)[172]. From this, it is clear that prediction of putative substrates for novel reductive dehalogenases based on sequence similarity cannot be considered reliable and the substrate specificity has to rely on purification and activity assays or transcriptome studies. Reductive dehalogenases characterized to date are listed in table 1.

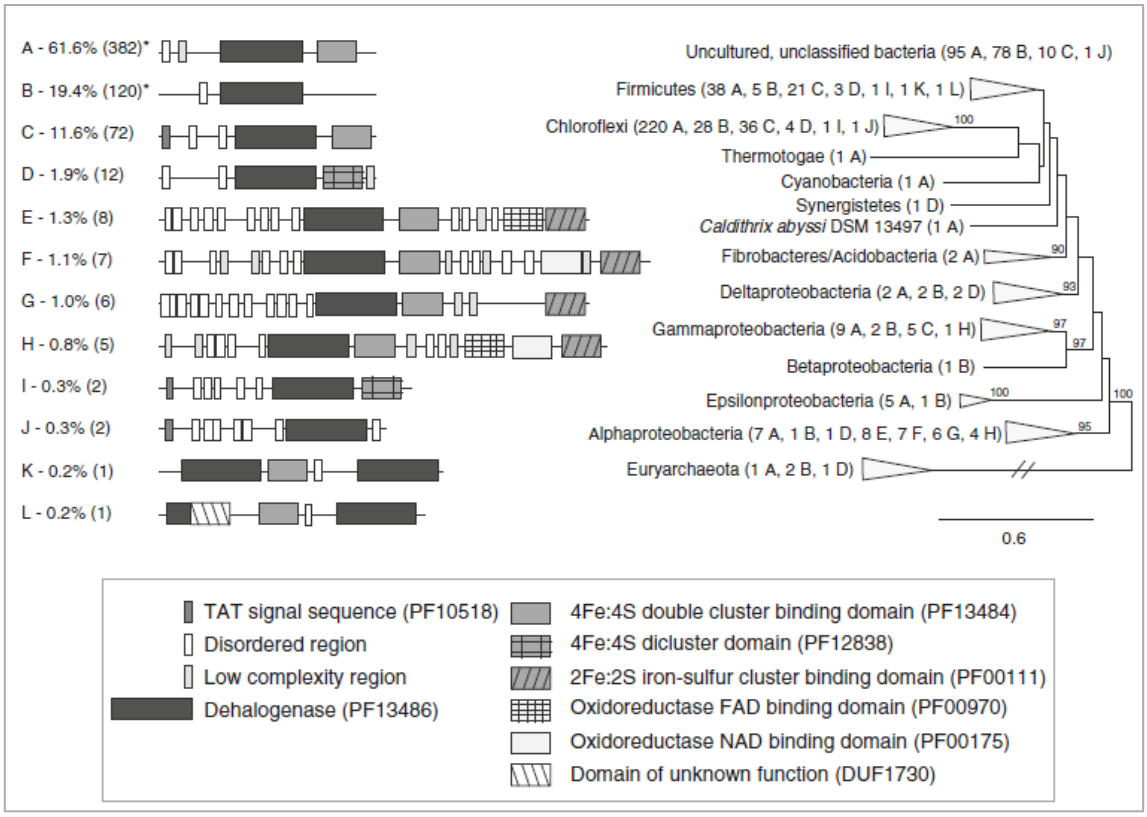


Figure 10 RdhA domain organizations and their phylogenetic affiliations. The number of sequences for each domain architecture is noted at left. Protein architectures were adapted from the reductive dehalogenase PFAM PF13486 and are not to scale (pfam.sanger.ac.uk). The phylogeny was generated with the 16S rRNA genes of all organisms with an RdhA in PF13486. rRNA sequences were mined from NCBI (ncbi.nlm.nih.gov) and the JCVI (moore.jcvi.org) and aligned with Muscle.

Microorganism	RDase(s) Characterized (Identification Approach)	UniProtKB Protein Accession	Substrates	Products	Refs
Dehalococcoides mccartyi CBDB1	CbrA (P)	Q3ZWF5	1,2,3,4-TeCB, 1,2,3-TCB	1,2,4-TCB; 1,3-DCB	[179,180]

Microorganism	RDase(s) Characterized (Identification Approach)	UniProtKB Protein Accession	Substrates	Products	Refs
Dehalococcoides mccartyi 195 (formerly *Dehalococcoides ethenogenes* 195)	PceA (P) TceA (P)	Q3Z9N3 Q3ZAB8	PCE, 2,3-DCP, TCE, All DCE isomers, VC	VC; Ethene	[181], [182], [183], [184], [185], [186], [187]
Dehalococcoides mccartyi BAV1	BvcA (T, P)	Q5YD55	VC, TCE, All DCE isomers, 1,2-DCA	Ethene	130, [188], [189]
Dehalococcoides mccartyi VS	VcrA (P)	D2BJ91	VC, All DCE isomers	Ethene	130, [190], [191]
Dehalococcoides mccartyi MB	MbrA (T, P)	C3RWJ4	PCE, TCE	*cis-/trans*-DCE	[192], 148
Dehalobacter restrictus PER-K23	PceA (T, P)	Q8GJ27	PCE, TCE	*cis*-1,2-DCE	[193], [177], [194], [194], [195]
Dehalobacter sp. CF	CfrA (P)	K4LFB7	TCM, 1,1,1-TCA	DCM; 1,1-DCA	[196], [173]
Dehalobacter sp. DCA	DcrA (P)	J7I1Z7	1,1-DCA	CA	
Dehalobacter sp. WL	DcaA (T)	B5TS67	1,2-DCA	Ethene	[197]
Desulfitbacterium sp. PR	CtrA (T, P)	R9YJY6	TCM, 1,1,1-TCA, 1,1-DCA	DCM CA	[198], [199]
Desulfitobacterium dehalogenans	CprA (P)	Q9XD04	Various *ortho*-chlorophenols (i.e., Cl-OHPA and 2,3-DCP)		[200], [197]
Desulfitobacterium hafniense DCB-2	CprA (P) RdhA3 (P)	P81594 B8FVX4	3Cl-4HPA 3,5-DCP	4-HPA -	[201], [202], [203]
Desulfitobacterium hafniense PCP-1 (formerly *Desulfitobacterium frappieri*)	CprA3 (P) CrdA (P) CprA5 (PceA) (P)	Q8RPG3 Q8GFE2 Q6V7J3	PCP, 2,3,4,5-TeCP, 2,3,4-TCP, 2,4,6-TCP, PCP Various di- and tri-chlorophenols	3,4,5-TCP; 2,4-DCP; 3-CP	[204], [205], [206]
Desulfitobacterium hafniense PCE-S	PceA (P)	Q848J2	PCE, TCE, *cis-/trans*-DBE	TCE; *cis*-1,2-DCE; VB	[207], [208], [209]
Desulfitobacterium sp. PCE1	CprA (P) PceA (P)	Q939S8 Q9APN4	Cl-OH-phenylacetate, PCE	TCE	[210]
Desulfitobacterium hafniense TCE1	PceA (P)	Q8GJ31	PCE, TCE	*cis*-1,2-DCE	[210], [211], [212]

Microorganism	RDase(s) Characterized (Identification Approach)	UniProtKB Protein Accession	Substrates	Products	Refs
(formerly *D. frappieri* TCE1)					
Desulfitobacterium hafniense Y51	PceA (P)	Q8L172	PCE, TCE	*cis*-1,2-DCE	[213], [202], [214]
Desulfitobacterium sp. KBC1	PrdA (T) CprA (T)	Q3LHG0 Q3LHG1	PCE Cl-OHPA		[178]
Desulfitobacterium chlororespirans Co23	CprA (P)	Q8RQC9	3Cl-4-HBA, Several ortho-chlorophenols	4-HBA	[215], [216]
Desulfomonile tiedjei DCB-1	3Cl-BA-RDase(P)	I4C3I3	3Cl-BA	Benzoate	[168]
Desulfitobacterium dichloroeliminans DCA1	DcaA (T)	Q08GR2	1,2-DCA	Ethene	[174],[217]
Dehalogenimonas lykanthroporepellens BL-DC-9	DcpA (T, P)	D8K2H9	1,2-DCP, 1,2-DCA, 1,2,3-TCP	Propene	[218]
Sulfurospirillum multivorans (formerly *Dehalospirillum*)	PceA (P)	W6EQP0	PCE, TCE, *cis-/trans*-DBE	*cis*-1,2-DCE;VB	[169],[171],[175],[208]
Shewanella sediminis[e]	PceA (T)	A8FZV0	PCE	TCE	147
Comamonas sp. 7D-2	BhbA (P)	K4MLZ9	3,5-DB-4-HBA, 3-bromo-4-HBA	4-HBA	[219]
Nitratireductor pacificus pht-3B	NpRdhA (P)	K2MB66	ortho-dibromophenol	Chlorophenol	[170],[220]
Flavobacterium sp strain ATCC 39723 aka Sphingobium chlorophenolicum	TeCH Rdase (P)	Q03520	Tetrachloro-p-hydroquinone (TeCH)	TCH ; DCH	[221]
Dehalobacter sp. strain TeCB1	TcbA (P)	-	1,2,4,5-TeCB, 1,2,4-TCB	1,3-DCB;1,4-DCB	[222]

Microorganism	RDase(s) Characterized (Identification Approach)	UniProtKB Protein Accession	Substrates	Products	Refs
Dehalobacter sp. strain UNSWDHB	TmrA (P)	-	Chloroform	DCM	[223]
Geobacter sp	PceA (P)	-	PCE	TCE and cis-DCE	[224]

Table 1 List of reductive dehalogenases characterized to date. The identification approach is reported: P for proteomics, T for transcriptional.

The identification of novel reductive dehalogenases genes and the characterization of the enzymes in terms of substrate specificity and catalytic mechanism proceed. While the latter investigation activity relies mostly on culture- and isolate-depending techniques, the first is performed both on laboratory cultures and environmental samples and is fostered by the vast number of rdhA sequences deposited in the publicly available databases. This makes it easier to annotate rdhA genes from genomic and metagenomics sequences and develop molecular tools for amplification with degenerate or specific PCR primers. A first degenerate primer pair designed as mentioned was B1R and RRF2[225]. . Primers RRF2 and B1R were designed to target conserved amino acid sequences based on a small set (14) of reductive dehalogenases homologous genes from the Dehalococcoides family. The forward primer RRF2 (5'-SHMGBMGWGATTTYATGAARR-3') was designed to target the RRXFXK motif, which is part of the Tat protein export pathway, while reverse primer B1R (5'-CHADHAGCCAYTCRTACCA-3') was designed to target a conserved twin arginine motif, WYEW, internal to the Dehalococcoides rdhB genes (Fig. 11). The primer pair was used in several works and allowed the identification of tens of novel rdhA genes[226,227,218,228].

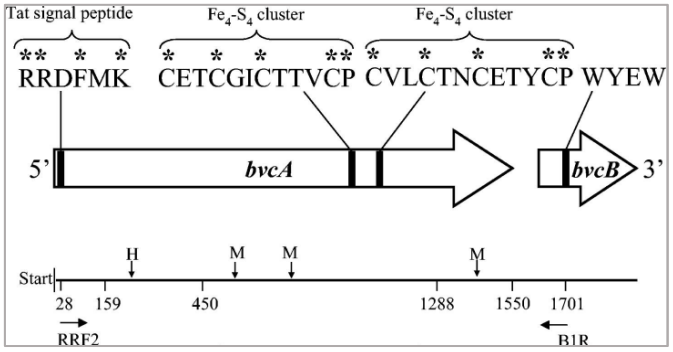


Figure 11 Arrangement of the bvcA gene and the corresponding B gene, bvcB. Also shown are conserved dehalogenase features including the Tat signal peptide RRDFMK, and two Fe4-S4 clusters near the C-terminal end. Amino acids conserved in RDases are labeled with an asterisk. RRF2 and B1R primer binding sites used for amplification of putative RDase genes are shown[225].

An outstanding work was carried out by Hug et al in 2013[229]: a suite of 44 degenerate PCR primer pairs targeting a comprehensive curated set of reductive dehalogenase genes was designed, targeting genes from several different taxonomic groups (Fig. 12) and brought to the identification of 798 novel rdhA homologous

genes from environmental samples, proving that the set of primers employed is a very powerful tool in the exploration of rdh diversity of the environment. Notably, most of the characterized reductive dehalogenases come from freshwater environments, from environmental sample and strain isolates. Nevertheless the rdhA genes diversity in marine environments has been explored and many novel genes have been identified. Specialized PCB-dechlorinase genes pcbA1, pcbA4 and pcbA5, for example, previously characterized in pure cultures of *D. mccartyi*, were here found for the first time in environmental samples.[230] The vast reductive dehalogenase diversity discovered in the marine environment is a promising source of interesting biocatalists and is being investigated further, this work is collocated in that line of research.

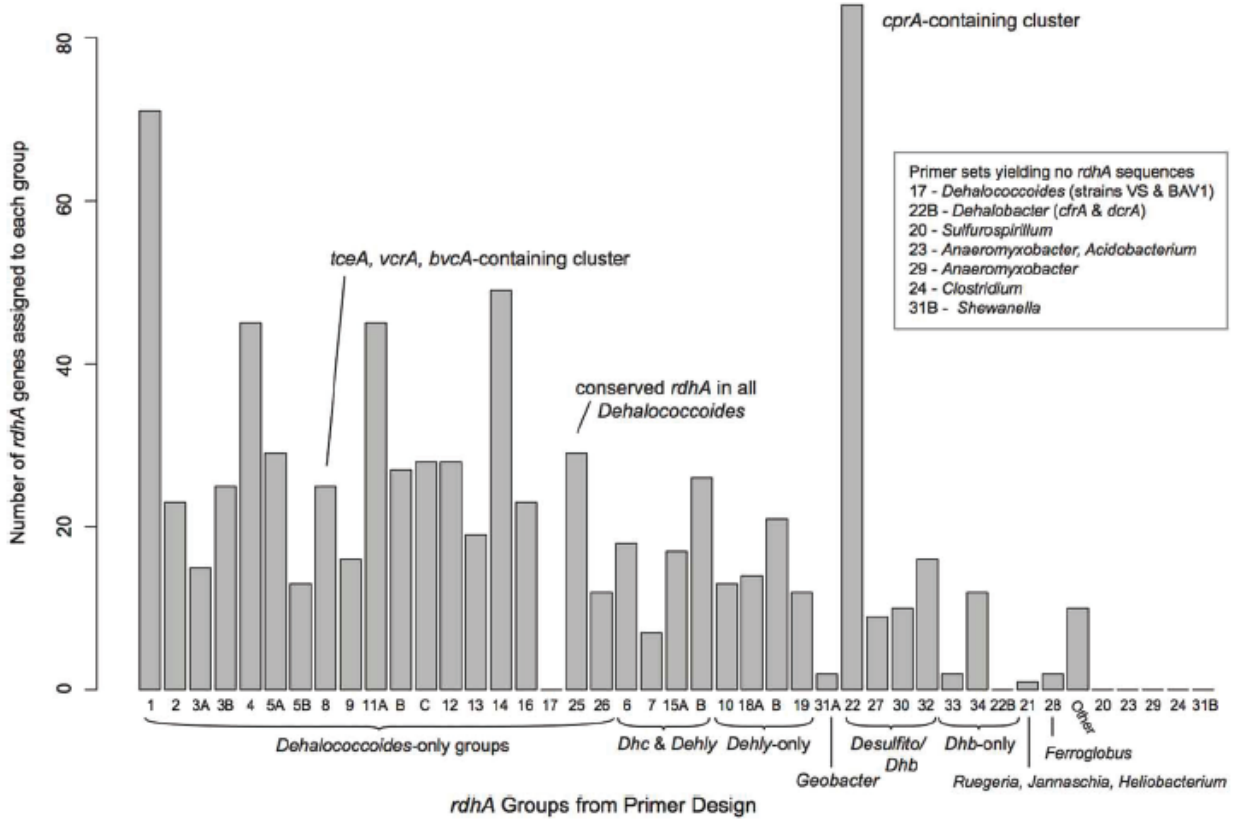


Figure 12 Taxonomic affiliation of rdh genes sequences amplified by degenerate primer pairs (X axis) PCR screens from Hug et al (2013)[229].

2 Aim of the work

Organohalide pollution of freshwater and marine sediments threatens human and environmental well-being. The understanding and exploitation of microbial reductive dehalogenation has made it a natural and powerful tool in the remediation of contaminated freshwater sites. The marine sediment is the final sink for such dangerous and persistent contaminants, which not only make the very environment noxious, but also bioaccumulate in living beings and reach our food. Therefore, the aim of this work is to gain more knowledge about reductive dehalogenation, organohalide respiring bacteria and reductive dehalogenases in the marine environment, which is crucial to better understand halogen biogeochemical cycles and provide information on how to employ such effective biocatalytic process in remediation strategies.

To investigate reductive dehalogenation in the marine environment, the project described in this thesis involved the following activities and goals:

1. Testing two native microbial communities and one from a PCB-dechlorinating enrichment for the ability of reductively dechlorinate several organochlorides to assess the presence of microorganisms capable of MRD and the extent, specificity and speed of their ability. This goal was achieved with the spike of organochlorides in slurry anaerobic microcosms mimicking the biogeochemical conditions of the site of origin. Organic solvent extraction of the spiked compounds and dechlorination products and gas chromatographic or HPLC analysis were used to evaluate the proceeding of the dechlorination;
2. Enrichment of dehalogenating bacteria in microcosm cultures. This was carried out by multiple subculturing of active cultures in a carefully designed defined mineral medium able to select a microbial community rich in dehalogenating species. This goal was important to study the specific expression (RNA and proteins) of rdh genes in active cultures because of these techniques' requirement of an abundant biomass to be used for extraction of samples;
3. Microbial population relative abundance quantification and dynamics analyses for the identification of the OHRB involved in the dechlorination process. Classic 16S rRNA PCR-DGGE was used to pursue this goal, along with 16S gene PCR and Next Generation Illumina sequencing, coupled to bioinformatics analysis of the results;
4. To deepen the knowledge about the biocatalysts involved in the MRD process taking place, the presence and diversity of reductive dehalogenases homologous genes in the microbial community was explored. The goal was accomplished by PCR screens with degenerate primers from literature designed on known rdhA genes, coupled to Next Generation Illumina sequencing, metagenomics software assembly and manual sequence annotation.
5. Linking identified reductive dehalogenases to dechlorination activity. This was carried out by rdh genes expression quantification and proteomics analyses, made possible by activities in point 2 of this list.

3 Materials and methods

3.1 Sediment samples

Anoxic sediment samples for microcosms preparation from Venice Lagoon were obtained by dredging from Brentella canal and Ravenna Harbour sediment was cored from Candiano canal. Sediment was stored in tanks in anoxic conditions at 4°C. Venice lagoon sediment was contaminated by PCBs at a concentration of 3.3 ± 2.8 mg/kg dw of sediment, mostly octa- and nonachlorinated (71.81% w/w) and less hexa- and heptachlorinated congeners (27.27% w/w).

3.2 Slurry microcosms set-up

Three sets of microcosms were prepared: Venice lagoon (VL) microcosms, Ravenna harbour (RH) microcosms and enriched microcosms. VL and enriched microcosms were set up with a sediment collected in Brentella Canal (Porto Marghera, Venice Lagoon, Italy), while RH microcosms were set up using a sediment collected in Candiano Canal (Ravenna harbour, Italy). For VL and enriched microcosms, each contaminant was spiked in four 120 ml anaerobic slurry microcosms (2 biologically active and 2 sterile controls), while for RH microcosms, each contaminant was spiked in five 120 ml anaerobic slurry microcosms (3 biologically active and 2 sterile controls). Primary slurry microcosms were set up with marine sediment collected from either Venice Lagoon or Ravenna Harbor at 20% dry w/v sediment suspension in their own site water without sterilization prepared in serum bottles starting from a preliminary sediment slurry. Aliquots of about 0.5 l of site water were placed into 1-l Erlen- meyer flask, where they were vigorously mixed through a magnetic stirrer and purged with 0.22µm filter-sterilized O_2 -free N_2 :CO_2 (70:30) (with a Hungate-similar apparatus) at room temperature for 2 h. Anaerobic sediment was added at 20% (w/v); then the sediment suspension was mixed and purged for another 2 h. While the slurries were stirred and purged, 70 ml aliquots of slurry were withdrawn, while mixing and purging, and transferred into sterile 120 ml serum bottles equipped with a magnetic bar; slurries were subjected to magnetic stirring and purging with filter-sterilized O_2-free N_2 :CO_2 (70:30) for 15 min before being sealed with Teflon-coated butyl stoppers and aluminum crimp sealers. Two bottles containing 50 ml of slurry were used to prepare sterile controls for each set by autoclave sterilization of sediment aliquots at 121°C for 1 h on 3 consecutive days with static incubation at 28°C between each autoclaving treatment. Finally sediment was resuspended in filter-sterilized site water under O_2-free N_2 :CO_2 (70:30) flow and subsequent spiked with the pollutants. Enriched microcosms were prepared starting from an autoclave-sterilized sediment slurry as described above. Biologically active microcosms were inoculated at 5% v/v with a previously obtained PCB-dechlorinating Venice lagoon sediment culture, whereas sterile controls were not inoculated and just spiked with the pollutant. All bottles were then spiked with the organohalide compounds and purged with filter-sterilized O_2-free N_2 :CO_2 (70:30) for 15 minutes. Contaminants were spiked in both biologically active and sterile microcosms at the concentrations in table 2.

Compound	Stock concentration	Concentration in the sediment
PCB	25 g/L in acetone	500 mg/kg-dw
1,2,3,4-TeCDD	310 µM in acetone	31µmol/kg-dw
HCBe	3.5 mM in acetone	70µmol/l slurry
1,2,3,5-TeCBe	4.6 mM in acetone	93µmol/l slurry
PCP	15 mM in methanol	75µmol/l slurry
2,3,5-TCP	20.3 mM in methanol	101µmol/l slurry
TCE	8.4 mM in water	152µmol/l slurry

Table 2 Concentration of spiked compounds and stock used.

Microbial PCB dechlorination had already been assessed in Venice lagoon in previous experiments (Zanaroli 2012[165]) so VL microcosms were not amended with PCBs. All microcosms were incubated statically in the dark at 30°C and sampled every 4weeks for 24 weeks as described in Fava, Zanaroli, and Young, 2003[231]. All TCE-spiked microcosms (VL, RH and enriched) and chlorobenzenes enriched microcosms were sampled every week after a second spike of the contaminant. In particular, each sealed microcosm was at first mixed vigorously via magnetic stirring for 5 min. The stopper of the sampled microcosm was then removed, and, while the microcosm content was being mixed magnetically and flushed with filter-sterilized O2 -free N2:CO2 (70:30), two identical samples of sediment slurry were removed: an aliquot was used for chemical analyses, following the procedures described below for every contaminant, while an aliquot of 2 ml was frozen at -20°C for DNA extraction until use. The head space was then flushed with filter-sterilized O_2-free N_2 :CO_2 (70:30) and the microcosm recapped with sterile, Teflon-coated butyl stoppers. Only headspace gaseous phase of TCE-spiked microcosms was sampled every 4 weeks, while 2-ml aliquots of slurry were removed only at the end of each incubation period, i.e. when all the trichloroethylene had been dehalogenated.

3.3 Sediment-free synthetic marine water and TCE-spiked cultures set-up

Subculturing in synthetic marine water without sediment was performed by inoculating 20% v/v in autoclave-sterilized Tropic marin obtained by dissolving the indicated quantity in distilled and de-ionized water to obtain the salts concentration expected[232] and aliquoted in 120 ml glass serum bottles. The total volume of liquid culture in every serum bottle was 70 ml. Carbon sources as short chain fatty acids (lactate, acetate, formate or pyruvate) were added to the final concentration of 20 mM from 200g/l stock solutions which were made anaerobic by 20 minute sparging with O_2-free N_2 and sterilized by filtration with sterile 0.22 µm filters. Vitamins were supplied to the concentration reported in section 5.3.1 in table 14 from a 1000X concentrated stock solution. Cultures headspace gas was O_2-free N_2 and bottles were sealed tight with Teflon-coated butyl stoppers and aluminum crimp sealers.

Supplementation of additional nutritional substances was done as follows: NH4Cl was added to a final concentration of 500 mg/l from a 50 g/l stock solution, Na_2HPO_4 was added to a final concentration of 600 mg/l from a 60g/l stock solution, cysteine hydrochloride was added to a final concentration 250 mg/l from a 25g/l stock solution. All stock solutions were made anaerobic by 20 minute sparging with O_2-free N_2 and sterilized by filtration with sterile 0.22 µm filters. Headspace gas was made a mixture of H_2 and CO_2 in a 4:1 ratio at 2 bar absolute pressure by first replacing N_2 with headspace with a flow of 1.6 bar H2 injected with a syringe needle through the stopper and with another needle to let the gas out. Outward needle was removed first and injection needle second. Pressure was then brought to 2 bar with the injection of 2 bar CO_2 through

a syringe needle through the stopper.

Defined mineral medium cultures were inoculated from synthetic marine cultures at 20% v/v in medium prepared by dissolving main salts reported in tab 3 at the indicated concentrations in distilled and de-ionized water in and adjusting pH to 6.8 with a 2 M HCl solution. pH-adjusted medium was made anoxic by 1 hour sparging with oxygen-free nitrogen and aliquoted in N_2-flushed 120 ml glass serum bottles. Other components were added from concentrated solutions directly in the microcosms. Reducing agents were added from a 25g/l stock solution. Trace minerals and vitamins were added from 100X stock solution. BES was added from a 50X concentrated stock solution. All stock solutions were made anaerobic by 20 minute sparging with O_2-free N_2 and sterilized by filtration with sterile 0.22 µm filters.

Medium component	**mmol/l**
Main salts	
Na_2CO_3	28.30
Na_2HPO_4	4.23
NH_4Cl	9.35
NaCl	300.21
$MgCl_2$ x $6H_2O$	19.43
$CaCl_2$ x $2H_2O$	0.34
KCl	9.10
Sodium lactate	20.00
Reducing agents	
cysteine-HCl x H_2O	1.42
Na_2S x $9H_2O$	1.04
Trace minerals	
$CoCl_2$	7.70E-02
$ZnCl_2$	6.19E-02
H_3BO_3	1.62E-01
$Na_2MoO_4x2H_2O$	4.86E-02
Li (LiCl)	2.90E-02
Si (Na_2SiO_3)	1.40E-02
Ba ($BaCl_2$)	3.20E-04
Ni ($NiCl_2$)	1.70E-03
Al ($AlCl_3x6H_2O$)	2.30E-01
Cu ($CuCl_2$)	4.61E-02
Zn ($ZnCl_2$)	6.19E-02
Mn ($MnCl_2$)	1.10E-03
Fe ($FeCl_2xH_2O$)	2.69E-04
Vitamins	

Vitamin B12	1.62E-05
Lipoic acid	2.42E-04
Sodium p-aminobenzoate	3.14E-04
Biotin	8.19E-05
Folic acid	4.53E-05
Pyridoxin-HCl	4.86E-04
Thyamin-HClx2H_2O	1.34E-04
Riboflavin	1.33E-04
Nicotinic acid	4.06E-04
Sodium pantothenate	2.07E-04
Antibiotics	
Bromoethanesulphonate (BES)	30

Table 3 Composition of defined mineral medium for growth of marine OHRB.

3.4 Chemical analyses

3.4.1 Poychlorobiphenyls (PCBs)

PCB analysis was performed as described in Fava, Zanaroli, and Young 2003[231]. Each 0.3 mL slurry sample collected from each microcosm was placed in 1.5 mL vials for gas chromatography (GC) (Agilent 6890 N) equipped with Teflon-coated screw caps; 900 µl of anhydrous diethyl ether was added along with 10 µl of a 40 mg/l stock solution of octachloronaphthalene (OCN, in hexane) and 150 µl of elemental mercury. GC vials were horizontally shaken on a rotary shaker at 250 rpm and 30°C for 15 h. Vials were then centrifuged at 1000 g for 10 min (Beckman J2-HS centrifuge, Beckman Coulter, Fullerton, CA, USA), placed at -20 °C for 30 min and then the liquid ether phase was transferred into an empty1.5-mL GC vial for gas chromatographic analysis. Elemental mercury was employed to remove elemental sulfur and other sulfur compounds present in the sediment, whereas OCN was used both as PCB recovery standard and internal standard for PCB analysis via GC. Qualitative and quantitative analyses of the extracted PCBs were performed with a Agilent GC (6890 N), equipped with a HP-5 capillary column (30 m by 0.25 mm), a ^{63}Ni electron capture detector (ECD) and a 6890 series II automatic sampler (Agilent Technologies, Milan, Italy) under the following conditions: initial temperature 60 °C; isothermal for 1 min; first temperature rate 40°C/min up to 140 °C; isothermal for 2 min; second temperature rate 1.5°C/min up to 185°C; third temperature rate 4.5 °C/min up to 275 °C; isothermal for 5 min; splitless injector; injector temperature 240 °C; ECD 320 °C; carrier gas (nitrogen) flow rate 60 mL/min; sample volume 3 µL. Qualitative analysis of the sediment-carried PCBs was performed by comparing the retention time (relative to OCN) of each of the GC peaks obtained from the analysis of the sediment organic extracts with those of pure congeners and PCBs of standard Aroclor 1242 and Aroclor 1254 (in the range 0.1 to 50 mg/L) analyzed under identical conditions. Standards were identified with confrontation between the analytical peaks and the chromatograph profiles reported by Frame 1996 and Frame 1997. Response factors were calculated for each peak, which was identified using the relative retention time to OCN as internal standards, and were verified monthly. Coeluted congeners were considered present at equal quantities. The degree of chlorination of the PCB mixture expresses the average number of chlorine substitution in the biphenyl molecule ad it is calculated as sum of each congener concentration (µmol/kg-dw) multiplied by the chlorination degree of that congener, all divided by the total concentration of PCBs

3.4.2 Chlorodibenzo-p-dioxins (CDDs)

Extraction was performed from 0.2 ml slurry sample placed in 1.5 ml vials for gas chromatography. Five volumes of a 9:1 mixture of toluene and acetone were added to each sample as well as 10 µl of a 400 mg/l stock solution of OCN, in hexane as internal standard. Samples were sonicated for 15 minutes and horizontally shaken on a rotary shaker at 150 rpm and 30°C for 15 h. After 15 minutes of sonication, samples were centrifuged by Beckman J2-HS centrifuge (Beckman Coulter, Fullerton, CA, USA) and frozen at -20°C for 30 min. Liquid phase was transferred into an empty1.5-ml GC vial and analyzed after dilution 1:25. PCDDs concentration and type were analyzed according to Vargas 2002. Samples were analyzed on a Agilent 6890 N GC with a 63 Ni electron capture detector (ECD) and 6890 series II automatic sampler (Agilent Technologies, Milan, Italy), using a DB-5MS fused silica column (30 m x 0.25mm x 200 nm). The injector and detector temperatures are 280 and 350 °C respectively. The oven temperature is held at 60 °C for 1 min, ramped at 40 °C/min to 150 °C, and then 10 °C/min to 280 °C, and held for 1 min.

3.4.3 Chlorobenzenes (CBes)

Extraction of chlorobenzenes was performed with the same protocol for PCBs extraction described above. A 63 Ni (ECD) electron capture detector GC (Agilent GC 6890 N) equipped with an Ultra-1 fused silica capillary column (0.2 mm x 25 m with a film thickness of 0.33 µm) was used for analysis. The oven temperature was maintained at 80 °C for 5 minutes, raised to 120 °C at 5 °C/min and maintained for 2 minutes, and then raised again to the final temperature of 200°C at 5 °C/min and held for 5 minutes, while temperatures of the injector and detector were 240 °C and 300 °C, respectively. Nitrogen was used for the carrier and makeup gases. The liner velocity was 16 cm/s, and the split ratio was kept at 10:1.

3.4.4 Chlorophenols (CPs)

Extraction was performed from 0.5 ml slurry sample placed in 1.5 ml vials for gas chromatography. Vials were centrifuged at 5000 rpm for 5 min using a Beckman J2-HS centrifuge (Beckman Coulter, Fullerton, CA, USA). Water is filtered with a 0.22 µm filter and analyzed by HPLC as described below; 1 ml pure ethanol is added to the sediment and horizontally shaken on a rotary shaker at 150 rpm and 30°C for 15 h. The sample is centrifuged by Beckman J2-HS centrifuge at 5000 rpm for 5 min and the supernatant is filtered by a 0.22 µm filter and analyzed. Qualitative and quantitative analyses of chlorophenols were performed using a 1260 Infinity series modular reverse-phase high performance liquid chromatograph (HPLC) (Agilent Technologies Palo Alto, CA, USA) with C18 column (0.46 by 15 cm) (Phenomenex, Castel Maggiore, Bologna, Italy) and UV diode array detector using 214 nanometer wavelength. Column temperature is maintained at 30 °C. The mobile phases consisted of (A) deionized water with 0.1% (v/v) of orthophosphoric acid (H_3PO_4); and (B) acetonitrile with 0.1% (v/v) of orthophosphoric acid ($H_3 PO_4$). Mobile phases were previously filtered using a mobile phase filtration assembly with a 0.22 µm filter. The run (A:B) was as follows: isocratic gradient from 70:30 mixture to a final 50:50 in 9 minutes; hold at 50:50 for 3 minutes; isocratic gradient down to mixture at 5:95 at minute 20; isocratic gradient up to mixture at 70:30 at minute 25; post-run for 3 minutes. Minimum pressure was 0.2 bar, maximum pressure 600 bar. The HPLC was calibrated with an equimolar mix of chlorophenols through linear six point calibration curves in the range of 0.2 to 20 mg/L each and with phenol through a linear six-points calibration curve in the range of 0.2 to 10 mg/l.

3.4.5 Chloroethenes (CEs)

Chlorobenzenes were analyzed via batch extraction with 3 volumes of hexane:acetone mixture (9:1) and analyzed via GC-ECD. Chlorophenols were analyzed via preliminary separation of water and sediment phase, extraction from sediment with 2 volumes of ethanol and injection of both aqueous phases in HPLC-UV-DAD. Chloroethenes were monitored by headspace analysis in GC-ECD and GC-FID using Henry's constant to infer concentrations in the liquid phase.

3.5 16S rRNA gene PCR-DGGE analysis

Metagenomic DNA was extracted from the wet sediment (approximately 250 mg) recovered from the centrifugation of 1 ml slurry samples at 10,000 g for 10 minutes with the UltraClean Soil DNA kit (MoBio Laboratories, Carlsbad, CA, USA). Sediment samples suspended in the bead solution supplied with the kit, and prior to cell lysis steps de- scribed in the provided protocol, were treated with 8.2 µl of a 100 mg/ml chicken egg lysozyme solution at 37°C under shaking at 150 rpm for 45 min and then with 4.5 µl of a 100 mg/ml Proteinase K solution from Streptomices Griseus and left under shaking at 150 rpm for 30 min. Extraction continued following manufacturer instructions, but one additional purification step with a solution of 70% ethanol in water was added before elution in 100 µl of solution C6 provided by the kit. DNA samples were quantified using nanophotometer P-330 (Implen GmbH, Munich, Germany).

For total 16S rRNA genes DGGE analysis, PCR amplification was performed with primers GC-357 F (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCGCCCCCTACGGGAGCAGCAG-3') and 907 R (5'-CCGTCAAATTCCTTTGAGTTT-3')[233] in 25 µl reaction mixtures containing 1 x PCR buffer (Promega corporation, USA), 1.5 mM MgCl2, 0.2 mM each dNTP, 0.5 µM each primer, 1.0 U of Taq polymerase (Promega corporation, USA) and 5 µl of template DNA. The reaction began with an initial 95°C denaturation for 10 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s and a final extension at 72°C for 10 min. PCR amplification of the 16S rRNA gene of the dechlorinating members of Chloroflexi was performed with the primers pair GC- Chl348 F (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GGA GGC AGC AGC AAG GAA-3') and Dehal 884 R (5'-GGC GGG ACA CTT AAA GCG-3') described by Watts[155] using the same reaction mixture but with the following cycle: initial 95°C denaturation for 10 min, followed by 40 cycles of 95 °C for 45 s, 55 °C for 85 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. DGGE of bacterial amplicons (approximately 600 ng DNA per lane) were performed with a D-Code apparatus (Bio-Rad, Milan, Italy) in 7% (w/v) polyacrylamide gels (acrylamide-N,N'-methylenebisacrylamide, 37:1) in 1% TAE buffer with a denaturing gradient from 40% to 60% for total 16S rRNAgenes and from 45% to 55% for Chloroflexi- specific 16S rRNA genes; 100% denaturant is 7 M urea and 40% (v/v) formamide. PCR reactions were carried out on DNA extracted from replicate microcosms and amplicons were pooled before loading on the gel. In both cases, run was held at 55 V for 16 h, 60°C (Zanaroli 2010[234]). Gels were stained with SYBR Green I 1x in TAE 1x for 20 minutes, destained in d-H2O water for additional 20 min and their image captured in UV transilluminator with a digital camera supported by a Gel Doc apparatus (Bio-Rad, Milan, Italy). For sequencing, the most prominent DGGE bands were cut from the polyacrylamide gel with a sterile scalpel and eluted overnight at 4°C in 50 µl sterile water. Eluted bands were then re-amplified in 25µl reaction mixtures containing 1 x PCR buffer (Promega corporation, USA), 2 mM MgCl2, 5% DMSO, 0.2 mM each dNTP, 0.5 µM each primer, 1.0 U of Taq polymerase (Promega corporation, USA) and 5 µl of template DNA, with the same cycles described above. Amplicons were resolved again in DGGE electrophore- sis under the conditions

described above; after elution from the second polyacrylamide gel, bands were amplified with non-GC-clamped primers and PCR products purified in the presence of 10 U of ExoI and 1 U of FastAP enzymes at 37°C for 15 minutes before sequencing with the corresponding forward primer. Sequencing was performed by BMR Genomics (Padova, Italy). Each 16S rRNA gene sequence obtained was aligned to the bacterial 16S rDNA database of the Ribosomal Database Project (RDP, release 11) and the closest relative and closest cultured relative retrieved with the Seqmatch tool. The phylogenetic affiliation of each sequence was obtained from the same website with the Classifier tool.

Cloning of reamplified DGGE bands for sequencing was done as follows. DNA extracted as described above was PCR-amplified with universal primers for total 16 S rRNA gene 27 F (5'- AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'- TACGGYTACCTTGTTACGACTT-3') with the PCR mixture described above and the following cycle: initial 95 °C denaturation for 10 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were checked on 1.0% (w/v) agarose gel stained with . and cloned in pCR© 4-TOPO™ vector (Thermofisher) following TOPO™-TA Cloning™Kit manufacturer instructions (ThermoFisher). Vector was transformed into 50µl OneShot ™ TOP 10 Chemically Competent E. coli cells (ThermoFisher), following manufacturer instructions. Colonies were grown for 15 h at 37°C on LB plates provided with ampicillin 50 µg/µl wuth a transformation efficiency of 97 106 CFU/µg plasmid DNA. Presence of Chloroflexi-specific rRNA gene was verified by colony PCR performed as decribed in the instructions provided with OneShot ™ TOP 10 Chemically Competent cells kit in 50 µl reactions containing primers 348 F and 884 R, in the conditions described before for Chloroflexi-specific rRNA gene amplification; colonies were added to PCR mixture aliquotes in sterile conditions. PCR-products were checked on 1.0% (w/v) agarose gel and colonies which gave positive results were grown 15 h at 37°C in horizontal agitation on a rotary shaker at 150 rpm, in 50 ml Erlenmayer flask provided with 10 ml LB medium with 50 µg/µl ampicillin. Plasmids were extracted from 0.8 ml of the culture, after centrifuging 10 minutes at 10000 g by PureLink© Quick Plasmid Miniprep Kit (ThermoFisher), according to manufacturer instructions. Plasmids were stored at -20°C in 75 µl TE buffer.

3.6 16S-rDNA Illumina sequencing and bacterial community analyses

Next Generation Sequencing was carried out by BMR genomics s.r.l. (Padova, Italy). Library from 16S rRNA gene PCR amplification was created with primer pair targeting the V1-V3 region 27 F: 5'-GAGTTTGATCNTGGCTCAG -3' and 519R: 5'-GWNTTACNGCGGCKGCTG -3'[235]. PCR reactions for libraries were done on DNA extracted from the 3 replicate microcosms and then pooled. Every library was analyzed in duplicate. Paired end sequencing was performed with an Illumina MiSeq platform. Raw data analysis to obtain microbial community profiles was carried out in our lab. Raw reads were quality filtered and trimmed joined and taxonomically assigned with a QIIME2 pipeline[236] and the SILVA database (release 128).

3.7 Reductive dehalogenases degenerate primers PCR screens, sequencing and annotation of novel rdh genes

Custom degenerate primers for the amplification of rdhA genes were purchased by BMR genomics s.r.l. (Padova, Italy). PCR products with degenerate primer pairs were pooled and sent to BMR genomics s.r.l. for tagmentation, library preparation and Illumina MiSeq sequencing. BMR genomics s.r.l. filtered reads and assembled contigs with SPAdes 3.7. Contigs were annotated with two set of softwares: i) the Entrez

Programming Utilities (E-utilities), a set server-side programs that provide a stable interface into the Entrez query and database system at the National Center for Biotechnology Information (NCBI); ii) the standalone BLAST executables, command line programs which run BLAST searches against local, downloaded copies of the NCBI BLAST databases, or against custom databases formatted for BLAST. The software esearch from E-utilities was used to search in the protein database of NCBI for any sequence containing in the name or description the words "reductive dehalogenase" and produce a list of UIDs (unique identifying numbers assigned to sequences in the NCBI database). The output of esearch was processed by the software efetch, which retrieved all fasta sequences connected to the UIDs previously found, creating a multifasta file. The multifasta file was processed with makeblastdb software, to create a database accessible by the standalone blast software suite. The resulting database contained 367181 sequences. At this point the 16288 assembled nucleotide sequences were blasted against the protein database with the command "blastall -p blastx", generating an "alignment results file". To extract reductive dehalogenase homologs from the 16288 sequences generated in the assemblies, a stepwise manual procedure was adopted on sequences from all four assemblies: i) alignment results file was screened for the term reductive dehalogenases, marking a hit; ii) the name of the sequence matching dehalogenases from the database with E values lower than 10^{-6} was searched in the assemblied sequences and saved in another multifasta file, the "hits file". The sequences from the the hits file were then manually blasted against the whole protein database of NCBI to verify the reductive dehalogenase annotation and identify possible chimeras mistakenly assembled. Chimeras were identified by having very good matches for reductive dehalogenases in different reading frames and portions of the same sequence. Nucleotide sequences from the hit file which again matched reductive dehalogenases genes and were not chimeras were saved in the "putative rdh genes" file. The sequences from the putative rdh genes file were manually translated into amino acid sequences with the expasy translate tool to the ORF which matched reductive dehalogenases from the database and the amino acid sequence was saved in the "amino acid sequences" file. Four amino acid sequences files were generated, one for each assembly. Amino acid sequences from the different assemblies were cross checked for similarity. With multiple sequence alignment function of Blastn and only unique ones were saved in a final multifasta file containing amino acid sequences of unique rdh genes.

3.8 Expression analysis of dehalogenases

For the specific detection of the newly identified rdhA genes in genomic DNA and RNA, 65 specific primer pairs were designed (Tab. 4), their specificity was tested in-silico with PrimerBlast website software. These primer pairs were employed in multiplex PCRs. Multiplex PCR cycle was as follows: 95 C°, 15min (1 cycle); 94°C, 30 sec - 60°C, 90 sec - 72°C, 90 sec (40 cycles); 72°C, 10 min (1 cycle). Total RNA was extracted for rdhA genes expression quantification from 70 ml of culture with PowerSoil RNA extraction kit from Qiagen. Coding DNA was retrotranscribed with iScript cDNA Synthesis Kit from Bio-Rad following the standard protocol. qPCR reactions on cDNA for rdhA genes expression quantification were set up with SsoAdvanced™ Universal SYBR® Green Supermix using primers from table 5. Absolute quantification of transcript was done by using gel-purified amplicons as template for calibration. Quantitative qPCR cycle was as follows: 95 C°, 5min (1 cycle); 95°C, 60 sec - 60°C, 60 sec (40 cycles); melting curve ramp.

rdhA sequence targeted	Primer pair (5'-3') Name - Sequence	Tm [C]	Amplified product Size [bp]
17, 34	F17 - CGTTGCCCCCAGTCATAAGT	60.04	454

rdhA sequence targeted	Primer pair (5'-3') Name - Sequence	Tm [C]	Amplified product Size [bp]
	R17 - AGTGGGATAAAACAGGCGCA	59.96	
24	F24 - ATTGCTCACCGCACCTGTTA	59.96	442
	R24 - GACGAAAACCGCTCCTACCA	60.04	
20	F20 - TTGTTACAGAGCCTGTCGGG	59.68	207
	R20 - TAGCACTGGGCGTTTTCTCC	60.32	
11	F11 - AACGGGAAGTGTTTCTGCCA	60.11	417
	R11 - AAAGGCTACCATGAGCCAGG	59.74	
32	F32 - AATGCGGTTCATGCCCCTTA	60.03	417
	R32 - GGCGTTGTAGAGCTTACCGA	59.83	
94	F94 - TCCAAGACTCGATAGGTGCC	58.89	190
	R94 - TTGCGTGGCAACTCATAACG	59.48	
21	F21 - TTTGCATGTTGGTTCGCCAG	59.97	606
	R21 - GTACCGGGGTGACACTTACG	60.11	
16	F16 - TCCTGACCATGCCGTACCTA	60.03	709
	R16 - TCCTAATGCTTTCTCCATATTGGT	57.73	
14, 5J	F14 - CAACGCAGGTGTTGCTTCAA	59.90	704
	R14 - CCATACAGCGAGCACAAAGC	59.90	
23	F23 - AGAGCTGCGGTTAACTCACG	60.39	397
	R23 - AAGTGGGCTCGTCAGAGAGA	60.25	
26, 76, 71	F76 - TGAAGATGTGCAGGGCTTTCT	59.93	400
	R76 - CAACCGCTAGCACCATTCAC	59.55	
72	F72 - AAACAACGGTGGCGACTACT	59.89	202
	R72 - CCTAATAATAACCCCACAAAACACA	57.53	
33	F33 - GTGCCCCACCGAATAGAACA	60.04	414
	R33 - GGCGGTTTTCAACTATGGCG	60.18	
50	F50 - GTTGGGAAGGCACACCTGAA	60.47	182
	R50 - GGTTTCTGTTTCGGCGAGAG	59.21	
52	F52 - AAGCCTTATCCGCAGGTCAC	60.11	408
	R52 - TCAGCCACAGAGGTTTGGTG	60.18	
82, 89	F82 - GGAATATGGCCTACCCCTGC	59.96	109
	R82 - AACCGCTGCACTGAGTTTCT	60.18	
27, 91	F91 - ACCTGCAGATGATTAGGGCG	59.89	134
	R91 - GAAACCAACTTCCAAGCCCG	59.69	
55, 2J, 80	F55 - CGAGGGTCTGGAAACCTGG	59.70	140
	R55 - CCACCGCCCTGACTAATGG	60.15	
44, 2	F44 - CCATTACAGGCAGAGCCAGG	60.47	576
	R44 - GAGCTGGCGTTCGGTTTAAG	59.56	
65	F65 - GAACATGGCTGCCCATTCAA	59.10	127
	R65 - GGCAAGCCATGCAGAAAGTA	58.82	
66	F66 - CGCCGCTCATGTCTCTTTTC	59.63	328
	R66 - CCCCGGATTACTACCTACGC	59.40	
58	F58 - ACCCGTTAACAAACCCTCCA	59.15	199
	R58 - GGAGGGTACCCCCGAAGAAA	60.91	

rdhA sequence targeted	Primer pair (5'-3') Name - Sequence	Tm [C]	Amplified product Size [bp]
68	F68 - TGTTAGCGAACTCACGCCAA	60.25	196
	R68 - ATGTGCCGAAGGCACTCTTA	59.39	
53	F53 - TCTATGCCGGACGCAATACC	59.97	292
	R53 - AAACCGTTGAGCGATGGGAT	60.04	
70	F70 - TGTGCGAAATGGCACGTAAC	59.76	269
	R70 - CATCTTCTTACCGGGGCCTG	60.18	
51	F51 - AGAAGTCGATCGGCGTAACC	59.90	116
	R51 - GACCAAAACCGGAGGCACTA	59.96	
60	F60 - GCGCCTCTGCATTTCTGTTC	60.18	105
	R60 - ATGGAGCGATTCCAGAAGCA	59.46	
90, 25	F90 - GGCAGGAGAGAGGTTGGATTC	60.13	100
	R90 - TCATATGCCCAGTCCAAGGG	59.15	
48	F48 - GCTCAAACCTCCAATTCGGC	59.83	418
	R48 - AATAAGGGGGTTACACCGGC	59.74	
61	F61 - AGTATTGAGCCTCCAGCCCT	60.33	115
	R61 - GTTCTGCCTGTGTGAGGCTT	60.54	
42	F42 - CACACACACTGAGAGGCACA	60.18	229
	R42 - GCCACCCATTGATGCTGGTA	60.40	
67	F67 - CCCTGTGGCTGTAGTAGTGC	60.11	100
	R67 - GAAGACCTGAGCCGGTGG	59.73	
64	F64 - AGGCAGGCATTTGTGACAGT	60.18	133
	R64 - GCCGCATCCAGAAGAGAGAA	59.82	
39	F39 - ATGGTGTTAGCACACCACCC	60.25	294
	R39 - CTACCGAACGCCCTAACCAT	59.54	
62	F62 - GCCCATGAACGTAAAACCCAC	60.07	113
	R62 - TTCAAACGCGCATGGTATGT	58.84	
45	F45 - ATACTTGAGTGCGTGCCTGT	59.68	306
	R45 - TGATGAGATCTGCCATGCGG	60.25	
47	F47 - AAGCGCTTAAGACAACGGGA	59.97	527
	R47 - AGTATGCCCACCTAGACCGT	60.03	
57	F57 - GTTTTGTGGGGGCTACAGGT	60.18	279
	R57 - ATATGTTTAAGCGCCCCGGT	59.82	
54	F54 - AAAAAGGTGTTGAATACTGGCGTA	59.18	100
	R54 - ATTGCCATACCGTTTGTCCATTTAC	60.39	
56	F56 - TACGGGCTATTCCCTAGCCT	59.51	327
	R56 - CTAAGGGCTCCAGAGGGACA	60.33	
49	F49 - GCGGCGGCGTTTGTATTAAA	60.18	213
	R49 - TGCAGACGCTTGTCAGGTAG	60.04	
85	F85 - AAGAGGGGTGTGCGTTCATC	60.32	102
	R85 - CCTCTGTTTCCCCCTGGTTG	60.25	
69	F69 - CGCTGAGCATCCTGGACATT	60.46	162
	R69 - CCCGCTGTTACGAGCATTTC	59.63	
30	F30 - GTACGACCGATGAGGGCAAT	59.90	515

rdhA sequence targeted	Primer pair (5'-3') Name - Sequence	Tm [C]	Amplified product Size [bp]
	R30 - TTCCGGAGCCATTACGACAC	60.11	
29	F29 - CCGCCGCCTGATATGCTAAA	60.60	485
	R29 - ATCCCACCAAGCCATGCAAA	60.54	
13	F13 - GGTACCCCGGAGGAAAACTG	60.04	310
	R13 - TTTCACTTTTGGGGCCCACT	60.03	
19	F19 - GACCGCCTTCTTTGCACTTG	60.04	398
	R19 - AGTGCTACCTGACCCATCCT	59.96	
95	F95 - TGACCCTGACCTCAACTGGA	60.11	326
	R95 - CACCCAGGTCTCTCTCCTCA	59.96	
1J	F1J - GGAAGACATCGAGCAGGGTT	59.75	288
	R1J - TTAGCTTGCATACCCGCCAT	59.82	
78,28,77	F78 - CATTTACCGCAGAGTTGCCG	59.90	274
	R78 - TGCCCATTTCGGTAATGCCT	60.03	
93, 15	F93 - AAACGGCCGGAAGTATGGTT	59.96	294
	R93 - TCCAAATTGCGTGGTAGCCT	59.96	
69	F69 - CGCTGAGCATCCTGGACATT	60.46	162
	R69 - CCCGCTGTTACGAGCATTTC	59.63	
38	F38 - GTGGCGATGAGGTAGGTTGT	59.75	322
	R38 - TGCATCACGCCACTTCCTAA	59.68	
63	F63 - TATGGCCATCAAACGGCGTA	59.82	184
	R63 - CGTGAAAGCCATGCTTCGTT	59.76	
73, 74, 10	F73 - GGCACTGAGGGATCTTGCT	59.39	103
	R73 - TGCAGGTGGACCTACCATGT	60.84	
1	F1 - TTGCCGGTGCATGTGAGTAA	60.25	217
	R1 - CGCTGGGTTTGGGAAGACA	60.23	
6	F6 - AGAACGGCTTTTTGACCCGA	60.18	393
	R6 - GAATGGAACGCACTTGCCTG	60.11	
22, 4J	F22 - GTGCCGTGTATTGATGCGTG	60.25	689
	R22 - TTTGGCCACCAAGCAGAGAT	59.89	
35	F35 - CTTGGTTCCAAGGCCCAGTA	59.60	448
	R35 - TGGACAGTGCAGCAACCAAT	60.47	
12	F12 - GCCGCTTTGTAGCCTAGTGA	60.11	577
	R12 - CTGCAGATACTGCTACCGCA	59.90	
81	F81 - TATCACAGTAGGGGCAGCCT	60.03	562
	R81 - ATGGACGGTGCGGTAAAAGT	59.96	
40	F40 - TGGTGAGGCGGGTTGTTATC	60.04	218
	R40 - CGTTTCCGTAGTCCGGTGAA	60.04	
41	F41 - CCGAATGCTAAGAAACGCCG	59.97	420
	R41 - CCGCTAAGCATACGGGACAT	59.97	
37	F37 - CCCGCATCAATCGGTTTTGT	59.47	380
	R37 - AATAGTTGCCAATGGGGGCT	59.66	
83	F83 - TGGAGCTTCTTCTTTCGCAGT	59.93	195
	R83 - CACGCTTCGGCACACTTTG	60.37	

rdhA sequence targeted	Primer pair (5'-3') Name - Sequence	Tm [C]	Amplified product Size [bp]
75,26	F75 - AACCGAAGGCTCTTCTTGGG	59.96	192
	R75 - GTGGTGCCAATGGTTGCTTT	59.89	

Table 4 Primer pairs employed in multiplex PCR screens for expression evaluation.

rdhA sequence targeted	Primer pair (5'-3') Name - Sequence	Tm [C]	Amplified product Size [bp]
17, 34	F17q - CGTTGCCCCCAGTCATAAGT	60.04	72
	R17q - TGTAGGCGCTGTGGAGATTG	60.11	
24	F24q - ATTGCTCACCGCACCTGTTA	59.96	72
	R24q - TCGCTCCTTCAAAGCCGATT	60.04	
20	F20q - CGGCAGAGGCATGTCTGTAA	60.11	78
	R20q - TCAGGGCAATCCATGTCACC	60.03	
11	F11q - CCTGGCTCATGGTAGCCTTT	59.74	74
	R11q - ACGTATAGGGAGCAGCAAGC	59.89	
32	F32q - GGTAAGCTCTACAACGCCCA	59.75	121
	R32q - TTCTGACCGCTACGGAAACC	60.04	
94	F94q - CCTTCCCAAAAGAGCCGAAG	58.83	145
	R94q - GATGAGCTTGAGCACACTGC	59.55	
21	F21q - CCTTCATGGGAAGGTGACCC	60.03	73
	R21q - GCCAGGTCTTGATTCCAGGT	59.67	
16	F16q - ATGTGTCAATCCTCCTGCCC	59.74	135
	R16q -TCCTAATGCTTTCTCCATATTGGT	57.73	
14, 5J	F14q - TCTTGCACCTAATACCCCGC	59.82	125
	R14q - CCATACAGCGAGCACAAAGC	59.9	
23	F23q - ATGCGCGGAAACTTGTCCTA	60.04	94
	R23q - AGTGGGCTCGTCAGAGAGAT	60.03	
26, 76,71	F76q - ACATACGGCTATTGTCGGGC	60.25	92
	R76q - CCGCTAGCACCATTCACAGA	60.11	
74, 10	F74q - GTGAGCTTGGGCGACTACAA	60.32	129
	R74q - AAAACCTCGATGCGCCAAAA	59.33	
75	F75q - TGCGAAACCTGTGGCATTTG	59.97	70
	R75q - CCCAAGAAGAGCCTTCGGTT	59.96	
72	F72q - AGGCACATGCCCGTTTAATG	59.18	81
	R72q - ATAGGAGTAGTCGCCACCGT	60.11	
33	F33q - ACCCATGGTATGTCGCTGAG	59.53	70
	R33q - CGCCATTTGTGGTCCCAATC	59.83	
50	F50q - AACGGTGCTACAGGTGCTTT	60.18	130
	R50q - CTTCAGGTGTGCCTTCCCAA	60.18	
52	F52q - GAACCCGGCTGGGATGTAAA	60.03	142
	R52q - CGCTGAATGCAGGTATCCCA	60.18	
77, 28	F77q - AAGAGAGTGGTTACGCCTGC	60.04	142

	R77q - GGGCTCTTAACTGGACCACC	60.04	
82, 89	F82q - GGAATATGGCCTACCCCTGC	59.96	109
	R82q - AACCGCTGCACTGAGTTTCT	60.18	
27, 91	F91q - ACCTGCAGATGATTAGGGCG	59.89	99
	R91q - GCCCCGCATCAAAAAGAGTC	59.83	
55, 2J, 80	F55q - CTGGTGGAACCGCGATACTT	60.11	148
	R55q - ACGGCGCCGAATATAATCCA	59.68	
44,2	F44q - ACTACCGCGGTGACAGAATG	60.11	79
	R44q - GCAGTTATGCCGTCAGCTTC	59.62	
65	F65q - GATTTGCGCAGAACATGGCT	59.83	140
	R65q - CTTGGCAAGCCATGCAGAAA	59.68	
66	F66q - TGATTCGGGCTGCATTCCAT	60.11	111
	R66q - CGTATGGTTTCCCTCTCCCG	59.9	
58	F58q - TGCCCGCACCTAAACCATAG	60.11	91
	R58q - ATTGGCAAGGCATACGACCA	60.03	
68	F68q - ACTTGGACTCATGCCGAACC	60.32	75
	R68q - AATGTGAGTGGAAGACCCGC	60.32	
53	F53q - ACCAGAGAGGTTACGTCCGA	59.96	137
	R53q - GGTATTGCGTCCGGCATAGA	59.97	
51	F51q - TAGTGCCTCCGGTTTTGGTC	59.96	70
	R51q - CGACACTTGCTTGGGAGCTA	60.04	
60	F60q - TTAGTCATGCGCCTCTGCAT	59.82	123
	R60q - CTGGACCAAGATGGAGCGAT	59.53	
90	F90q - CGCTTGACCCAACTTACCCT	59.96	124
	R90q - TAGTTGGCAGCTTCTTCGGG	60.04	
25	F25q - ATGGTGAATTGGGGCGTACC	60.4	139
	R25q - GATTTGCAGAACTCACGCCC	59.83	
48	F48q - AATGTATCAGATGGGGCGGC	60.25	88
	R48GGGTGGATGCTAGCGTCTTT	60.11	
61	F61q - ACGGGCAGAAAGCCATACAA	59.96	73
	R61q - ACTGGTTTCAAGGGCTGGAG	59.89	
42	F42q - CTGAGAGGCACATACCGCAA	60.11	139
	R42q - ATGGAAACGGAACCGTCCTG	60.32	
67	F67q - CAACGGGTTCGCCGTAAAAT	59.48	72
	R67q - CAGTTCGCATGAAGCCTGAG	59.27	
64	F64q - GATCAAGTCAAAGGCGTGGC	59.83	72
	R64q - CCATCTTTCATCAAGAGCTGCAA	59.56	
39	F39q - GGCTCCCCTTCGGGTATAGA	60.18	92
	R39q - AAGCCCGTTGATTTTGGTGC	59.97	
62	F62q - GCCCATGAACGTAAAACCCAC	60.07	78
	R62q - ACCCACCACAGAAGTTGACTG	60.13	
45	F45q - GGCATTAAGCCCCAGTGTCT	60.03	144

	R45q - GCAAGGCGACAGCATTTTGA	60.04	
47	F47q - CCAGTTGGACAAGTGACGGA	59.89	116
	R47q - AGTATGCCCACCTAGACCGT	60.03	
57	F57q - GCCCTGAAGGGATTGCAAGA	60.32	136
	R57q - TATGTTTAAGCGCCCCGGTT	60.04	
54	F54q - GGCGTAATAGCTGAAGTGGC	59.06	81
	R54q - TTGCCATACCGTTTGTCCAT	57.79	
56	F56q - TCAGTCCGACATTTGGCACA	59.89	147
	R56q - TAAGGGCTCCAGAGGGACAA	59.88	
49	F49q - CAAACAGGTTCAGCGCTTGG	60.32	96
	R49TGCAGACGCTTGTCAGGTAG	60.04	
85	F85q - TAAGGGTCAGCGGGAGAAGA	59.96	93
	R85q - GATTTGCCCTCATCGCACAC	59.9	
69	F69q - GAAATGCTCGTAACAGCGGG	59.63	81
	R69q - TTCTATGTGCCCGGCTCAAC	60.39	
30	F30q - GTTTGACTTTGGTCAGGCGG	59.69	71
	R30q - GTCGCCTCTGGTCTCTATGC	59.97	
29	F29q - CCACGGTACCCGAAACAAGT	60.25	132
	R29q - ATCCATAACCGCACGCTTGA	60.11	
13	F13q - GCAGAAGCCTGCCCTTATGA	60.11	122
	R13q - TGGCGCTGGCAATGAGAATA	60.11	
19	F19q - GACGCCTCAACAAGCAACAG	60.04	121
	R19q - CCTCAGGTGTTCCTTGCCAT	59.96	
95	F95q - GGACACCTGGCCATACGAAA	60.04	91
	R95q - AGTCCAGTTGAGGTCAGGGT	60.1	
1J	F1Jq - GCGTGGGCAAAACCATTAGG	60.11	141
	R1Jq - AGCTTGCATACCCGCCATAA	59.82	
78	F78q - GGGCTCTTAACTGGACCACC	60.04	142
	R78q - AAGAGAGTGGTTACGCCTGC	60.04	
93	F93q - AGACCCTGATGACCCTTGGT	60.18	134
	R93q - TCCAAATTGCGTGGTAGCCT	59.96	
69	F69q - GAAATGCTCGTAACAGCGGG	59.63	81
	R69q - TTCTATGTGCCCGGCTCAAC	60.39	
4J	F4Jq - CCAACGTGGGATCTTGCTGA	60.32	106
	R4Jq - TGCGGCATGTGGCATAGTTA	60.11	
38	F38q - GGGCAAAATGCAGCCGTAAA	60.04	113
	R38q - TGCATCACGCCACTTCCTAA	59.68	
63	F63q - CGAAGCATGGCTTTCACGAG	59.9	114
	R63q - ACTGCCCTGGTACTGCTAAC	59.39	
73, 10, 74	F73q - CGGCAGGTTGGTATGGTGTAA	60.34	77
	R73q - TGCAGGTGGACCTACCATGT	60.84	
15	F15q - TCCAAATTGCGTGGTAGCCT	59.96	134

	R15q - AGACCCTGATGACCCTTGGT	60.18	
1	F1q - GCCAGCCATAAAACGAGCAC	60.18	90
	R1q - TACTCACATGCACCGGCAAT	60.04	
71	F71q - CCGCTAGCACCATTCACAGA	60.11	92
	R71q - ACATACGGCTATTGTCGGGC	60.25	
6	F6q - GAGGCGCATACCATACACCA	59.89	149
	R6q - CCGAGTGGAGCAATAAGCGA	60.18	
22, 4J	F22q - TGCGGCATGTGGCATAGTTA	60.11	106
	R22q - CCAACGTGGGATCTTGCTGA	60.32	
35	F35q - ACATTGCGTCGGCAGTTCTA	60.04	127
	R35q - ACATGGACTTGGCGCATACA	60.04	
12	F12q - ATAGGGCCCCATTCAGGAGT	60.03	111
	R12q - CATAGGCCCGTGGACATACC	59.97	
81	F81q - TATCACAGTAGGGGCAGCCT	60.03	76
	R81q - AATACTTGGAACCGCCCAGG	60.03	
40	F40q - TGGTGAGGCGGGTTGTTATC	60.04	115
	R40q - GGGCCCAAGTGTTCTATCCC	60.11	
70	F70q - TGACTATTCAGGCCCCGGTA	60.03	114
	R70q - ACGTTAAACACGCAGTTGCC	59.97	
41	F41q - GCCTATTTCCGGCCCTACTC	59.96	127
	R41q - ACAGGCATGGGAGCATTTCA	59.96	
37	F37q - CCAAGGTGAACCCCAATCCC	60.61	110
	R37q - TAAGCGGCGAGACGAAACAA	60.32	
83	F83q - GGGGATGCAGCAACTTGGAT	60.69	91
	R83q - GACACTGATACCCCAGTCCG	59.54	
74	F74q - GCAACAACGGGACCTTGGGA	62.97	71
	R74q - TCGGGTGTCCCGTTCCA	60.18	
10	F10q - TAGGTCCACCTGCAACGGG	61.59	76
	R10q - CTATTCTCCTCGGGTGTCCC	58.96	
73	F73q - TCAACCTGGACTTCCCCTGT	60.4	106
	R73q - GCGAAGAACGTTGGTAAGCA	59.14	

Table 5 Primer pairs employed for expression quantification.

3.9 Proteomics analyses of dehalogenases

For whole cells shotgun proteomics from extracts cells were harvested by consecutive centrifugation at 16°C for 1 hour followed by removal of the top half of the volume until a volume of 750 µl were reached, then supernatant was removed and cells were resuspended in 30 µl of 10mM ammonium bicarbonate (Ambic) buffer at pH 8. Cells wer disrupted by freeze thawing and 30 seconds ultrasonication, the first consisting of repeating 3 times the cycle: 1 minute in liquid nitrogen, 1 minute at 40°C. Cysteine-SH bonds were reduced with dithiothreitol (DTT) at a final concentration of 50mM and incubation for 1 hour at 30°C. Subsequently, cysteines were acetamidylated with iodoacetamide (IAA) at a final concentration of 130 mM and incubation of 1 hour at room temperature. Trypsin digestion was done by adding 0.6 µg of enzyme per sample and

incubating at 37°C overnight. Trypsin digestion was stopped by adding 1 µl of 100% formic acid to each sample. Samples were centrifuged at 13'000 rpm for 10 minutes and supernatant was isolated.Sample was dried with a vacuum centrifuge and resuspended in 20 µl of a 1% formic acid solution in MS grade water. Desalting of peptides was performed by Zip Tipping with $ZipTip_{\mu-C18}$, Millipore, 2 µg capacity tips. Samples containing peptides were then loaded (3 µl) in a Thermo nano-Liquid Chromatography device with a C18 reverse phase column and the effluent loaded and ionized into a Thermo Orbitrap Fusion device by an Advion NanoMate. Devices and analytical help were supplied by ProVIS – Centre for Chemical Microscopy, Helmholtz Centre for Environmental Research – UFZ, Permoserstrasse 15, 04318 Leipzig. Identified peptides were aligned to a database with Proteome Discoverer software (Thermo Fisher Scientific) created with the list of identified dehalogenases formatted by the same software. SDS PAGE for other MS analyses was performed by harvesting cells in the same way described for whole cells shotgun proteomics until the removal of the supernatant and by centrifugation of 10 ml of culture. Instead of resuspending cells in ambic buffer they were resuspended in SDS buffer. Cells disruption was done by heating the sample with SDS buffer at 95°C for 10 minutes right before loading 30 µl on a 10% polyacrylamide gel. Gel was silver stained according to Nesterenko (2014)[237] and a gel band between 40 and 70 kDa was cut for in gel trypsin digestion. In-gel trypsin digestion followed these steps: gel piece was washed for 5 min with 200 µl ddH2O, it was added to the gel piece 200 µl of a freshly prepared mixture of 1:1 v/v 30 mM potassium hexacyanoferrate (III) (100 µl) and 100 mM sodium thiosulfate(100 µl), the gel piece was washed two times for 5 min with 200 µl double-distilled H2O (ddH2O), supernatant was discarded, the gel piece was washed for 10 min with 200 µl 50 mM Ambic buffer, supernatant was discarded, 200 µl of acetonitrile were added to the gel piece and incubated for 5 min, the gel piece was dried in the vacuum centrifuge for 5 min. For reduction of SH protein bonds 50 µl 10 mM DTT were added to the gel piece, sample was incubated 30 minutes shaking 450 rpm at room temperature and supernatant was removed. For SH groups protection 50 µl 100 mM 2-iodacetamide was added to the gel piece and sample was incubated shaken at 450 prm at room temperature, supernatant was discarded. 200 µl of acetonitrile was added to the gel piece and sample was incubated for 5 min, supernatant was discarded. 200 µl of 10 mM Ambic buffer was added to the gel piece and sample incubated for 10 min, supernatant was discarded. 200 µl of acetonitrile was added to the gel piece and sample was incubated for 5 min, supernatant was discarded. The gel piece was dried in the vacuum centrifuge for 5 min. 20 µl diluted trypsin in buffer C was added to the gel piece (Trypsin/Lys-C Mix, Mass Spec Grade, Promega). Sample was incubated overnight. 30 µl of 5 mM Ambic buffer were added to the gel piece and sample was incubated shaking at 700 rpm at room temperature for 10 minutes, supernatant was collected. 30 µl of extraction buffer were added to the gel piece and sample was incubated for 15 min shaking at 700 rpm at room temperature, supernatant was collected with the one from the previous step. Last step was repeated once. The three combined supernatants containing the extracted peptides were dried with a vacuum centrifuge and resuspended in 20 µl of a 1% formic acid solution in MS grade water. Resuspended peptides were analyzed by MS like previously described.

3.10 Chemicals

Aroclor 1242 (for calibration) and 1254 (for calibration and spikes), chlorodioxins ,chlorophenols and chlorobenzenes were supplied by ULTRA Scientific Italia S.r.l, Bologna Italy. Proteinase K from S. griseus, Lysozyme from chicken egg, urea, formamide, acrylamide-bisacrylamide solution, agarose, SYBR Green I, medium components, chloroethenes and organic solvents acetone were supplied by Sigma Aldrich Italia S.r.l,

Milan, Italy. FastAP and ExoI enzyme stock solutions were provided by Thermo Scientific, part of Thermo Fisher Scientific Italia S.r.l, Milan, Italy

4 Results and discussion

4.1 Reductive dechlorination capabilities of microbial communities in marine sediments of two sites of the Adriatic Sea

4.1.1 Organochloride compounds dehalogenation

Slurry microcosms were set up with marine sediment collected from either Venice Lagoon or Ravenna Harbor in their own site water without sterilization in serum bottles. HCBe, TeCBe, PCP, TCP and TCE were spiked at final concentration of 20 mg/l of slurry, PCB were spiked only in RH microcosms to a concentration of 500 mg/Kg of dry sediment. Concentration of organochlorides was monitored periodically as described in the Materials and methods section.

4.1.1.1 Chlorophenols reductive dehalogenation in primary cultures from Venice Lagoon and Ravenna Harbor

As far as chlorophenols are concerned, 2,3,5-trichloro phenol (TCP) was dechlorinated in both RH and VL microcosms. In the former, dechlorination started only after 2 months of lag phase and, once started, it stopped after 59% of TCP was converted to 3,5-DCP after three out of 6 months of incubation and no accumulation of other reductive dehalogenation products was observed (Fig. 13A). In the latter, a 2 months lag phase was observed likewise, but 77% of spiked TCP was dechlorinated at 6 months to 3,5-DCP (27%) and 3-CP (70.6%), with an initial accumulation of 3,5-DCP at month 3 (Fig. 13B). PCP reductive dehalogenation was not detected in VL nor RH microcosms, not in the active ones nor in the sterile negative controls (Tab. 7). TCP dechlorination pattern in RH sediment is like what have been observed previously in literature, while in VL sediment, where dechlorination proceeds to 3-CP, a less common pattern is observed, with removal of a meta-unflanked chlorine (Fig. 14). Dechlorination rates of TCP are similar in the two sediments, although in the RH microcosms the conversion to 3,5-DCP happens faster (Tab. 6). This might be due to a different set of reductive dehalogenases in the microbial community, but with RH dehalogenases highly specific and more active. The dechlorination speed is lower by one order of magnitude (16% RH, 12% VL) than the one observed in a pure culture of *Dehalococcoides mccartyi* strain DCMB5, which is expected as growing conditions are optimized in the pure culture.

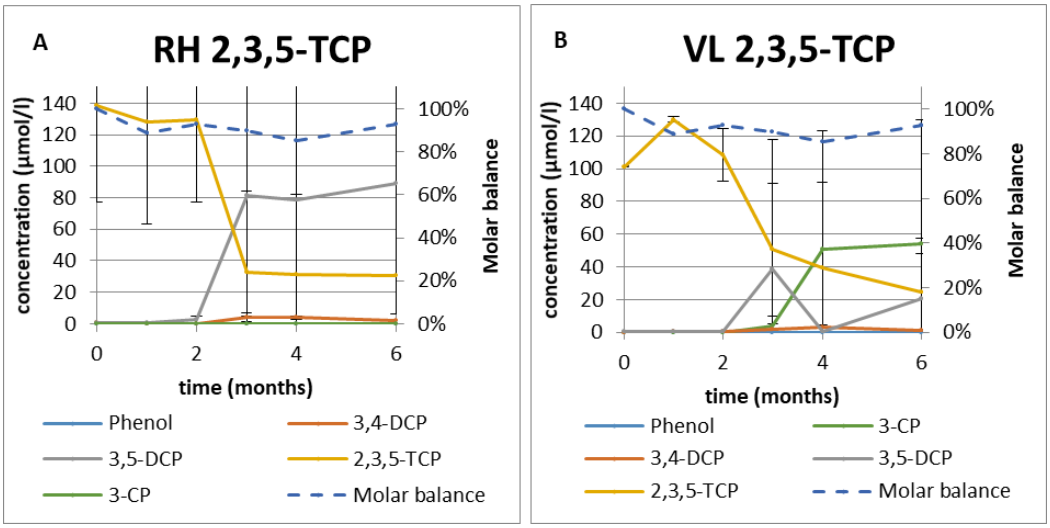


Figure 11 Change in concentration of 2,3,5-trichlorophenol and its dechlorination products in primary microcosms with sediment from Ravenna Harbor (A) and Venice Lagoon (B). The molar balance is shown, calculated as the sum of moles of spiked compound and products, expressed in percentage of the initial quantity of 2,3,5-TCP.

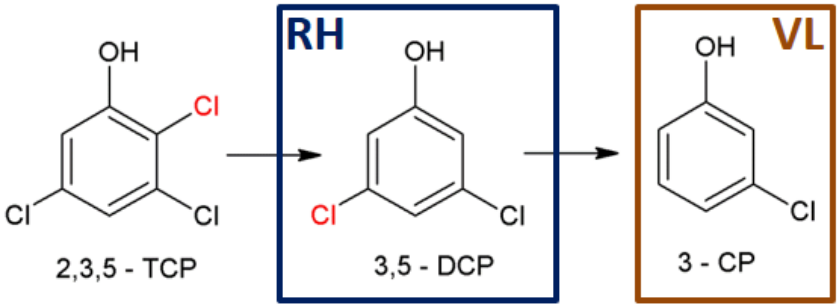


Figure 12 Dechlorination pattern of 2,3,5-trichlorophenol in Venice Lagoon (VL) and Ravenna Harbor (RH). Chlorine removed at every step are enlightened in red. Microbial community from RH sediment is able to remove only ortho-single flanked chlorines, while VL's can performed also removal of at least one of the two meta unflanked chlorines.

	Rate of organic chlorine removal (µmol*day^{-1}*l^{-1})	
Source of OHRB	**2,3,5-TCP**	**PCP**
RH	0.97	-
VL	0.70	-
VL ENR	7.45	1.60
Yang et al, 2008[238]	-	10.29
D. mccartyi Strain JNA[239]	-	2.50
D. mccartyi Strain DCMB5[240]	6.00	7.27

Table 6 Rate of daily dechlorination of 2,3,5-TCP and PCP in different studies compared with values obtained in our set of different cultures. Speed is calculated in the whole period monitored (6 months), although the two months lag phase obviously plays a big role in reducing the speed to almost half and most of the biotransformation happens during the third month of incubation.

		Time(months)	
		0	6
VL (Sterile)	2,3,5-TCP	98.3 ± 15.3	89.0 ± 0.6
RH (Sterile)		98.0 ± 7.5	87.2 ± 4.2
VL ENR (Sterile)		99.1 ± 3.1	110.6 ± 1.9
VL (Sterile)	PCP	36.0 ± 14.9	26.2 ± 16.2
RH (Sterile)		44.8 ± 2.4	44.9 ± 3.7
VL (Active)		66.5 ± 0.1	55.8 ± 4.2
RH (Active)		82.7 ± 19.8	70.4 ± 16.4
VL ENR (Sterile)		49.5 ± 17.1	47.3 ± 17.4
		0	3
VL (Sterile)	HCBe	94.7 ± 8.6	108.9 ± 4.3
RH (Sterile)		89.0 ± 8.0	115.8 ± 6.9
VL ENR (Sterile)		80.0 ± 7.0	85.8 ± 3.2
VL (Sterile)	TeCBe	60.4 ± 2.0	60.0 ± 20.4
RH (Sterile)		103.5 ± 3.5	100.0 ± 33.1

VL ENR (Sterile)		84.1 ± 12.9	84.1 ± 5.0

Table 7 Concentrations of spiked chlorophenols and chlorobenzenes at the beginning and end of incubation period in: i) sterile negative controls from all microcosms sets: primary cultures and Venice Lagoon enrichments, ii) alive controls of primary cultures spiked with PCP which did not show any dechlorination.

4.1.1.2 Chlorobenzenes reductive dehalogenation in primary cultures from Venice Lagoon and Ravenna Harbor

Both chlorobenzene compounds spiked separately, HCBe and 1,2,3,5-TecBe, were dechlorinated in both RH and VL sediments, sterile negative controls, on the other hand, did not show any dehalogenation of spiked compounds nor accumulation of less chlorinated benzenes (Tab. 7). After a 1 month lag phase, in VL microcosms HCBe disappeared in only one month, while accumulation of 1,3,5-TCBe and 1,3-DCBe was observed at month 2. At month 3 the tri-chlorinated benzene was not detected anymore, while the 1,3-DCBe concentration was observed to have risen further, with the detection of low levels of 1-chloro benzene and benzene (Fig. 15A). The non-stoichiometric transformation of HCBe into 1,3-DCBe, 1-CBe and benzene might be due to the mineralization of benzene by the microbial community. TeCBe VL microcosms spiked with 1,2,3,5-TeCBe showed the same dechlorination pattern and timing of the microcosms spiked with HCBe, although no lag phase was observed, which could be probably explained by the higher water solubility and therefore bioavailability of TeCBe over HCBe's (Fig. 15C). In RH microcosms spiked with HCBe a 1 month lag phase precedes the complete stoichiometric biotransformation of the organochloride into 1,3,5-TCBe at month 3 (Fig. 15B). TeCBe spiked in RH microcosms was dechlorinated in the same way but, like in VL microcosms, without lag phase (Fig. 15D). Interestingly, in HCBe spiked RH and VL microcosms very low levels of intermediate products were detected (< 2 µmol/l), namely penta and 1,2,3,5-tetrachloro benzene, most likely because they were further dehalogenated immediately. Dechlorination patterns of chlorobenzenes in literature record a general preference towards double-flanked chlorines, which are removed more quickly than single or unflanked ones. This is consistent to what we observed in both RH and VL sediments, with the difference that, in the latter, dechlorination proceeds forward, targeting unflanked chlorines (Fig. 16 and 17). This is not common, as in other studies were identified microbial communities or OHRB isolates which could dechlorinate HCB to dichlorobenzenes and no further. In particular, pattern observed in VL is the same observed in Sohn et al., studying primary cultures with river sediments in New Jersey (USA). Conversely, Dehalococcoides mccartyi strains CBDB1 and DCMB5 both have a chlorine removal specificity towards single-flanked chlorines, accumulating mostly 1,2,4-TCB and 1,4-TCB, while strain 195 dechlorinated HCB only to 1,3,5-TCB and in a minor proportion to 1,2,4-TCB[241]. Reductive dehalogenation speed of the spiked compounds in both sediments was similar to the one observed in other studies, in particular to the one of a consortium originally derived from contaminated groundwater from the historically contaminated area of Bitterfeld-Wolfen[242] (Saxony-Anhalt, Germany) highly enriched in Dehalococcoides (84% of clones) and a pure culture of Dehalococcoides mccartyi DCMB5[239] (Tab. 8), although pure cultures of D.mccartyi strains are more active, this might depend on the optimization of the growing medium. These results come from highly enriched cultures, suggesting that the original marine microbial communities of RH and VL sediments have very active reductive dehalogenases towards these compounds.

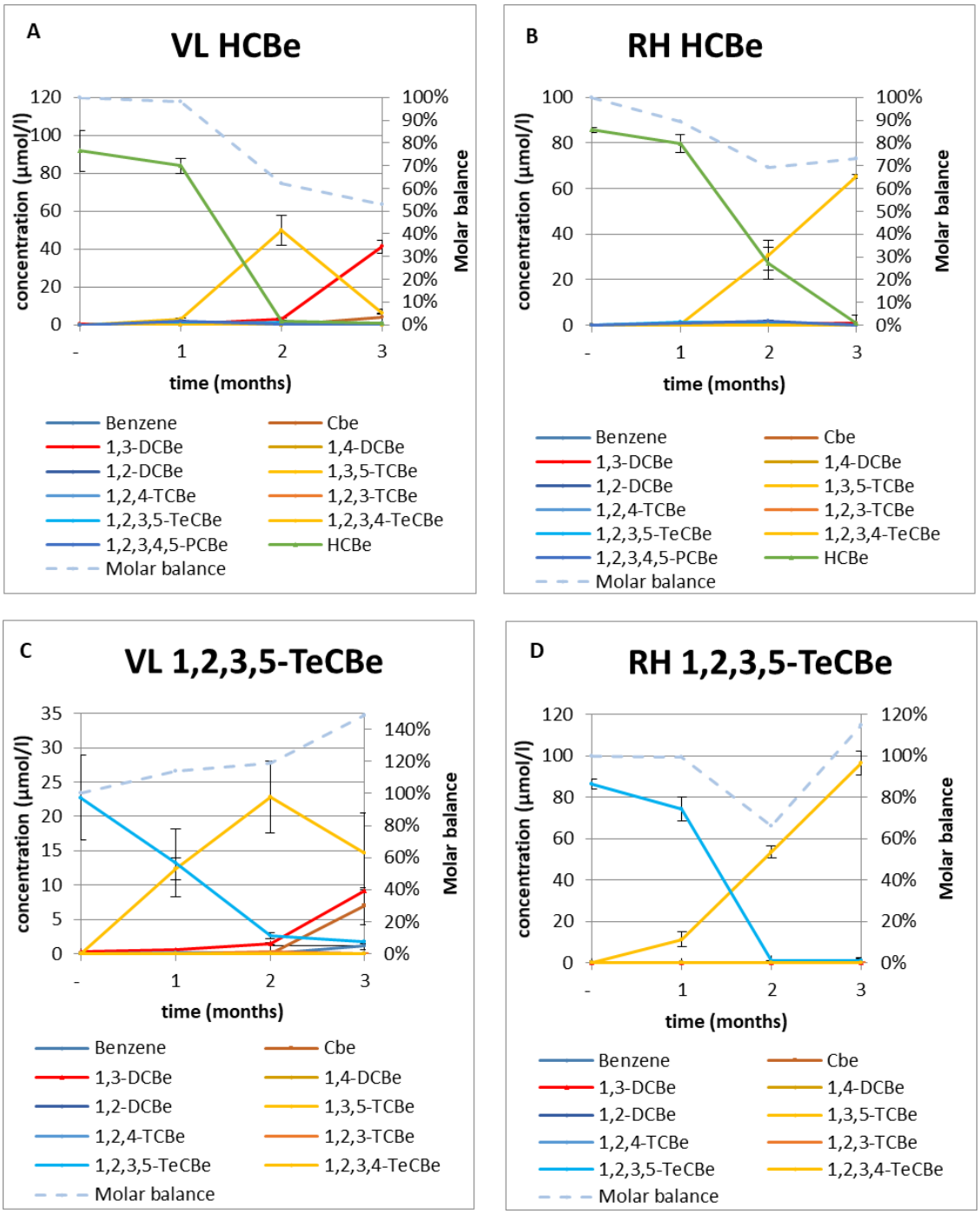


Figure 13 Change in concentration of spiked HCB and 1,2,3,5-TeCB and their dechlorination products in primary microcosms with sediment from Venice Lagoon (A and C, respectively) and Ravenna Harbor (B and D, respectively). The molar balance is shown, calculated as the sum of moles of spiked compound and products, expressed in percentage of the initial quantity of the spiked chlorobenzene (HCBe or 1,2,3,5-TeCBe).

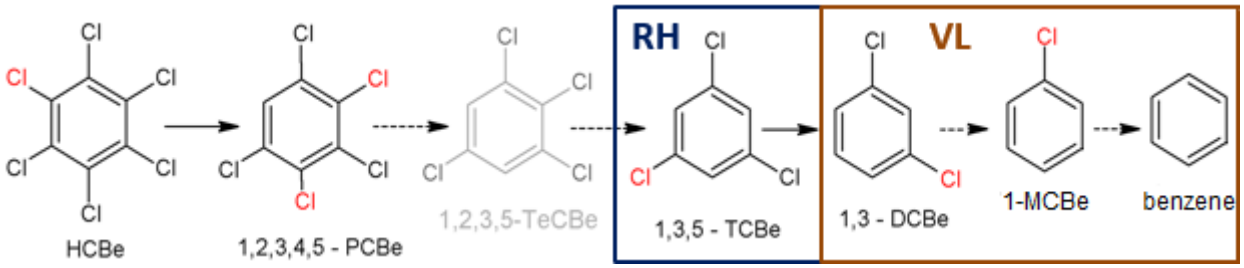


Figure 14 Dechlorination pattern of hexachlorobenzene in Venice Lagoon (VL) and Ravenna Harbor (RH). Chlorine removed at every step are enlightened in red. Undetected intermediate product 1,2,3,5-tetrachlorobenzene is pale because it was not detected. VL's microbial community was able to remove both double flanked and unflanked chlorine on the benzene ring, whereas RH's chlorine removal ability is limited to double flanked ones.

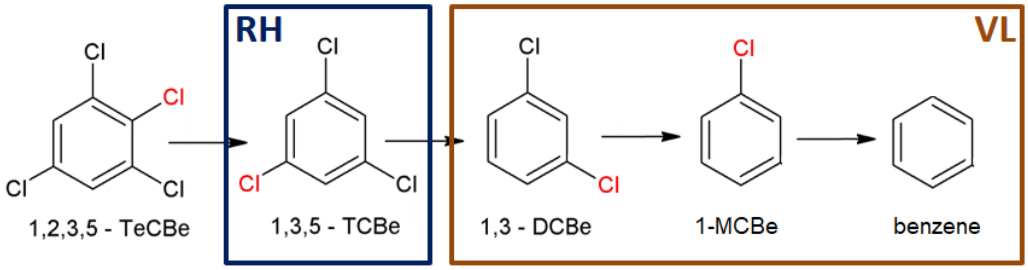


Figure 15 Dechlorination pattern of 1,2,3,5-tetrachlorobenzene in Venice Lagoon (VL) and Ravenna Harbor (RH). Chlorine removed at every step are enlightened in red. The chlorine removal specificity is the same observed towards hexachlorobenzene, with removal of unflanked and double-flanked chlorines in VL's sediment and only double-flanked in RH's.

	Rate of organic chlorine removal (µmol*day^{-1}*l^{-1})	
Source of OHRB	**HCBe**	**1,2,3,5-TeCBe**
RH	3.88	2.84
VL	5.09	1.35
VL ENR Spike1	18.07	6.73
VL ENR Spike2	24.16	3.03
Kaufhold et al, 2013[242]	5.44	0.38
Sohn et al. 2016[243]	0.8	-
D. mccartyi Strain CBDB1[244]	9.86	-
D. mccartyi Strain 195[241]	40.00	-
D. mccartyi Strain DCMB5[240]	4.63	3.17

Table 8 Rate of daily dechlorination of 1,2,3,5-TeCBE ad HCBe in different studies compared with values obtained in our set of different cultures. Speed is calculated in the period between the beginning and the end of the 3rd month, when dechlorination had stopped.

4.1.1.3 Chloroethenes reductive dehalogenation in primary cultures from Venice Lagoon and Ravenna Harbor

Concerning chloroethenes, TCE was dechlorinated in sediments from both VL and RH, albeit with the accumulation of different products (Fig. 19). Both sets from the two different sites were respiked over time. In Venice lagoon primary microcosms, after the first spike of TCE, no dechlorination was observed after 87 days. The microcosms were stripped from TCE by sparging with oxygen-free nitrogen and respiked. The second TCE spike was depleted by 65% in 27 days, leading to the accumulation of mainly *trans*-DCE and to

less extent *cis*-DCE (Fig. 18B). RH sediment microcobial community depleted TCE completely in 60 days. Stripped and respiked, TCE was completely dechlorinated in 13 days accumulating stoichiometrically *cis*-DCE only. The microcosms were stripped and respiked two other times (Fig. 18A). No dechlorination was detected in any of the sterile controls, the decrease of TCE over time is of abiotic source and is related to the high volatility and hydrophobicity of the compound, from which the ability to diffuse through BTFE coated caps (Tab. 5).

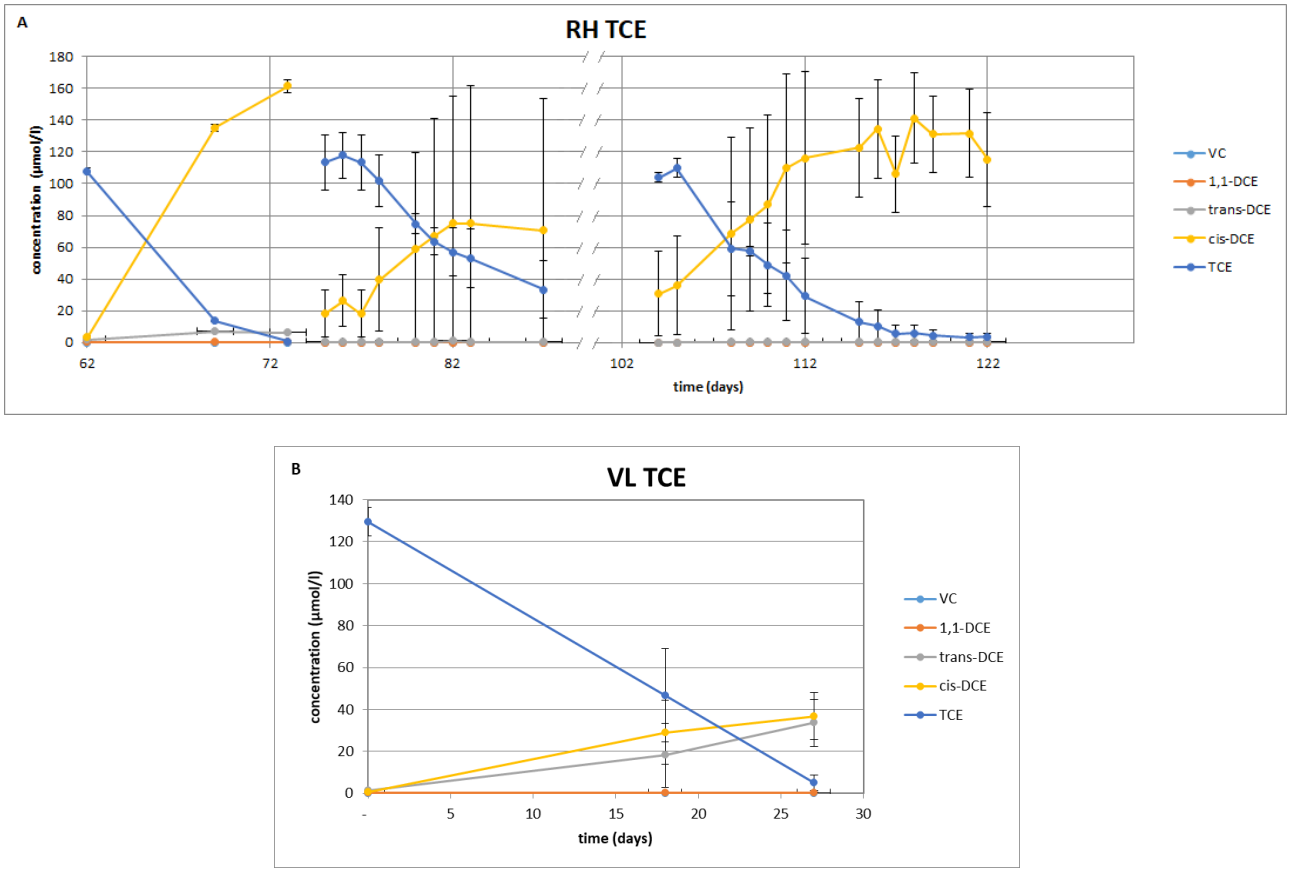


Figure 16 Change in concentration of spiked TCE its dechlorination products in primary microcosms with sediment from Ravenna Harbor (A) and Venice Lagoon (B).

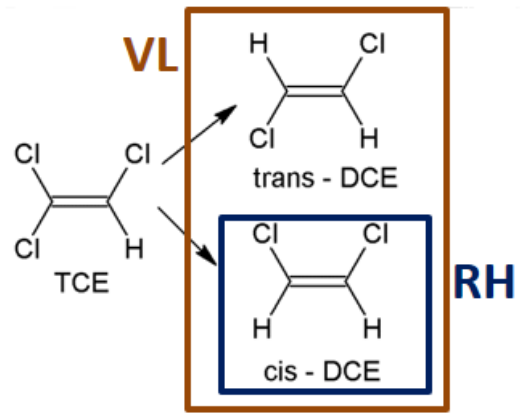


Figure 17 Dechlorination pattern of trichloroethylene in Venice Lagoon (VL) and Ravenna Harbor (RH). While both cis- and trans-DCE were produced in VL's cultures, in RH cultures only cis-DCE accumulation happened.

Reductive dehalogenation rate of TCE was less than half the one observed in other studies[245,246] where growing conditions were optimized. Interestingly, in both VL, and especially in RH microcosms, speed was observed to increase and then stabilize spike after spike of TCE (Tab. 9). This could mean that after the second spike the OHRB have decreased their metabolism after an initial growth to then settle to a lower dechlorination activity which can be then maintained for other spikes. This could be due to the decrease in other important nutrients necessary for exponential growth of cell number other than the organohalide electron acceptor, or to cell density signaling and growth inhibition.

	Rate of organic chlorine removal from TCE (µmol*day^{-1}*l^{-1})			
	Spike number			
Source of OHRB	1	2	3	4
RH	1.09	16.51	15.75	8.14
VL	0.00	1.51	-	-
VL ENR	8.29	125.68	119.89	-
D. mccartyi Strain GT[245]	40.00	-	-	-
He et al, 2003[246]	36.32	-	-	-

Table 9 Rate of daily dechlorination of TCE in different studies compared with values obtained in our set of different cultures. Speed is calculated in the period between the spike and the end of dechlorination products accumulation.

4.1.1.4 Reductive dehalogenation of chlorodioxins and PCBs in primary cultures from Venice Lagoon and Ravenna Harbor

Dechlorination of PCBs did not occur in Ravenna harbor microcosms after 6 months of incubation, whereas previous studies demonstrate that the process is carried out in Venice Lagoon sediment[247]. 1,2,3,4-tetrachloro dibenzo-*p*-dioxin dechlorination was not observed neither in RH nor in VL after 6 months.

4.1.2 Microbial population dynamics

Samples from primary microcosms with VL and RH sediment were taken for DNA extraction and analysis at several times during incubation. Total DNA was extracted from samples at the end of the dechlorination phase of each compound set and 16S rDNA genes were PCR-amplified from the metagenomic DNA with the GC-357f and 907r primers; amplicons from each replicate culture of the same conditions were then pooled together and run on 40-60% DGGE gels. The analyses showed that peculiar differences are present between cultures, amongst both the different conditions (spiked compounds) and between the two sediment sets (Fig. 20).

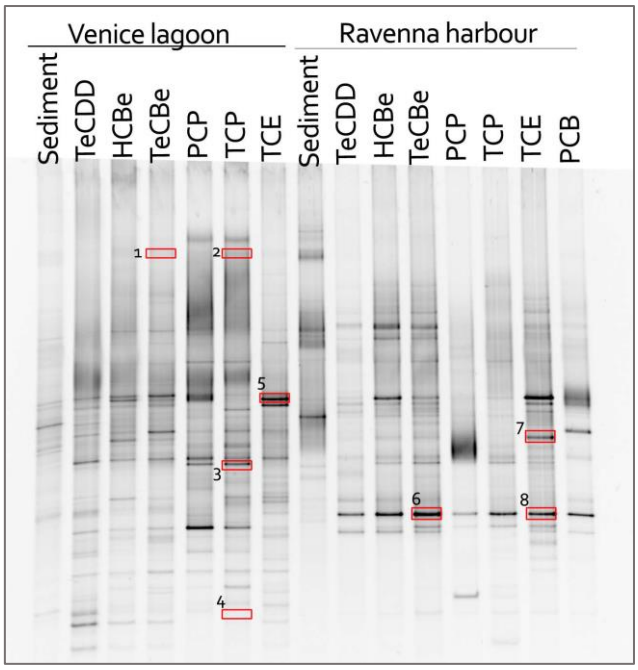


Figure 20 PCR-DGGE analysis of the rRNA gene of the total bacterial community of the primary cultures(VL or RH) spiked with the different organohalides at the beginning of the incubation ("sediment" lane) and at the end of dechlorination. Squared bands have bees sequenced and annotated phylogenetically, results are in table 8.

Results of alignment of sequences from DGGE with universal primers for total 16 S rRNA gene of primary microcosms against RDP database are shown in table 10. The only species associated with reductive dehalogenation was *Dehalogenimonas lykanthroporepellens* BL-DC-9, the presence of which in a certain sample did not correlate perfectly with the observation of reductive dehalogenation, it is enriched in VL PCP and TeCDD, which were not dechlorinated, whereas it is absent in RH and VL TCP, both dechlorinated.

Band	Similarity score (%ID)	S_ab score	Unique common oligomers	Genbank code	Microorganism
1	0.994	0.922	1380	KP172208	*Lutimonas sp. KHS05*
2	0.914	0.623	1295	JQ661158	*Lutimonas saemankumensis*
3	0.867	0.399	1262	KJ535409	*Atribacteria bacterium JGI 0000059-I14*
4	0.983	0.790	1334	AF295656	*Desulfotomaculum sp. 175*
5	0.878	0.474	1338	EU679418	*Dehalogenimonas lykanthroporepellens BL-DC-9*
6	0.959	0.773	1262	KJ535409	*Atribacteria bacterium JGI 0000059-I14*
7	0.875	0.424	1429	CP009415	*Candidatus Izimaplasma sp. HR1*
8	0.921	0.532	1262	KJ535409	*Atribacteria bacterium JGI 0000059-I14*

Table 10 Results of alignment of sequences from DGGE with universal primers for total 16 S rRNA gene of primary microcosms against RDP database (environmental and isolates).

To gain more insight on the microbiome dynamics of the cultures and understand better which microbial species could be correlated with reductive dehalogenation of spiked compounds, DNA was used for 16S Illumina sequencing and QIIME2 phylogenetic and relative abundance analysis of microbial groups. Original sediments analysis at the phylum level revealed that different phyla are represented in the bacterial community, in particular RH sediment hosts a much diverse microbial community, harboring also *Cyanobacteria*, *Plantomycetes* and *Synergistetes* (Fig. 21).

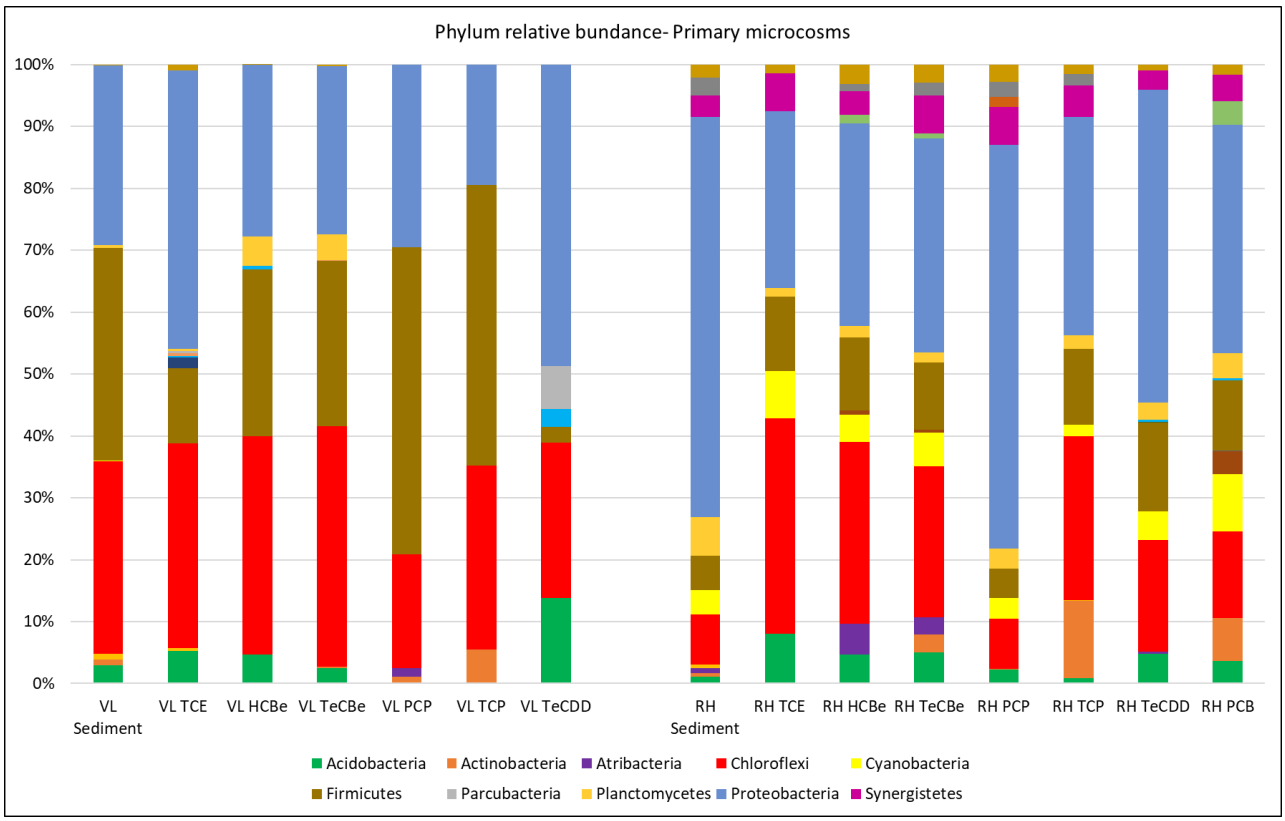


Figure 21 Taxa relative abundance plot based on the 16S rDNA amplification and sequencing in samples from primary cultures from VL and RH. Phylogenetic analysis at Phylum level.

To identify the bacteria responsible of the dehalogenation we looked for the clades that specifically and relevantly enriched when dehalogenation was observed and compared our information with the list of phylogenetic groups known to contain OHRB. At the phylum level no correlation was observed between the observation of dehalogenation and a specific enrichment. Of the most represented classes, the ones that showed enrichment in the microcosms with dehalogenation from both sediments were *Dehalococcoidia* and *Anaerolinae* from the *Chloroflexi* phylum, *Epsilonproteobacteria* (only in VL sediments) and *Clostridia* (Fig. 22). Of these classes only the *Dehalococcoidia* and the *Epsilonproteobacteria* contain known OHRB, in particular the order *Dehalococcoidales* and *Campylobacterales*. Microbial members of the order *Campylobacterales* show enrichment in all VL microcosms and most of RH (Fig. 23) but a deeper analysis at the genus level revealed that no member of the genus *Sulfurospirillum* was detected, the only OHRB-containing genus of *Epsilonbacteriales*. The *Campylobacteriales* detected were from the genus *Sulfurovum*, which has never been directly linked do reductive dehalogenation, although some studies observed its enrichment in PCB degradation and decabromodiphenyl ether degradation[134]. The *Dehalococcoidia* class and, in particular, its orders GIF9 and *Dehalococcoidales* showed (Fig. 23) enrichment in all the microcosms where dehalogenation of the organochloride was detected except in VL TeCBe and RH TCE. Interestingly, in both

cases the *Epsilonproteobacteria* class *Campylobacteriales* and its genus *Sulfurovum* showed enrichment.

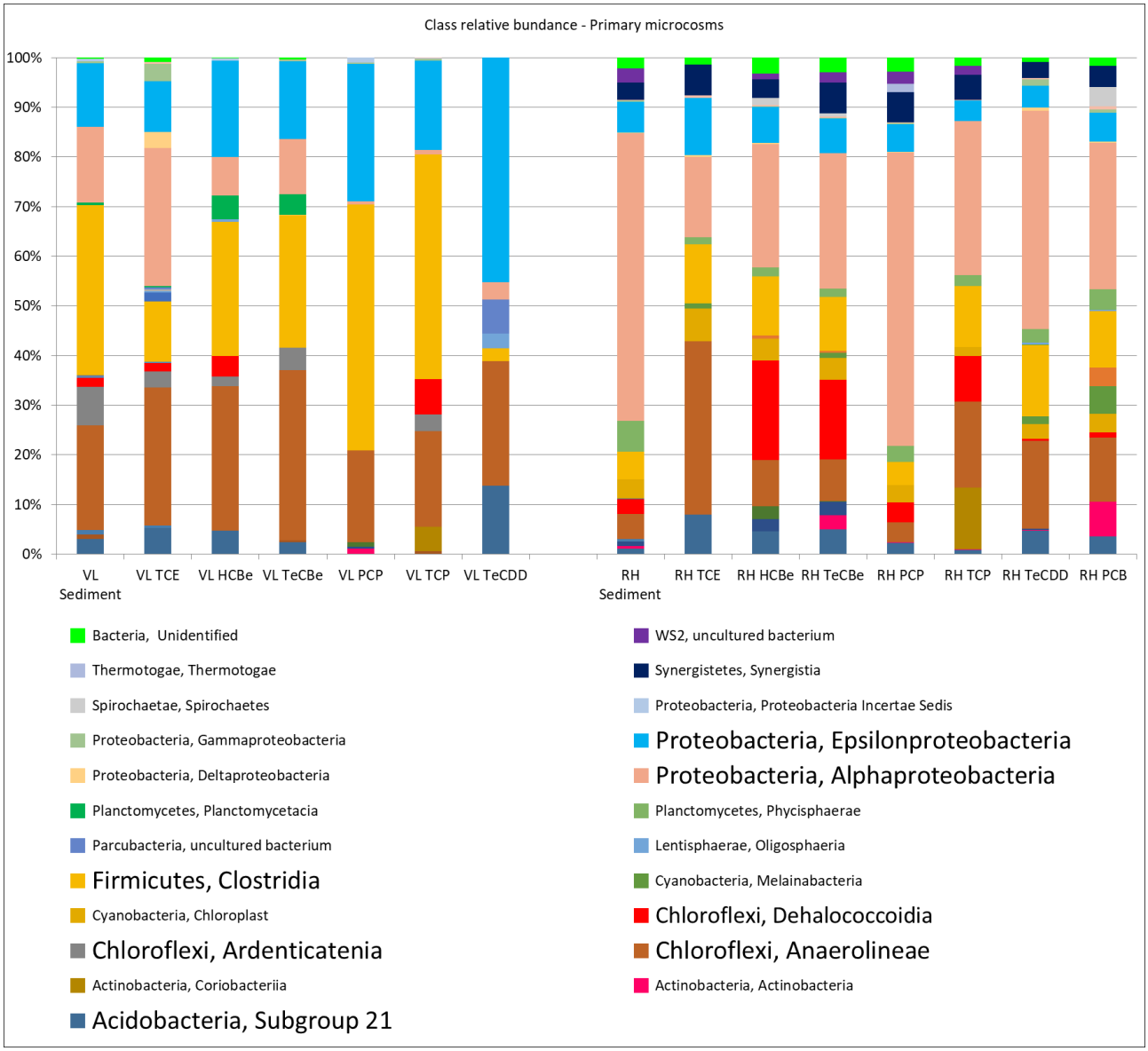


Figure 22 Taxa relative abundance plot based on the 16S rDNA amplification and sequencing in samples from primary cultures from VL and RH. Phylogenetic analysis at Class level.

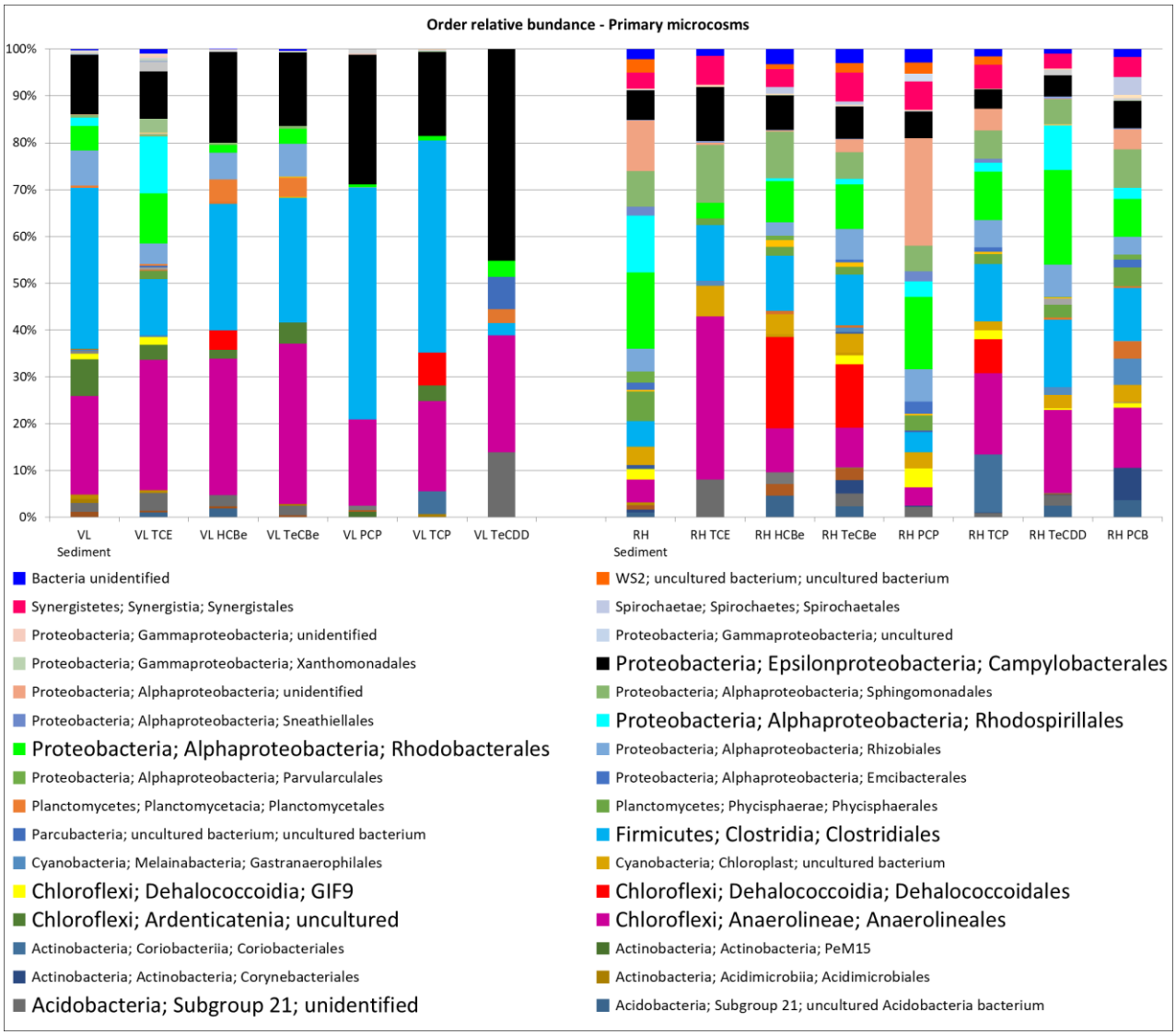


Figure 18 Taxa relative abundance plot based on the 16S rDNA amplification and sequencing in samples from primary cultures from VL and RH. Phylogenetic analysis at Order level.

As Chloroflexi phylum and the Dehalococcoidia class were the phylogenetic group whose enrichment best correlated with reductive dehalogenation, PCR-DGGE with DNA amplified by primers specific for dechlorinating Chloroflexi bacteria (GC348 F and 884 R) was performed (Fig. 24). Compared to the sediment culture profile at the beginning of the incubation, two intense bands were visible in all the microcosms in which dehalogenation activity occurred: in RH microcosms, only one band at low denaturing concentration is detected where dechlorination occurred, while in VL microcosms an additional band is visible at higher denaturing concentration, together with another band in chlorobenzenes and TCE-amended microcosms. Unexpectedly, the lower band is observed at high intensity also in TeCDD spiked microcosms. In RH microcosms, an intermediate band at lower intensity is observed in all the microcosms. These two main bands were cut out, re-amplified and sequenced (Tab. 11).

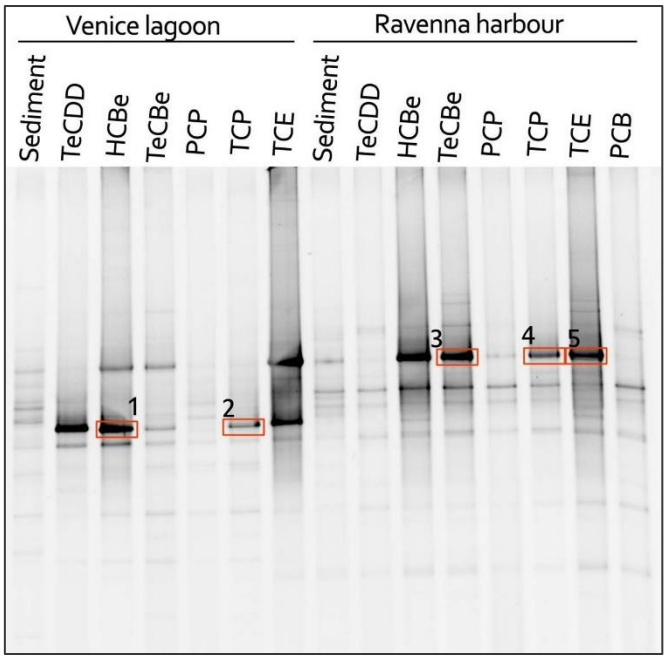


Figure 19 DGGE analysis of the dehalogenating-Chloroflexi bacterial community in sediment spiked with the different organohalides at the beginning of the incubation ("sediment" lane) and at the end of dechlorination.

Band	Similarity score	S ab Score	Unique common oligomers	Genbank code	Microorganism
1	1.000	0.932	1148	AY356384	uncultured bacterium N3-9
2	1.000	0.922	1148	AY356384	uncultured bacterium N3-9
3	0.992	0.878	1150	AF393781	*Dehalobium chlorocoercia DF-1*
4	0.992	0.889	1150	AF393781	*Dehalobium chlorocoercia DF-1*
5	0.992	0.880	1150	AF393781	*Dehalobium chlorocoercia DF-1*

Table 11 Results of alignment of sequences from DGGE with dehalogenating-Chloroflexi-specific primers for 16S rRNA gene of primary microcosms against RDP database (environmental and isolates).

4.2 PCB-dechlorinating slurry enrichments from Venice Lagoon dechlorination spectrum

4.2.1 Organochloride compounds dehalogenation

To test the capabilities of PCB-dehalogenating enriched community from the Venice lagoon sediment against other organochlorides, active dehalogenating cultures were pooled together and transferred at 5% v/v in sterile water and sediment of the Venice lagoon and spiked with the same compounds tested on the primary microcosms at the same concentrations. These new cultures are referred to as Venice lagoon slurry enrichments (VL-ENR).

4.2.1.1 Chlorobenzenes reductive dehalogenation in PCB-dechlorinating OHRB-enriched cultures from Venice Lagoon sediment

Depletion of HCBe and TeCBe and accumulation of dechlorination products occurred within the first sampling point at 1 month, leading to the accumulation of only 1,3-DCBe (Fig. 25A and 25B, respectively). To gain further insight on the dechlorination specificities, the cultures were re-spiked with the same compound at week 18. Only 1,3,5-TCBe could be observed before 1,3-DCBe accumulation, suggesting that double flanked chlorines are removed first and quickly, and slowly meta flanked attack occurs (Fig. 25A). At the 4th week

from the second spike only 1,3-DCBe and benzene were detected, but not in quantities high enough to justify stoichiometrically the decrease of HCBe or TeCBe spiked. This dehalogenation pattern (Fig. 26) is similar to what observed in the primary cultures of the Venice Lagoon, spiked with the same compounds, hinting at the mineralization of benzene by the microbial community. As previously discussed in section 4.1.1.2, removal of double flanked chlorines is common in literature, whereas unflanked chlorines removal is rare. The reductive dehalogenation speed of the added compounds was higher than the primary microcosms in this study, from both VL and RH sediments. Remarkably, the dechlorination rate of HCBe was almost 4 times higher in VL-ENR than in the respective VL primary microcosms and the pure culture of *Dehalococcoides mccartyi* DCMB5, CBDB1 and the consortium from Kaufhold et al, 2013, nevertheless, pure cultures of *D. mccartyi* 195 and CBDB1 were almost twice as fast in HCBe dechlorination (Tab. 8). Conversely, dechlorination speed increased from the second to the second respike towards HCBe, whereas TeCBe dechlorination, while still higher than in primary microcosms and comparison studies, decrease during the second spike.

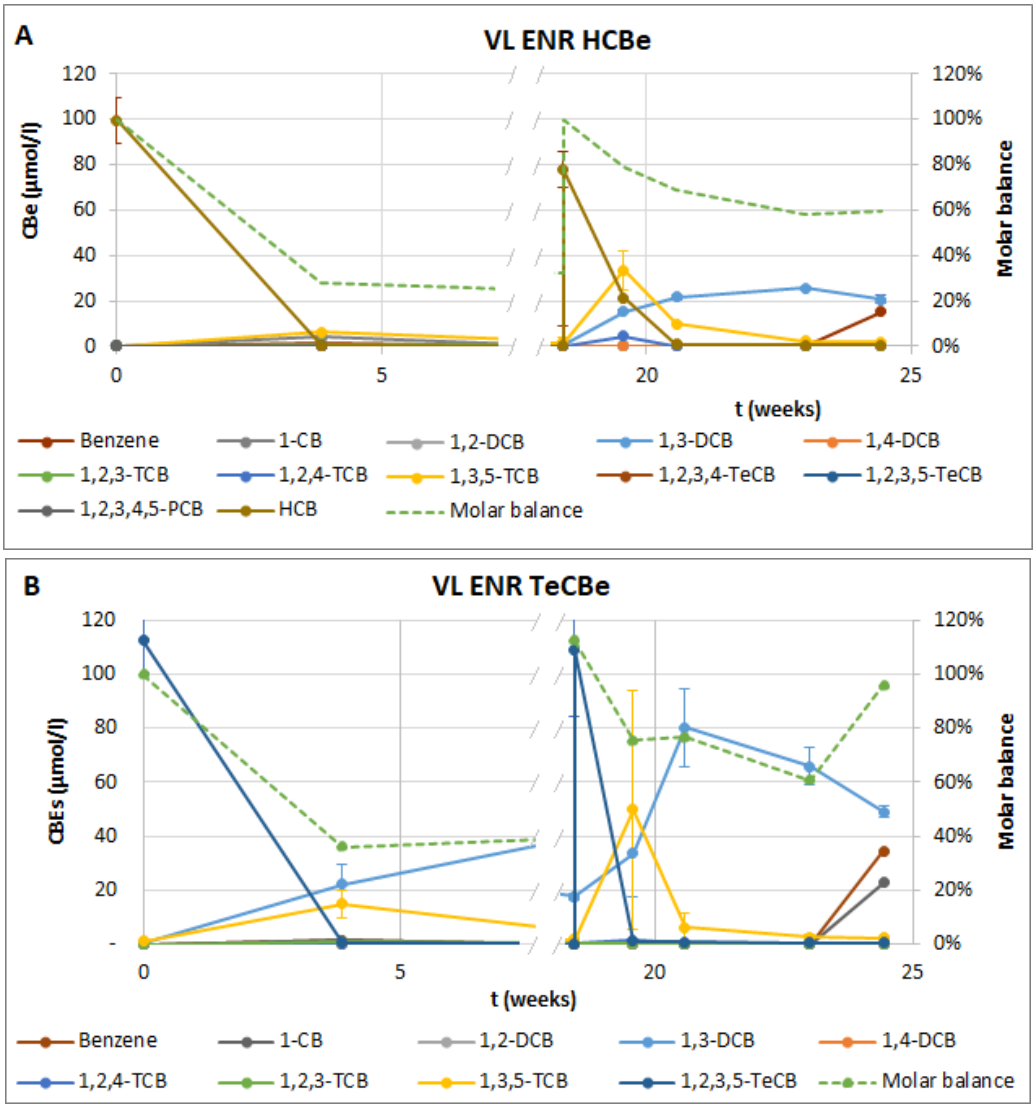


Figure 20 Change in concentration of spiked compounds HCBe (A) and 1,2,3,5-TeCBe (B) and their dechlorination products in VL enrichments. The molar balance is shown, calculated as the sum of moles of spiked compound and products, expressed in percentage of the initial quantity of the spiked chlorobenzene (HCBe or 1,2,3,5-TeCBe).

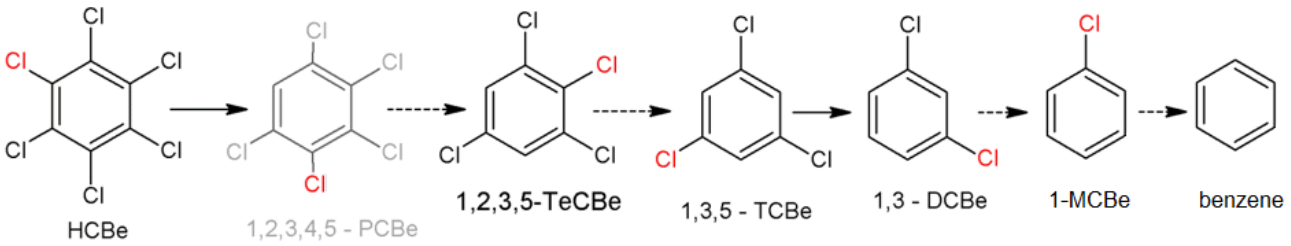


Figure 21 Dechlorination pattern of hexachlorobenzene and 1,2,3,5-tetrachlorobenzene in VL enrichments. Chlorine removed at every step are enlightened in red. Undetected intermediate product 1,2,3,4,5-pentachlorobenzene is pale because it was not detected.

4.2.1.2 Chlorophenols reductive dehalogenation in PCB-dechlorinating OHRB-enriched cultures from Venice Lagoon sediment

As far as chlorophenols are concerned, contrary to what happened in the primary cultures from VL and RH sediment, both PCP and TCP could be dehalogenated by the PCB-dehalogenating community. In particular, PCP was depleted in 14 weeks of incubation, with the accumulation of mostly 2,4,6-TCP (Fig. 27). A temporary accumulation of 2,3,5,6-TeCP is observed between weeks 5 and 10, its decrease is coupled to a small accumulation of 3,5-DCP and phenol, possibly indicating two separate dechlorination pathways being followed in different proportions (Fig. 28). Dehalogenation seems thus to be directed mostly against meta double flanked chlorines and in minor percentage to para double flanked and ortho positions, suggesting that the hydroxyl group may play a substantial role in directing the dehalogenation towards preferred sites of the molecule. On the other hand, TCP was only dehalogenated to 3,5-DCP in only 5 weeks, without the formation of lower chlorinated phenols (Fig. 29), thus favouring ortho removal and suggesting that PCP and TCP selected different microorganisms capable of different dechlorination activities. Comparison of PCP dechlorination pattern observed in VL ENR cultures and in literature highlights a major difference, as obligate OHRB tend to accumulate mostly 3,5-DCP[238,240], removing preferentially ortho-single flanked and para-double flanked chlorines. On the other hand, dechlorination pattern of 2,3,5-TCP is identical to the one observed in other studies (Fig. 30) and different to the dechlorination pattern of PCP reinforcing the hypothesis that 2,3,5-TCP dechlorination might have been carried out by a separate minority microbial species unable to dechlorinate PCP to 2,3,5-TCP and then to 3,5-DCP. Interestingly, although faster (Tab. 4), TCP dechlorination does not proceed further to 3-CP, unlike in primary cultures from the same site, hinting at a loss of microbial species involved in meta-unflanked chlorines removal during subculturing in presence of PCBs. Reductive dechlorination speed of TCP, while more than 7 times the one observed in primary microcosms in this study is perfectly comparable with the one of a pure culture of *Dehalococcoides mccartyi* strain DCMB5. PCP dechlorination speed was lower than the one observed in other studies[238,240], but similar to a *Dehalococcoides mccartyi* Strain JNA pure culture (Tab. 6).

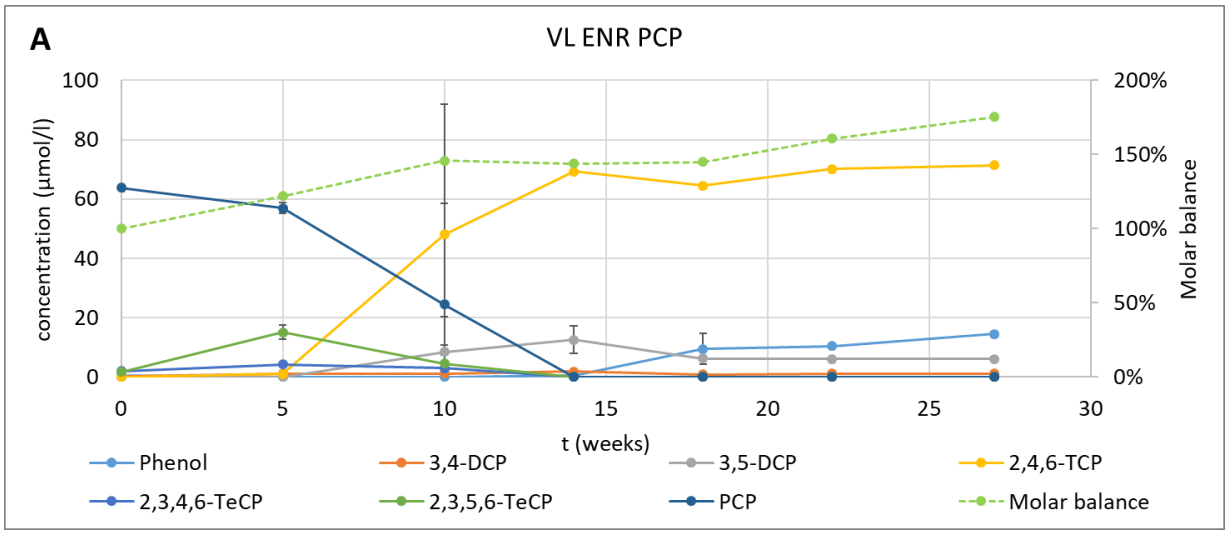


Figure 22 Change in concentration of spiked PCP in VL enrichments and its dechlorination products. The molar balance is shown, calculated as the sum of moles of spiked compound and products, expressed in percentage of the initial quantity of the spiked PCP.

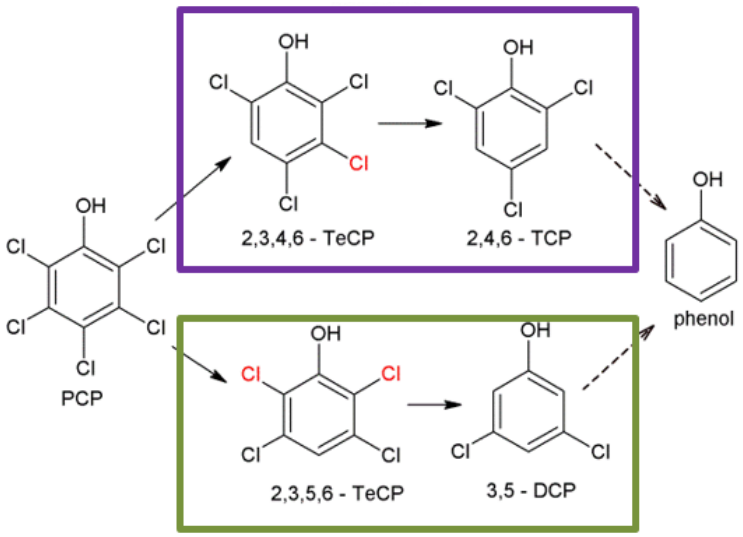


Figure 23 Dechlorination pattern of PCP in VL enrichments. Chlorine removed at every step are enlightened in red. Two separate dechlorination routes are taken as products from both are detected. The preferential route (violet) involves the removal of meta-double flanked chlorines to 2,4,6-TCP, the secondary one (green) starts with a para double-flanked chlorine removal and continues with the removal of ortho-single flanked chlorines. The detection of phenol suggests a further removal of chlorines, but the route of provenience is unclear, although phenol increase in concentration is corresponded by a decrease in 3,5-DCP concentration, suggesting its provenience from the secondary route.

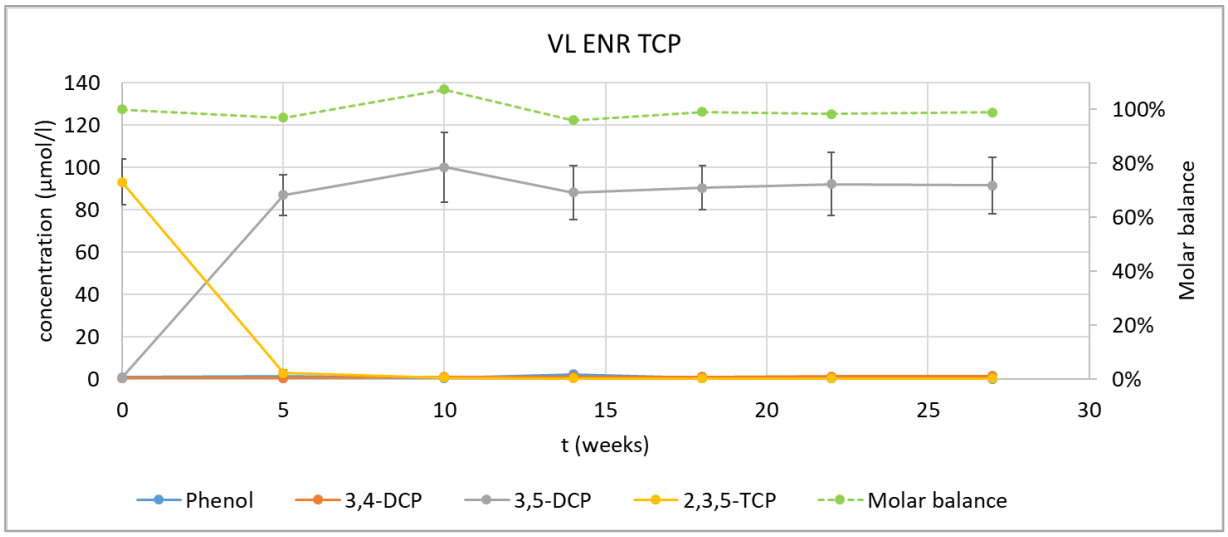


Figure 24 Change in concentration of spiked 2,3,5-TCP in VL enrichments and its dechlorination product 3,5-DCP. The molar balance is shown, calculated as the sum of moles of spiked compound and products, expressed in percentage of the initial quantity of the spiked TCP.

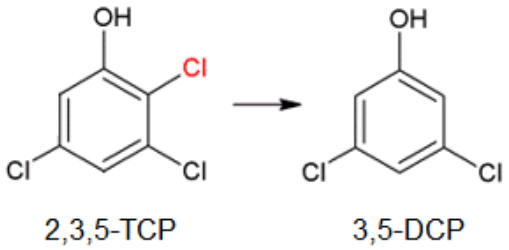


Figure 30 Dechlorination pattern of 2,3,5-TCP in VL enrichments. Ortho-single flanked chlorine is the only one being removed.

4.2.1.3 Chlorodioxin reductive dehalogenation in PCB-dechlorinating OHRB-enriched cultures from Venice Lagoon sediment

PCB-dehalogenating enriched cultures were effective also in dehalogenating 1,2,3,4-TeCDD to 1,2,4-TCDD, (lateral double-flanked chlorine removed). The dehalogenation started with no lag phase but stopped when 79% of the spiked dioxin was converted stoichiometrically to 1,2,4-TCDD at 4 months. The reductive dehalogenation speed was higher although less extensive than what was observed in three other studies: a pure culture of *Dehalococcoides mccartyi* DMB5[240] and the microbial community of an estuarine sediment in Ahn *et al*, 2007[156] (Tab. 12). While in our study, dechlorination pattern by 1,2,3,4-TeCDD consisted in the removal of meta-double flanked chlorines (Fig. 32), *D. mccartyi* DCMB5 dechlorinated the same compound to mostly 2,3-DCDD and, less, to 1,3-DCDD and 2-CDD, targeting also ortho and meta-single flanked chlorines. In Ahn *et al*. instead, dechlorination pattern of TeCDD depended on the site of origin of the sediment, but the main product was 2-DCDD. The structural similarity between PCBs and chlorodioxins, together with the absence of a lag phase and the high dehalogenation activity suggest that the reductive dehalogenases expressed on PCBs could play a role in the dehalogenation of TeCDD as well.

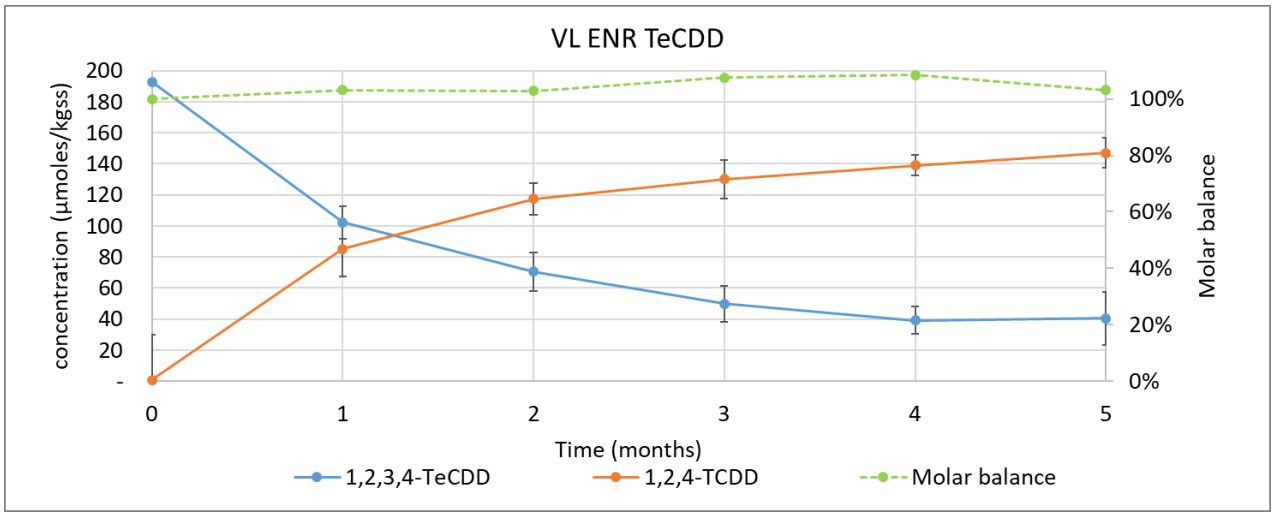


Figure 31 Change in concentration of spiked 1,2,3,4-TeCDD in VL enrichments and its dechlorination product 1,2,4-TCDD. The molar balance is shown, calculated as the sum of moles of spiked compound and products, expressed in percentage of the initial quantity of the spike.

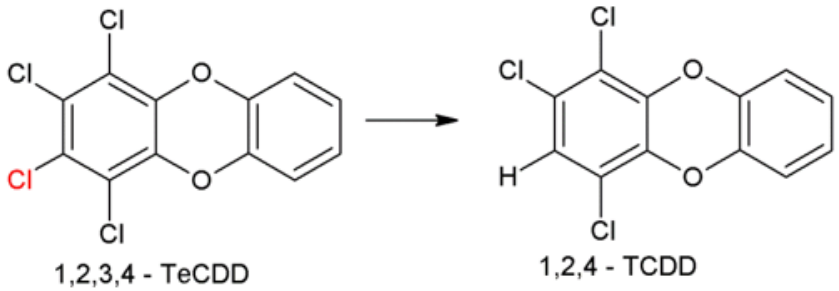


Figure 32 Dechlorination pattern of 1,2,3,4-TeCDD in VL enrichments. Double flanked chlorines are the only target, without further dechlorination.

	Rate of organic chlorine removal (µmol*day^{-1}*l^{-1})
VL ENR	5.0
D. mccartyi DCMB5[240]	2.2
D.mccartyi Strain 195[241]	0.1
Ahn et al, 2007[156]	0.1

Table 12 Rate of daily dechlorination of 1,2,3,4-TeCDD in different studies compared with values obtained from our set of enriched cultures from VL. Speed is calculated in the whole period monitored (6 months).

4.2.1.4 PCBs reductive dehalogenation in PCB-dechlorinating OHRB-enriched cultures from Venice Lagoon sediment

PCB dechlorination activities were also tested and confirmed, as the average number of chlorine atoms in the PCB mixture was decreased from 5.1 to 3.8 in 14 weeks of incubation, while no accumulation of dechlorination products was observed in sterile negative controls (Fig 33). The reductive dechlorination speed of PCBs was very similar to the one registered in similar studies. In Kaya et al. (2018)[248] three different sediments from river (GR, FR) and estuarine (BH) environments showed similar activities (Tab. 13). The most important similarity is anyway the almost identical dechlorination rate between our study and a pure culture of *Dehalobium chlorocoercia*, which is exactly the closest relative to VLD-1 bacterium described in this study.

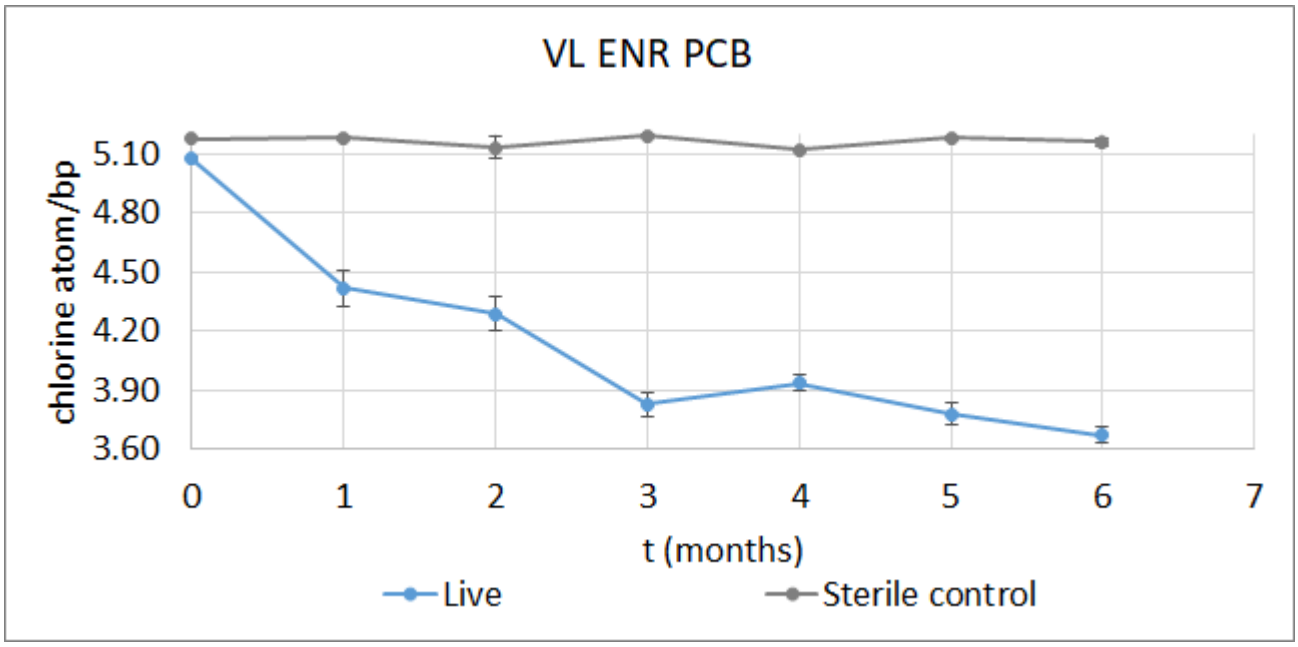


Figure 33 Change in dechlorination grade of the byphenyl molecule in PCB-spiked VL enrichments, live cultures compared to sterile negative control, expressed in number of chlorine atoms per byphenyl molecule.

Rate of organic chlorine removal ($Cl*bp^{-1}*day^{-1}$)	
VL ENR	9.40E-03
Kaya et al, 2018 (GR)	9.70E-03
Kaya et al, 2018 (FR)	6.90E-03
Kaya et al, 2018 (BH)	3.20E-03
Dehalobium chlorocoercia	9.52E-03

Table 13 Rate of daily dechlorination of Aroclor 1254, a commercial mixture of PCB's in another study compared with values obtained from our set of enriched cultures from VL. Speed is calculated in the whole period monitored (6 months).

4.2.1.5 Chloroethenes reductive dehalogenation in PCB-dechlorinating OHRB-enriched cultures from Venice Lagoon sediment

PCB-enriched cultures were also able to dechlorinate TCE, with rates increasing after each TCE spiking. Similarly to the enrichment from Venice Lagoon sediment from which they origin, PCB-enriched cultures dechlorinated TCE mainly to equal ratio of *cis*- and *trans*-DCE. (Fig. 34).

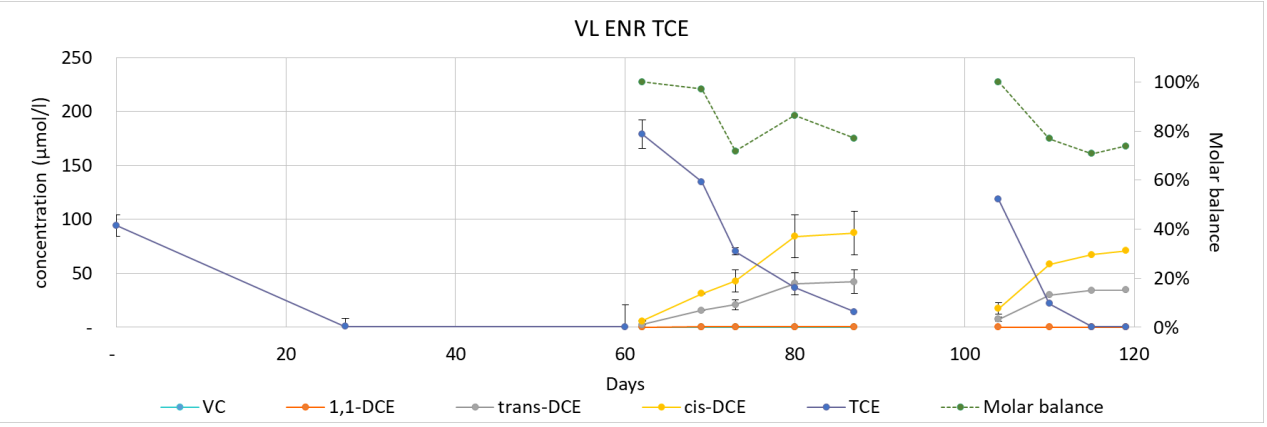


Figure 25 Change of TCE and its dechlorination products in VL enrichment cultures during three different spikes. Molar balance is shown, calculated as the sum of moles of spiked compound and products, expressed in percentage of the initial quantity of the spike.

TCE was dechlorinated faster at every spike, reaching speeds even an order of magnitude greater than in the respective primary sediment (VL) (Tab. 9), and stabilizing at around 110 µmol of Cl per liter per day after the second spike, an overall high dechlorination speed if compared to other studies, marking a great efficiency.

4.2.2 Microbial population dynamics and OHRB species involved in reductive dehalogenation

In order, PCR-DGGE analysis of 16S rRNA genes of the microbial communities were carried out to investigate if the onset of the dechlorination activity is associated to the enrichment of specific bacterial species. DNA was extracted from all active microcosms, from slurry sampled at times in which all the spiked compounds had been dechlorinated (i.e. after 24 weeks for PCB, TeCDD and TCE-spiked microcosms and after 12 weeks for chlorobenzenes-spiked microcosms) and PCR-amplified using either primers targeting all bacterial 16S rRNA genes or *Chloroflexi*-specific 16S rRNA gene. For chlorophenols-spiked microcosms, two different sampling times were analyzed to look for differences in microbial population as different dechlorination products were observed accumulating in chemical analyses. DGGE of total 16 S rRNA genes is shown in figure 35.

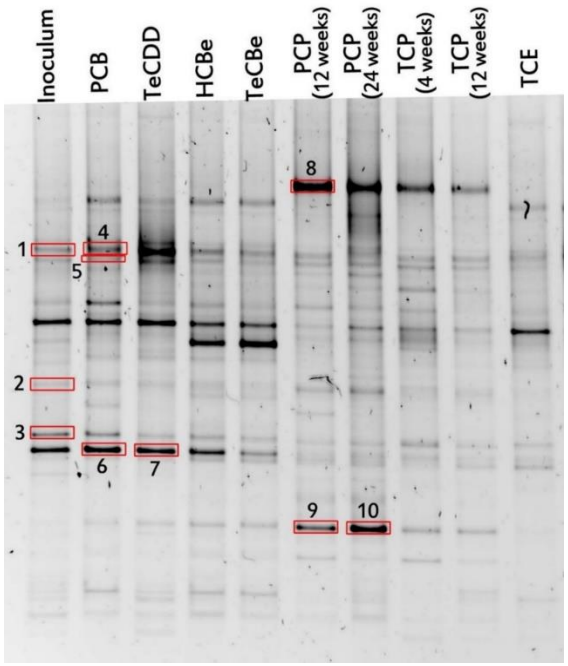


Figure 26 PCR-DGGE analysis of the rRNA gene of the total bacterial community of the enrichment VL cultures spiked with the different organohalides at the beginning of the incubation ("inoculum" lane) and at the end of dechlorination or at another time (where indicated). Squared bands have been sequenced and taxonomically assigned, see table 13 for results.

As expected, the profile in PCBs-amended microcosms was nearly identical to the inoculum, which was indeed a sediment culture enriched in PCB-dechlorinating bacteria. In the picture, bands whose DNA was sequenced are highlighted by red squares. Sequences were aligned against RDP database (Tab. 12).

Band	Similarity score	S ab score	Unique common oligomers	Genbank code	Microorganism
1	1.000	0.991	1422	JX391342	uncultured *Tissierella*
2	0.978	0.836	1296	JN525824	uncultured *Firmicutes*
3	0.986	0.893	1444	AF418173	*Desulfococcus multivorans*
4	0.998	0.964	1267	JN490189	uncultured *Bacteroidales*
5	0.997	0.981	524	LC026366	uncultured *Dethiosulfatibacter*
6	1.000	0.986	1361	EU522638	uncultured *Desulfobacteraceae*
7	0.998	0.925	1361	EU522638	uncultured *Desulfobacteraceae*
8	0.991	0.931	519	AB062687	uncultured *Bacteroidales*
9	0.974	0.878	1416	FJ264755	uncultured *Clostridiales*
10	0.974	0.867	1416	FJ264755	uncultured *Clostridiales*

Table 14 Results of alignment of sequences from DGGE with universal primers for total 16 S rRNA gene of VL enrichment cultures against RDP database (environmental and isolates).

TeCDD-spiked microcosms show a very similar profile to PCB-spiked conditions, with a more intense band at lower denaturing concentration, whereas an additional band is detected at intermediate denaturing concentration in cholorobenzenes-spiked microcosms. A band, whose sequence shares 97% similarity with Desulfatibacillum alkenivorans, is very intense in PCBs, TeCDD and chlorobenzenes-spiked microcosms, but it is hardly detected in the other cultures. On the other hand, in both chlorophenols-spiked microcosms, a

phylotype, having 99% similarity with an uncultured Bacteroidales enriched notably, whereas the contemporary presence of another phylotype which shares 94% similarity with Alkaliphilus peptidifermentans could be detected only in PCP-spiked conditions. Overall, the community appears less rich than that observed in primary microcosms. DGGE with DNA amplified with primers for dechlorinating Chloroflexi-specific 16S rRNA gene (GC348 F and 884 R) is shown in figure 36. The same time points analyzed in the DGGE for total bacteria rRNA genes were used. Sequences alignment results against RDP database are listed in table 13. Three bands, similar to the same bacteria found in primary microcosms, are observed in all the microcosms, except in TCE-amended ones, where only the two lower bands at higher denaturing concentration were present and the PCP-amended ones that showed an enrichment of the higher band. These same three bands become hardly detectable in chlorophenols-amended microcosms at the end of the incubation, when all the spiked organohalide has probably been reduced, showing the great dynamicity of microbial populations even in controlled environments.

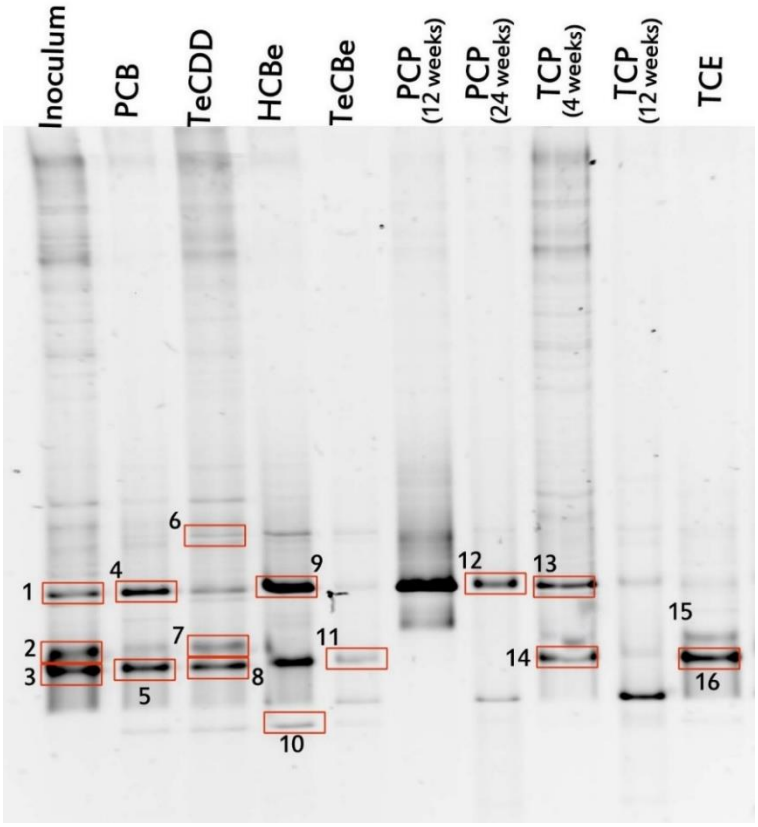


Figure 27 DGGE analysis of the dehalogenating-Chloroflexi bacterial community in VL enrichment cultures spiked with the different organohalides at the beginning of the incubation ("inoculum" lane) and at the end of dechlorination or at another time (where indicated). Squared bands have been sequenced and taxonomically assigned, see table 13 for results.

Band	Similarity score	S ab score	Unique common oligomer	Genbank code	Microorganism
1	0.990	0.890	1150	AF393781	*Dehalobium chlorocoercia DF-1*
4	0.991	0.888	1150		
6	0.980	0.839	1150		
9	0.990	0.869	1150		
12	0.993	0.888	1150		
13	0.992	0.895	1150		
2	1.000	0.929	1148	AY356384	uncultured bacterium N3-9
3	1.000	0.931	1148		
5	1.000	0.932	1148		
7	1.000	0.932	1148		
8	1.000	0.928	1148		
11	1.000	0.920	1148		
14	1.000	0.926	1148		
15	1.000	0.932	1148		
16	1.000	0.922	1148		
10	0.993	0.951	1255	DQ330175	uncultured Chloroflexi bacterium

Table 15 Results of alignment of sequences from DGGE with dehalogenating-Chloroflexi-specific primers for 16S rRNA gene of primary microcosms against RDP database (environmental and isolates). Only thwo phylotypes were identified.

The sequences of these bands and the ones in the PCR-DGGE of the primary microcosms matched perfectly the sequences of two species of bacteria identified in another PCB-dechlorinating culture from our lab and published in another work[166], the higher band corresponded to the VLD-1 phylotype, while the two lower bands matched the VLD-2 phylotype. Both phylotypes are from the Class Dehalococcoidia of the phylum Chloroflexi, a phylogenetic tree with the latest most relevant close relatives is shown in fig 37.

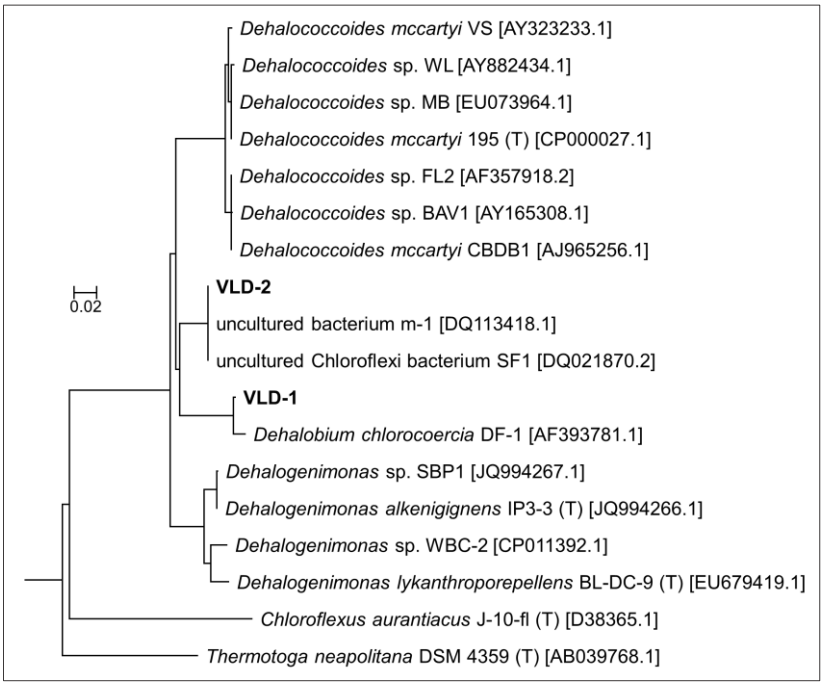


Figure 28 Phylogenetic tree of members from the Dehalococcoidia class. Phylotypes VLD-1 and VLD-2 are highlighted with a bold font.

4.3 Development of sediment-free TCE-dechlorinating cultures and enrichment of OHRB species

4.3.1 Identification of minimal carbon and energy source necessary for OHRB growth

New cultures were started in order to further enrich the reductive dehalogenating members in the microbial community. To be able to specifically enrich our bacteria of interest, new growing conditions had to be tailored on the metabolic needs expected from them. The most important features to be decided were, namely, i) the organohalide to be used as electron acceptor, ii) the source of the inoculum , iii) the composition of the growing medium. The organohalide compound of choice were TCE and PCP, for the ability to enrich one specific OHRB over the other one, in particular PCP seemed to favor the enrichment of VLD-1, whereas TCE appeared to cause the enrichment of VLD-2. Furthermore, TCE had one great advantage: after dechlorination of the spiked compound was over, dechlorination products could be stripped away by sparging the cultures with oxygen-free nitrogen and the TCE could be re-added, allowing for multiple spikes on the same culture, without the limitation effect on OHRB growth caused by the accumulation of products. The source of the inoculum for the two microcosm sets (PCP and TCE) were the respective VL ENR cultures and the cultures were inoculated 5% v/v. The growing medium of choice was commercial synthetic marine water (Tropic Marin) commonly used for fish tanks, a set of vitamins comprising of B12, fundamental for the biosynthesis of reductive dehalogenases (Tab. 16), no sediment from the site and only one among several short chain fatty acids which were identified in literature as possible carbon and energy source for the OHRB[132], namely, sodium salts of formate, acetate, pyruvate and lactate. Negative controls with TCE-spiked synthetic marine water without inoculum and with inoculum but without any carbon source. These cultures were named ENR2 PCP and ENR2 TCE, respectively.

Vitamins (DSMZ #141)	mg/l
B12	0.022
Lipoic acid	0.05
Para-aminobenzoate	0.05
Biotin	0.02
Folic acid	0.02
Pyridoxin-HCl	0.1
Thyamin-HCl	0.05
Riboflavin	0.05
Nicotinic acid	0.05
Panthotenate	0.05

Table 16 Concentration of vitamins in medium for sediment-free, synthetic marine water cultures.

ENR2 PCP were set up, spiked with 50 µmol/l PCP and monitored for four months Unfortunately no dechlorination of PCP was observed in any of the controls, not decrease of the initial amount of spiked compound (initial concentration 46.4 ± 2.5, final concentration 50.5 ± 10.6) nor accumulation of less chlorinated compounds. ENR2 TCE were set up and spiked with 40 mg/l of TCE and monitored over time. During the first spike a stable decrease in TCE was observed in all microcosm sets, due to abiotic losses caused by the high volatility of the compound (Fig. 38A). With a normalization for the abiotic losses in the negative

control (containing just synthetic marine water) a more accurate picture of the situation appeared (Fig. 38B), revealing that the bets carbon source allowing TCE dechlorination was pyruvate, followed by lactate, then acetate and formate. While only traces of dechlorination products (cis-DCE and trans-DCE) were detected in acetate and formate microcosms, in lactate and pyruvate-amended controls cis-DCE and trans-DCE are produced in amounts that sum up to the spiked amount of TCE after normalization for abiotic losses (fig. 38 C and D, respectively). Abiotic loss was reduced by applying a silicon paste between the Teflon stopper and the glass, therefore this strategy was applied henceforth.

More spikes of TCE were performed in all microcosms sets, but conversion of TCE to less chlorinated compounds was only observed in lactate- (Fig. 39) and pyruvate-amended (Fig. 40) microcosms. In these sets TCE dechlorination was carried out to cis-DCE and trans-DCE stably over time in a *cis/trans* ratio of 2.36 ± 0.29 (lactate) and 2.13 ± 0.18 (pyruvate).

Carbon source concentration was measured at the beginning and at the end of the spikes, starting from the second spike. Both pyruvate and lactate were quasi-stoichiometrically converted to acetate during the time of one spike, figure 41 A and B show accumulation of acetate during TCE spikes 2,3 and 4 in lactate- and pyruvate-amended cultures.

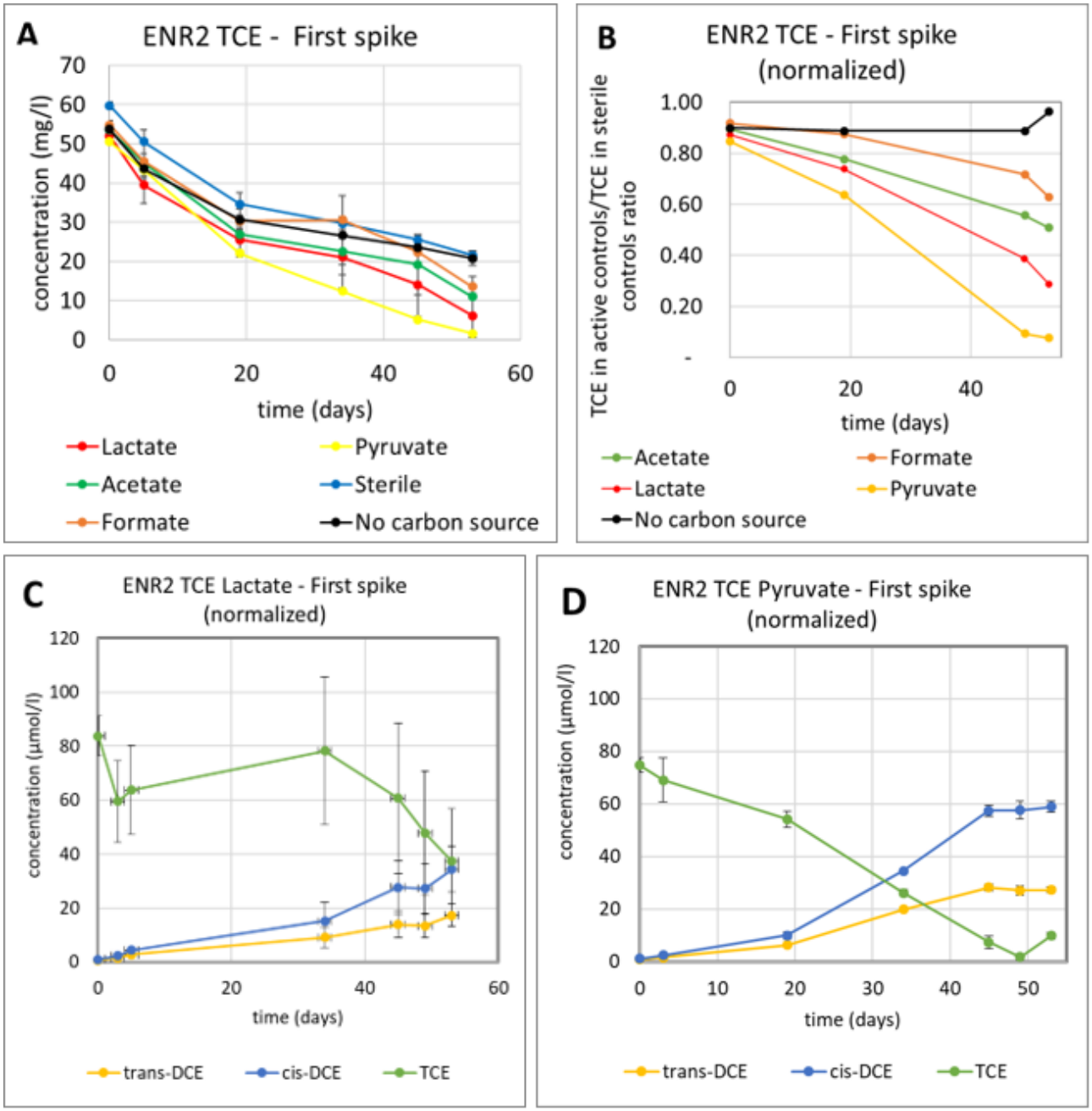


Figure 29 Change of TCE concentration during the first spike in sediment-free synthetic marine water cultures ENR2 amended with only one carbon source among formate, acetate, lactate or pyruvate. In figure A TCE concentration (mg/l) over time in all sets of microcosms is reported. Figure B shows data from figure A, but normalized for abiotic losses by division for the concentration in sterile negative controls. Figures C and D represent change of DCEs dechlorination products concentrations and TCE concentration normalized for abiotic losses in, respectively lactate- ad pyruvate-amended cultures.

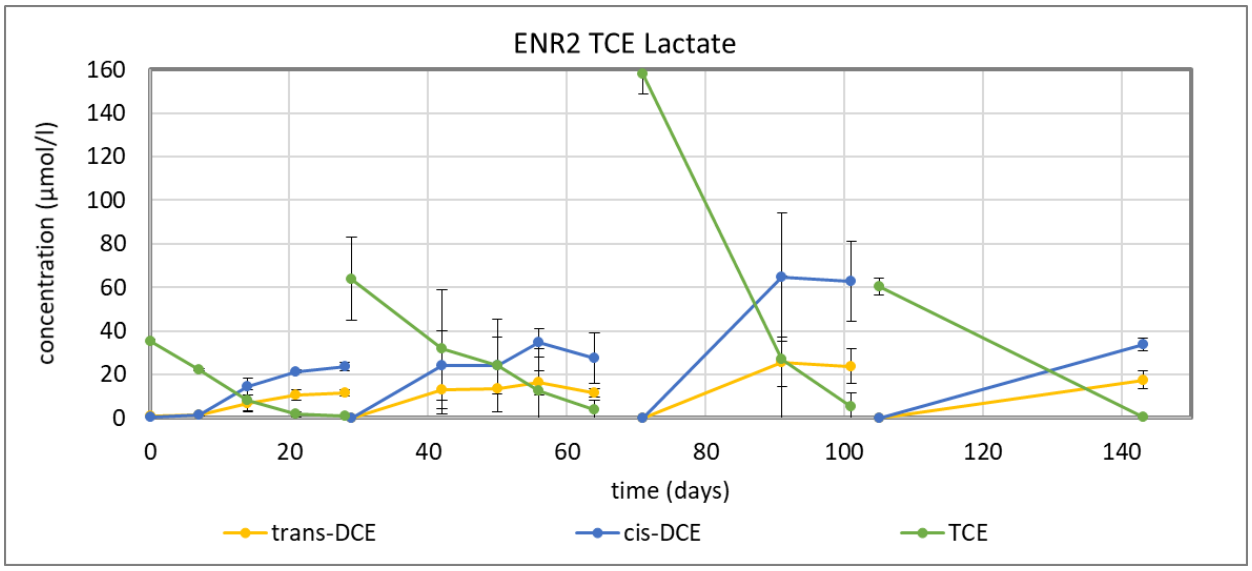


Figure 30 Change of concentration of TCE and its dechlorination products in synthetic marine water ENR2 cultures amended with lactate during four different spikes.

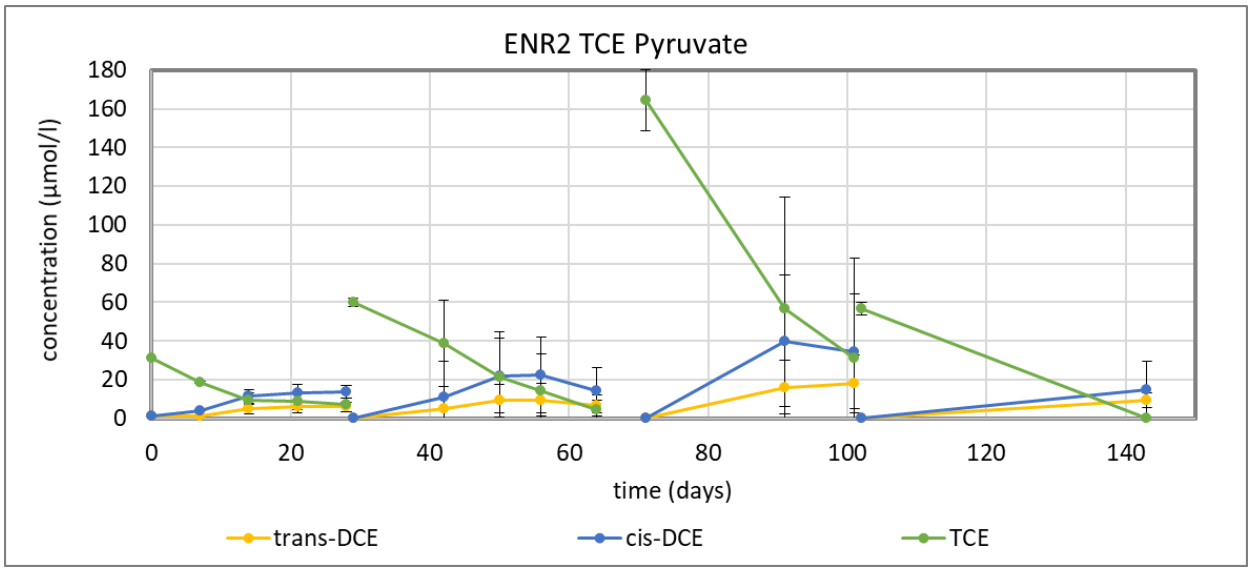


Figure 40 Change of concentration of TCE and its dechlorination products in synthetic marine water ENR2 cultures amended with pyruvate during four different spikes.

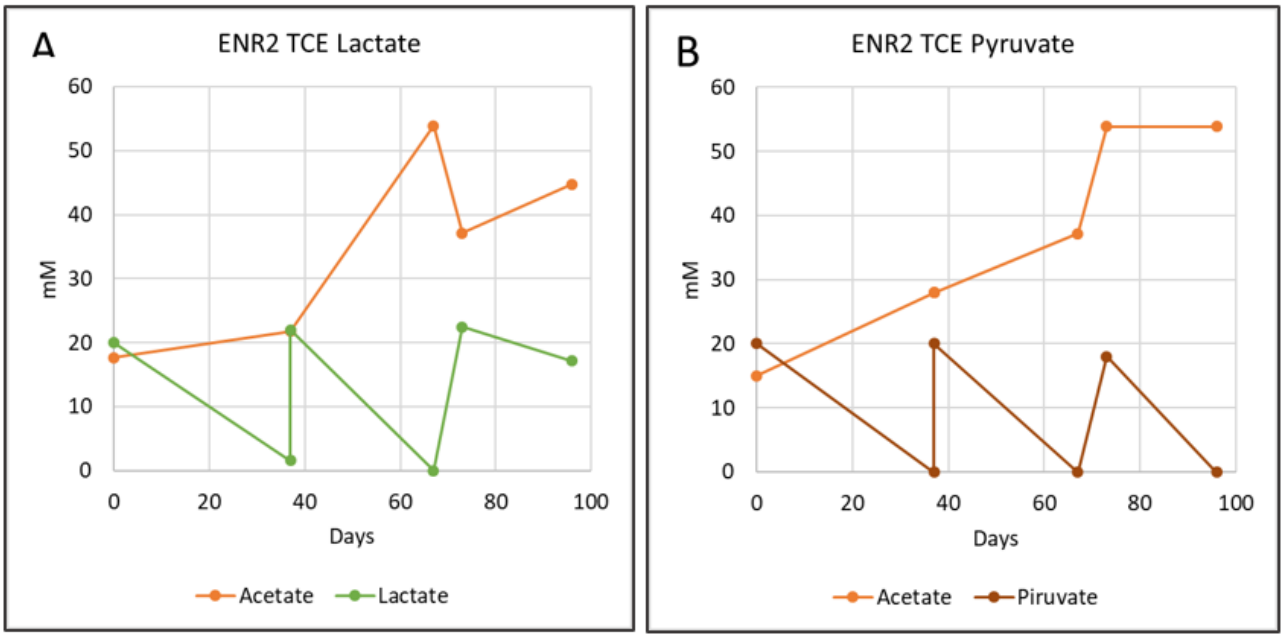


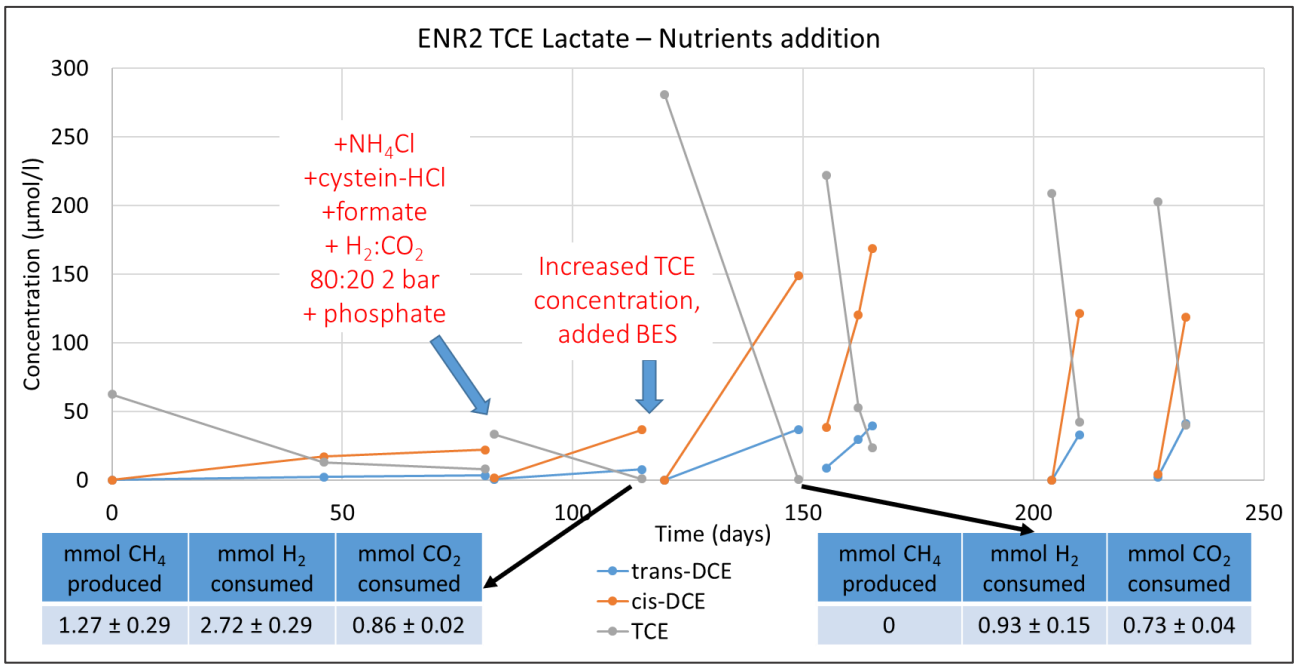
Figure 41 Concentration of amended carbon source and acetate in, respectively, lactate- (A) or pyruvate-amended cultures (B). At every TCE spike carbon source was readded and monitored at the end of TCE dechlorination.

After each spike headspace gas. analyses were performed to measure the concentration of the following gases: oxygen (to ensure that the microcosms were still anaerobic, hydrogen (necessary to OHRB for reductive dehalogenation and product of the organic carbon fermentation), carbon dioxide (to assess possible accumulation after degradation of carbon source) and methane (to measure the methanogenic activity of Archaea members of the microbial community). Headspace gas measurements detected that, after TCE and the carbon source were consumed, hydrogen, methane and carbon dioxide were produced. As for hydrogen, in ENR2 TCE Lactate an average of 6.48E-2 mmoles of hydrogen were produced, while in ENR2 TCE Pyruvate the amount was 3.65E-2 mmoles. Every addition of 20 mM of lactate or pyruvate, the cultures produced on average 8.21E-2 mmoles (lactate) and 70.35E-2 (pyruvate).

4.3.2 Identification of additional nutritional requirements for OHRB growth

Given the accumulation of acetate to concentrations as high as 60 mM, carbon source addition to the cultures after every spike was suspended. Suspension in lactate or pyruvate addition resulted in very low rates of dechlorination (first spike in fig 42 and 43). Probably because of the absence of hydrogen production by fermentation of the carbon source by fermenting bacteria in the community. New nutrients to enhance growth and TCE dechlorination were supplied: i) ammonium chloride ad nitrogen source, ii) cysteine hydrochloride as reducing agent to keep the redox potential low, iii) formate as possible alternative carbon source, iv) hydrogen and carbon dioxide in a 4 to 1 ratio at a pressure of 2 bar, to make up for the absence of carbon and electron source, v) sodium phosphate necessary for biomass. The addition of the extra nutrients after spike 8 rescued the dechlorination in all the cultures during TCE spike number 9 (Fig. 44). Unfortunately, during spike number 9 the hydrogen and carbon dioxide boosted the methanogenic activity, increasing the production of methane more than 10-fold in both lactate and pyruvate-amended microcosms. To avoid an excessive enrichment of methanogenic microbes, during spike 10 bromoethansulphonate (BES) was added and TCE concentration during spikes was increase to create even more selective growing conditions favouring OHRB. BES is a structural analog of coenzyme M of methanogens and a competitive

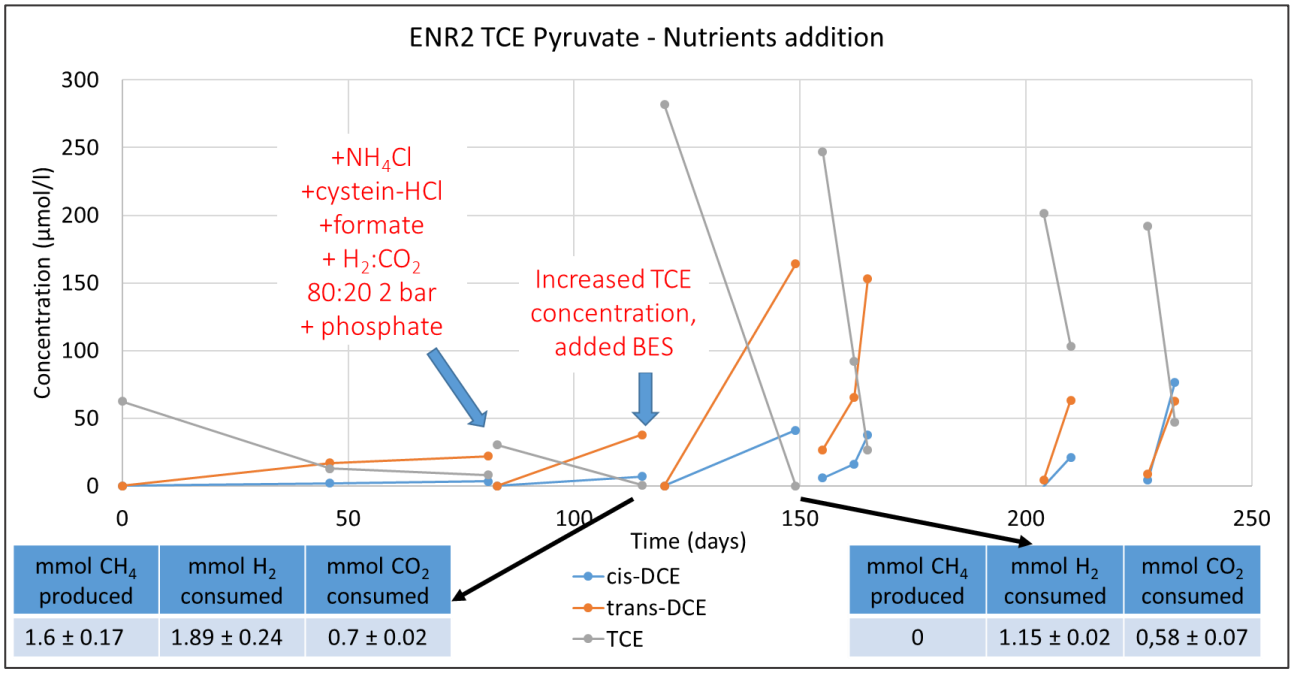
inhibitor of methyl-coenzyme M reductase and acts as antibiotic inhibiting methanogenesis. BES was effective in stopping the methane production (Fig. 42 and 43).



mmol CH_4 produced	mmol H_2 consumed	mmol CO_2 consumed
1.27 ± 0.29	2.72 ± 0.29	0.86 ± 0.02

mmol CH_4 produced	mmol H_2 consumed	mmol CO_2 consumed
0	0.93 ± 0.15	0.73 ± 0.04

Figure 42 Change of concentration of TCE and its dechlorination products in synthetic marine water ENR2 cultures amended with lactate during six different spikes. It is marked the moment of the addition of nutrients, bromoethanesulphonate (BES) and increase of TCE concentration. Consumption of hydrogen and carbon dioxide and production of methane before and after addition of BES are reported.



mmol CH_4 produced	mmol H_2 consumed	mmol CO_2 consumed
1.6 ± 0.17	1.89 ± 0.24	0.7 ± 0.02

mmol CH_4 produced	mmol H_2 consumed	mmol CO_2 consumed
0	1.15 ± 0.02	0,58 ± 0.07

Figure 31 Change of concentration of TCE and its dechlorination products in synthetic marine water ENR2 cultures amended with pyruvate during six different spikes. It is marked the moment of the addition of nutrients, bromoethanesulphonate (BES) and increase of TCE concentration. Consumption of hydrogen and carbon dioxide and production of methane before and after addition of BES are reported.

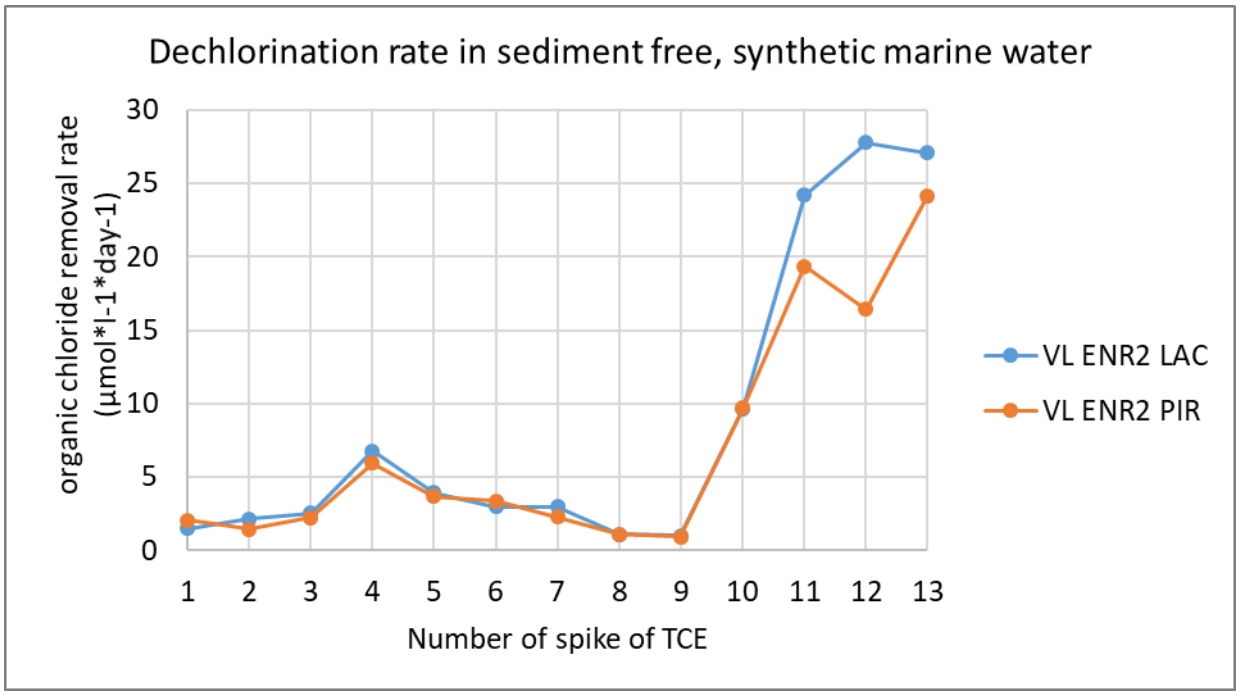


Figure 32 TCE dechlorination speed in consecutive spikes in synthetic marine water amended with lactate or pyruvate. After spike 9 TCE concentration was increased.

Molecular analysis to assess variations in the organohalide respiring community (composed originally by phylotypes VLD-1 and VLD-2) were performed both by 16S PCR-DGGE with dehalogenating *Chloroflexi*-specific primers and by Illumina sequencing of the 16S on VL ENR2 Lactate, which was the best performing control. The DGGE in figure 45 shows how, in contrast to what observed previously, TCE together with sediment-free growing medium brought to the selection of VLD-1 over VLD-2. The Illumina sequencing reveals that at the 12th spike in VL ENR2 TCE Lactate the microbial community diversity is much lower than in the respective VL primary microcosms. Moreover, *Dehalococcoidia* family showed an enrichment from 2% to 17% of the total bacterial community and a composition of 87% of VLD-1 and 13% of VLD-2 (Fig. 46).

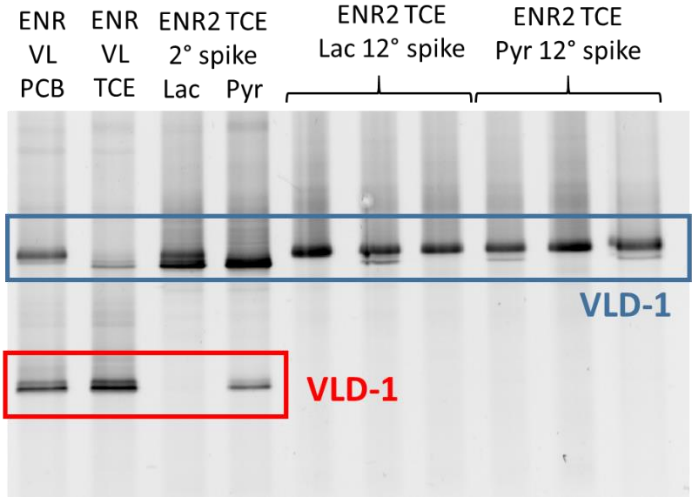


Figure 33 PCR-DGGE with dehalogenating-Chloroflexi-specific primers. Bands belonging to phylotypes VLD-1 and VLD-2 are highlighted.

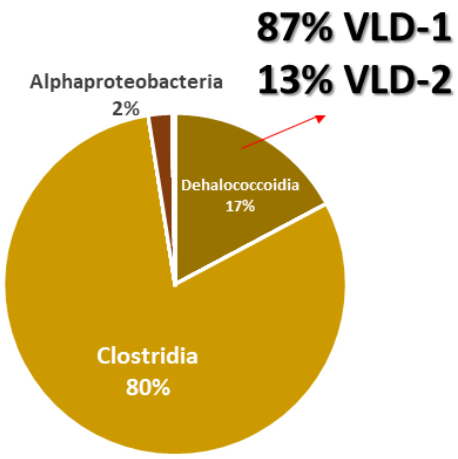


Figure 34 Taxa relative abundance at Class level of synthetic marine water TCE dechlorinating lactate amended cultures. Relative abundance of the two main phylotypes from the Dehalococcoidia class are shown.

4.4 Establishment of a defined mineral medium for growth of a stable TCE-dechlorinating consortium

4.4.1 Stability of TCE dechlorination during subculturing

Further efforts to increase the growth of OHRB were done following the VL ENR2 TCE cultures. The goal was to obtain enough biomass for expression studies based on RNA ad protein extraction, techniques which require a lot of starting material. A defined mineral medium was designed taking into consideration several aspects: i) Sulphate salts preciously present in Tropic marin synthetic marine water, were avoided as they serve as electron acceptor for sulphates reducing bacteria able to grow on lactate as carbon and energy source; ii) E-Cl medium used to cultivate VLD-1 closest relative *Dehalobium chlorocoercia*[111] was used as model for nutrients concentration and compared with Tropic marin. The resulting composition of the defined mineral medium is shown in table 3 in section 3.3 of this thesis; iii) nutrients which rescued growth and TCE dechlorination in VL ENR2 TCE cultures were included. Anaerobic microcosms with such medium were inoculated 20% v/v, spiked with TCE and the headspace gas was composed of H_2 and CO_2 at an absolute pressure of 2 bar.

Cultures in the new medium were able to stably convert TCE to cis- and trans-DCE as similar rates observed before, in a cis/trans ratio of 3.3 ± 0.5, showing the maximum chlorine removal rate of 20 $\mu mol*l^{-1}*day^{-1}$ at the second spike. From the 4th spike on, dechlorination rate stabilized at around 5 $\mu mol*l^{-1}*day^{-1}$, indicating that OHRB maximum growth was reached and the limiting factor for growth was finished (Fig. 47). Ammonium chloride is probably the limiting factor as carbon and energy sources and other elements are in excess. Carbon source for VLD-1 can be identified in the CO_2 in the headspace gas (members of the *Dehalococcoides* family can use it) or in acetate, the former is continuously added at every spike, the latter is never consumed completely. After the 4th spike total cell concentration in cultures was 5.1±2.0E+6 cells/ml and VLD-1 cell concentration was 1.99±0.87E+6 cells/ml, 39% of the total. VLD-2 presence was not detected.

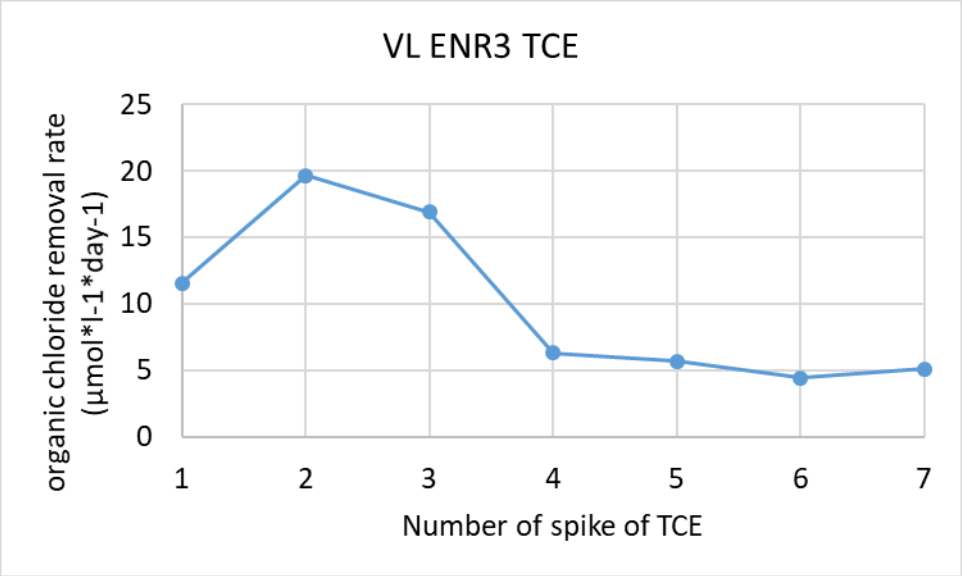


Figure 35 Dechlorination speed of seven consecutive TCE spikes in defined mineral medium cultures inoculated with synthetic marine water TCE-dechlorinating cultures amended with extra nutrients.

Further subculturing at 20% v/v or 10% v/v of these cultures was performed in defined mineral medium (VL ENR4) and TCE dechlorination was maintained at a rate of, respectively, 4.1 and 6.3 µmol*l^{-1}*day^{-1}, showing that the established medium allows repeated subculturing.

4.4.2 Metagenome of the consortium

The DNA from a TCE-dechlorinating culture in defined mineral medium was used to do a metagenome sequencing of the stable microbial community. The metagenome sequencing was carried out with a PacBio technology which yielded 5.3 Gbp. Raw sequences were filtered for quality and assembled with CANU into 295 contigs for a total of 24545258 bp, mean length is 83204.26 bp and N50 is 143683. The longest contig was 895584 bp long and harbored a 100% match with VLD-1 16S gene. An MG-RAST analysis of the contigs was performed and 32 contigs with a total length of 3'070'075 bp were taxonomically assigned to the Dehalococcoidia class. The size is comparable to the genome length of other OHRB (Tab. 17) and most importantly, is the first instance of genome sequencing of a species closely related to *Dehalobium chlorocoercia DF-1* to date. Automatic and manual annotation of contigs and ORFs is in progress.

OHRB Species	**Accession**	**Length**
Dehalococcoides mccartyi strain KBDCA3	NZ_CP019946	22456520
Dehalococcoides mccartyi strain 195[181]	NC_002936.3	2939440
Dehalococcoides mccartyi strain BAV1	NC_009455	2683784
Dehalococcoides mccartyi strain BTF08[249]	NC_020387.1	2904670
Dehalococcoides mccartyi strain CBDB1[179]	NC_007356.1	2791004
Dehalococcoides mccartyi strain CG1[250]	NZ_CP006949	1486678
Dehalococcoides mccartyi strain CG4[250]	NZ_CP006950	1382308
Dehalococcoides mccartyi strain CG5[250]	NZ_CP006951	1362151
Dehalococcoides mccartyi strain DCMB5[249]	NC_020386.1	2863804
Dehalococcoides mccartyi strain GT	NC_013890.1	2720308
Dehalococcoides mccartyi strain GY50	NC_022964.1	2814836
Dehalococcoides mccartyi strain IBARAKI	NZ_AP014563	1451056

OHRB Species	Accession	Length
Dehalococcoides mccartyi strain VS	NC_013552.1	2826924
Dehalococcoides strain UCH007	NZ_AP014722	1473548
Dehalogenimonas alkenigignens strain BRE15M	NZ_QEFQ00000000.1	3504329
Dehalogenimonas formicexedens strain NSZ-14	NZ_CP018258	2092789
Dehalogenimonas lykanthroporepellens strain BL-DC-9	NC_014314.1	3373020
Dehalogenimonas strain GP	NZ_JQAN00000000	2023475

Table 17 Sequenced genomes from the Dehalococcoidia class to date with their length. NCBI Genome accession is indicated when available.

Other contigs were assigned to different Orders: *Marinobacter*, *Bacillus*, *Staphylococcus*, *Vibrio*, *Spirochaeta*, *Candidatus Phytoplasma*, *Streptomyces*, *Pseudanabaena*, *Paenibacillus*. *Ruminococcus*, unclassified *Ruminococcaceae*, *Moorella*, *Desulfococcus*.

4.5 OHRB antibiotic resistance and efforts towards isolation

4.5.1 Ampicillin and vancomycin resistance of enriched OHRB

To attempt the further enrichment and isolation of the dechlorinating bacteria VL ENR2 TCE Lactate microcosms were used as inoculum for four sets of cultures (VL ENR3) with the defined mineral medium described above: i) one with only BES as antibiotic against methanogens (present also in the inoculum) representing the positive control for dechlorination and growth(VL ENR3 BES); ii) one with ampicillin and vancomycin (BES+AV), to which Dehalococcoidia have been observed to be resistant (VL ENR3 BES+AV). During the first spike only cultures without ampicillin and vancomycin showed dechlorination activity, decrease in TCE in the BES+AV was due to abiotic losses (Fig. 48). From the second spike on, dechlorination of TCE to cis- and trans-DCE was observed both in the antibiotic-amended cultures and in the unamended controls. Dechlorination speed in unamended controls reached 24.1 µmol*l-1*day-1 at the 3rd spike, while BES+AV cultures only dechlorinated TCE at a rate of 9.1 µmol*l-1*day-1, with an average cis/trans ratio of 4.3± 0.6, interestingly higher than in unamended controls (3.8±0.31) (Fig. 49). Therefore it can be affirmed that VLD-1 is resistant to ampicillin and vancomycin, most probably because of the lack of cell wall observed in other members of the Dehalococcoidia family[251–253]. A cell concentration of 5.49±2.16E+6 cells/ml was reached.

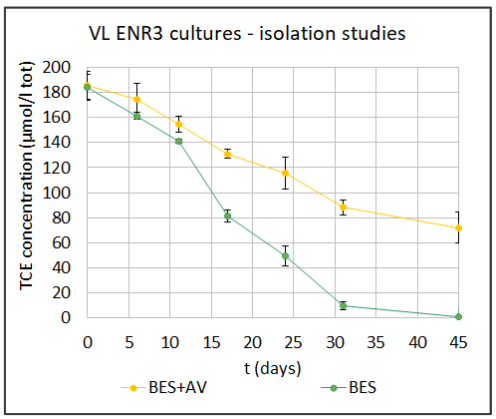


Figure 36 Change in TCE concentration in cultures with BES as the only antibiotic or amended also with ampicillin and vancomycin (BES+AV) during the first spike.

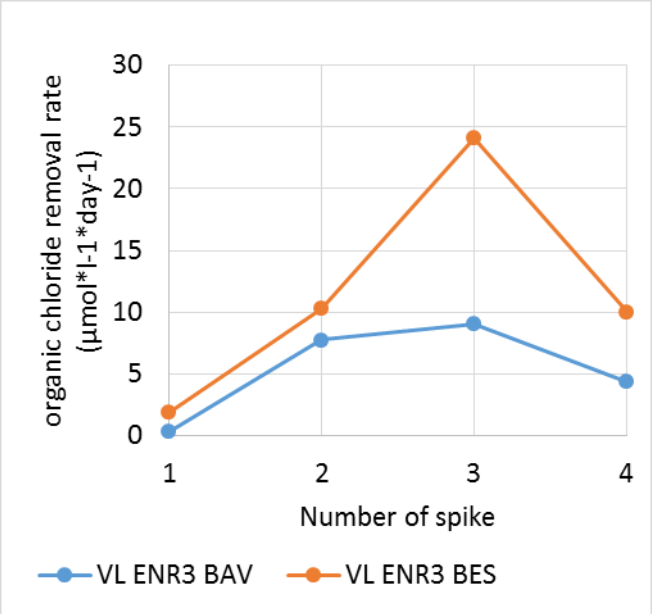
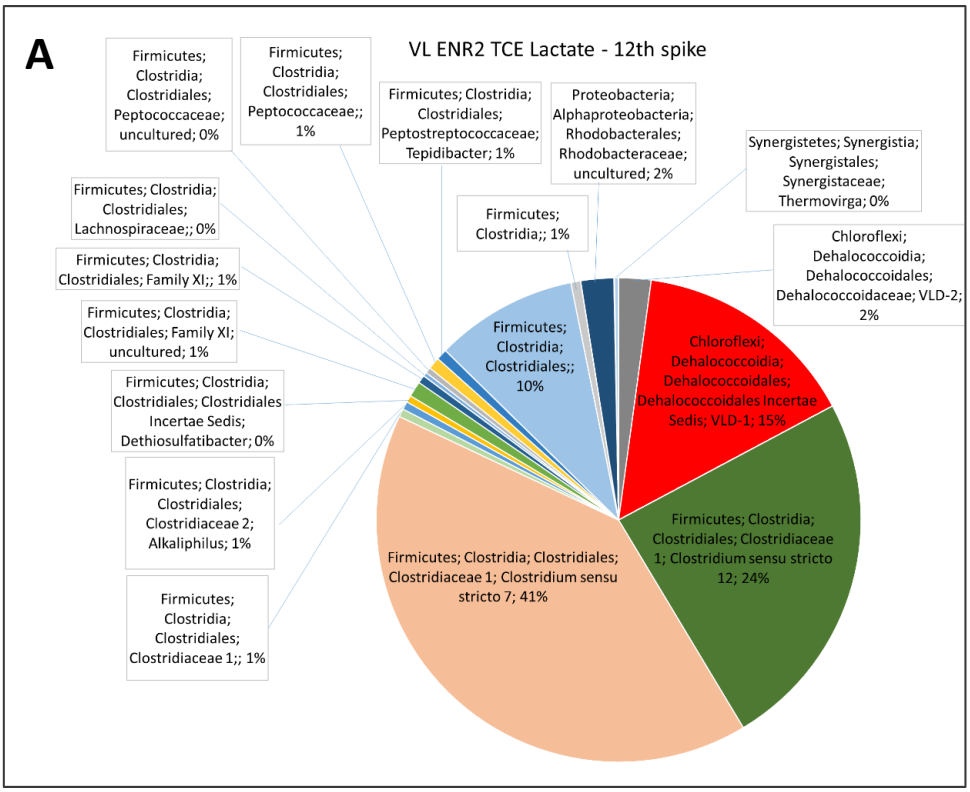


Figure 37 Dechlorination speed of TCE in defined mineral medium amended with BES as only antibiotic or with ampicillin and vancomycin (BAV) too during 4 different TCE spikes.

Illumina sequencing and analysis of the microbial population was performed both on DNA samples from VL ENR2 at the 12th spike and from the B+AV microcosms (Fig. 50A and 50B, respectively). A profound change in the microbial community is observed, with a strong decrease in the microbial community diversity. Changes in relative abundance are reported in table 18. Members of the bacterial community came from 14 different Genera in VL ENR2 TCE, while in the antibiotic-amended cultures, Genera with members were only 8. VLD-1 relative abundance made up 17% of the total bacterial community in VL ENR2 TCE cultures, while in the antibiotic-amended ones that value rose to 69. VLD-2 presence, on the other hand, was confirmed to be lost. Antibiotics showed to have a high impact on the Firmicutes phylum, every genus showed a decrease in relative abundance except for Dethiosulfatibacter, which showed a 16-fold increase in relative abundance. The Genus Marinobacter, from the class Gammaproteobacteria showed a strong enrichment, from undetected to 7% of the total bacterial community. Exact values are compared in table 16. The enrichment of Marinobacter, a facultative anaerobic, denitrifying bacterium can be explained by its ability to grow on acetate (available in the growing medium as product of lactate fermentation) and alkanes (ethane from debromination of bromoethanesulphonate, bromide ions being detected up to a 2 mM concentration)[254].



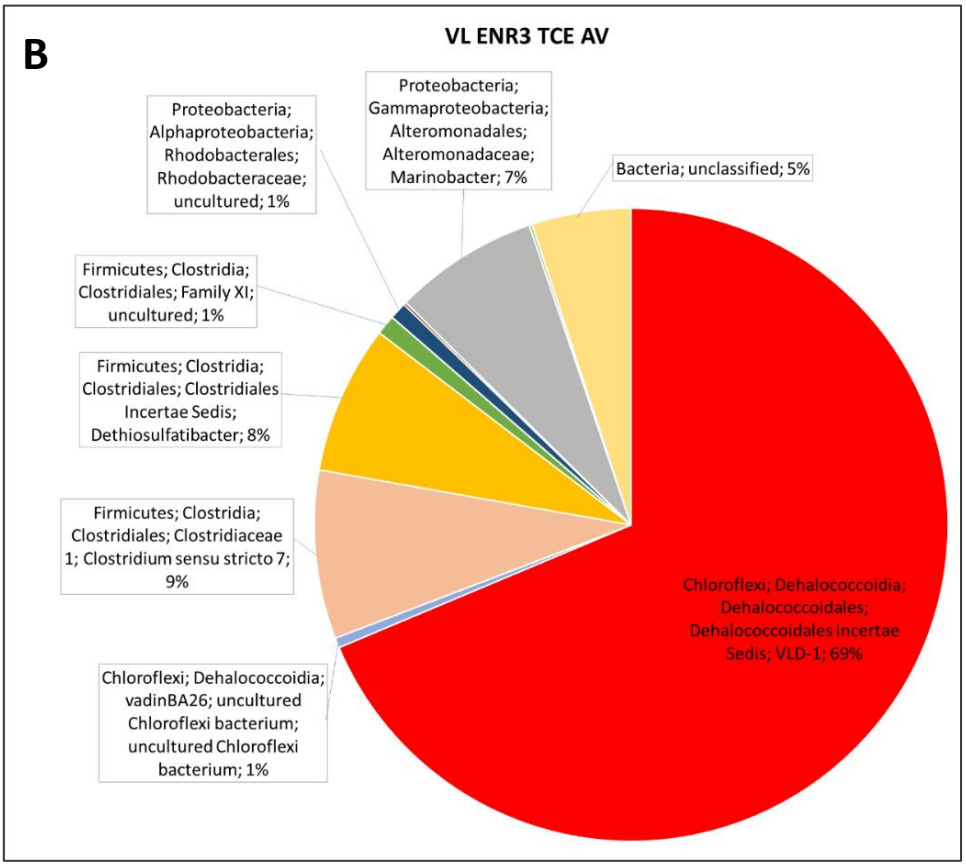


Figure 50 Taxa relative abundance at Genus level of TCE-dechlorinating synthetic marine water ENR2 cultures (A) and defined mineral medium ENR3 cultures amended with antibiotics (B).

Genus	Relative abundance	
Chloroflexi; Dehalococcoidia; Dehalococcoidales; Dehalococcoidaceae; VLD-2	2.15%	0.00%
Chloroflexi; Dehalococcoidia; Dehalococcoidales; Dehalococcoidales Incertae Sedis; VLD-1	15.04%	68.67%
Chloroflexi; Dehalococcoidia; vadinBA26; uncultured Chloroflexi bacterium; uncultured Chloroflexi bacterium	0.00%	0.51%
Firmicutes; Clostridia; Clostridiales; Clostridiaceae 1; Clostridium sensu stricto 12	24.20%	0.00%
Firmicutes; Clostridia; Clostridiales; Clostridiaceae 1; Clostridium sensu stricto 7	40.63%	8.61%
Firmicutes; Clostridia; Clostridiales; Clostridiaceae 1;	0.54%	0.00%
Firmicutes; Clostridia; Clostridiales; Clostridiaceae 2; Alkaliphilus	0.54%	0.00%
Firmicutes; Clostridia; Clostridiales; Clostridiales Incertae Sedis; Dethiosulfatibacter	0.46%	7.60%
Firmicutes; Clostridia; Clostridiales; Family XI; uncultured	1.04%	1.01%
Firmicutes; Clostridia; Clostridiales; Family XI;	0.54%	0.00%
Firmicutes; Clostridia; Clostridiales; Peptococcaceae;	0.81%	0.00%
Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae; Tepidibacter	0.73%	0.00%
Firmicutes; Clostridia; Clostridiales;	9.50%	0.00%
Firmicutes; Clostridia;	0.65%	0.00%
Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; uncultured	2.19%	0.87%
Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Marinobacter	0.00%	7.38%
Bacteria; unclassified	0.00%	5.07%

Table 18 Taxa relative abundance change between TCE-dechlorinating synthetic marine water ENR2 cultures (first column) and defined mineral medium ENR3 cultures amended with antibiotics (second column). Red-green coloring indicates an increase or decrease between the two conditions. Uncultured bacteria from Family XI of Clostridia are yellow as no significant change was observed between the two conditions in relative abundance.

4.6 Identification of a set of novel reductive dehalogenases homologous genes

4.6.1 Identification of suitable degenerate primer pairs from literature targeting rdh genes

To identify reductive dehalogenases homologous genes in the cultures examined an approach was used involving degenerate PCR primer pairs from literature. Different sets of degenerate primer pairs available in the literature, allowing the amplification of a wide range of reductive dehalogenase genes, have been used, namely: primers RRF2 and B1R[225] and a set of 27 primer pairs from Hug et al[229], see section 3.7 for more details. The presence and diversity of reductive dehalogenase genes has been investigated in the orgahonalide respiring VL and RH primary cultures and in the sediment-free enrichment ENR2 TCE Lac. For our study, knowing our OHRB of interest belonged to the Dehalococcoidia class, we used only 28 primer pairs, in particular those designed to target rdh genes from: i) Dehalococcoides-only groups, ii) Dhc & Dehly, iii) Dehly-only. Primary cultures from VL and RH produced positive amplicons with the primer pair RRF2-B1R and

only with the primer pairs n. 4 and 14 from Hug et al[229] (Fig. 51, 52, 53). Conversely, the enrichment culture produced positive amplicons both with RRF2-B1R primers and 7 primer pairs (n. 5, 11B, 14, 15B, 18A, 18B, 26) from Hug et al[229] (Fig. 54). This suggested that the enrichment culture is characterized by a higher diversity of reductive dehalogenase homologous genes, which is consistent with its broader range of organohalide dechlorination. For this reason, the amplicons from the enrichment were selected for sequencing.

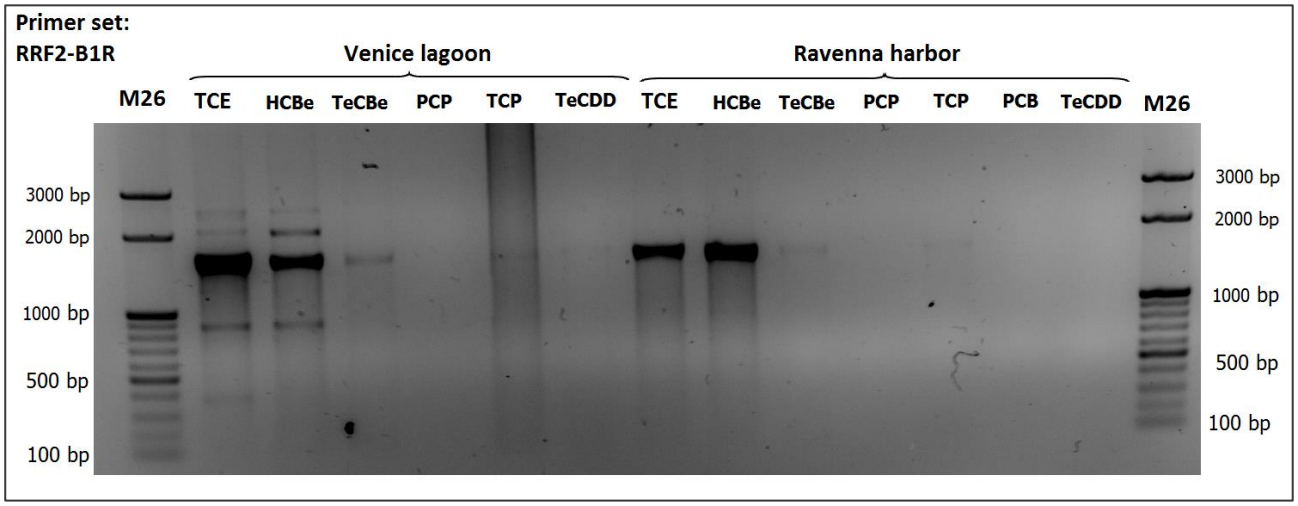


Figure 51 Agarose gel electrophoresis of PCR reactions of DNA from primary cultures with VL and RH sediment spiked with different organochloride compounds with primer pair RRF2 and B1R.

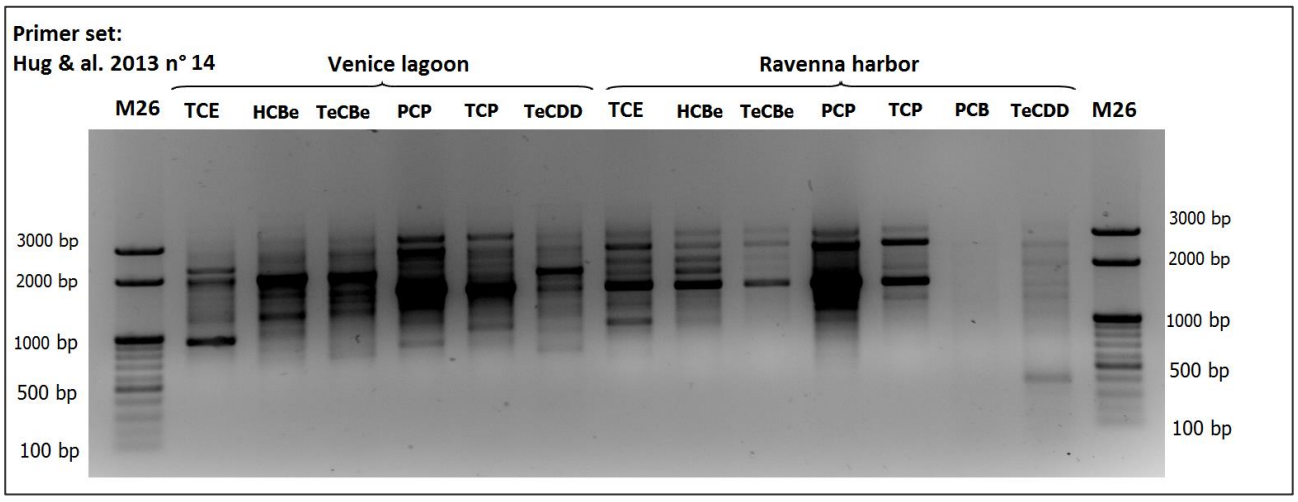


Figure 52 Agarose gel electrophoresis of PCR reactions of DNA from primary cultures with VL and RH sediment spiked with different organochloride compounds with primer pair n°14 from Hug et al 2013[229].

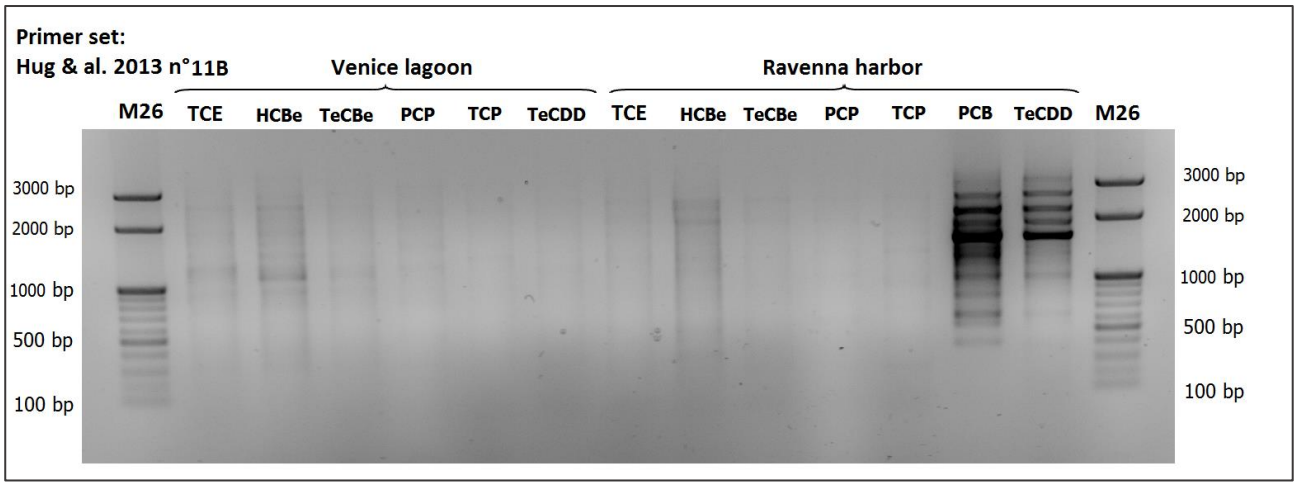


Figure 38 Agarose gel electrophoresis of PCR reactions of DNA from primary cultures with VL and RH sediment spiked with different organochloride compounds with primer pair n°11B from Hug et al 2013[229].

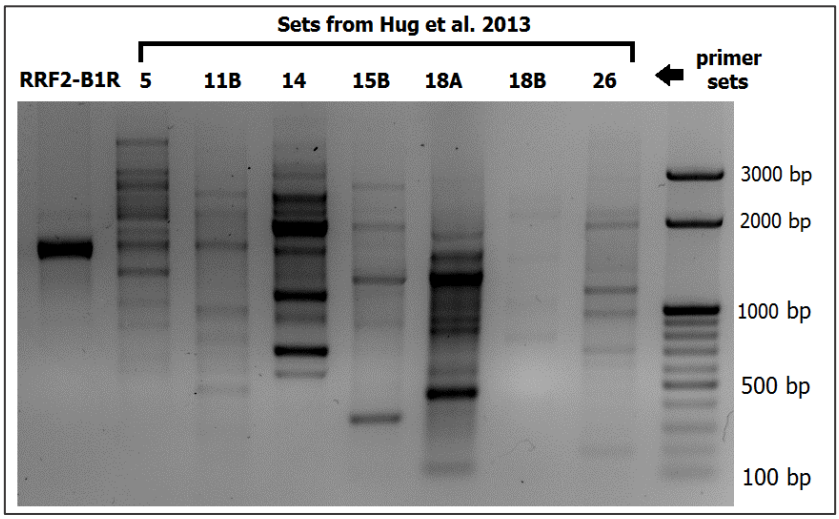


Figure 39 Agarose gel electrophoresis of PCR reactions of DNA from synthetic marine water TCE-dechlorinating lactate amended ENR2 cultures with primer pairs from Hug et al 2013[229] which yielded positive amplification.

4.6.2 Rdh genes amplicon sequencing with a Next Generation Sequencing approach, functional annotation and parsing of sequences of interest

To be sequenced, amplicons resulting from successful PCR reactions with primers from Hug et al.[229] and RRF2-B1R pair on genomic DNA from VL ENR2 Lactate, were pooled and sent to BMR Genomics S.r.l. (Padova, Italy), where amplified DNA was subjected to Tagmentation, barcoding, and sequencing with an Illumina MiSeq platform. The sequencing yielded 162338 paired ends raw reads, which were assembled with the metagenomics software SPAdes 3.7 with three different algorithms, the results are shown in table 19: i) ass-meta: raw reads were assembled assuming different genomes of origin; ii) ass-nometa: assembly of raw reads assuming the same genome of origin; iii) ass-merge-nometa: paired ends raw reads were merged before assembly assuming the same genome of origin iv) ass-merge-platanus: paired ends raw reads were merged before assembly with an alternative algorithm.

Assembly	Sequences	Total length	Minumum length	Average length	Maximum length	N50	L50
ass-meta	8086	4914362	9	607.8	4645	568	2444
ass-merge-platanus	317	326016	337	1028.4	3097	1081	108
ass-merge-nometa	1228	1399738	104	1139.9	4604	1158	416
ass-nometa	6657	4640139	61	697	4712	684	1967

Table 19 Comparison of assembly outputs of SPAdes with different algorithms of the raw reads obtained by tagmentation and Illumina MiSeq sequencing of rdh genes amplicons with degenerate primer pairs.

Annotation of sequences was necessary for extrapolating the reductive dehalogenases homologs from the 16288 sequences generated in the assemblies. To do so, it was necessary to compare the assembled sequences to a database containing the sequences of all the reductive dehalogenases identified to date. This task was carried out with two set of softwares: i) the Entrez Programming Utilities (E-utilities) and the standalone BLAST executables as described in the Materials and methods section (subsection 4.8).

81 rdh sequences were identified, with an average length of 1059 bp, a longest sequence of 2473 bp and a shortest 274 bp. Amino acid sequence similarity between each of these sequences and previously identified ones in literature was low (Fig. 55).

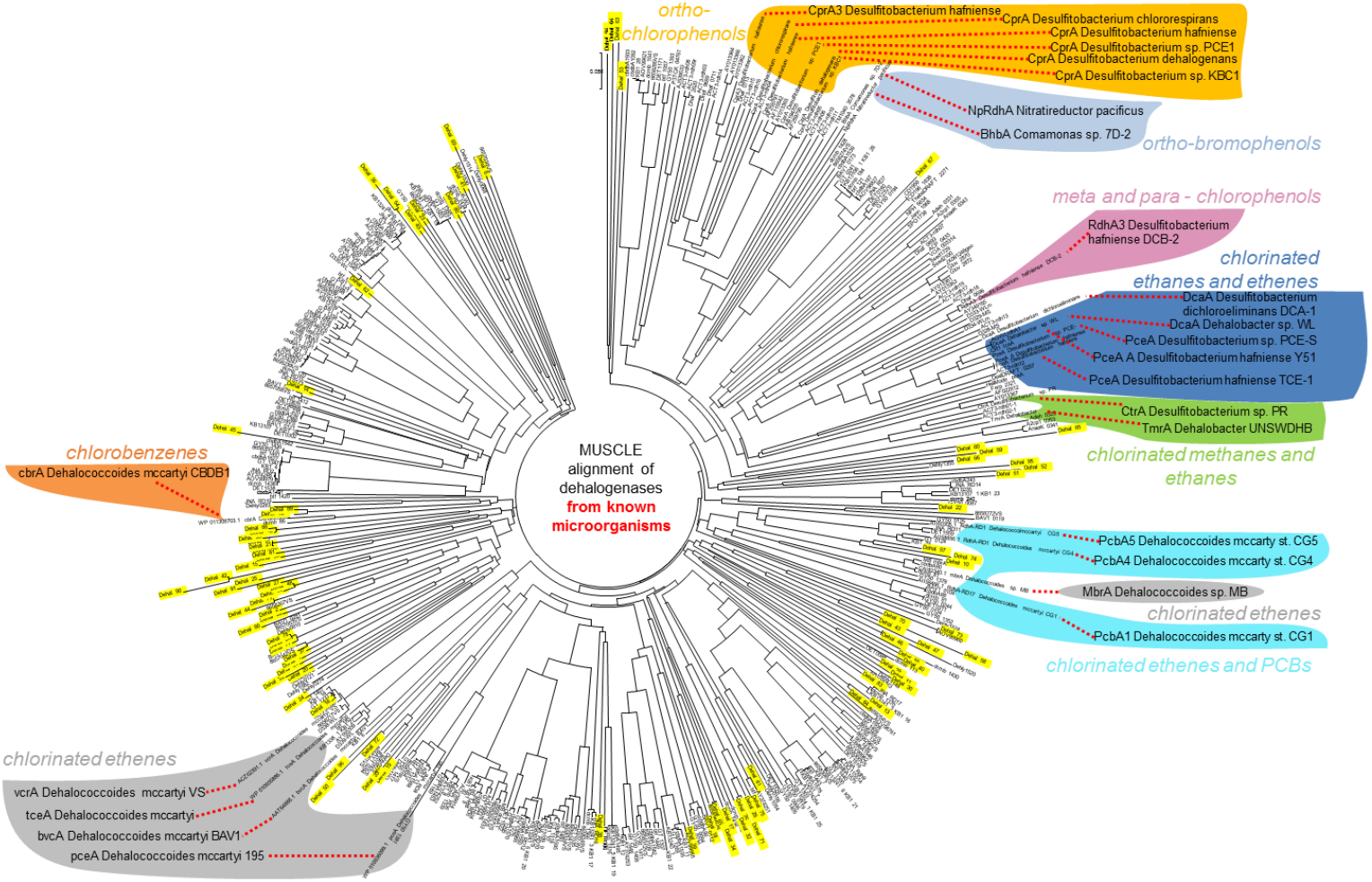


Figure 40 MUSCLE alignment of amino acid sequences of the newly identified rdh genes from this study and previously identified reductive dehalogenases. Sequences from this study are highlighted in yellow. Clusters of reductive dehalogenases characterized in terms of substrate specificity are shown.

Blastx alignment of sequences agains a database of all non-redundant GenBank CDS translations and entries from PDB, SwissProt, PIR, PRF, yielded results shown in table 20. 13 sequences high similarity (> 80%) with rdhA homologs from other studies. In particular, 5 identified sequences have identities higher than 99% with their best matches: Dehal15, Dehal17, Dehal27 and Dehal34 matche sequences from a Pentachlorobenzene-dechlorinating estuarine sediment from the Arakawa River, Japan. Dehal16 matches vinyl chloride dehalogenase VcrA from Dehalococcoides mccartyi. 53 sequences have a varying identity with other rdh genes between 90% and 50% and only 22 share less than 50% identity with other entries in the database. With an average identity of 61%, the great majority of rdh genes identified proved to be novel. The phylogenetic proveniences of most of the matches clusters in the Dehalococcoidia class, mostly genes from *D. mccartyi* or *Dehalogenimonas* species, furtherly reinforcing information about the phylogeny of the microbial species performing the reductive dehalogenation.

rdh homolog	Best match - ID	Best match - Description	Identity (%)	Amino acid sequence alignment length	Total score	E value
Dehal65	WP_049765553.1	reductive dehalogenase *[Dehalogenimonas sp. GP]*	74.803	127	467	2.78e-63
Dehal66	WP_013218779.1	reductive dehalogenase *[Dehalogenimonas lykanthroporepellens]*	83.206	131	301	7.34e-74
Dehal67	WP_014162463.1	4Fe-4S dicluster domain-containing protein *[Thermovirga lienii]*	63.710	124	214	1.86e-41
Dehal68	WP_052465210.1	reductive dehalogenase *[Dehalococcoides mccartyi]*	65.116	129	267	1.63e-53
Dehal69	WP_041220236.1	reductive dehalogenase *[Dehalogenimonas lykanthroporepellens]*	86.719	128	297	3.54e-73
Dehal70	PKH47714.1	reductive dehalogenase *[Dehalococcoides mccartyi]*	57.692	182	280	9.31e-71
Dehal71	BAS32390.1	reductive dehalogenase *[Dehalococcoides mccartyi IBARAKI]*	63.951	491	498	0.0
Dehal72	CCA41202.1	reductive dehalogenase subunit A [uncultured bacterium]	98.571	70	489	5.29e-40
Dehal73	RAL68873.1	Tetrachloroethene reductive dehalogenase TceA *[Dehalococcoides mccartyi]*	48.544	103	163	6.01e-20
Dehal74	WP_102331321.1	reductive dehalogenase *[Dehalogenimonas sp. GP]*	54.279	409	479	2.03e-162
Dehal75	WP_061977517.1	reductive dehalogenase *[Dehalococcoides mccartyi]*	63.441	372	482	2.90e-161
Dehal76	BAS32390.1	reductive dehalogenase *[Dehalococcoides mccartyi*	64.155	491	498	0.0

rdh homolog	Best match - ID	Best match - Description	Identity (%)	Amino acid sequence alignment length	Total score	E value
		IBARAKI]				
Dehal1	BAI47844.1	putative reductive dehalogenase [uncultured bacterium]	46.462	424	449	7.10e-130
Dehal77	BAI47830.1	putative reductive dehalogenase [uncultured bacterium]	64.220	436	453	0.0
Dehal78	BAI47830.1	putative reductive dehalogenase [uncultured bacterium]	64.450	436	453	0.0
Dehal80	BAI47852.1	putative reductive dehalogenase [uncultured bacterium]	63.504	137	459	4.16e-56
Dehal2	WP_012882579.1	reductive dehalogenase *[Dehalococcoides mccartyi]*	64.941	425	473	0.0
Dehal81	BAI47804.1	putative reductive dehalogenase [uncultured bacterium]	62.998	427	441	0.0
Dehal82	WP_013217763.1	reductive dehalogenase *[Dehalogenimonas lykanthroporepellens]*	46.767	464	462	6.02e-124
Dehal83	AGY78437.1	putative reductive dehalogenase subunit A [uncultured bacterium]	51.429	140	283	5.81e-35
Dehal85	KUK91590.1	reductive dehalogenase *[Marinimicrobia bacterium 46_43]*	45.890	146	146	2.03e-17
Dehal6	WP_076004771.1	reductive dehalogenase *[Dehalogenimonas formicexedens]*	53.053	475	475	1.08e-170
Dehal89	WP_013217763.1	reductive dehalogenase *[Dehalogenimonas lykanthroporepellens]*	46.767	464	462	4.22e-123
Dehal90	AGY79045.1	putative reductive dehalogenase subunit A [uncultured bacterium]	28.814	118	137	0.024
Dehal94	OBW62540.1	dehalogenase [*Dehalococcoides mccartyi*]	65.556	90	90	5.41e-19
Dehal4J	WP_061977086.1	reductive dehalogenase *[Dehalococcoides mccartyi]*	42.765	463	490	1.13e-115
Dehal1J	BAI47844.1	putative reductive dehalogenase [uncultured bacterium]	47.748	444	449	7.24e-139
Dehal2J	BAI47852.1	putative reductive dehalogenase [uncultured bacterium]	67.793	444	459	0.0

rdh homolog	Best match - ID	Best match - Description	Identity (%)	Amino acid sequence alignment length	Total score	E value
Dehal6&9	BAF34632.1	reductive dehalogenase homologue [uncultured bacterium]	41.513	489	462	9.43e-118
Dehal10	PPD58316.1	reductive dehalogenase [*Dehalogenimonas sp. GP*]	55.034	447	479	0.0
Dehal11	BAI47798.1	putative reductive dehalogenase [uncultured bacterium]	40.988	405	455	1.46e-88
Dehal12	BAI47810.1	putative reductive dehalogenase [uncultured bacterium]	53.913	460	474	5.44e-179
Dehal13	BAI47828.1	putative reductive dehalogenase [uncultured bacterium]	66.234	462	469	0.0
Dehal14	OBW62536.1	reductive dehalogenase [*Dehalococcoides mccartyi*]	53.908	499	502	1.11e-171
Dehal15	BAF34634.1	reductive dehalogenase homologue [uncultured bacterium]	99.788	472	492	0.0
Dehal16	WP_079398699.1	reductive dehalogenase [*Dehalococcoides mccartyi*]	99.016	508	519	0.0
Dehal17	BAF34631.1	reductive dehalogenase homologue [uncultured bacterium]	99.588	486	486	0.0
Dehal19	BAI47846.1	putative reductive dehalogenase [uncultured bacterium]	81.210	463	472	0.0
Dehal20	ACZ62443.1	reductive dehalogenase [*Dehalococcoides mccartyi VS*]	62.338	462	488	0.0
Dehal21	WP_083774184.1	reductive dehalogenase [*Dehalogenimonas lykanthroporepellens*]	44.954	436	488	7.62e-123
Dehal22	WP_023651751.1	reductive dehalogenase *[Dehalococcoides mccartyi*]	43.534	464	490	6.77e-120
Dehal23	WP_041331433.1	reductive dehalogenase [*Dehalococcoides mccartyi*]	57.819	486	499	0.0
Dehal24	PPD57553.1	reductive dehalogenase [*Dehalogenimonas sp. GP*]	50.835	419	465	8.69e-159
Dehal25	WP_011308708.1	reductive dehalogenase [*Dehalococcoides mccartyi*]	33.049	469	486	3.92e-65
Dehal26	BAS32390.1	reductive dehalogenase [*Dehalococcoides mccartyi IBARAKI]*	64.562	491	498	0.0
Dehal27	BAF34633.1	reductive dehalogenase homologue [uncultured	99.780	455	457	0.0

rdh homolog	Best match - ID	Best match - Description	Identity (%)	Amino acid sequence alignment length	Total score	E value
		bacterium]				
Dehal28	BAI47830.1	putative reductive dehalogenase [uncultured bacterium]	63.801	442	453	0.0
Dehal29	BAI47798.1	putative reductive dehalogenase [uncultured bacterium]	50.000	450	455	1.88e-132
Dehal30	BAI47798.1	putative reductive dehalogenase [uncultured bacterium]	41.685	463	455	1.93e-101
Dehal32	WP_015407269.1	reductive dehalogenase [*Dehalococcoides mccartyi*]	48.421	475	505	4.60e-142
Dehal33	WP_012881445.1	reductive dehalogenase [Dehalococcoides mccartyi]	77.641	407	418	0.0
Dehal34	BAF34631.1	reductive dehalogenase homologue [uncultured bacterium]	99.706	340	340	0.0
Dehal35	BAI47810.1	putative reductive dehalogenase [uncultured bacterium]	49.829	293	454	2.69e-94
Dehal95	WP_083635413.1	reductive dehalogenase [*Dehalogenimonas formicexedens*]	75.424	354	392	0.0
Dehal37	WP_012881438.1	reductive dehalogenase [Dehalococcoides mccartyi]	64.012	339	497	1.59e-164
Dehal38	WP_041341161.1	reductive dehalogenase [Dehalococcoides mccartyi]	51.536	293	504	9.95e-104
Dehal39	WP_041344588.1	reductive dehalogenase [Dehalococcoides mccartyi]	54.698	298	494	3.18e-114
Dehal40	AGY79065.1	putative reductive dehalogenase subunit A [uncultured bacterium]	69.951	203	203	4.99e-105
Dehal41	WP_041220236.1	reductive dehalogenase [*Dehalogenimonas lykanthroporepellens*]	82.105	285	422	2.48e-180
Dehal42	WP_076003486.1	reductive dehalogenase [*Dehalogenimonas formicexedens*]	59.130	115	392	5.82e-58
Dehal44	WP_012882579.1	reductive dehalogenase [*Dehalococcoides mccartyi*]	63.985	261	309	4.79e-113
Dehal45	AGY78562.1	putative reductive dehalogenase subunit A [uncultured bacterium]	72.398	221	284	3.23e-117
Dehal47	WP_023652808.1	reductive dehalogenase [*Dehalococcoides mccartyi*]	43.145	248	358	2.40e-59
Dehal48	WP_012882579.1	reductive dehalogenase	62.441	213	473	6.44e-100

rdh homolog	Best match - ID	Best match - Description	Identity (%)	Amino acid sequence alignment length	Total score	E value
		[*Dehalococcoides mccartyi*]				
Dehal49	PKH47753.1	reductive dehalogenase *[Dehalococcoides mccartyi*]	74.126	143	494	1.42e-72
Dehal50	WP_013218498.1	reductive dehalogenase *[Dehalogenimonas lykanthroporepellens]*	86.124	209	243	1.00e-130
Dehal51	WP_049760344.1	twin-arginine translocation signal domain-containing protein [*Dehalogenimonas lykanthroporepellens]*	45.860	157	211	5.31e-40
Dehal52	ATB17171.1	reductive dehalogenase [uncultured bacterium]	52.717	184	223	2.44e-61
Dehal53	BAI47798.1	putative reductive dehalogenase [uncultured bacterium]	41.081	185	180	3.81e-30
Dehal54	WP_076003492.1	reductive dehalogenase *[Dehalogenimonas formicexedens*]	54.545	77	464	1.17e-24
Dehal55	BAI47852.1	putative reductive dehalogenase [uncultured bacterium]	72.321	112	459	5.70e-53
Dehal56	WP_076004771.1	reductive dehalogenase [*Dehalogenimonas formicexedens*]	44.295	149	395	1.76e-35
Dehal57	WP_102331321.1	reductive dehalogenase [*Dehalogenimonas* sp. GP]	60.759	158	373	6.68e-64
Dehal58	WP_023652808.1	reductive dehalogenase [*Dehalococcoides mccartyi*]	42.690	171	315	9.41e-39
Dehal59	AGY79055.1	putative reductive dehalogenase subunit A [uncultured bacterium]	67.949	156	232	3.14e-70
Dehal60	WP_102331321.1	reductive dehalogenase [*Dehalogenimonas sp. GP*]	51.974	152	203	5.21e-46
Dehal61	WP_013218498.1	reductive dehalogenase [*Dehalogenimonas lykanthroporepellens]*	90.845	142	469	4.27e-92
Dehal62	OBW62536.1	reductive dehalogenase [*Dehalococcoides mccartyi]*	59.167	120	120	3.22e-29
Dehal96	BAG55936.1	reductive dehalogenase [uncultured bacterium]	66.187	139	145	7.97e-63
Dehal63	AGY78591.1	putative reductive dehalogenase subunit A [uncultured bacterium]	51.852	135	164	8.62e-43
Dehal64	WP_023387223.1	reductive dehalogenase [*Youngiibacter fragilis*]	39.048	105	150	7.11e-15

Table 20 Blastx alignment results of the 81 rdh sequences identified by local alignment.

Aminoacid sequences analyses with HMMER/hmmscan revealed a general conservation of domain organization, with most of the sequences possessing no TAT signal, but a general transmembrane signal peptide predicted (Phobius software) at the N-terminal, central catalytic domain for the reductive dehalogenase and a 4Fe-4S double cluster binding domain compatible sequence at the C-terminal, lack of disordered and low complexity regions, compatible with most of the domain organizations of reductive dehalogenases identified from the Chloroflexi phylum (Fig 56).

Figure 41 Predicted domain organization common to most of the identified rdh translated genes.

4.7 Assessment of reductive dehalogenases expression: RNA and protein-based approaches

4.7.1 Multiplex PCR screens for identification of rdh genes present in genomic DNA and in cDNA

To link rdh genes to function, VL ENR3 TCE cultures, which could stably dechlorinate TCE to cis and trans-DCE, were subject to expression analysis to identify which reductive dehalogenase genes were transcribed the most into RNA. 65 end-point multiplex PCR primer pairs were designed for specific amplification of one or more (in case of high sequence similarity) rdh genes and are reported in section 3.8 in table 4. The 65 pairs were designed to be used in 23 different sets in multiplex end-point PCR. Primer pairs from the same set or "primer mix" (to be used in the same multiplex PCR mix) had different expected amplicon sizes to allow the recognition of specific amplification. Composition of multiplex PCR primer mixes, in terms of primer pairs comprised, are reported in table 21. Table 21 also reports the expected size of the PCR product. PCR products were run on an agarose gel and the size of the amplicon made it possible to identify which primer pair found its target. This allowed to screen DNA faster, performing 23 multiplex PCR instead of 65. DNA from VL ENR3 TCE cultures was screened with the 23 sets of primers in multiplex end-point PCR. Most of the primer pairs yielded PCR products, although 18 of them did not, targeting 21 genes (red cells in table 21), meaning absence from the metagenome of the microbial community.

	Length of expected amplicon						
Multiplex PCR Primer Mix	100	200	300	400	500	600	700
1	91,27		78, 28, 77	17, 34			
2	64		13	32			
3	90,25	40			30		
4	73, 74, 10	83	93,15				
5		94		33	47		
6			70	37			22, 4J
7	82,89	20	66				

	Length of expected amplicon						
Multiplex PCR Primer Mix	100	200	300	400	500	600	700
8	55,2J,80		53				14,5J
9		72	39		29		
10	65		45			81	
11	51		57	76,26,71			
12	60	42		11			
13	61		38	24			
14	67		1J	23			
15	62	1		52			
16	54			48		44,2	
17	85	58					16
18		75,26		19		21	
19		50		6		12	
20		63	56	41			
21		69	95	35			
22		49					
23		68					

Table 21 Schematic sum-up of end-point multiplex PCR performed on TCE-dechlorinating defined mineral medium ENR3 cultures. Number of primer mix are non-consequential numbers identifying a mix of three primer pairs used together in the same multiplex PCR. Numbers in cells correspond to the rdh genes (one to three) targeted by the primer pair. Red cells identify primer pairs which did not amplify the template in genomic DNA from the culture. Yellow cells contain primer pairs which yielded amplicons from genomic DNA, but not from coding DNA retrotranscribed from RNA. Green cells indicate that the primer pair has successfully amplified the template coding DNA.

To assess which of the rdh genes which was in the template DNA was expressed, primer pairs which showed successful amplification of template DNA were used to perform the same multiplex end-point PCRs on cDNA from the same cultures. Most of the primer pairs (36) yielded PCR products on the cDNA (green cells in table 21), demonstrating expression of those genes, but 19 did not (yellow cells in table 21).

4.7.2 Quantification of rdh genes expression by qPCR on cDNA

Primers suitable for quantitative PCR were designed targeting the sequences detected in the cDNA (for a list of primers see table 5 in section 3.8). Absolute quantification of copied of mRNA/ml of culture was carried out on the first set of genes covered by 12 different primer pairs. Results are shown in figure 57.

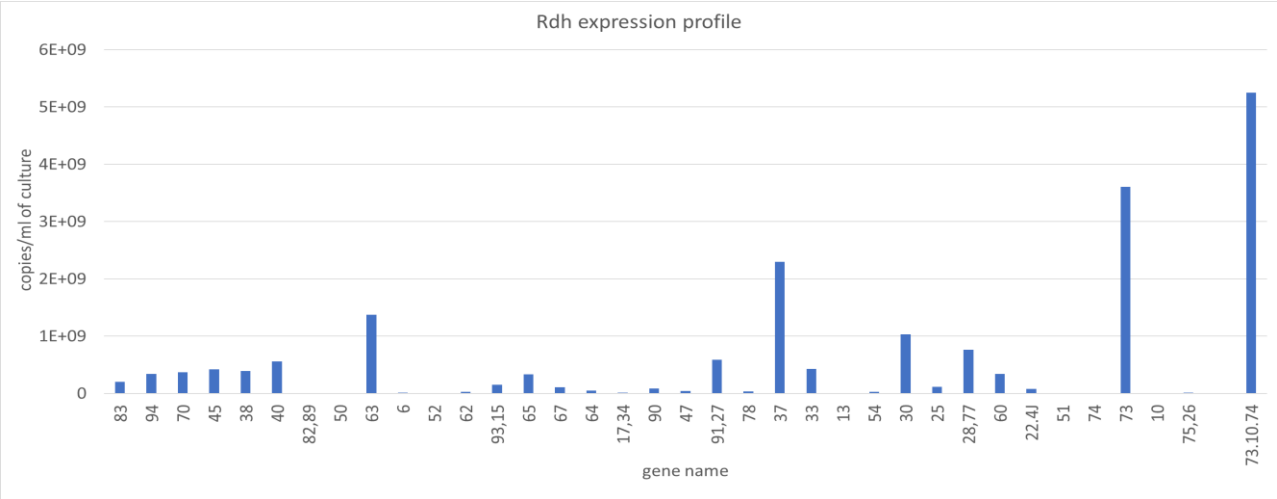


Figure 42 Transcription quantification of a set of rdh genes expressed in copies of transcript per milliliter of cell culture.

The three different genes 73, 74 and 10 share high sequence similarity, therefore a unique primer pair was used for their expression quantification at first. The expression of these three rdh genes surpasses the average expression of the other rdh genes, therefore suggesting that one or more of the three is responsible for TCE dechlorination. Specific primer pairs for the quantification of each of the three rdh genes were designed and used for a specific quantification. The analyses revealed that only rdh number 73 had a high level of expression, suggesting it could be the one responsible for TCE dechlorination activity. With a cell concentration of the VLD-1 bacterium measured in the microcosms at 1.65E+08 cell/ml during the analysis, the amount of transcript for the 73 is 21.84 transcript/cell. Although very low, the measured value is in line with what observed for another dehalogenating culture by Wagner et al (2009)[255]. Interestingly, in Wagner et al it is noted that althoug the mRNA concentration can indeed change 100 fold in time during induction by spike, the higher concentrations are between 1 and 10 transcripts per 16S gene of the bacterium.

4.7.3 Protein analyses of reductive dehalogenase presence by mass spectrometry proteomics

Proteomics analyses were carried out on the same TCE-dechlorinating cultures analyzed with q-PCR to measure the expression of the rdh identified with a mass spectrometry(MS)-based approach. Cells for protein extraction were harvested by centrifugation from cultures as described in the materials and methods section of this thesis. Proteins were extracted with different approaches and subsequently digested into polypeptides with trypsin (see materials and methods section for protocol). Polypeptides were then separated by nano liquid chromatography and their presence and abundance measured by MS-MS analysis. Identification of the reductive dehalogenases present in samples required the comparison of the polypeptides detected with a database composed of the list of amino acid sequences of the 81 genes identified in genomic DNA. Separate approaches were tried for the recovery of proteins to be tripsyn-digested: i) whole cell extract with detergent and bead beating, ii) gel extraction from SDS-PAGE. The first analysis identified 11 polypeptides with matches among the identified reductive dehalogenases (Tab. 22), the second 25 peptides (Tab. 23).

Polypeptide	Matched proteins	Positions in Matched Proteins
KSAGWYGVRY	73	73 [16-23]
RFFGASSVGFVKL	10; 73; 74	10 [140-150]; 73 [82-92]; 74 [100-113]
KWNGTPEENSKM	10; 73; 74; 60	10 [123-132]; 73 [65-74]; 74 [83-95]; 60 [105-117]
RFCINCSKC	10; 74	10 [309-315]; 74 [269-276]
KEPSWETTPKY	10; 74	10 [330-338]; 74 [290-301]
KEDFASIHDVVKGVVAKT	10; 74	10 [386-401]; 74 [346-364]
KEDFASIHDVVKG	10; 74	10 [386-401]; 74 [346-360]
KCSDACPSGALKGEKEPSWETTPKY	10; 74	10 [316-338];74 [276-301]
KCSDACPSGALKGEKE	10; 74	10 [316-329];74 [276-301]
KCSDACPSGALKG	10; 74	10 [316-326];74 [276-301]

Table 22 Peptides detected by nLC-MS after whole protein extraction and matching rdh among the ones identified in this study. Matched sequences and position is reported.

Polypeptide	Matched proteins	Positions in Matched Proteins
KYNNFNAHLPEEEATQIRA	10	10 [41-57]
RLQQLITPEWGPMIRE	10	10 [272-285]
RLQQLITPEWGPMIRE*	10	10 [272-285]
RESVVVLMDLPVAPTKPVDFGASRF	10	10 [286-308]
RESVVVLMDLPVAPTKPVDFGASRF*	10	10 [286-308]
RLLGAFDVGCVPFTENIRKL	26	26 [185-202]
RLLGAFDVGCVPFTENIRK	26	26 [185-201]
KHQGTPEENLKM	26	26 [168-177]
KNFGYGFKN	26	26 [460-466]
RTPEGYGYQKW	33	33 [134-142]
KSAGWYGVRY	73	73 [16-23]
RMANNDTPGWALRD	74	74 [25-36]
RFFGASSVGFVKL	10; 73; 74	10 [140-150]; 73 [82-92]; 74 [102-112]
KWNGTPEENSKM	10; 73; 74	10 [123-132]; 73 [65-74]; 74 [85-94]
KMLTNVLRF	10; 73; 74	10 [133-139]; 73 [75-81]; 74 [95-101]
KYWQETDSYCGFCNAVCVFSKE	10; 74	10 [366-385]; 74 [328-347]
KLFDGFFVEMDKM	10; 74	10 [404-414]; 74 [366-376]
KRPGWISDGAAGAAYDNCDIIQYRL	10; 74	10 [212-234]; 74 [174-196]
KEDFASIHDVVKG	10; 74	10 [386-396]; 74 [348-358]
KCSDACPSGALKGEKEPSWETTPKY	10; 74	10 [316-338]; 74 [278-300]
KYNDDIQPQLFNNPGIKS	10; 74	10 [339-354]; 74 [301-316]
KSWYFDHFKC	10; 74	10 [355-362]; 74 [317-324]
RFCINCSKC	10; 74	10 [309-315]; 74 [271-277]
RQSLDMFKR	10; 74	10 [205-211]; 74 [167-173]
KEPSWETTPKY	10; 74	10 [330-338]; 74 [292-300]

Table 23 Peptides detected by nLC-MS after SDS PAGE of proteins and in-gel trypsin digestion and matching rdh among the ones identified in this study. Matched sequences and position is reported. Peptides marked with asterisks were detected with acetamidized methionines.

The first analysis identified only one polypeptide specific for one dehalogenase, while all the others matched more than one. Peptides matched rdh sequences of dehalogenase 10,73, 74, 60. The second analysis carried out by an in-gel digestion of an SDS PAGE allowed to detect more peptides from dehalogenase 10, 73, 74 and also 26, 33. A semi-quantitative analysis based on peak intensity of peptides allowes to have a relative abundance of peptides matching the same protein (Fig. 58). In figure 58 the intensity of peptide peaks matching specifically one or more dehalogenases are summed and put together. The rdh cluster 10-73-74 is the most represented, with intensities of the 10 peptides specific both for dehalogenases 10 and 74 making up for 42.3% of total intensity and 3 dehal_10-specific peptides contributing by 17.4%. Data is not unequivocally pointing towards one specific dehalogenase being overexpressed compared to others, although data about Dehal_10 hints at a higher presence of it.

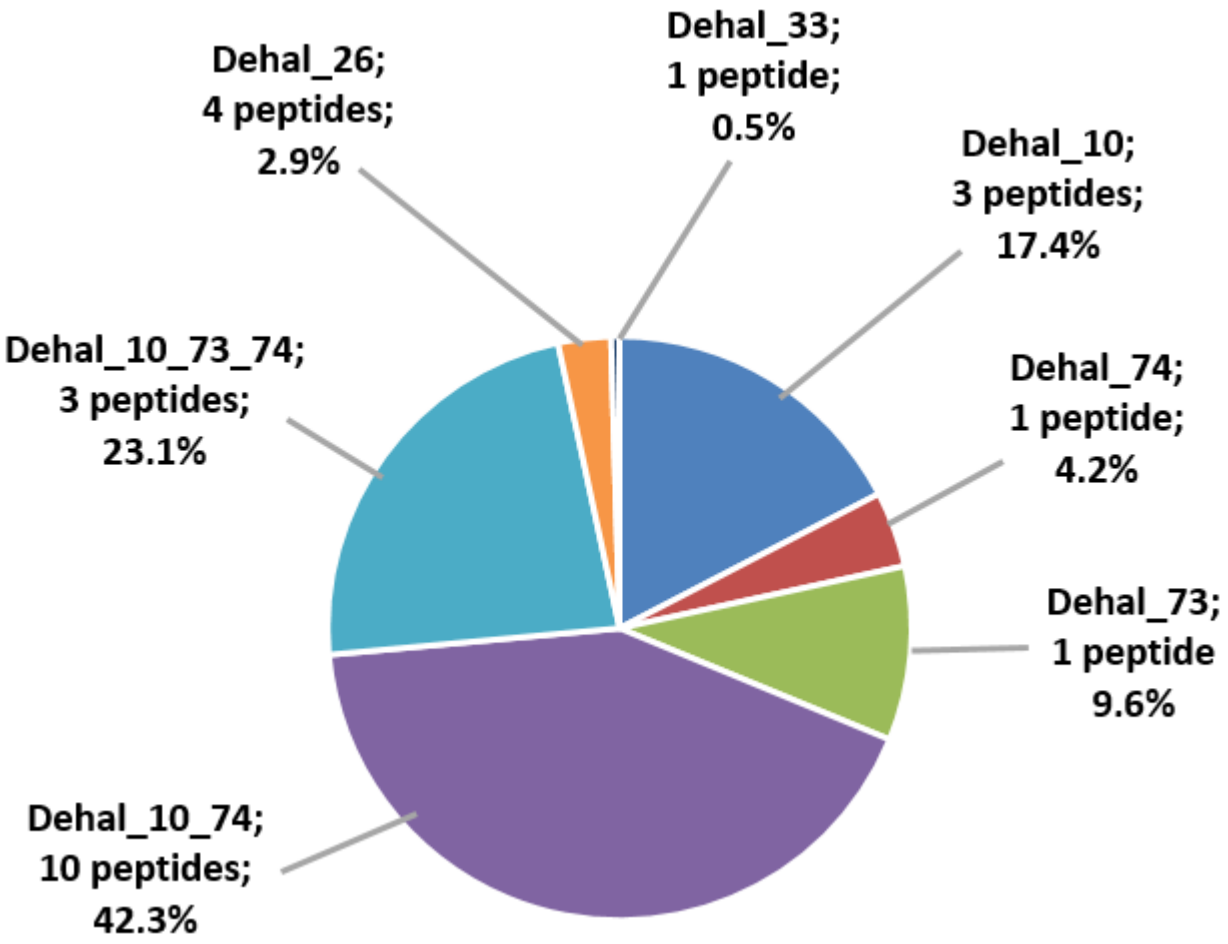


Figure 43 Intensities of peptides matching different reductive dehalogenases expressed as percentage of the overall sum of peak intensities.

5 Conclusions and outlook

Two indigenous microbial community from marine sediments of the Adriatic Sea are able to dechlorinate several dangerous contaminants. Although pentachlorophenol was not dechlorinated, 2,3,5-TCP was, especially by VL microbial community which showed the rare ability to remove unflanked meta chlorines in chlorophenols. Dechlorination of chlorobenzenes was also different and more extensive in VL sediment, where complete dechlorination was observed and benzene detected. These differences in dechlorination patterns, along with the specific dechlorination of TCE to cis-DCE only in RH sediment and not both DCEs, marks a smaller dechlorination range of the RH community, clearly harboring a smaller or less able OHRB community.

Members of the Dehalococcoidia class are most probably responsible for reductive dehalogenation of most the tested compounds, although co-metabolism with members of other taxa cannot be excluded. The specific enrichment of the Dehalococcoidia classhighlighted by NGS data and, in particular, its orders GIF9 and Dehalococcoidales in all the microcosms where dehalogenation of the organochloride was detected, except in VL TeCBe and RH TCE, along with the dehalogenating Chloroflexi-specific PCR-DGGE data, suggests that Dehalococcoidia and, specifically the phylotypes VLD-1 and VLD-2, might be the main OHRB involved, the former in both RH and VL, while the latter only in VL sediment. The peculiar enrichment of the Sulfurovum order of the Epsilonproteobacteria is reported when Dehalococcoidia members did not show enrichment and reductive dechlorination was observed.

OHRB enriched cultures from VL exhibit greater dechlorinating capabilities, indicating that original OHRB concentration in primary sediments could be below a threshold for growth and extensive dechlorination of contaminant and an artificial enrichment could be necessary. VL PCB-dechlorinating enrichment cultures in which the two main OHRB are VLD-1 and VLD-2 can have a higher dechlorination speed and a broader range of substrate compared with indigenous communities from VL and RH as pentachlorophenol and 1,2,3,4-tetrachloro dibenzo-p-dioxin were also dechlorinated.

TCE proved efficient in heavily enrich a marine OHRB (VLD-1 for the main part) in a sediment-free culture and its concentration was tolerated at concentrations as high as 250 µmol/l. In absence of organic matter from the sediment, lactate and pyruvate (and their decarboxylation product acetate) were carbon sources that sustained the reductive dechlorinating community. Bromoethanesulphonate was necessary to avoid cultures take-over by methanogens. Ammonium chloride was enough as a nitrogen source for bacterial growth. Lactate fermentation by fermenting bacteria (reasonably from the Clostridia class) and the production of hydrogen was important, as TCE dechlorination could only be carried out by re-adding lactate to the culture or substituting headspace gas with a 4:1 mixture of hydrogen and carbon dioxide at a pressure higher than the atmospheric one. VLD-1's resistance to ampicillin and vancomycin was demonstrated. These pieces of knowledge made it possible to establish medium allowing growth of a stable TCE-dechlorinating consortium whose metagenome was sequenced and which annotation will reveal more about the biochemical networks involved in marine organohalide respiration.

A relatively simple OHRB community composed of mainly VLD-1 and VLD-2 harbors at least 81 reductive dehalogenase homologous genes. Global alignment of proteins derived from the nucleotide sequences revealed a high diversity among the identified sequences and with previously described ones. Only few (13)

have a % identity higher than 80% with rdh's from other studies. The use of degenerate PCR primers from literature, NGS technology and publicly available tools was sufficient to explore the great rdh genes diversity in our cultures and made it possible to link genes to function by transcriptomic data.

Transcriptomics analyses identified a specific rdh gene to be overexpressed during TCE dechlorination, hinting at its importance in the process. Proteomics analyses confirmed the involvement of a cluster of three reductive dehalogenases comprising the overexpressed one in the process. Further proteomic studies with native purification of active enzymes and mass spectrometry identification could clear doubts on the specific activity of the proteins and their role in TCE dechlorination.

Future developments of this research will be necessarily focused on the quantification of the expression of the remaining genes in presence of TCE to be able to confirm that the cluster of 3 rdh genes overexpressed is the only one which can be correlated with TCE dechlorination. Moreover, reductive dechlorination of other organohalide by VLD-1-containing consortium could be assessed in new cultures and expression analysis could be used to link other rdh genes to their substrate with a transcriptomic approach. Other proteomics analysis will also be able to characterize reductive dehalogenases expressed in terms of specific enzymatic activity and substrate specificity.

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