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ANAEROBIC REDUCTIVE DEHALOGENATION OF ORGANOHALIDES IN ENRICHMENT CULTURES FROM MARINE SEDIMENTS OF VENICE LAGOON

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ABSTRACT

Particular attention in recent years has been given to organohalide pollution of subsurface environments in industrialized areas. Microbial reductive dehalogenation plays an important role in decontamination of soil and sediments as strictly anaerobic microorganisms have evolved using naturally occurring halogenated organics as their terminal electron acceptor. To investigate the activity of these unusual organisms, an indigenous microbial community of a PCB-impacted sediment from Brentella Canal (Venice Lagoon, Italy) was enriched in defined mineral media through subculturing from TCE- amended slurry microcosms. Reductive dechlorination process was tested initially in the presence of lactate and pyruvate, two organic acids with the ability to serve as substrates during cometabolic anaerobic reduction. Bacteria's activity was stimulated as they completely bioconverted TCE simultaneously to cis- and trans-DCE within 6 weeks with no lag phase. The cis-isomer was the major intermediate product at the beginning of chemical monitoring but after a few weeks the trans-isomer took the lead role in TCE dechlorination. This change could be ascribed as the action of a *non-Dehalococcoides* phylotype of the class Dehalococcoidia, namely phylotype VLD-1, which was considered as the main phylotype for the dehalogenation activity. For further test and enrichment, subculturing of lactate-fed microcosms was performed using the same defined mineral medium but amended with antibiotics. The presence of ampicillin, vancomycin and 2-bromoethanesulfonate did not inhibit TCE dechlorination activity since the dechlorinating VLD-1, which turned out to be tolerant to the antibiotics, was enriched. Finally, another set of microcosms from cultures with lactate was prepared to test dehalogenation in the presence of different electron acceptors including TCE in the defined mineral medium. In contrast with the positive results cultures showed before subculturing while in sediment-slurry medium, in the defined medium none of the cultures exhibited dehalogenation activity except for the ones amended with TCE. This finding suggested that due to subculturing and enrichment certain synergistic bacteria were lost whose presence was significant for the production of enzymes catalyzing dehalogenation on several organohalides.

ΠΕΡΙΛΗΨΗ

Ιδιαίτερη προσοχή τα τελευταία χρόνια έχει δοθεί στη ρύπανση από οργανοαλογονίδια στο υπέδαφος σε βιομηχανικές περιοχές. Η μικροβιακή αναγωγική αφαλογόνωση διαδραματίζει σημαντικό ρόλο στην απορρύπανση του ανοξικού εδάφους και των ιζημάτων καθώς αυστηρώς αναερόβιοι μικροοργανισμοί έχουν εξελιχθεί χρησιμοποιώντας φυσικώς παραγώμενα αλογονωμένα οργανικά ως τελικό δέκτη ηλεκτρονίων. Για να διερευνηθεί η δραστηριότητα αυτών των ασυνήθιστων οργανισμών, μια ενδογενής μικροβιακή κοινότητα ενός ρυπασμένου ιζήματος με πολυχλωριωμένα διφαινύλια (PCB) από το κανάλι Brentella (Λιμνοθάλασσα της Βενετίας, Ιταλία) εμπλουτίστηκε σε καθαρό μεταλλικό μέσο διαμέσου υποκαλλιεργειών από μικρόκοσμους αποτελούμενους από ίζημα και θαλασσινό νερό της περιοχής ενδιαφέροντος στους οποίους προστέθηκε τριχλωροαιθένιο (TCE).

Η διεργασία της αναγωγικής αποχλωρίωσης εξετάστηκε αρχικά υπό την παρουσία γαλακτικού και πυροσταφυλικού άλατος, δύο οργανικές ενώσεις οι οποίες χρησιμοποιούνται ως υποστρώματα κατά τη διάρκεια συμμεταβολικής αναερόβιας αναγωγής. Η δραστηριότητα των βακτηριδίων διεγέρθηκε καθώς βιομετέτρεψαν πλήρως το TCE σε cis- και trans-DCE εντός 6 εβδομάδων χωρίς τη μεσολάβηση διαστήματος προσαρμογής. Στους μικρόκοσμους με πυροσταφυλικό άλας ο μέγιστος ρυθμός αποχλωρίωσης του TCE ήταν 19.4±2.9 μmol/L/d ενώ ο λόγος παραγωγής cis-DCE προς trans-DCE ήταν 4.0±0.6. Αντίστοιχα, οι μικρόκοσμοι με γαλακτικό άλας επέδειξαν μέγιστο ρυθμό αποχλωρίωσης 21.2±1.4 μmol/L/d με λόγο παραγωγής cis- προς trans-DCE 4.2±0.2. Σε καμία από τις καλλιέργειες δεν παρατηρήθηκε συσσώρευση 1,1-DCE. Τριχλωροαιθένιο προστέθηκε αρκετές φορές κατά τη διάρκεια της επώασης των μικρόκοσμων. Έπειτα από την βιομετατροπή του σε cis- και trans-DCE, τα τελευταία μαζί με το υπολειπόμενο TCE απογυμνώνονταν από τις καλλιέργειες με χρήση ροής αζώτου. Παρατηρήθηκε ότι οι ρυθμοί βιοαποδόμησης στους μικρόκοσμους γίνονταν μέγιστοι 5 με 8 ημέρες έπειτα από την προσθήκη τριχλωροαιθενίου. Κατά την 15η εβδομάδα επώασης των μικρόκοσμων παρουσιάστηκε μέγιστη ανάπτυξη των μικροοργανισμών με μέγιστο ρυθμό

αποχλωρίωσης 55.0±7.5 μmol/L/d για τους μικρόκοσμους με το γαλακτικό και 36.1±15.8 μmol/L/d για εκείνους με το πυροσταφυλικό άλας. Παρ'όλα αυτά, μέτα από την πλήρη βιομετατροπή των οργανικών ενώσεων, οι ρυθμοί αποχλωρίωσης του TCE ελαττώθηκαν κατά την 18η εβδομάδα επώασης (12.0±0.3 μmol/L/d για τις καλλιέργειες που περιείχαν το γαλακτικό άλας και 5.2±3.3 μmol/L/d για εκείνες με το πυροσταφυλικό άλας). Ασυνήθιστη μεταβολή παρουσιάστηκε στην παραγωγή των DCE ισομερών. Ενώ τις πρώτες εβδομάδες το cis-DCE ήταν το κυρίαρχο προϊόν της αποχλωρίωσης του TCE, οι ρόλοι εντέλει αντιστράφηκαν καθώς μεγαλύτερες ποσότητες του trans-ισομερούς παρήχθησαν πρώτα στους μικρόκοσμους με το πυροσταφυλικό άλας και έπειτα σε αυτούς με το γαλακτικό. Υπεύθυνο θεωρήθηκε ένα είδος βακτηρίων του γένους *non-Dehalococcoides*, ονόματι VLD-1, το οποίο έχει 98.8% γενετική ομοιότητα με το *Dehalobium chlorocoercia* DF-1 το οποίο παράγει περισσότερα trans- από cis-DCE κατά την αφαλογόνωση του TCE.

Από τους μικρόκοσμους με το γαλακτικό άλας δημιουργήθηκαν δύο σετ υποκαλλιεργειών. Στο ένα εκ των δύο προστέθηκαν αντιβιοτικά για τον εμπλουτισμό των μικροοργανισμών του γένους *non-Dehalococcoides*, οι οποίοι αποτελούσαν το 17% της συνολικής βακτηριακής κοινότητας στις προηγούμενες καλλιέργειες εμπλουτισμού, και δύναται να παρουσιάσουν ανθεκτικότητα σε αυτά. Οι μισοί μικρόκοσμοι περιείχαν αμπικιλλίνη, βανκομυκίνη και 2-βρωμοαιθάνιο-σουλφονικό (BES) και οι υπόλοιποι μόνο BES. Κατά τις πρώτες εβδομάδες επώασης έπειτα από προσθήκη TCE διαμεσολάβησε περίοδος προσαρμογής των μικροοργανισμών στα αντιβιοτικά. Από τη δεύτερη φορά και έπειτα τα βακτήρια εκτέλεσαν αναγωγική αποχλωρίωση του TCE με μέσο λόγο παραγωγής cis- προς trans-DCE 4.2±0.8 στους μικρόκοσμους με τα τρία αντιβιοτικά και 4.0±0.4 στους μικρόκοσμους με το BES. Μοριακή ανάλυση της μικροβιακής κοινότητας πραγματοποιήθηκε στους μικρόκοσμους με τα τρία αντιβιοτικά. Το VLD-1 ήταν το βακτήριο που παρουσίασε ανθεκτικότητα στα αντιβιοτικά και εμπλουτίστηκε αποτελώντας το 69% της συνολικής μικροβιακής κοινότητας.

Το δεύτερο σετ υποκαλλιεργειών που δημιουργήθηκε αποτελείται από μικρόκοσμους στον καθένα εκ των οποίων προστέθηκε ξεχωριστά

εξαχλωροβενζόλιο (HCBe), μίγμα πολυχλωριωμένων διαφαινυλίων (PCB, Aroclor 1254), 1,2,3,4-τετραχλωροδιβενζοπαραδιοξίνη (1,2,3,4-TeCDD), TCE και πενταχλωροφαινόλη (PCP). Υπό την παρουσία ιζήματος και νερού από την περιοχή ενδιαφέροντος στις καλλιέργειες εμπλουτισμού οι μικροοργανισμοί μπορούσαν να εκτελέσουν αναγωγική αφαλογόνωση σε όλους τους υπό εξέταση ρύπους. Αντίθετα, στο καθαρό μεταλλικό μέσο, αφαλογόνωση εκτελέστηκε μόνο στους μικρόκοσμους με το τριχλωροαιθένιο. Αυτό συνέβει είτε γιατί κατά το διαδοχικό σχηματισμό υποκαλλιεργειών τα βακτήρια έχασαν την ικανότητα παραγωγής ενζύμων που καταλύουν αποχλωρίωση των υπόλοιπων ρύπων είτε γιατί δίχως την παρουσία ιζήματος τα βακτήρια δε μπορούσαν να προστατευτούν από πιθανές υψηλές συγκεντρώσεις των ουσιών καθώς η μετέπειτα προσθήκη TCE στους μικρόκοσμους με τους αρωματικούς ρύπους έδειξε ότι τα υπεύθυνα για την αποχλωρίωση βακτήρια δε ζούσαν.

Συμπερασματικά, σχηματίστηκε καθαρό μεταλλικό μέσο το οποίο υποστήριξε την αναγωγική αφαλογόνωση τριχλωροαιθενίου, η οποία διεγέρθηκε με την προσθήκη γαλακτικού και πυροσταφυλικού άλατος. Ο φυλότυπος VLD-1, κυρίαρχος της διεργασίας αποχλωρίωσης, αποδείχθηκε ανθεκτικός υπό την παρουσία των αντιβιοτικών αμπικιλλίνη, βανκομυκίνη και BES και εμπλουτίστηκε σημαντικά. Στους μικρόκοσμους με χλωριωμένους αρωματικούς ρύπους δεν παρατηρήθηκε αναγωγική αποχλωρίωση. Χρειάζεται να διεξαχθούν επιπλέον πειραματισμοί με αρωματικούς και αλειφατικούς οργανικούς ρύπους στο ίδιο ή και σε διαφορετικό μεταλλικό μέσο εμπλουτισμού καθώς και έρευνες των ενζύμων που εκφράζονται κατά τη διεργασία της αναγωγικής αφαλογόνωσης για την κατανόηση της δράσης των βακτηρίων υπό διαφορετικές συνθήκες ώστε να χρησιμοποιηθούν σωστά σε εφαρμογές βιοεξυγίανσης περιβάλλοντος.

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CHAPTER 1

INTRODUCTION

1.1. Halogenated organics and reductive dehalogenation

In the last few decades, groundwater and marine contamination from halogenated organics poses a relatively modern threat due to anthropogenic activities. Pesticides, industrial chemicals and by-products have significant effects such as acute and chronic toxicity to aquatic organisms, accumulation in the ecosystem as well as hazards to human health. They consisted of one or more halogen atoms and their structure is ranging from a simple aliphatic to complicated polyaromatic such as dioxins (**Figure 1**). The physical and chemical characteristics of these compounds have enabled them to be used in a large range of applications. Some of the most notable examples include: Polychlorinated biphenyls (PCBs) which were formerly used widely as heat transfer agents in electrical transformers and capacitors, as hydraulic fluids, and in carbonless copy paper, chlorobenzenes in the manufacture of other organic chemicals, dyestuffs and insecticides, perchloroethene (PCE) as dry cleaning solvent (Mohn & Tiedje, 1992; Lee, Marquis, Judger & Manefield, 2015). Because of their utility, these compounds have been synthesized in enormous quantities. They are subject to long-range atmospheric transport and thus they can deposit in oceans by dry-deposition of particulate-bound pollutants, diffusive gas exchange between the lower atmospheric layer and the water surface or scavenging by rain (Jurado et al., 2004). After their enter in the water, their low solubility allows them to bound to the particulate materials of the sediments and become accessible to marine biota and therefore enter the food web.

The majority of halogenated compounds are chlorinated, but brominated and fluorinated aromatic compounds are also in use. Marine sediments are among the most relevant long-term reservoirs of these compounds. A diverse range of marine organisms such as phytoplankton, mollusks, algae, polychaetes, jellyfish

and sponges are known to produce organohalide compounds (Häggblom & Bossert, 2003). As these compounds are generally recalcitrant and microorganisms in the seawater aren't able to metabolize them, they end up buried in marine subsurface sediments. A few centimeters below sediments' surface, in the absence of oxygen, microbial processes based on the metabolic activities of anaerobic bacteria are very effective in the degradation of the halogenated organics (Doble & Kumar, 2005; Zanaroli, Negroni, Häggblom & Fava, 2015). Due to their electronegative nature, organohalides tend to behave like electron acceptors and to be dehalogenated reductively. The degradation of organohalides by anaerobic microorganisms leads to the formation of less toxic compounds able to be degraded under aerobic conditions. These strictly anaerobic bacteria have evolved using naturally occurring halogenated organics as their terminal electron acceptor, in a respiratory manner, known as organohalide respiration (OHR). Essentially, this process is based on organohalide-respiring bacteria's capability to derive energy for growth from dehalogenation of aromatic and aliphatic halogenated compounds (Jackson, 2004). Alternatively, reductive dehalogenation is carried out co-metabolically, meaning the transformation of the halogenated contaminant occurs fortuitously during microorganism growth on another substrate.

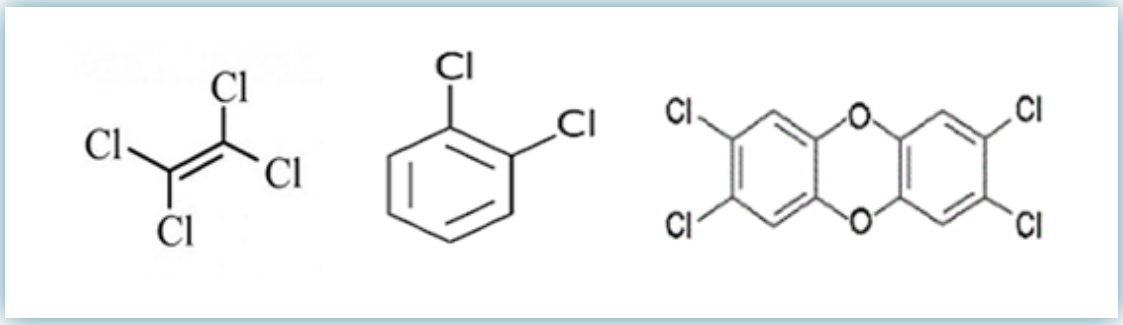

Figure 1: Examples of organohalides. From left to right: Perchloroethene (PCE); ortho-dichlorobenzene;2,3,7,8-tetrachlorodibenzo-p-dioxin (TeCDD)

Organohalides that will be used for reductive dehalogenation in this project are chlorinated compounds as they constitute the majority. Some of them are included in the initial list of 12 persistent organic pollutants (POPs) covered under the Stockholm Convention, which calls for direct measures and international cooperation to control the production, use, trade and disposal of

these substances due to their toxicity and persistence in the environment. Thus, it is considered necessary to describe their properties and how they end up in the marine sediments.

1.1.1. Polychlorinated biphenyls.

Polychlorinated biphenyls (PCBs) are synthetic chlorinated organic compounds (Figure 2), that were widely used in industry from 1929 as heat-transfer fluids, dielectric fluids in capacitors and transformers because of their non-flammable nature and chemical stability. Also they were used as additives in paints, pesticides, adhesives, copying paper, sealants and plastics. They became world-renowned with a variety of commercial names such as Aroclor (USA), Clophen (Germany), Phenoclor & Pyralene (France), Kanechlor (Japan), Fenclor (Italy), Soval (Russia) and Delor (Czech Republic). Studies documenting PCB degradation, and thus in this study, frequently refer to Aroclor which is specified with a four-digit code. The first two numbers in the code refer to the parent structure (12 indicating biphenyl) and the second two digits refers to the weight percentage of chlorine. For instance, Aroclor 1260 refer to PCB mixture with an average weight percentage of chlorine 60% (Field & Sierra-Alvarez, 2008a).

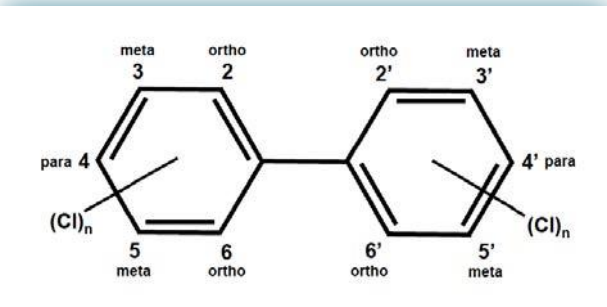

Figure 2: PCB molecule

A PCB is one of 209 compounds having the formula $C_{12}H_{10-n}Cl_n$, where n=1-10, since chlorine atoms have theoretically the possibility to bond with any of the 10 available carbon atoms of the biphenyl. Nevertheless, chlorine atoms usually occupy 4 to 6 from the 10 available positions and therefore in practice only 130 compounds have been synthesized and used. The entire set of 209 PCBs forms a set of congeners.

PCBs have entered the environment through both use and disposal. They are highly chemically stable and resist microbial, photochemical, chemical and thermal degradation. They are physically stable with very low vapor pressures and water solubility. Thus, PCBs do not readily degrade in the environment and are lipophilic, so they persist and tend to bioaccumulate (Erickson, 2001). Because of these properties, PCBs belong in the list of the chemical substances considered as POPs. Moreover, PCBs are transported by air, water, fish ,birds and other routes. They are deposited from air by rain, snow, dry fallout and vapor-phase deposition. They tend to migrate from the lower latitudes where evaporation predominates to the Polar regions where deposition predominates, but most of them are retained in a sink such as deep oceanic sediments (Wania & Mackay, 1996).

Microbial degradation of PCBs can be an important environmental endpoint for PCBs. The kinetics of degradation is primarily dependent on PCB concentration, as well as microbe type and population, temperature, nutrients and a host of other conditions, but is generally slow with half-lives on the order of days-to-weeks under optimal conditions and obviously very much longer under non-conducive conditions. The kinetics of degradation also depend on the degree of chlorination and the position of the chlorine atom on the biphenyl molecule. Under anaerobic conditions such as aquatic sediments, microorganisms partially dechlorinate the more highly chlorinated congeners. Under aerobic conditions, microorganisms prefer to attack the lower chlorinated congeners, ultimately breaking open the phenyl rings to produce carbon dioxide, water and chloride ions through a chlorinated benzoic acid intermediate, although intermediate degradation products are often observed. Under natural conditions, microbial degradation appears to be not only slow, but also generally incomplete. For example, 2% to 45% of the original congeners were reductively dechlorinated in St. Lawrence River (NY) sediments (Sokol, Kwon, Bethoney & Rhee, 1994). As a result, accumulation of weathered PCBs is often documented in aquatic sediments (Moret et al., 2001; ATSDR, 2006; OAP, 2017).

1.1.2. Polychlorinated phenols.

Phenol is an aromatic compound derived from benzene and can have one to five chlorines (**Figure 3**). There are 5 basic types of chlorophenols and 19 different chlorophenols. The 5 basic types are:

- Pentachlorophenol (PCP)
- Tetrachlorophenol (TeCP)
- Trichlorophenol (TCP)
- Dichlorophenol (DCP)
- Chlorophenol (CP)

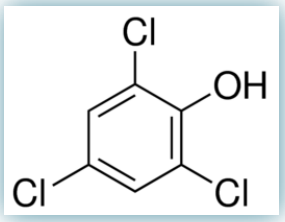

Figure 3: 2,4,6-trichlorophenol

Some chlorophenols such as 2,4-DCP and 2,4,5-TCP are used in the production of herbicides. Other are used as antiseptics. Small amounts are produced when water is disinfected with chlorine. They are also produced while bleaching wood pulp with chlorine to make paper (Field & Sierra-Alvarez, 2008b).

General toxicity of PCP is caused by its ability to act as an uncoupler of oxidative phosphorylation and when inhaled it can cause neurological, blood and liver defects and eye irritation in humans. Long-term exposures by inhalation may also result in damage of the respiratory tract, blood, kidney, liver, immune system, eyes, nose and skin (Lopez-Echartea, Macek, Demnerova & Uhlik, 2016).

Chlorophenols can enter the environment when they are being made or used as pesticides. Most of them go into water whereas small amounts enter the air. In the air, sunlight helps destroy these compounds and rain washes them out of the air, whereas in water chlorophenols stick to sediments. Low levels of chlorophenols in sediments, water or soil are broken down by microorganisms in a few days to weeks (ATSDR, 1999).

Several studies evaluated the anaerobic degradation of chlorinated phenols in marine and estuarine sediments. For example, reductive dechlorination of 2,4-DCP to 4-CP and 3-CP in slurries of marine sediments was reported by Boothe, Rogers & Wiegel in 1997. Estuarine sediments from the lower Hudson River in New York were able to degrade 2-CP, 3-CP and 4-CP under sulphate reducing conditions (Häggblom, Rivera & Young, 1993).

1.1.3. Chlorobenzenes.

The chlorobenzenes are important environmental contaminants that are used for both private and industrial applications. They have been used as pesticide compounds, components of dielectric fluids and solvents and their wide application spectrum has led to environmental contamination worldwide. This class of compounds is formed by the chlorine substitution of a single benzene ring and it is comprised of 12 chlorobenzenes isomers ranging from mono- to hexa-substituted. Due to their hydrophobic nature, chlorobenzenes especially accumulate in river and harbour sediments (Barber, Sweetman, van Wijk & Jones, 2005), where microbial dechlorination occurs. While lower chlorinated benzenes can be oxidized under aerobic conditions (Field & Sierra-Alvarez, 2008c), highly chlorinated benzenes are primarily degraded under anaerobic conditions involving reductive dechlorination processes.

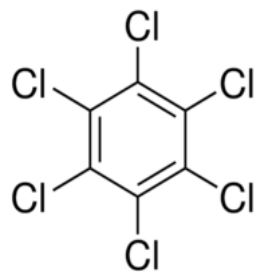

Figure 4: Hexachlorobenzene (HCBe)

Figure 4 depicts the most chlorinated benzene (HCBe) which is also the type of chlorobenzene used in this project. Hexachlorobenzene was listed as one of the 12 persistent organic pollutants (POPs) by the United Nations Stockholm Convention due to its toxicity and persistence. Although HCBe is no longer manufactured as a commercial end product in most countries, it is still being released as a by-product during the synthesis of other chemicals and pesticides, as a result of partial combustion, or from old landfills (Barber et al., 2005). Therefore it can be found in air, water, soils and sediments, where it accumulates and enter the food chain (ATSDR, 2015).

1.1.4. Polychlorinated dibenzo-p-dioxins.

Polychlorinated dibenzo-p-dioxins (PCDDs), commonly known as dioxins, is a group comprised of 75 different compounds (congeners). They have a planar aromatic tricyclic structure with 1-8 chlorine atoms as substituents. The most

recognized example is the so-called “dioxin of Seveso”, since it was the main contaminant in the Seveso contamination incident (Eskenazi et al., 2001), of which official nomenclature is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Figure 1 shows the structure of this compound. 2,3,7,8-TCDD is the most toxic congener, the one most studied and it has been characterized by World Health Organization as carcinogenic.

PCDDs can be emitted by different human activities and industrial processes where they can be present as unwanted by-products. They can also result from natural processes like volcanic eruptions and forest fires (Srogi, 2008) but with very little contribution to the observed levels of the industrialized countries. Most industrial countries have restricted or stopped their use since 1970s, leading to decreasing concentrations in long-term environmental surveillance programmes (Thompson et al., 2007). However, compounds remained in ecosystems either due to their persistency or transport from developing countries where use in agricultural and industrial purposes is still current. Since they can be transported over long distances from the source of emission, PCDDs are characterized as persistent organic pollutants (POPs). Due to their chemical stability and hydrophobic nature, these compounds are adsorbed onto sediment particles, accumulated in aquatic organisms and highly biomagnified through the aquatic food webs (Pereira, 2004).

Adverse effects induced by PCDD exposure include dermal toxicity, immunotoxicity, reproductive deficits, teratogenicity, endocrine toxicity, and carcinogenicity/tumor promotion (ATSDR, 1998).

1.1.5. Chloroethenes.

Chloroethenes are aliphatic compounds that are produced in great amounts in nature, most of which are produced by biota in the marine environment (Gribble 2003). One of the main sources is marine algae, but also abiotic sources such as volcanoes and biomass burning produce considerable emissions. However, some chlorinated derivatives of ethene are not produced naturally in the environment. Tetrachloroethene (PCE) and trichloroethene (TCE), which for years were considered to be compounds of anthropogenic origin only, can be produced by

seaweed (Ballschmiter 2003). Also, vinyl chloride (VC) may be produced naturally in reaction of humic acids, iron (III) and chlorides.

Chloroethenes are used as degreasing and cleaning agents, paint removers and industrial solvents, also in the production of pesticides, electronic components and polymers. VC can be leached from PVC piping (Walter, Lin, Edwards & Richardson, 2011). Highly chlorinated ethenes such as tetrachloroethene (PCE) and trichloroethene (TCE) are less soluble, denser and have the tendency to be adsorbed onto bottom sediments. PCE and TCE are commonly occurring chloroethene contaminants due to their extensive usage along with careless handling and storage (Moran, Zogorski & Squillace, 2007). Therefore, from those common pollutants, TCE is chosen to be used in the experiments of this thesis.

Trichloroethene or trichloroethylene (TCE) is a colorless, nonflammable, volatile liquid and known human carcinogen. It can usually be found in high levels in the water and soils near industrial sites where it is produced or discarded (ATSDR, 2014).

Anaerobic degradation of both PCE and TCE by microorganisms takes place through a series of steps, each consisting of progressively less chlorinated ethenes, i.e. via cis-DCE (dichloroethene), trans-DCE, and 1,1-DCE and VC to ethene. The main dechlorination pathways of chlorinated ethenes are presented in Figure 5.

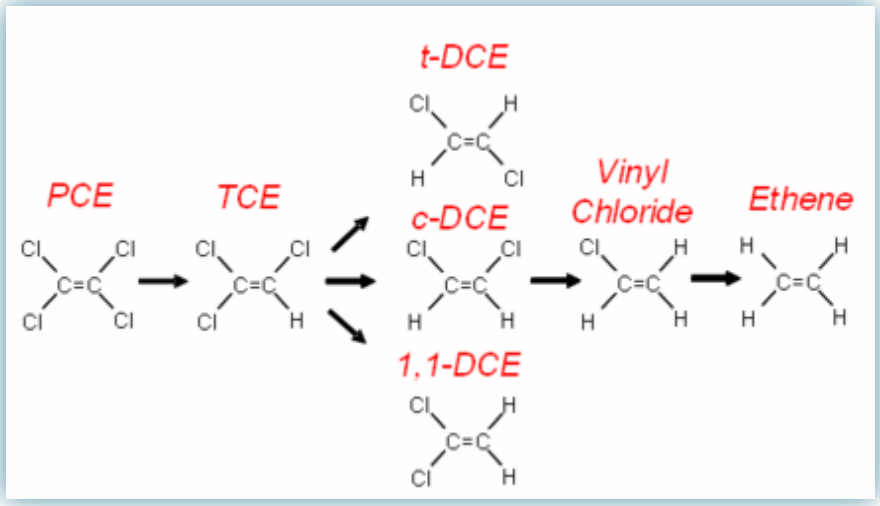

Figure 5: Pathway for anaerobic microbial degradation of chloroethenes.

1.2. Organohalide-respiring bacteria and reductive dehalogenases

The dehalogenation of halogenated organics like the contaminants mentioned above can be catalyzed by anaerobic bacteria called organohalide-respiring bacteria (OHRB), which are using these contaminants to derive energy for growth. They have been identified from diverse bacterial phyla, including the Proteobacteria, Firmicutes and Chloroflexi (Holliger et al., 1998; Sung et al., 2006; Nonaka et al., 2006; Atashagi, Lu & Smidt, 2016). The known OHRB can be grouped as either obligate or non-obligate organohalide respirers based on their capacity to use a broader range of electron acceptors and donors. (Maphosa, de Vos & Smidt, 2010). The proteobacterial OHRB, including *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter* and *Sulfurospirillum,* are all non-obligate organohalide respirers with versatile metabolisms encoded on relatively large genomes. In contrast, the organohalide respiring Chloroflexi (e.g. *Dehalococcoides* and *Dehalogenimonas*) are all obligate organohalide respirers with a very restricted metabolism. The Firmicutes contain non-obligate organohalide-respiring *Desulfitobacterium* spp. as well as metabolically restricted *Dehalobacter* (Maphosa et al., 2010).

The OHRB conserve energy by utilizing H_2 or organic compounds as electron donors and organohalides as electron acceptors. The key enzyme that catalyzes halogen removal is called reductive dehalogenase (RDase), which is encoded by reductive dehalogenase homologous gene (rdh). Rdh denotes all proteins inferred to catalyze a reductive dehalogenation reaction based on sequence information or experimental validation (Fincker & Spormann, 2017). A typical *rdh* gene cluster consists of the *rdhA* (encoding the catalytic subunit A of the enzyme, RdhA) and *rdhB* (encoding a putative membrane-anchoring protein for RdhA) genes, as well as other regulator genes (Maphosa et al., 2010). Purified RdhA enzymes are highly oxygen sensitive and composed of approximately 500 amino acids. With few exceptions, the catalytic subunit contains a corrinoid and two Fe–S clusters (either two 4Fe–4S or one 4Fe–4S and one 3Fe–4S) and has been localized at the outer side of the cytoplasmic membrane (Futagami, Goto & Furukawa, 2008). The Fe–S clusters are thought to be involved in electron

transfer from the physiological electron donor to the catalytic site and the corrinoid cofactor is assumed to play a central part in substrate activation and reduction (Figure 6).

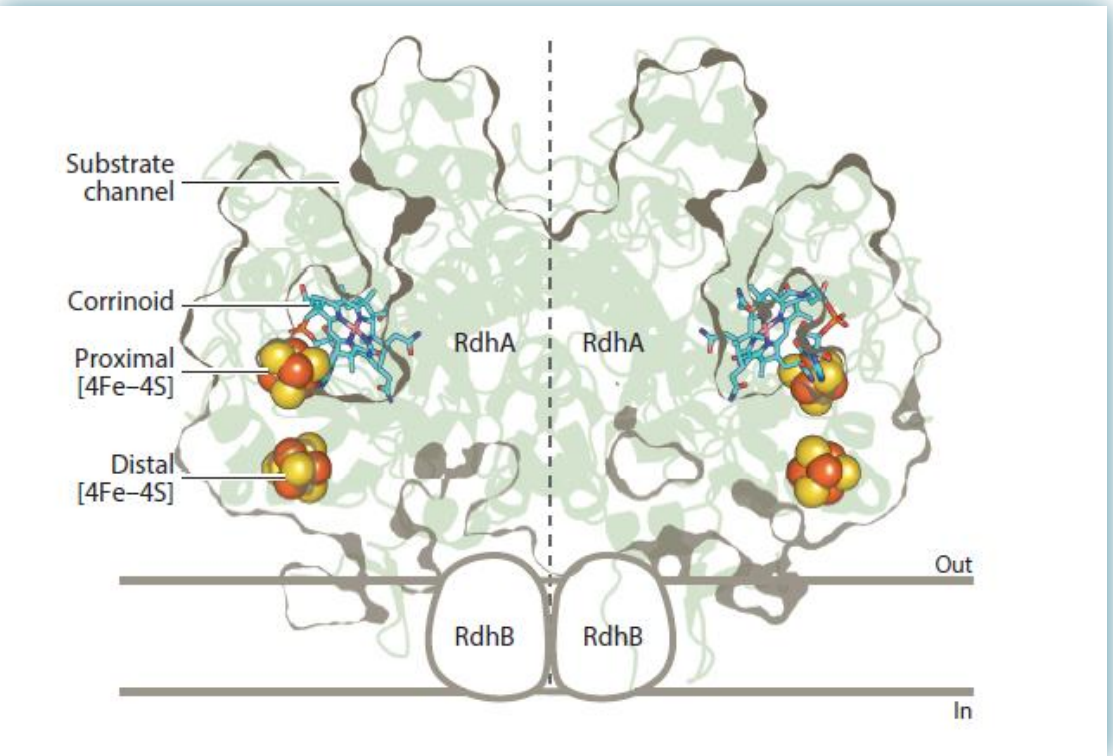

Figure 6: Proposed structure of a generic RDase based on the X-ray crystallographic structure of dimeric PceA of *Sulfurospirillum multivorans* (Bommer et al., 2014). Each monomer seems to be catalytically independent. RdhB, the putative membrane anchoring subunit, is shown to illustrate the association of RdhA with the outer surface of the cytoplasmic membrane.

Completion of the genome sequences of dehalorespiring species is an ongoing situation which has greatly increased over the years and it intends to foster better understanding of their physiology and metabolism as well as to identify similarities and differences between these ecologically important organohalide-respiring bacteria.

Research findings indicate that pure cultures of obligate species, such as *Dehalobacter* and *Dehalococcoides*, can be onerous to cultivate due to their slow growth rate and fastidious nutritional requirements and therefore the use of mixed enrichment cultures is preferable (Jugder et al., 2016). In enrichment cultures, the biochemical characterization of the responsible RDases catalyzing dehalogenation is more accessible. Identification of functionality of RDases,

which are coded by abundant *rdh* genes, is a major step towards optimizing remediation approaches by selecting the most effective and efficient enzymes.

Marine environment is a huge resource of marine organisms and the marine microorganisms, like OHRB, are highly abundant. However, there are not plenty information about the diversity of OHRB and their *rdh* genes as many of them fall under viable but unculturable group. To overcome this problem and to study the diversity pattern of these marine bacterial species, many advanced techniques like metagenomics, 16S rRNA gene amplification, denaturing gradient gel electrophoresis and cloning have been used.

Figure 7 shows genetic diversity among cultured isolates from different species and depending on genome size, they are classified as metabolically versatile or restricted OHRB.

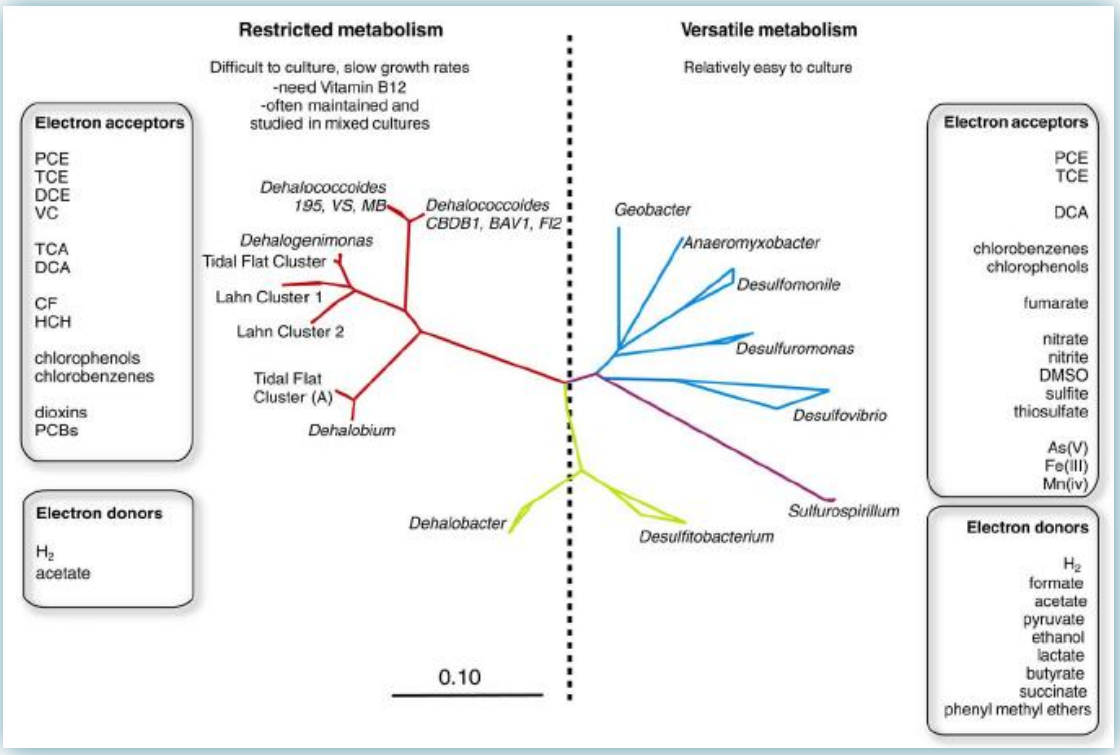

Figure 7: Phylogenetic tree of OHRB based on bacterial 16S rRNA sequences (Maphosa, de Vos & Smidt, 2010). The reference bar at the bottom centre indicates the branch length that represents 10% sequence divergence. Electron donors and acceptors are listed in the flanking text boxes, and are grouped according to their chemical nature and complexity. Halogenated electron acceptors listed are restricted to chlorinated compounds, however, there are many other organohalide compounds that these OHRBs can respire. Color key: Chloroflexi (red), Deltaproteobacteria (blue), Epsilonproteobacteria (purple), Firmicutes (green).

Isolates belonging to the genera *Anaeromyxobacter*, *Desulfitobacterium*, *Sulfurospirillum*, *Desulfomonile*, *Desulfuromonas*, *Desulfovibrio* and *Geobacter* are metabolically versatile with respect to their spectrum of electron donors and acceptors, and can dehalogenate a wide range of halogenated aromatic and aliphatic compounds. *Dehalococcoides* and *Dehalobacter spp.* isolates appear as highly specialized bacteria that strictly depend on organohalide respiration for growth, in most cases coupled to hydrogen as the sole electron donor. The fact that many OHRB are notoriously difficult to culture and do not grow to high density poses a great challenge for their detailed physiological and genetic characterization. For example, *Dehalococcoides spp.* are difficult to maintain in pure culture, but are more easily grown in a microbial community on which they depend for H_2 supply (Holmes, He, Lee & Alvarez-Cohen, 2006). Similarly, several *Dehalobacter spp.* have now been reported to grow only in strict coculture or consortia (Grostern & Edwards, 2009; Yoshida, Ye, Baba & Katayama, 2009), raising thoughtful questions about the possible role of syntrophic interactions that could act as drivers for dehalogenation. Knowledge of the metabolic interactions of species able to perform dehalogenation as well as their specific degradation abilities in indigenous microbial communities sets the ground for the design of successful bioaugmentation and biostimulation approaches.

As it was mentioned before, in the marine environment, organohalides are produced either naturally or due to anthropogenic activities and they end up accumulating in the subsurface sediments. A small fraction of the bacterial community of the sediment has the ability to degrade the naturally occurring compounds and some of industrial relevance. It is reported that in marine sediments there is a high diversity of reductive dehalogenase homologous (*rdhA*) genes, indicating that marine microorganisms are able to dehalogenate more halogenated compounds (Futagami, Morono, Terada, Kaksonen & Inagaki, 2013).

One objective of this project is to test dehalospiring microbes ability, from marine contaminated habitat, to dehalogenate different aliphatic and aromatic organohalides. Therefore, collected marine samples (water + sediment) will be used to set up enrichment cultures and check bacteria's dehalogenating activity over several halogenated organics. Patterns and rates of dehalogenation will be estimated by analyzing the concentrations of the pollutants and their products in the cultures.

Despite the fact that reductive dehalogenases have been discovered more than 30 years ago, their substrate range and specificity are not completely understood mainly due to slow growth of OHRB. In this project dehalogenation activity will be correlated with the use of different fermentable organic acids provided to the cultures as electron donors as well as other nutrients and elements. The aim of this, is to find the proper conditions to stimulate the growth and dehalogenation activity of organohalide-respiring bacteria.

At the end of this work, it is expected to acquire insight on the conditions that favor the growth of OHRB and preserve it, the organohalides degraded and the biodegradation patterns. The selection of the electron acceptor to be used is a significant factor to be also investigated and it is aimed to form sustainable cultures with good dehalogenation performance. This experiment will contribute to gene expression analyses to gain functional view of dehalorespirers' biology and improvement of the use of OHRB for *in situ* bioremediation.

The following chapter discusses about marine bioremediation strategies and delves into the anaerobic biodegradation of organohalides. Section 2.2.4. is directly connected to this project and provides substantial information, and thus it is suggested not to be omitted. Chapter 3 outlines the experimental work carried out and Chapter 4 discusses in detail the results. The final chapter states the conclusions of this study while summing up the main results.

CHAPTER 2

LITERATURE REVIEW

2.1. Bioremediation of marine sediments contaminated with recalcitrant compounds

2.1.1. Introduction

The focus in this chapter is in bioremediation approaches, not only because of the nature of this study but also because these methods, contrastingly of others, are cost-effective and non-invasive, leaving the ecosystem intact (Perelo, 2010). Bacteria have a huge potential for environmental restoration and thus the use of them for biodegradation of various natural and synthetic substances is increasingly drawing attention. Especially, remediation of aquatic sediments is of high importance since they are usually recognized as both the carrier and potential source of toxic organic compounds like POPs. Marine bacteria possess a wide variety of bioremediation potentials which are beneficial from both environmental and economic point of view (Dash, Mangwani, Chakraborty, Kumari & Das, 2013). It is the metabolic ability of the microorganisms to transform organic contaminants into less harmful substances which can be integrated into natural biogeochemical cycles. The process of metabolic pathways requires transfer of electrons from electron donors to electron acceptors. The electron donors act as food for microbes which is usually limited in a non-contaminated site. However, in a contaminated site release of an organic electron donor may stimulate microbes to compete for available acceptors to restore the balance of the system. In seawater, microbes use oxygen as final electron acceptor to convert organic and inorganic pollutant into harmless products, often carbon dioxide and water. In sediments, oxygen is not present or limited and the microbes use other electron acceptor such as nitrate, iron, sulphate, etc. to break down organic compounds often into carbon dioxide and methane.

For bioremediation strategies, the metabolic capabilities of marine bacteria for aerobic and anaerobic degradation are exploited. In case of pollutants in the seawater, aerobic bacteria are responsible for the contaminant transformation whereas in the marine sediments the degradation is taking place because of the metabolic or co-metabolic activity of anaerobic bacteria.

2.1.2. Bioremediation strategies

Sediment remediation techniques are commonly classified as *in situ* (i.e., treatments operating where the contamination is present with no sediment dredging) and *ex situ* (i.e., treatments typically take place off-site and require sediment dredging. Generally, dredging activities or natural resuspension phenomena like strongly adverse weather conditions can remobilize pollution releasing it into the water column. Thus, *ex situ* remediation activities can be hazardous compared to *in situ* techniques that try to keep to a minimum sediment mobilization, unless dredging is compulsory to reach a desired bathymetric level (Lofrano et al., 2017).

When the level of contaminant is relatively low, there is a passive *in situ* bioremediation strategy, called monitored natural attenuation (MNA), which requires indigenous microbes able to degrade, transform or immobilize certain pollutants under favorable conditions without any human intervention, but with monitoring at defined times. This method is considered the most appropriate when it can be assured that contaminants will be reduced or transformed in an acceptable time. Usually, there is need to enhance the growth of the microbial degraders in order to have more efficient remediation results since in the marine environment the microbes ability to effectively stimulate biodegradation is generally low. Therefore, enhanced bioremediation can be performed, which relies on inoculation of specific competent microbes or consortia of microbes in contaminated sites with the ability of degradation (Tyagi, da Fonseca & de Carvalho, 2011). Such microbial consortium can be stored under laboratory conditions and subsequently added to contaminated sites for cleaning the pollutant. However, it is important to know the substrates and nutrients required to be added in order to assist the degrading process.

The two approaches, often complementary, able to enhance the cleaning action of microorganisms are bioaugmentation and biostimulation. In bioaugmentation, the addition of degrading bacteria and nutrients boosts bioremediation rates whereas in biostimulation, the growth of indigenous microbial degraders is stimulated by the addition of electron donors, especially the addition of growth-limiting nutrients. In bioaugmentation the added bacteria are pre-cultured with known degradation activity and usually in order to be successful the use of biostimulation approach is necessary (Ellis, Lutz, Odom, Buchanan & Bartlett, 2000). Despite this, the decision to implement either or both of these techniques for bioremediation largely depends on the degrading capability of the indigenous microbes and the extent of contamination of the site to be treated.

When the concentration of contaminants is very high, *ex situ* bioremediation is required. A good option to restore sediments contaminated with high concentrations of toxic organic compounds is by using slurry bioreactors. In slurry bioreactors the sediments are mixed with equal or greater amounts of water and mixed with microbes and nutrients to form a sediment slurry. The treatment mechanism is natural degradation by existing and/or added populations of microorganisms. Except for *in situ* application, the previous strategies mentioned above (natural attenuation, biostimulation and bioaugmentation) can be also applied within the bioreactors. They are designed to operate anaerobic and aerobic processes. Polychlorinated biphenyls, chlorophenols, chlorinated ethenes and other organics can be partially or fully degraded in bioreactors by providing the proper conditions (Robles-González, Fava & Poggi-Varaldo, 2008). The use of anaerobic and aerobic steps in series offers a method to treat substances that do not respond to conventional treatment. An example is highly chlorinated organic pollutants. Anaerobic bacteria can dechlorinate the substance to a point where aerobic bacteria can completely degrade it. This method has been observed in natural environment (Beeman & Bleckmann, 2002; Abe, Aravena, Zopfi, Parker & Hunkeler, 2009; Tiehm & Schmidt, 2011) and has been undertaken in batch reactors (Gerritse, Renard, Gottschal & Visser, 1995; Tartakovsky, Manuel & Guiot, 2003; Frascari Fraraccio, Nocentini & Pinelli, 2013). Using sequential anaerobic and aerobic

degradation, complete dechlorination of PCE is possible (Gerritse et al., 1995). Nevertheless, the use of slurry bioreactors for remediation has a few disadvantages, as they are related to requirements for sediment excavation and handling, and also bioreactor construction/operation that typically increase treatment costs compared to most simple bioremediation techniques.

Generally, *in situ* bioremediation of sediments contaminated with toxic and persistent substances like halogenated organic compounds is an attractive choice because it can avoid the problematic redistribution of contaminants that is associated with dredging and would decrease the cost of sediment management. Reductive dehalogenation appears to be a potentially powerful process for achieving bioremediation of marine and estuarine sites contaminated with organic halogenated pollutants, if mechanisms and pathways of degradation are known and can be managed to achieve removal of the compounds of interest as well as potentially toxic metabolic degradation products. Microbial degradation requires the presence of enzymes that cleave the carbon-halogen bond under physiological conditions and microorganisms have evolved a variety of metabolic strategies for cleaving this bond. Dehalogenation reactions comprise different strategies, where organohalides serve either as electron donors (and carbon sources) or as electron acceptors. However, application of *in situ* marine bioremediation of organohalide-contaminated sediments has been limited by lack of fundamental knowledge about the microorganisms responsible for contaminant degradation, including anaerobic reductive dehalogenation, which is the first step required for the ultimate complete degradation of highly halogenated compounds. In addition, application of bioremediation requires the availability of suitable electrons donors and microbes to effectively stimulate biodegradation. In order for this to be accomplished, the use of certain growth media to favor the growth of particular autochthonous microorganisms with the ability to degrade recalcitrant compounds is required.

2.1.3. Incubation and enrichments

The most straightforward option to enrich and cultivate microorganisms of interest is to use the local environment as a “natural lab” and take the enriched

culture for analysis. This approach is mainly suited for marine microorganisms whose optimal growth conditions are not well defined but whose growth locations are. The composition of the medium and the incubation conditions that are applied are critical for the cultivation success. When cultivating any kind of microorganism, conditions should be adapted to natural environmental conditions, by at least adjusting the pH, salinity, temperature and in some cases pressure to simulate environmental conditions. The degree of specificity increases with the specificity of the requirements and tolerances of the organism. The smaller the ecological niche where the microorganism can thrive is defined, the more specifically the medium has to be prepared and the environmental conditions have to be simulated to allow cultivation. Thus, for any bacterium to be propagated in the labs, the conduction of thorough investigations of the ecological niche it belongs is necessary to provide the appropriate biochemical and biophysical environment. However, microbial interactions with the environment is not the only relation that should be considered. As far as isolation of marine bacteria is concerned, microbial interactions with the microbial community should not be omitted during cultivation in the laboratory. A mechanism for interaction that may be crucial to successful isolation of marine bacteria involves metabolic consortia. In a microbial consortium, the metabolism of one species results in the production of a compound or series of compounds, that can be metabolized by other species. Bacteria are capable of concerted action, driven by the different metabolic capabilities of different species (Joint, Mühling & Querellou, 2010). Clearly, these types of processes must operate in the natural environment and it must be common for one bacterium to depend on other bacteria for essential metabolites. Therefore, enrichments have to be carefully examined in order to be able to let the important for biodegradation species to grow without the elimination of the synergistic species.

2.2. Microbial degradation of organohalides in anaerobic sediments

2.2.1. Factors affecting organohalide dehalogenation

In anaerobic environments, in terms of microbial respiration and carbon flow, the most important electron acceptors are nitrate, sulfate, Fe(III), Mn(IV), and carbonate, which result in the processes of denitrification, sulfidogenesis, iron reduction, manganese reduction, and methanogenesis, respectively (Häggblom, Ahn, Fennel, Kerkhof & Rhee, 2003). Monserrate and Häggblom (1997) have demonstrated that reductive dehalogenation is the initial step in the biodegradation of halogenated phenols under sulfate-reducing and iron-reducing conditions, using enrichment cultures from anaerobic sediments collected at different marine sites. They concluded that dehalogenation was allowed under all reducing conditions tested without meaning that a single reducing condition necessarily permits the metabolism of all compounds tested. Dehalogenation rates depend on the reducing equivalents but also on the addition of auxiliary carbon sources. The addition of auxiliary carbon sources as an electron donor usually stimulates dehalogenation. The final electron donor is most of the times hydrogen and many different hydrogen-releasing substrates may be used as primary electron donors such as pyruvate, lactate, hydrogen and formate.

Organohalide respiration rates can be influenced through interaction with biogeochemical processes and through competition for hydrogen in diverse microbial populations (Azizian, Marshall, Behrens, Spormann & Semprini, 2010; Chambon et al., 2013). Thus, addition of an electron donor may not only stimulate the activity of dehalogenating microorganisms but also stimulate the activity of competing microbial populations, such as methanogens, acetogens and sulphate, nitrate, iron and manganese reducers. Methanogens, for example, can compete for hydrogen if there are high hydrogen concentrations and use it for methanogenesis (Aulenta, Gossett, Papini, Rossetti & Majone, 2005). At low hydrogen concentrations, dechlorinating bacteria have a higher affinity for the hydrogen and may outcompete the methanogens (Yang & McCarty, 1998). Thus, slow release of hydrogen donors favors dehalogenating bacteria. Consequently, the relationship between reductive dehalogenation and redox processes, such as

sulphate and iron reduction, has an important impact on the degradation process. Several studies have shown that sulphate and iron reduction compete for hydrogen and as a consequence anaerobic reductive dechlorination is inhibited (Aulenta, Beccari, Majone, Papini & Tandoi, 2008; Azizian et al., 2010; Paul, Jakobsen, Smolders, Albrechtsen & Bjerg, 2016).

Anaerobic degradation's dependency on the position of the halogen substituent is another factor to be considered. Bacteria tend to be less inclined to undertake reductive dehalogenation as the number of halogen substituents decreases due to the lower energetic yield of the metabolic reaction. That is the reason why fewer bacterial species are capable to degrading low-chlorinated hydrocarbons through organohalide respiration and thus such compounds often accumulate at sites where high-chlorinated ones are degraded (Aeppli et al., 2010; Imfeld et al., 2011). For chlorinated compounds, the rule of thumb is that the chlorine substituent that has the most neighboring chlorine substituents is the one most likely to be removed. Thermodynamically speaking and based on the assumption that dehalogenation pathway of a poly-chlorinated compound will follow sequentially, the steps that release the most energy then provide the most likely route for dechlorination (Häggblom & Bossert, 2003).

Another factor that determines biodegradation is the competency of the microbial population. Häggblom, Rivera and Young (1993) made this observation given that the pattern of dehalogenation activity appeared different for the chlorinated compounds utilized under each reducing condition tested, resulting in the conclusion that biodegradability is not determined by the position of the halogen substituent but rather by the presence of competent microbial populations.

An additional factor affecting transformation of halogenated organics in the sediments is the presence of toxic or inhibitory compounds like heavy metals that are harmful towards dehalogenating microorganisms. This is an important factor that needs to take into consideration when under the same conditions slower dehalogenation activity is observed in samples with different origin than others where the activity is more rapid. To function at a site in which natural or

synthetic inhibitors are present at injurious levels, the organism to be used must be resistant to the toxins.

Finally, another site-specific factors such as the geochemical features of the site and the low concentration of indigenous dehalorespiring populations may affect the effectiveness of dehalogenation, so for the performance of bioremediation techniques the optimization of the site conditions is required in terms of selecting the appropriate type of electron donors and the proper concentration.

2.2.2. Approaches for biostimulation and bioaugmentation under marine in situ mimicking conditions

In marine and estuarine sediments the enhancement of reductive dehalogenation of organohalides has been investigated in order to successfully stimulate the growth and activity of autochthonous dehalorespirers. In particular, the addition of alternative halogenated compounds as supplemented electron acceptors leads to the stimulation of PCB and PCDD reductive dechlorination of spiked contaminants in marine and estuarine sediment cultures established in synthetic media (Ahn, Häggblom & Fennell, 2005). This "priming" process is based on the idea that high concentrations of an appropriate dehalogenation substrate will selectively promote the growth of dehalogenating microorganisms that use the compound as an electron acceptor. The enhanced dehalogenating population will then dechlorinate the organohalides in the sediment. However, structural similarity of supplemented halogenated compounds towards the organohalides needed to dehalogenate plays an important role in order to successfully stimulate the bacteria's activity. Therefore, in the presence of different organohalides in the sediments, the halopriming technique will not be enough. Moreover, it is a process that takes time to observe apparent activity as shown by Ahn et al. (2005).

A second promising approach to biostimulation involves the addition of organic electron donors or hydrogen. A series of experiments in anaerobic contaminated sediments have provided evidence that reductive dechlorination by indigenous microorganisms is supported with the addition of fermentable

organic acids (Matturro, Ubaldi & Rossetti, 2016; Xu, Gregory & VanBriesen, 2018). The addition of carbon sources can benefit dehalogenation by providing sufficient carbon and energy sources for dehalogenating microorganisms or by stimulating the growth of essential co-species of OHRB. Yet, in case of polychlorinated biphenyls, supplementary carbon can result in the rapid growth of non-PCB dechlorinating microorganisms, which may ultimately inhibit PCB dechlorinating bacteria through competition (Wiegel & Wu, 2000; Zanaroli et al., 2012). With that being said, this is an approach that needs further experimentations with several organohalides and with several combinations of carbon sources in order to draw certain conclusions.

Another potential candidate for bioremediation on site is bioaugmentation, if the ecological and biogeochemical conditions of a site contaminated with organohalides are suitable and do not impede its application. It was shown that sediment geochemical properties can affect dechlorination activity of bioaugmented dehalorespiring PCB cultures. In particular, harbor sediments, rich in organic carbon and trace metals such as zinc, nickel and cobalt, support effectively the competition between indigenous and inoculated microorganisms for the dechlorination of PCBs (Yan, LaPara & Novak, 2006; Fagervold, Watts, May & Sowers, 2011). The availability of trace metals in sediment might be critical for the expression and production of dehalogenases. For instance, it has previously been demonstrated that cobalt is significant for the synthesis of corrinoids (Schumacher, Holliger, Zehnder, & Hagen, 1997) in corrinoid-dependent reductive dehalogenases (**Figure 6**). So, in order to insert a dehalogenating strain to the target environment, the latter has to have conditions that allow the bioaugmented microbes survival and growth.

Moreover, bioaugmentation seems to be a good approach for remediation when the target site misses certain strains able to assist dehalogenation process. For instance, for complete dechlorination of PCE to ethene, the addition of a microbial consortium that contained phylogenetic relatives of *Dehalococcoides ethenogenes*, capable of transforming intermediates (cis-DCE) to ethene (last step of dechlorination) is required (Major et al., 2002). It is the presence of proper *rdhA* genes inside the strains that supports dehalogenation activity in the

target environment. Thus, it can be said, that choosing bioaugmentation as remediation strategy is viable if the limiting factor of biodegradation is the absence of relevant catabolic genes within the indigenous microbial community and this lack of genetic information will be filled by the introduced strain.

2.2.3. Isolates of organohalide respiring bacteria in marine and estuarine environment

Of all known OHRB, the obligate organohalide-respiring bacteria of the Chloroflexi phylum seem to play the most prominent role in organohalide respiration in marine and estuarine sediments. The *ortho*-PCB-dechlorinating strain *o*-17 was isolated from a PCB dechlorinating culture enriched from estuarine sediments of Baltimore Harbor (USA) (Cutter, Watts, Sowers & May, 2001). This strain is able to dechlorinate PCBs, chlorobenzenes and PCE. Similarly, The PCB-dechlorinating *Dehalobium chlorocoercia* DF-1 is indigenous to estuarine sediment and was isolated from Charleston harbor in South Carolina (Wu, Watts, Sowers & May, 2002). *Dehalobium chlorocoercia* DF-1 couples the oxidation of either formate or hydrogen to the reductive dechlorination of PCE and TCE (Miller, Milliken, Sowers & May, 2005), hexa-chlorobenzene and pentachlorobenzene (Wu, Milliken et al., 2002), and PCB congeners with double-flanked chlorines (Wu, Watts et al. 2002; , May, Miller, Kjellerup, & Sowers, 2008). An active role of these isolates and other Chloroflexi members that are distinct from *Dehalococcoides mccartyi* species were shown in PCB dechlorination in sediment microcosms from Baltimore Harbor, USA (Watts, Fagervold, May & Sowers, 2005). Similar Chloroflexi phylotypes were reported for PCB dechlorination in enrichment from contaminated marine sediment of the Venice lagoon, Italy (Zanaroli et al., 2012). Furthermore, another obligate dehalorespirer, *Dehalococcoides mccartyi* MB, uses only hydrogen as electron donor and PCE and TCE as electron acceptors, that are reduced to trans-DCE and cis-DCE. Non-obligate OHRB, like *Desulfomonile limimaris* and *Desulfovibrio* of Proteobacteria phylum, can couple the oxidation of several organic electron donors to the reductive dehalogenation of less hydrophobic, hydroxylated or carboxylated monoaromatic organohalides, including *ortho*-substituted bromophenols commonly produced by marine organisms (Zanaroli et al., 2015).

Table 1 shows a summary of the isolated marine and estuarine microorganisms able of reductive dehalogenation. Contrary to the large number of OHRB obtained from groundwater, aquifer, river sediments and soils, only a few OHRB are isolated from marine and estuarine environment (Atashagi et al., 2016).

Table 1: Dehalorespiring bacteria isolated from marine and estuarine environments-Zanaroli et al., 2015.

Microorganism	Isolation Source	Organohalide electron acceptors	Reference
Desulfovibrio dechloracetivorans SF3	Marine sediment	2-Chlorophenol and 2,6-chlorophenol	(Sun, Cole, Sanford & Tiedje, 2000)
Desulfomonile limimaris DCB-M and DCB-F	Marine sediment	Chlorobenzoates	(Sun, Cole & Tiedje, 2001)
Dehalococcoides mccartyi MB	Marine sediment	PCE and TCE	(Cheng & He, 2009)
Desulfoluna spongiiphila AA1	Marine sponge	2,4,6-TBP and its debromination intermediates, 2-iodophenol and 3-iodophenol	(Ahn, Kerkhof & Häggblom, 2009)
Desulfovibrio sp. TBP-1	Estuarine sediment	Bromophenols with only unflanked *ortho* and *para* bromines	(Boyle, Phelps & Young, 1999)
Dehalobium chlorocoercia DF-1	Estuarine sediment	HCBe and PeCB, PCE, TCE, PCB congeners (double-flanked chlorines)	(Miller et al. ,2005; Wu, Milliken et al. ,2002; Wu, Watts, 2002; May et al., 2008)

2.2.4. Reductive dechlorination of PCBs in Venice Lagoon

PCBs [in the range 0.2-5 mg·(kg·drywt)$^{-1}$] in sediments of the Porto Marghera area of the Venice Lagoon (Italy) have been documented (Moret et al., 2001). The occurrence of reductive dechlorination processes of endogenous PCBs in three contaminated marine sediments of the Brentella Canal of Porto Marghera has been previously demonstrated under laboratory *in situ* mimicking

conditions by Fava, Zanaroli and Young (2003). It was shown that dechlorination was more extensive in the presence of the site water than with mineral medium. Thus, a series of slurry-phase anaerobic microcosms consisting of the sediment suspended in water collected from the same contaminated area was developed where a significant and generally quantitative conversion of the endogenous high-chlorinated biphenyls into tri- and di-chlorinated, ortho-substituted congeners was observed from the 11th week of incubation. After 20 weeks, dehalogenation was observed in microcosms amended with eubacteria-inhibiting antibiotics, even when provided with methanol, formate and acetate as exogenous substrates. Dehalogenation was hardly detected in microcosms amended either with molybdate, which inhibits methanogenesis, or with 2-bromoethanosulfonate (BES), inhibitor of sulfate-reducing bacteria.

In subsequent studies, all microcosms were prepared from those preliminary sediment slurry. Coplanar PCBs were added in the sediment slurries in an effort to prime the dechlorination of the endogenous pre-existing PCBs, but it did not significantly influence their dehalogenation rate. Nonetheless, coplanar PCBs were extensively transformed (Zanaroli, Pérez-Jiménez, Young, Marchetti & Fava, 2006). In order to investigate further the dechlorinating abilities of microbial community and enrich it, the same sediment was cultured in 25% (v/v) slurry microcosms with the same exogenous coplanar congeners used in the previous experiment. In exponential phase, culture was transferred as inoculum at 25% (v/v) in a second slurry microcosm (M2C), which in turn was used as inoculum at 10% (v/v) in 6% (v/v) (M3C-6) and 12% (M3C-12) slurry microcosms (**Figure 8**). Dechlorination towards *meta* and *para* positions of PCB molecules was observed in all the microcosms, while unflanked *meta* dechlorination activity was lost with subculturing (Zanaroli et al., 2010). In comparison to M1C microcosms, M2C and M3C ones did not have any lag phase and increased dechlorination rate from 112±26 μmol/kg-dw per week observed in M1C to 179±25 μmol/kg-dw per week in M2C and to 232±36 μmol/kg-dw and 219±48 μmol/kg-dw per week in M3C-12 and M3C-6 respectively. 16S rRNA gene PCR-DGGE analysis showed that phylotypes having high sequence similarity with genus *Sulfurovum* of Epsilonproteobacteria and with an

uncultured Alphaproteobacterium enriched in M2C, M3C-6 and M3C-12 cultures, while a phylotype with high sequence similarity to an uncultured Chloroflexi was detected in all subculturing steps.

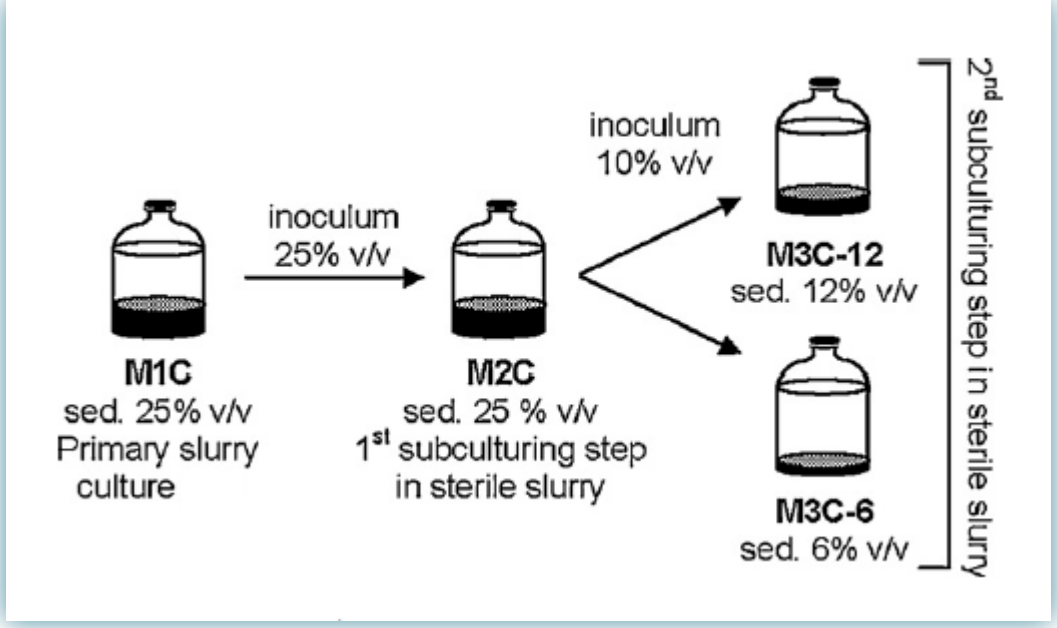

Figure 8: Flow sheet of subculturing steps and cultures developed- Zanaroli et al., (2010).

Further studies on slurry microcosms (Nuzzo, Negroni, Zanaroli & Fava, 2017), prepared by suspending six different sediment samples from Venice lagoon and spiking them with Aroclor 1254 at a final concentration of 1g-PCBs/ kg, identified two dechlorinating *non-Dehalococcoides* phylotypes of the class Dehalococcoidia (**Figure 9**): one, named VLD-1, had 98.8% sequence identity with the dechlorinating Chloroflexi bacterium *Dehalobium chlorocoercia* DF-1 and one, named VLD-2, had 100% sequence identity with the uncultured Chloroflexi bacteria SF1 and m-1. Three out of six marine sediments used in the study showed PCB-dechlorination activities under laboratory *in situ* mimicking conditions, suggesting that a potential for dehalogenation is present, although not ubiquitously, in the microbial communities of the Porto Marghera area of Venice Lagoon. The two *non-Dehalococcoides* phylotypes were associated to two distinct dechlorination activities. Phylotype VLD-1 is the one capable of unflanked *meta* and *para* chlorines removal, and thus potentially able to achieve

extensive decontamination and detoxification of sediments impacted by complex PCB mixtures.

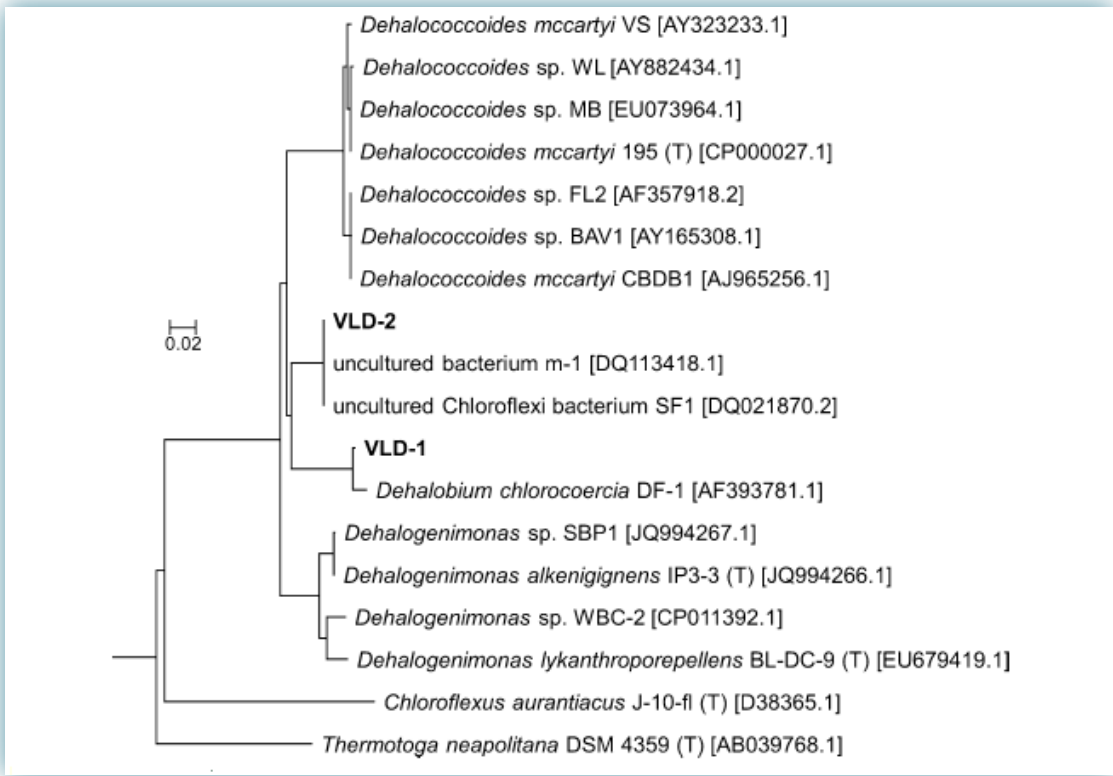

Figure 9: Phylogenetic placement of VLD-1 and VLD-2 within the class Dehalococcoidia of the phylum Chloroflexi-Nuzzo et al., 2017.

Phylogenetic and functional characteristics of microbial communities of the same sediment from Venice Lagoon and of sediment from Ravenna Harbour were evaluated in presence of different chlorinated pollutants, investigating their ability to dehalogenate these contaminants and the potential involvement of VLD-1 and VLD-2 in the dechlorination. Moreover, the ability of the same phylotypes to dehalogenate organohalides different from PCBs was investigated in Venice Lagoon sediment inoculated with the enriched PCB-dechlorinating culture. Most of the pollutants (PCBs, chlorobenzenes, TCP and TCE) added to both of Venice Lagoon and Ravenna Harbour slurry microcosms were extensively dehalogenated, while others (TeCDD and PCP) were dechlorinated only in enriched microcosms. Dehalogenation could indeed be attributed to VLD-1 and VLD-2, since they were the main phylotypes in Chloroflexi-specific 16S rRNA gene DGGE of DNA of these microcosms.

For further enrichment and biomass growth, sediment-free cultures were produced from enriched TCE ones in synthetic marine water. A commercial mixture of seasalts, named Tropic Marin, that is used to replicate marine conditions in aquariums, was used in order to make the medium. Its composition can be found in the literature (Atkinson & Bingman, n.d.). This set of subcultures, named VL2 TCE, consisted of five sets of microcosms. Four of them were amended each with a different carbon source at a concentration of 20mM and the other one without one. Only the cultures amended with lactate and pyruvate allowed growth and dehalogenation, but dehalogenation activity could not be sustained indefinitely. Yet, with addition of vitamins and trace minerals the activity was restored.

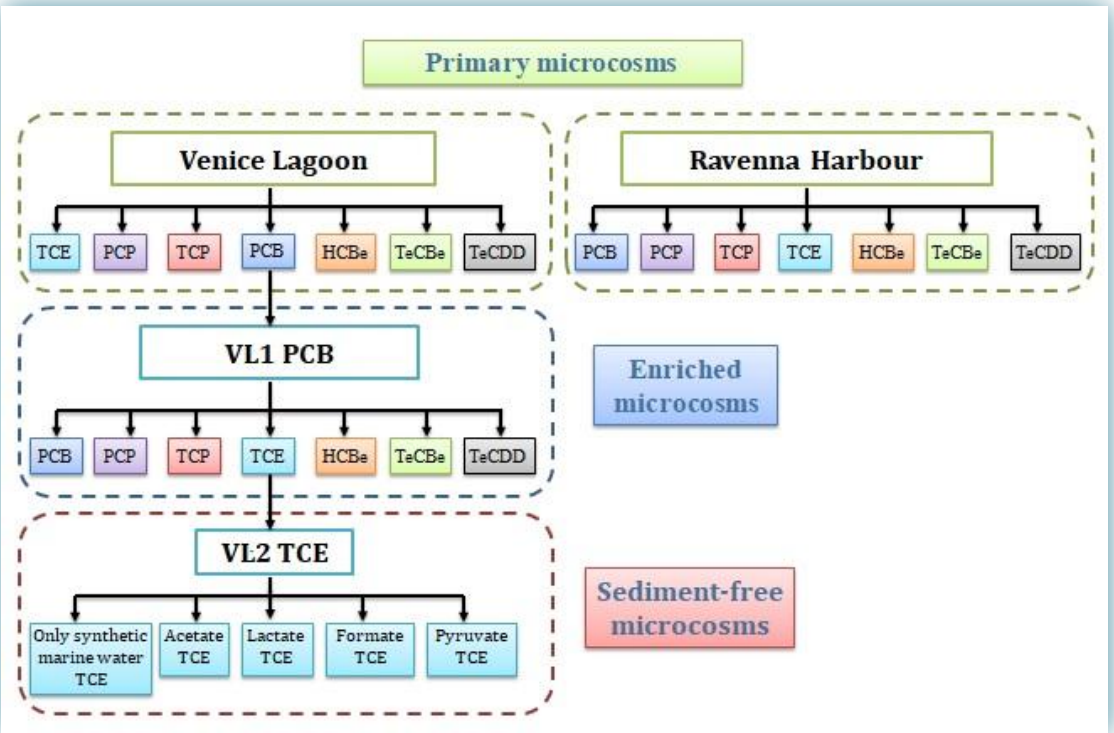

Figure 10: Sediment primary and enrichment cultures and subcultures in synthetic marine water.

Molecular analysis of the bacterial community in cultures in synthetic marine water revealed an enrichment of the main two phylotypes of the class Dehalococcoidia, VLD-1 and VLD-2, with the former reaching 87% of the class and the latter 13%.

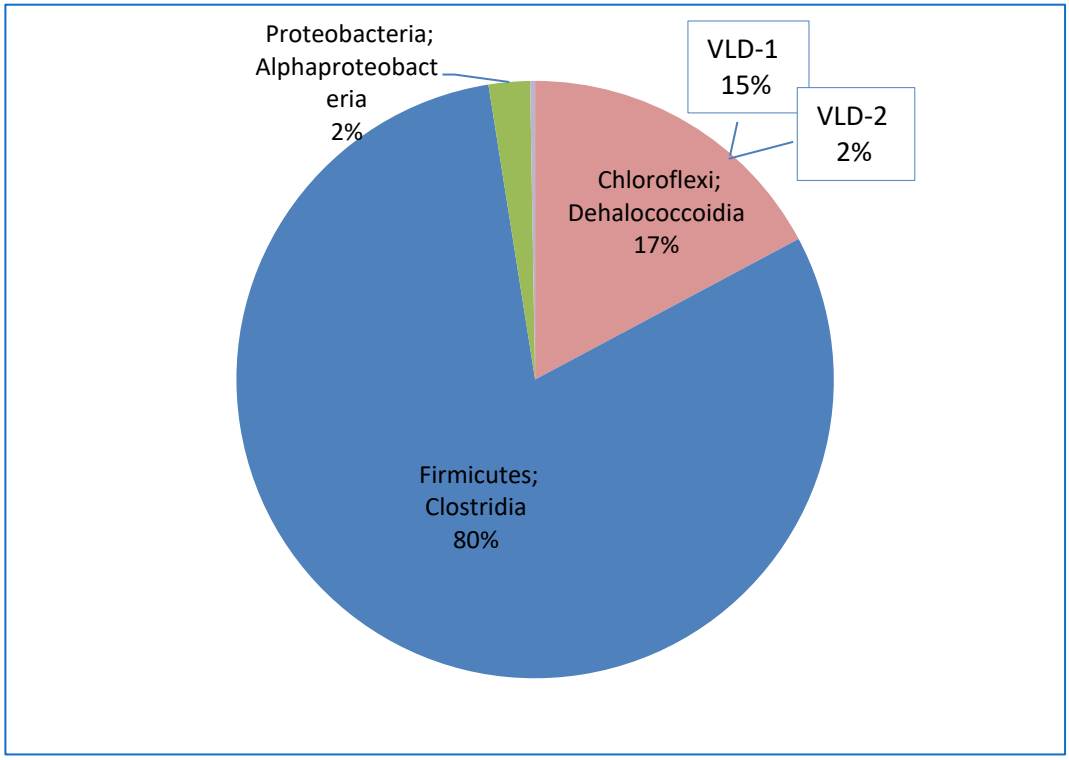

Figure 11: Pie chart of percent mean relative abundance of microbial composition at Class level in cultures amended with BES, ampicillin, vancomycin as determined by 16S rDNA Illumina Sequencing.

In this study, further enrichment of the Venice Lagoon microcosms, that were inoculated with the enriched PCB-dechlorinating culture, took place with a sediment-free form in order to see if the cultures maintained or altered their dehalogenating ability.

2.3. Conclusions

Bioremediation has been extensively investigated due to its advantages as a cost-effective and environmentally friendly method for treating sediments contaminated with halogenated compounds over methods entailing sediment excavation and the possibility of altering the biogeochemical conditions of the target environment. In marine sediments, organohalides can be anaerobically dehalogenated to less-halogenated compounds that are generally more amenable to further biodegradation or even be completely dehalogenated, thus eliminating the need for additional treatment. Strategies like biostimulation and bioaugmentation have received increasing attention mainly for the treatment of

petroleum products, halogenated organic solvents and herbicides. Their successfulness depends on the chemical conditions of the sediment, including total organic carbon and the availability of critical trace metals, on the competition between bacteria for electron donors as it might inhibit or accelerate dehalogenation and on the availability or amount of the autochthonous dehalorespirers. To increase dehalogenation efficiency, sediments can be enriched with the addition of alternate halogenated compounds and exogenous carbon sources. Another way to enhance reductive dehalogenation is the addition of pure or mixed cultures of bacteria with known dehalogenating ability. The increasing availability of phylogenetic information about OHRB permits detailed analysis of indigenous microbial communities and monitoring of their development during the organohalide remediation process. Therefore, the absence of known dehalogenating bacteria at a particular contaminated site, or the failure to enrich indigenous dechlorinating bacteria *in situ*, can be observed and mitigated by introducing pre-enriched OHRB at a high population density. The creation of enrichment cultures with competent dehalorespiring microbes is a promising approach for enhanced bioremediation processes. As it was mentioned in the previous section, enrichment cultures were obtained from PCB-impacted marine sediments of Venice Lagoon, tested in sediment and sediment-free media and dominant OHRB were phylogenetically characterized. In this study, these cultures were transferred in a different medium where reductive dehalogenation will be tested under different conditions that will hopefully stimulate or further enrich dehalogenating bacteria.

CHAPTER 3

MATERIALS AND METHODS

3.1. Enriched sediment-free cultures

Propagation of microorganisms capable of reductive dehalogenation is continued with further enrichment of previous cultures (see section 2.2.4.).

The following organohalides were used in this study:

- TCE (1,1,2-trichloroethene)
- HCBe (hexaclorobenzene)
- TeCDD (1,2,3,4-tetrachlorodibenzodioxin)
- PCP (pentachlorophenol)
- PCB (polychlorobiphenyls in Aroclor 1254™)

Table 2: Spike stock solutions.

Contaminants	Concentration
TCE	1.4g/L
HCBe	1g/L in acetone
TeCDD	1g/L in acetone
PCP	1g/L in methanol
PCB	20g/L in acetone

3.1.1.Cultures in defined mineral media

New cultures were produced in 120mL serum bottles using inocula (20%v/v) of VL2 TCE microcosms with best dehalogenation rates. These sets of microcosms were cultured a different media where synthetic marine water components were replaced with individual salts. Sulphate salts were avoided to avoid growth of sulphate reducing bacteria. Nutrients and microelements were added in quantity according to previous isolation studies.

3.1.1.1. Preparation of medium components

Medium was prepared starting from a solution of the main salts. For every set of microcosms made, the procedure of the medium preparation was the following: the main salts shown in **Table 3** were dissolved in deionized water; pH was measured and adjusted to 6.8 with a 2M HCl solution; afterwards, solution was filter-sterilized with 0.22µm filters and sparged with nitrogen for 20min to remove oxygen before use.

Table 3: Concentration of the main salts used for medium's preparation.

Salts	Concentration [mg/L]
Na_2CO_3	3000.0
Na_2HPO_4	600.0
NH_4Cl	500.0
NaCl	17532.0
KCl	678.4
$CaCl_2 \cdot 2H_2O$	50.0
$MgCl_2 \cdot 6H_2O$	3950.0

A series of stock solutions were prepared in serum bottles:

- 100x concentrated vitamin solution
- 50x concentrated solution of trace minerals
- 100x concentrated solution of reducing agents
- 1000x concentrated solution of antibiotics
- 50x concentrated solution of sodium 2-bromoethansulfonate (BES)

Vitamin stock solution (100x concentrated) was prepared using the concentrations in **Table 4** and according to the following procedure: vitamins were dissolved in bidistilled water; pH was adjusted to 7; solution was filter-sterilized, sparged with nitrogen to remove oxygen and then stored at 4°C in the dark.

Table 4: Vitamins solution at 100x concentration.

Vitamins 100x Solution	100xConcentration [mg/L]
Biotin	2.0
Folic acid	2.0
Pyridoxine HCl	10.0
Thiamine HClx2H_2O	5.0
Riboflavin	5.0
Nicotinic acid	5.0
Sodium pantothenate	5.0
Vitamin B_{12}	2.2
Sodium aminobenzoate	5.0
Lipoic acid	5.0

Trace mineral solution (50x concentrated) was prepared as follows: the $FeCl_2 \cdot H_2O$ was dissolved in 20-25% HCl bidistilled water solution and then all the other minerals were added; dilution 1:10 with bidistilled water was performed; the solution was filtered-sterilized and stored at room temperature; nitrogen sparging for 1h.

Table 5: Trace minerals solution 50x concentrated.

Element	Chemical formula	50xConcentration [mg/L]
B	H_3BO_3	500.0
Mn	$MnCl_2$	6.9
Co	$CoCl_2$	500.0
Ni	$NiCl_2$	11.0
Cu	$CuCl_2$	310.2
Zn	$ZnCl_2$	422.0
Li	LiCl	61.5
Si	Na_2SiO_3	85.4
Ba	$BaCl_2$	3.3
Mo	$Na_2MoO_4 \cdot 2H_2O$	587.4
Al	$AlCl_3 \cdot 6H_2O$	2775.4
Element	**Chemical formula**	**10000xConcentration [mg/L]**
Fe	$FeCl_2 \cdot H_2O$	400.0

Reducing agents (used to keep the redox potential low) were dissolved in bidistilled anoxic water separately; they were sterilized by filtration, sparged with nitrogen for 30min and then stored in the dark at 4°C. The same procedure was applied for antibiotics and BES solutions.

All stock solutions after being autoclaved and let to cool down at room temperature overnight, were added in autoclaved, anaerobic, serum bottles; they were autoclaved and then let to cool down at room temperature overnight; finally, they were stored in the fridge at 4°C.

Table 6: Concentrations of reducing, antibiotics and BES solutions.

Reducing Agents Solution	**100xConcentration [g/L]**
Cysteine $HCl \cdot H_2O$	25.0
$Na_2S \cdot 9H_2O$	24.9
Antibiotic Solutions	**1000xConcentration [g/L]**
Ampicillin	0.1
Vancomycin	0.1
Solution	**50xConcentration [g/L]**
Sodium 2-bromoethanesulfonate (BES)	316.5

3.1.1.2. Microcosms set up

Figure 12 shows the subcultures originated from VL2 TCE ones with each set named according to the substances they contain.

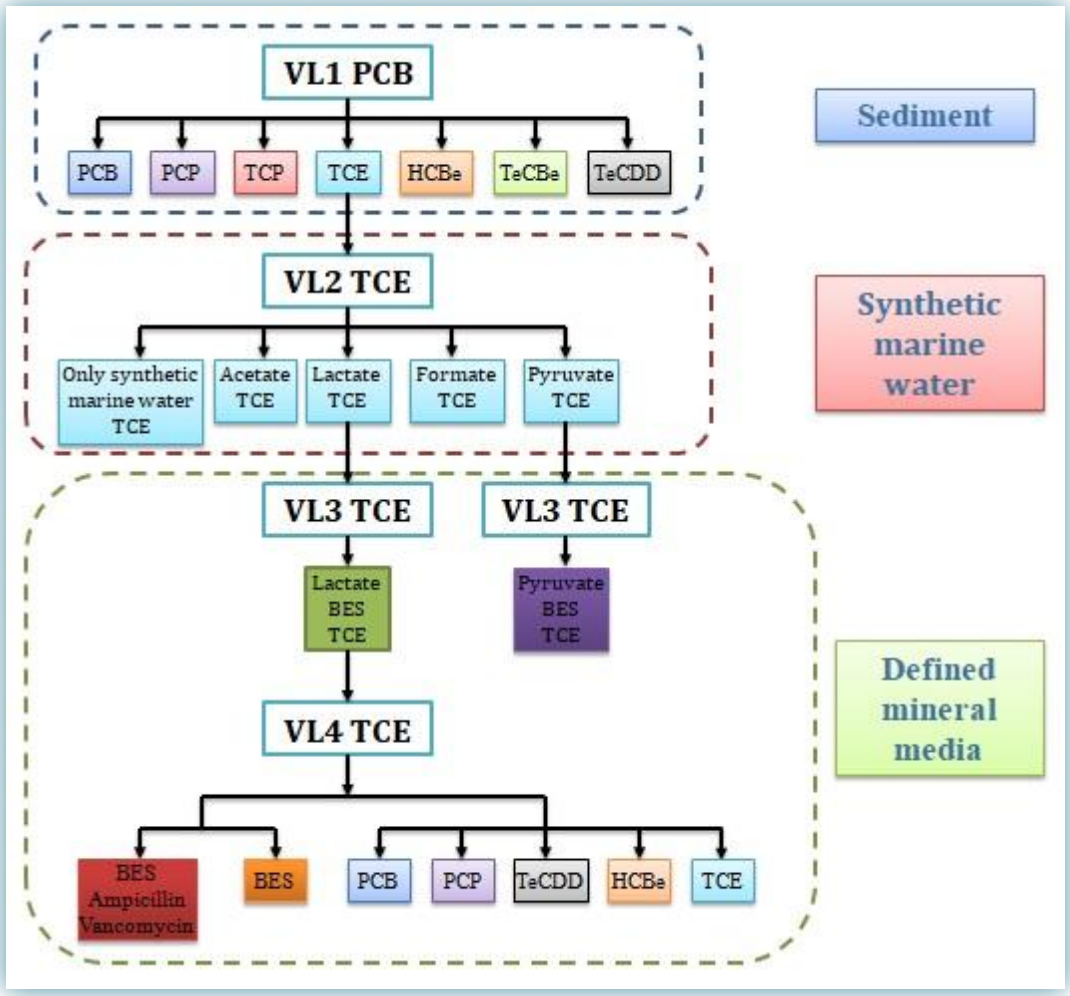

Figure 12: Enrichment cultures tree.

All serum bottles used for every set of microcosms were autoclaved empty for all the cultures except for the ones that would be amended with PCB, 1,2,3,4-TeCDD and HCBe, which they were autoclaved with 14g of silica. Silica was provided as sediment substitute for hydrophobic substances. All bottles were put under nitrogen flow to create anaerobic conditions. Inoculation percentage was 20%(v/v) for all the cultures made. All medium components mentioned previously were added into the serum bottles via a deoxygenated syringe under nitrogen flow. Sodium lactate (20mM) was also added to all sets of microcosms except for VL3 TCE amended with 20mM of sodium pyruvate. Afterwards, all microcosms were spiked with their corresponding contaminant solvent from Table 2. Finally, the bottles were capped and crimped with aluminum sealers. Hydrogen and carbon dioxide were provided at a ratio 4:1 and pressure of 2 bar

in order to fill the rest of the bottles volume and create an overpressure. Essentially, because organic acids (lactate and pyruvate) were provided one time at VL3 TCE sets' preparation to avoid excessive accumulation of acetate through their fermentation, H_2 and CO_2 (products of carbon sources consumption and necessary to dehalorespiring bacteria for reductive dehalogenation) were added.

3.2. Analytical methods

3.2.1. Extraction and analysis of PCB microcosms

The procedure was performed according to the protocol described by Fava et al. (2003) with some modifications. Duplicate samples of 0.3mL collected for PCBs analysis from each slurry microcosm were placed in 2mL-vials for gas chromatography (GC) equipped with Teflon-coated screw cups. Afterwards, 0.9mL of hexane: acetone (9:1) were added in each of them. Vials were put again into vortex for 30s. Subsequently, they were horizontally shaken on a rotary shaker at 150rpm and 30°C overnight. The next day, vials were centrifuged at 5000rpm for 10min (Beckman J2-HS centrifuge, Beckman Coulter, Fullerton, CA, USA), then placed in the freezer at -20°C for 2h. After this time, the liquid organic phase was recovered into an empty 2mL-GC vial and 10μL of a 40mg/L stock solution of octachloronaphthalene (OCN, in hexane) were added as PCB 'recovery standard' and internal standard for PCB analysis via GC.

Qualitative and quantitative analyses of the extracted PCBs were performed with a Hewlett Packard GC (6890, series II), equipped with a HP-5 capillary column (30m x 0.25mm x 250nm), a ^{63}Ni electron capture detector (ECD) and a 6890 series II automatic sampler (Hewlett Packard) under the following conditions: initial temperature 60°C; isothermal for 1min; first temperature rate 40°C/min ; final temperature 140°C; isothermal for 2min; second temperature rate 1.5°C/min ; final temperature 185°C; third temperature rate 4.5°C/min ; final temperature 275°C; isothermal for 5min; injector (splitless mode) 240°C; ECD 320°C; carrier gas flow rate (nitrogen) 60mL/min ; sample volume 3μL. Qualitative analysis of PCBs was performed by comparing the retention time (relative to OCN) of each of the GC peaks obtained from the analysis of the organic phase of PCBs samples with PCBs of standard Aroclor 1242 and Aroclor

1254 (in the range 0.5 to 30 mg/L) analyzed under identical conditions. Standards were identified with comparison between the analytical peaks and the chromatograms reported by Frame et al. (1996). Response factors were calculated for each peak, which was identified using the relative retention time to OCN as internal standards, and were verified monthly.

3.2.2. Extraction and analysis of chlorobenzene microcosms

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 (0.2mm x 25 m with a film thickness of 0.33µm) was used for analysis. The oven temperature was maintained at 80°C for 5min, raised to 120°C at 5°C/min and maintained for 2min, and then raised again to the final temperature of 200°C at 5°C/min and held for 5min, 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 16cm/s, and the split ratio was kept at 10:1.

3.2.3. Extraction and analysis of TeCDD microcosms

Extraction was performed from 0.2mL slurry sample placed in 2mL-vials for gas chromatography. Afterwards, 1mL of a 9:1 mixture of toluene and acetone was added to each sample. GC vials were put into vortex for 30s. Subsequently, they were sonicated for 15min and horizontally shaken on a rotary shaker at 150rpm and 30°C overnight. The next day, they were sonicated again for 15min and then they were centrifuged at 5000rpm for 10min by Beckman J2-HS centrifuge (Beckman Coulter, Fullerton, CA, USA) and frozen at -20°C for 2h. Liquid phase from the samples was transferred into empty 2mL-GC vials. Before the analysis, samples were diluted 5 times using the 9:1 mixture of toluene: acetone and 10µL OCN in hexane were added as internal standard.

TeCDDs concentration and type were analyzed according to Vargas, Fennell and Häggblom (2001). 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 (30m x 0.25mm x 200nm). The injector and detector temperatures are 280 and 350°C respectively. The oven temperature is held at 60°C for 1min, ramped at 40°C/min to 150°C, and then 10 °C/min to 280°C, and held for 1min.

3.2.4. Extraction and analysis of chlorophenol microcosms

Extraction was performed from 1mL slurry sample placed in 2mL-GC vials that were centrifuged at 5000rpm for 5min using a Beckman J2-HS centrifuge (Beckman Coulter, Fullerton, CA, USA). Water is filtered with a 0.2µm PTFE filters and analyzed by HPLC as described below; 2mL pure ethanol is added to the sediment and horizontally shaken on a rotary shaker at 150rpm and 30°C overnight. The next day, samples are centrifuged by Beckman J2-HS centrifuge at 5000rpm for 5min and the supernatant is filtered by a 0.2µm filter and analyzed by HPLC.

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 x 15cm) (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_3PO_4). Mobile phases were previously filtered using a mobile phase filtration assembly with a 0.22µm filter. The run (A:B) was as follows: isocratic gradient from 70:30 mixture to a final 50:50 in 9 min; hold at 50:50 for 3 min; 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 min. Minimum pressure was 0.2bar, maximum pressure 600bar. The HPLC was calibrated with an equimolar mix of chlorophenols through linear six point calibration curves in the range of 0.2 to 20mg/L each and with phenol through a linear six-points calibration curve in the range of 0.2 to 10mg/L.

3.2.5. Analysis of TCE microcosms

TCE was analyzed according to Frascari et al. (2006) with a Hewlett Packard 6890 FID ECD GC equipped with a capillary HP-VOC column (30m x 0.32mm) connected to a flame ionization detector (250°C) for the analysis of methane, propane, VC and to an electron capture detector (250°C) for the analysis of the remaining aliphatic hydrocarbons (injector temperature 250°C; injection volume 100µL; split ratio 10:1; oven temperature 3min at 60°C, ramp to 230°C at 20°C/min, 5min at 230°C; carrier gas He at 0.9mL/min). To avoid microbial cross contamination, before each analysis the syringe was kept in the GC injector (250°C) for about 1min and subsequently filled twice with hot gas from the GC injector. Detection limits were (µM in the aq. phase): methane: propane, 0.007; VC, 1.2; trans-DCE, 0.08; cis-DCE, 0.15; TCE, $4 \cdot 10^{-6}$; 1,1,2-TCA, 0.01; 1,1,2,2-TeCA, 0.02. Total masses and aqueous phase concentrations were calculated utilizing the gas/liquid and solid/liquid equilibrium constants estimated at 30°C (Sander, 1999).

Calibration was performed as follows: injection of small volumes (<200µL) of a mixture of chloroethenes and hexane of known concentration in closed serum bottles; vigorous shaking for 30s; injection of 100µL gas from serum bottles into GC. For TCE data analysis, signal is integrated and peak areas are transformed in gas concentrations by excel calibration. Concentration in water of microcosms is derived by the dimensionless Henry constant of compounds and gas phase concentration. The equation used to calculate the concentration of TCE and its dehalogenation products is:

$$C_{tot} = \frac{C_{gas} \cdot \left(V_{gas} + V_{overpressure}\right) + C_{liq} \cdot V_{liq}}{V_{tot}}$$

where,

- C_{tot} : total concentration [µmol/L]
- C_{gas} : concentration of gas phase [µmol/L]
- V_{gas} : gas volume, 0.05L
- $V_{overpressure}$: overpressure gas volume, 0.025L

- C_{liq} : concentration of liquid phase [μmol/L]
- V_{liq} : liquid volume, 0.07L
- V_{tot} : total volume, 0,12L

Dechlorination rate is expressed in μmoles of chlorine released per liter per day and it is calculated according to the following equation:

$$r_d = \frac{C_{initial} - C_{final}}{t}$$

Every time trichloroethene was depleted, formation products and remaining TCE would be stripped with oxygen-free nitrogen and then new TCE would be spiked again and thereby dechlorination would be monitored overtime.

3.2.6. Gas analysis

Gas production was measured before every TCE stripping with an air-tight syringe and the collected head-space gas was analyzed for CH_4, CO_2, H_2 and O_2 with μGC-TCD model 3000A (Agilent Technologies, Milano, Italy) equipped with a 10m x 0.32mm MolSieve 5A column under the following conditions: injector temperature 90°C; column temperature 60°C; sampling time 30s; injection time 50ms; column pressure 25 psi; run time 44s, carrier gas nitrogen.

Methane production was measured as it implies activity of methanogenetic microbes and along with carbon dioxide and hydrogen are products of microbial activity towards TCE and carbon sources' consumption. As the conditions in this work were strict anaerobic, oxygen was monitored to ensure that.

3.2.7. Anions analysis

Anions concentration in the water phase was determined, after slurry centrifugation at 10000g for 10min and 1:100 dilution of the supernatant, with a Dionex DX-120 ion chromatograph equipped with an IonPac AS14 4x250 mm column and a conductivity detector combined to an ASRS-II Ultra conductivity suppressor system (Dionex, Sunnyvale, CA, USA), as described in Fava et al. (2003). Linear 4-point calibration curves (1-50 mg/L) for SO_4^{-2} . The eluent was

a solution of 8mM Na_2CO_3 and 1mM $NaHCO_3$ prepared in ultra-resi-analyzed water; the flow rate was 1mL/min.

3.3. Microbial community analysis

Genomic DNA was extracted with UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) as follows: 1 mL aliquots of enrichment cultures were centrifuged 10,000 x *g*, 10 min. The cell pellets were suspended in the bead solution supplied with the kit according to the manufacturer's instructions with some modification. In particular, 4.5 µL of a 100 mg/mL Proteinase K solution in sterile deionized water plus 8.2 µL of a 100mg/mL Lysozyme solution in sterile deionized water were added to the suspension in the bead solution. Pellet suspension was then incubated at 37°C on a rotary shaker at 150rpm for 45 min prior to cell lysis. Extraction continued following manufacturer instructions. DNA samples were quantified using nanophotometer P-330 (Implen GmbH, Munich, Germany).

Extracted DNA was PCR-amplified using primers targeting total bacterial 16S rRNA genes. 16S PCR amplification was performed with primers GC-357F (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CGC CCC CTA CGG GAG CAG CAG-3') and 907R (5'-CCG TCA AAT TCC TTT GAG TTT-3') (Sass, Sass, Coolen, Cypionka & Overmann, 2001). The amplification was performed in 25 µL reaction mixtures containing 1 x PCR buffer (Promega corporation, USA), 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.5 µM of each primer, 1.0 U of Taq polymerase (Promega corporation, USA), and 5 µL of template DNA, and used the following PCR conditions: 95 °C for 10 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 10 min.

Next Generation Sequencing was performed by BMR Genomics (Padova, Italy) using the Illumina MiSeq System. Primer pairs 27F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GWNTTACNGCGGCKGCTG-3') were used for library preparation by 16S rRNA PCR gene amplification. From Illumina MiSeq System, generated demultiplexed paired-end FASTQ files were used as input files. The sequences were quality filtered and analyzed using the computational pipeline QIIME2. DADA2 software package, wrapped in QIIME2,

was used for dereplicating and denoising Illumina sequences (Callahan et al., 2016). FASTQ files were processed with `qiime dada2 denoise-paired` command, which merges and denoises paired-end reads.

3.4. Chemicals

Hexachlorobenzene, 1,2,3,4-tetrachlorodibenzodioxin, pentachlorophenol and polychlorobiphenyls in Aroclor 1254 and in Aroclor 1242 used in this work were supplied by ULTRA Scientific Italia s.r.l., Bologna, Italy. 1,1,2-trichloroethene, Proteinase K from *S. griseus*, Lysozyme from chicken egg and all organic solvents as well as medium components used in the experiment were supplied by Sigma-Aldrich Italia S.r.l, Milan, Italy.

CHAPTER 4

RESULTS

4.1. Microcosms amended with lactate and pyruvate

The first day of chemical analysis, concentrations of TCE in the gas phase of the microcosms were estimated at 222.0±6.0 μmol/L in lactate-amended and at 247.0±9.1 μmol/L in pyruvate-amended microcosms. TCE was completely dechlorinated in approximately 6 weeks in both sets with no lag phase and accumulation of TCE intermediates was detected. The following figures depict dechlorination and products formation at the first time of analysis until TCE was depleted.

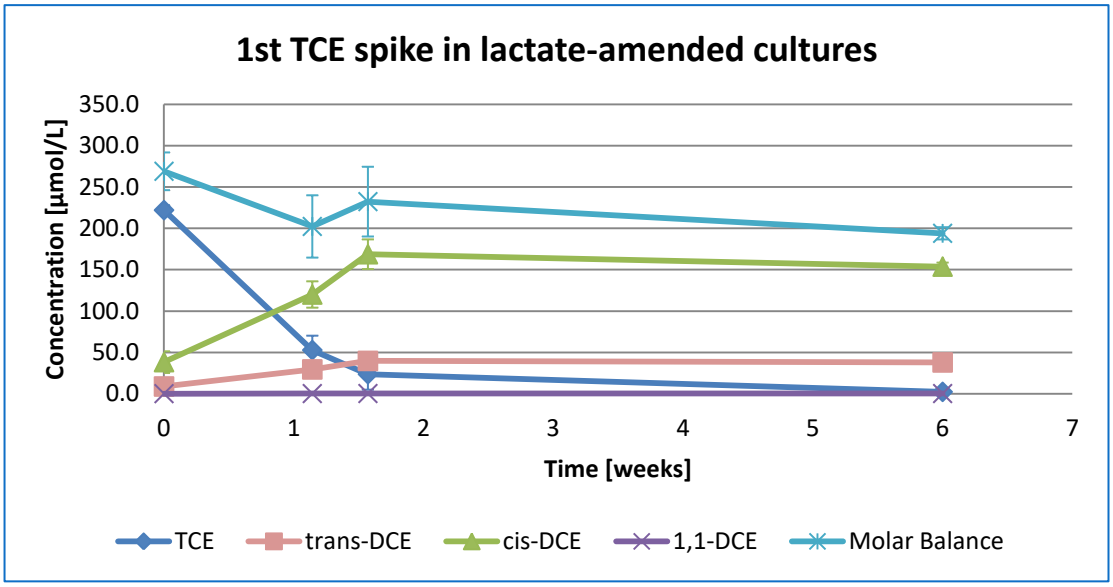

Figure 13: TCE dechlorination in lactate-amended cultures in defined mineral media. Data is the average of six replicates. Standard error bars shown.

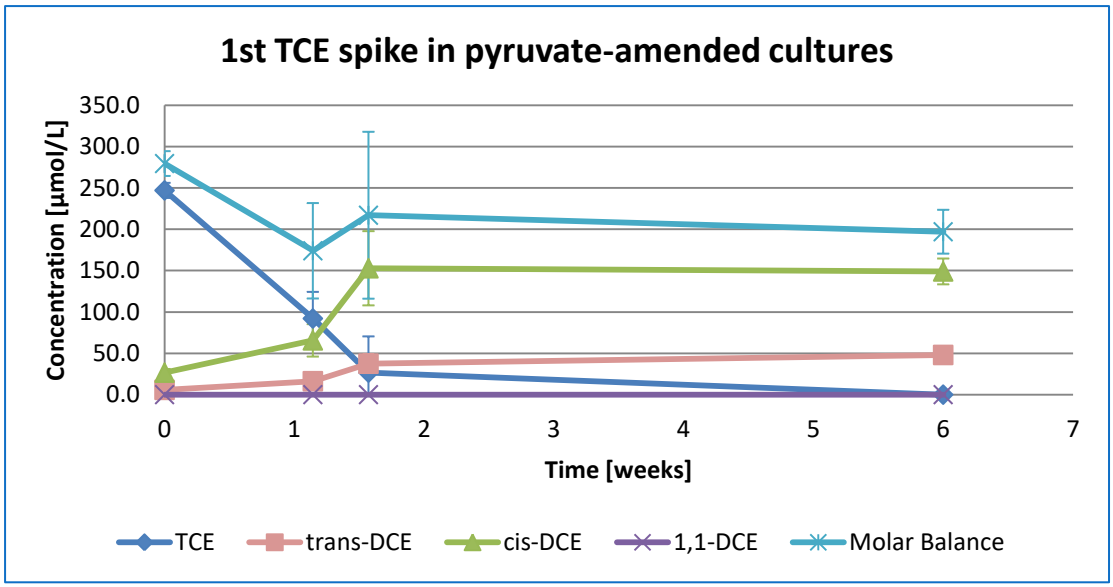

Figure 14: TCE dechlorination in pyruvate-amended cultures in defined mineral media. Data are the average of six replicates. Standard error bars shown.

The results in both sets of cultures are similar. While no accumulation of 1,1-DCE was observed, cis-DCE and trans-DCE isomers were detected at a ratio of 4.2±0.2 for cultures with lactate and 4.0±0.6 for cultures with pyruvate. Maximum TCE depletion rate and products formation rates (values depicted in **Table 7**) were observed at the beginning of the second week.

Table 7: Maximum TCE depletion and products formation rates during the first TCE spike in cultures amended with lactate and pyruvate.

VL3 TCE cultures	TCE [µmol/(L·d)]	cis-DCE [µmol/(L·d)]	trans-DCE [µmol/(L·d)]
Lactate-amended	21.2±1.4	11.9±0.4	2.8±0.1
Pyruvate-amended	19.4±2.9	11.5±3.7	1.9±1.0

However, the rate values shown in **Table 7** are not entirely representing dechlorination activity. Because of chloroethenes' highly volatile nature, abiotic losses were also observed in the cultures. At every chemical analysis the amounts of cis-DCE and trans-DCE should be equal to the spiked TCE amount that was degraded. However, the balance fluctuated every time of analysis, meaning part of the contaminant was lost due to possible leak from the bottles rubber stopper as it was pierced with the syringe several times for chemical analyses.

Each time TCE was depleted, products were stripped and TCE was re-added for new monitoring of dechlorination. Maximum biodegradation rates were reached around 5 to 8 days after each spike. Figures 15, 16 and 17 depict the rate values of TCE depletion and products formation throughout chemical monitoring for both of cultures. These values include possible occurrence of abiotic losses inside the cultures and thus the values representing biotransformation and bioaccumulation are expected to be equal or lower than the ones depicted on the following graphs. Moreover, these values resulted from data taken from two subsequent chemical analyses every time trichloroethene was spiked and in which maximum biodegradation was reached.

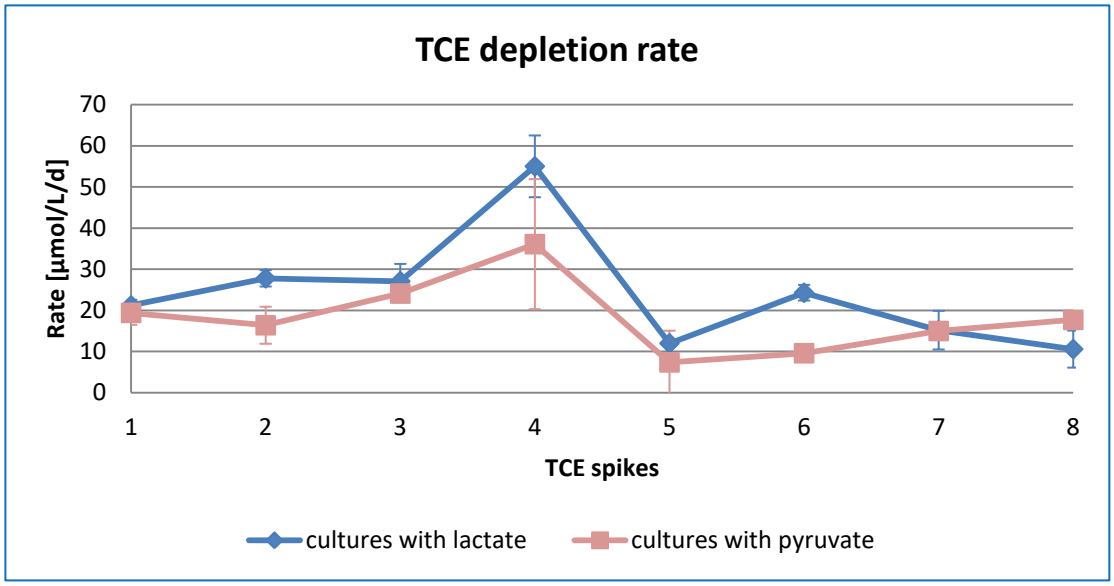

Figure 15: TCE depletion rate in lactate- and pyruvate-amended cultures in defined mineral media for eight consecutive TCE spikes. Data shown include possible abiotic losses and are the average of six replicates. Standard error bars shown.

Figure 15 shows that the maximum trichloroethene's depletion rate was detected at the 4th TCE spike in both sets of cultures during the 15th week of incubation. At this time, it is expected that dechlorinating bacteria reached their growth peak. At the next TCE spike during the 18th week of incubation, the depletion rates decreased, meaning that something changed inside the microcosms. The drop in values can be ascribed to the depletion of the organic acids within the microcosms. The prominent product of organic acids' fermentation is hydrogen which is necessary for reductive dehalogenation, and

therefore, with the organic acids running out, hydrogen's concentration within the cultures decreased causing trichloroethene's dechlorination activity to drop. Nonetheless, the ongoing TCE biotransformation indicate that the other elements along with the spiked hydrogen were enough to maintain biodegradation and biomass within these cultures.

Trichloroethene's ongoing biotransformation is visible in the figures below, as products of trichloroethene's chlorine removal were formed throughout chemical monitoring. Figure 16 and Figure 17 were created to compare the formation rates of cis-DCE and trans-DCE, respectively, in both sets of cultures.

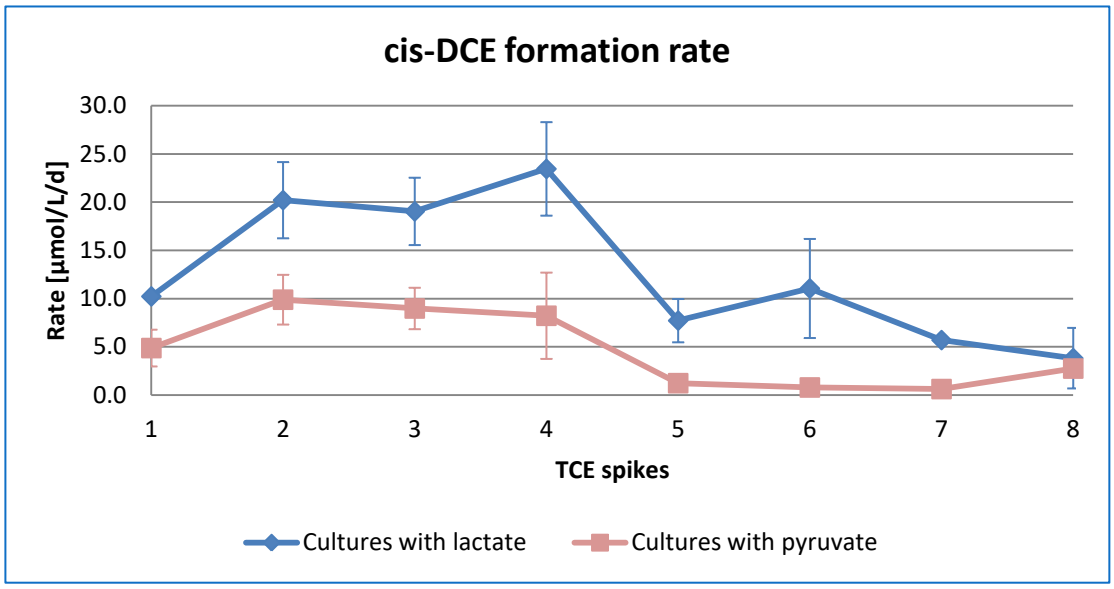

Figure 16: Rate of cis-DCE production in lactate- and pyruvate-amended cultures in defined mineral media for eight consecutive TCE spikes. Data shown include possible abiotic losses and are the average of six replicates. Standard error bars shown.

Apparently, accumulation rate of cis-DCE is higher in cultures amended with lactate. At the fourth TCE spike, the maximum value of the rate also indicates that organohalide respiring bacteria's activity and growth have reached their peak, following by a reduction caused by carbon sources' depletion. In pyruvate-amended cultures, cis-DCE rate dropped almost to zero. Yet, an interesting occurrence, depicted at Figure 18, is that trans-DCE formation rate, surpassed the one of cis-DCE whereas as it was shown in the beginning of chemical monitoring, average production ratio of cis-DCE to trans-DCE was 4.0±0.6. So dechlorinating bacteria's preference regarding the position of chlorine's substitution on TCE molecule has changed throughout the analysis.

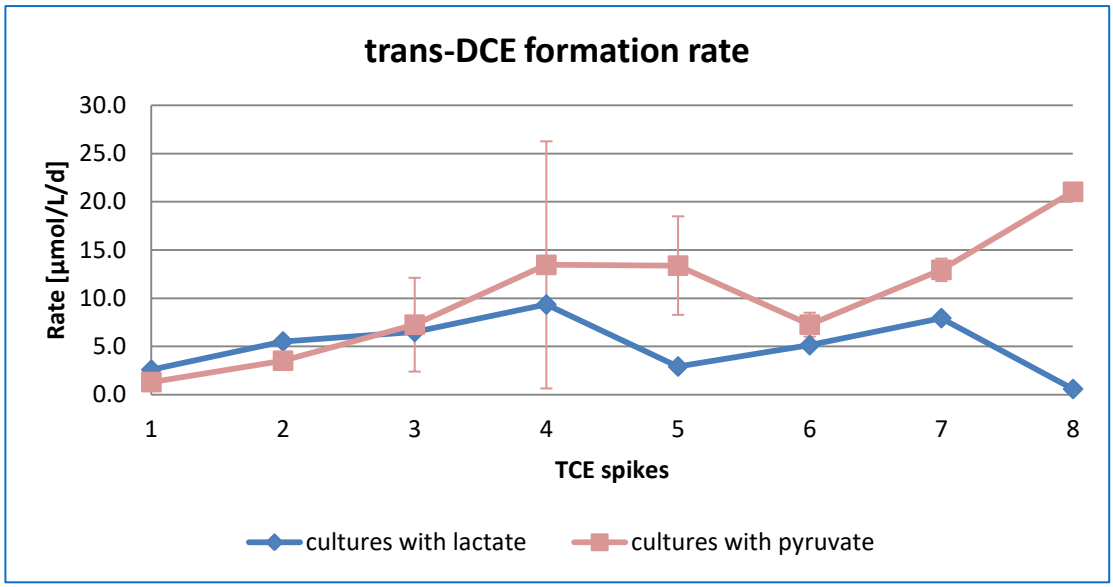

Figure 17: Rate of cis-DCE production in lactate- and pyruvate-amended cultures in defined mineral media for eight consecutive TCE spikes. Data shown include possible abiotic losses and are the average of six replicates. Standard error bars shown.

Figure 17 shows a higher trans-DCE formation rate in cultures with pyruvate than in the other set from the 4th TCE spike. The change of cis- and trans-DCE ratio is more visible in Figures 18 and 19.

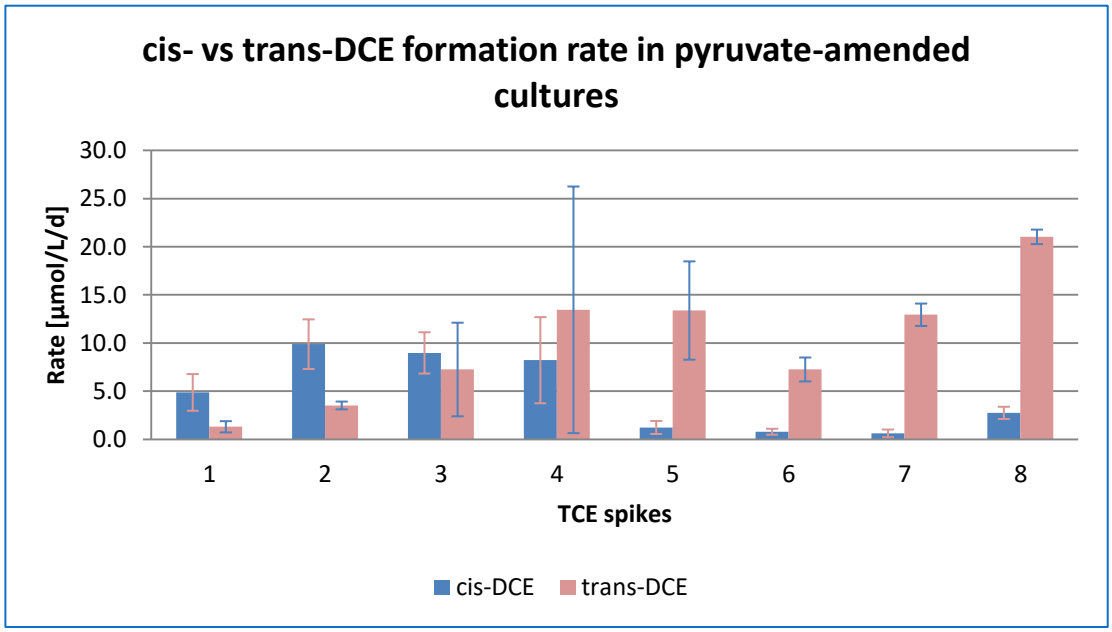

Figure 18: Comparison of cis- and trans-DCE formation rates in pyruvate-amended cultures in defined mineral media for eight consecutive TCE spikes. Data shown include possible abiotic losses and are the average of six replicates. Standard error bars shown.

Figure 18 depicts a significant change in the production of cis- and trans-DCE. At the 4th TCE spike, looking at the large error bar, it shows how much the deviance of the results obtained from the chemical analysis of each microcosm is. However, from the subsequent TCE spikes, the change in the formation products is apparent. The following figure shows how the ratio of products formation ranges in lactate-amended cultures.

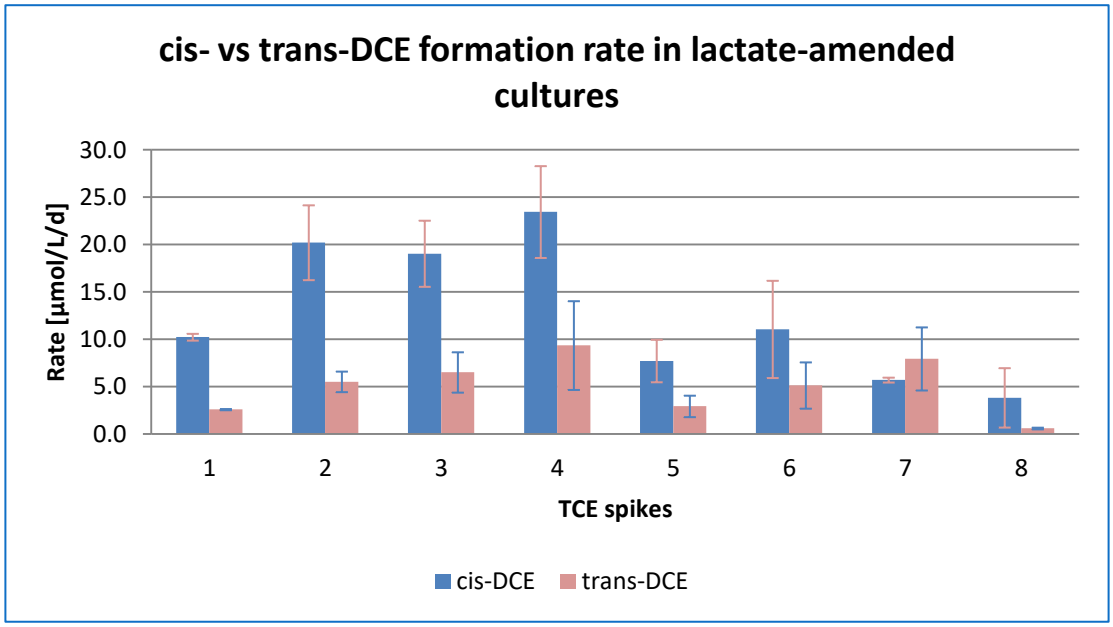

Figure 19: Comparison of cis- and trans-DCE formation rates in lactate-amended cultures in defined mineral media for eight consecutive TCE spikes. Data shown include possible abiotic losses and are the average of six replicates. Standard error bars shown.

Although, in pyruvate-amended cultures, production of trans-DCE was bigger than cis-DCE's one, the figure above shows different results for cultures with lactate. The ratio fluctuates but only at the 7th spike the formation rate of trans-DCE is higher than the one of cis-DCE.

Overall, similar results obtained from both sets of microcosms regarding biotransformation of trichloroethene. The differences, though, were found to be in the products formation. Apart from the fact that no formation of 1,1-DCE was observed, production of cis- and trans-DCE took place but not with a stable ratio, eventually leading to significant changes in cultures with pyruvate. Initially, these cultures appear to prefer the removal of chlorines that are on the same side of trichloroethene's molecule but throughout the analysis the major TCE intermediate formed inside these microcosms was trans-DCE. Usually, for non-

aromatic alkenes, trans-isomers are favorable over cis-isomers as they usually present a higher thermochemical stability (Smith, 2001). Thus, this change could be ascribed to that. Although, microbes in lactate-amended cultures showed preferentially production of cis-DCE throughout the analysis, there was a time that they exhibit a higher rate of formation towards trans-DCE. Therefore, chemical monitoring of cultures with lactate was continued in order to observe possible changes in dechlorination products. After five consecutive TCE spikes, the following results, shown in Figure 20, regarding the formation of cis- and trans-DCE, were obtained in the cultures.

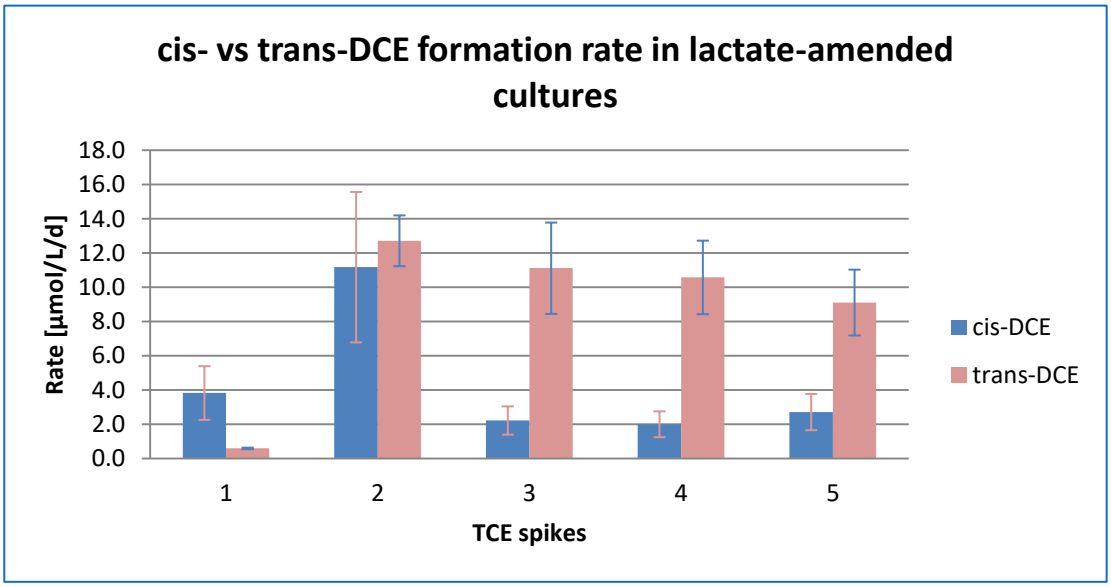

Figure 20: Comparison of cis- and trans-DCE formation rates in lactate-amended cultures in defined mineral media for another five consecutive TCE spikes. Data shown include possible abiotic losses and are the average of six replicates. Standard error bars shown.

Indeed, the change in formation of products rate occurred eventually in these cultures too. Higher amounts of trans-DCE were formed at the second TCE spike, a situation maintained until the end of chemical monitoring that established trans-DCE as the major intermediate product, or end product since no reduction of its concentration was observed, in TCE reductive dechlorination process.

Previous studies based on the sediment cultures from which these ones originated, mentioned in section 2.2.4., identified two dechlorinating *non-Dehalococcoides* phylotypes of the class Dehalococcoidia (Figure 9): one, named VLD-1, had 98.8% sequence identity with the dechlorinating Chloroflexi

bacterium *Dehalobium chlorocoercia* DF-1 and one, named VLD-2, had 100% sequence identity with the uncultured Chloroflexi bacteria SF1 and m-1. *D. chlorocoercia* DF-1 dechlorinates highly chlorinated benzenes (Wu, Milliken, et al., 2002) and PCE and TCE producing higher amounts of *trans*-DCE than *cis*-DCE compared with canonical PCE/TCE dechlorinators (Miller et al., 2005). That could be an indicator that the presence of VLD-1 in the cultures could be responsible for having the same effect on dehalogenation products.

Moving forward to new tests, lactate-amended cultures were selected for subculturing in order to study the performance and resilience of organohalide-respiring bacteria. New sets of microcosms (named VL4 TCE, see **Figure 12**) were prepared, some of them amended with growth inhibitors for further enrichment of dehalogenating bacteria and the rest of them were amended with several organohalides to test bacteria's dehalogenation capabilities.

4.2. Microcosms amended with growth inhibitors

The substances used in this part of the experiment were 2-bromoethanesulfonate (BES), ampicillin and vancomycin. BES, except for the fact that acts as methanogenesis inhibitor during anaerobic digestion, have also the ability to inhibit dechlorination of chloroethenes in the absence of methanogens. Ampicillin and vancomycin act by inhibiting cell wall synthesis of bacteria. However, resistance to these cell wall biosynthesis inhibitors is characteristic trait of several *Dehalococcoides* isolates (Zinder, 2016) and possibly of the *non-Dehalococcoides* VLD-1 and VLD-2. This test could lead to further enrichment of the bacteria involved in reductive dechlorination and it will show how antibiotics affect dehalogenation activity.

One set of the lactate-amended subcultures was amended with BES, ampicillin and vancomycin and another set with BES only. Trichloroethene was spiked in both sets of cultures and monitored as in the former sets. Within the first 7 weeks of incubation, cultures amended with antibiotics, at an initial trichloroethene concentration of 185.3±10.0 μmol/L, exhibited resistance to the antibiotics as the concentration was decreasing with a slow rate of 2.5±0.0 μmol/L/d. At the end of this period, cis-DCE and trans-DCE were detected at

concentrations of 34.4±9.9 and 6.1±2.1 μmol/L, respectively. Formation of 1,1,-DCE was not detected. From the figure below it is quite visible from the drop in molar balance that abiotic losses have a significant part in trichloroethene's concentration reduction.

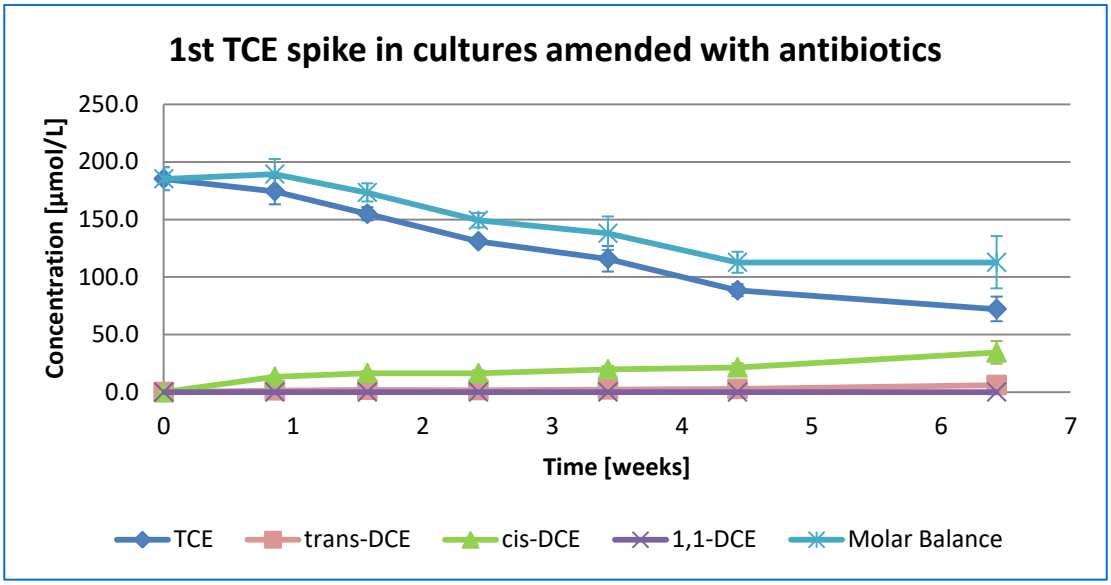

Figure 21: TCE dechlorination in cultures amended with ampicillin, vancomycin and BES in defined mineral media. Data are the average of three replicates. Standard error bars shown.

TCE, at an initial concentration of 184.3±9.0 μmol/L, in cultures with BES, was biotransformed to cis- and trans-DCE as shown in **Figure 22**. The rate of TCE depletion was estimated at 4.1±0.2 μmol/L per day. The average ratio of cis- to trans-DCE was 5.1±0.9. This result was something that was expected as these subcultures were prepared when the major product of TCE dechlorination in lactate-amended cultures was cis-DCE. Abiotic losses seem to had a role to the dechlorination process in these cultures too as the graph below shows a decline in molar balance throughout the analysis.

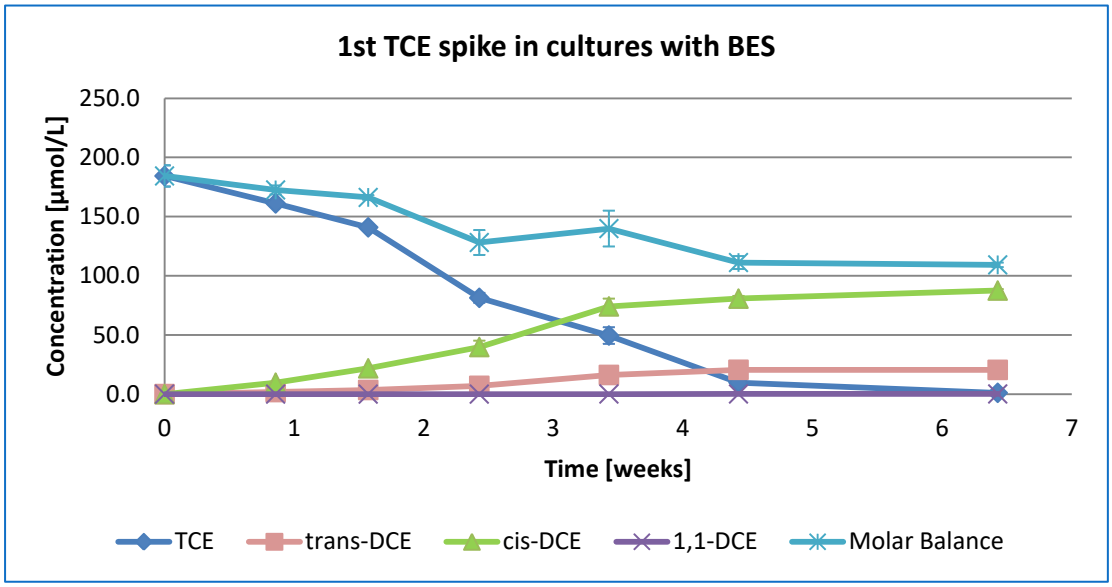

Figure 22: TCE dechlorination in cultures amended with BES in defined mineral media. Data are the average of three replicates. Standard error bars shown.

In subsequent spikes, biotransformation rates increased in both sets of cultures. The figures below depict the rate values of TCE depletion and products formation throughout chemical monitoring for both of cultures. These values include possible occurrence of abiotic losses inside the cultures and thus the values representing biotransformation and bioaccumulation are expected to be equal or lower than the ones depicted below. Moreover, these values resulted from data taken from several consecutive chemical analyses every time trichloroethene was spiked and thereby average rates could be estimated.

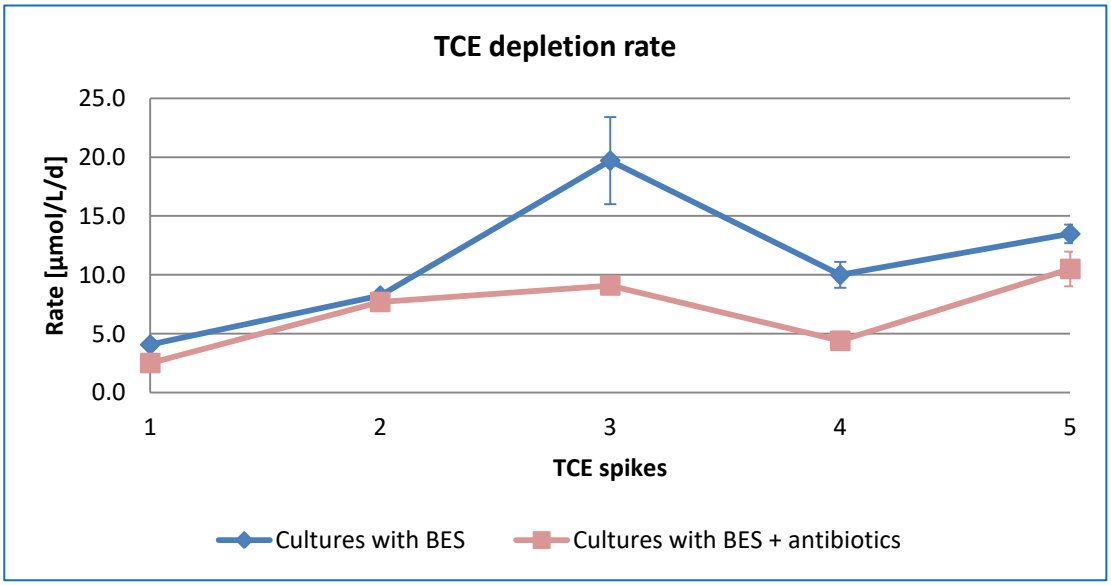

Figure 23: TCE depletion rate in cultures with antibiotics in defined mineral media for five consecutive TCE spikes. Data shown include possible abiotic losses and are the average of three replicates. Standard error bars shown.

In Figure 23, the depicted rates of trichloroethene's depletion fluctuate similarly throughout the analyses but in cultures with BES is quite higher from the third spike. Dechlorination activity was visibly confined in cultures with antibiotics but the fact that activity was continuous means that resistant to antibiotics microbes were present. Formation of cis-DCE and trans-DCE in higher rates than in the first TCE spike implies growth inside the microcosms. Figures 24 and 25 shows the comparison in products' rates between the two sets of cultures.

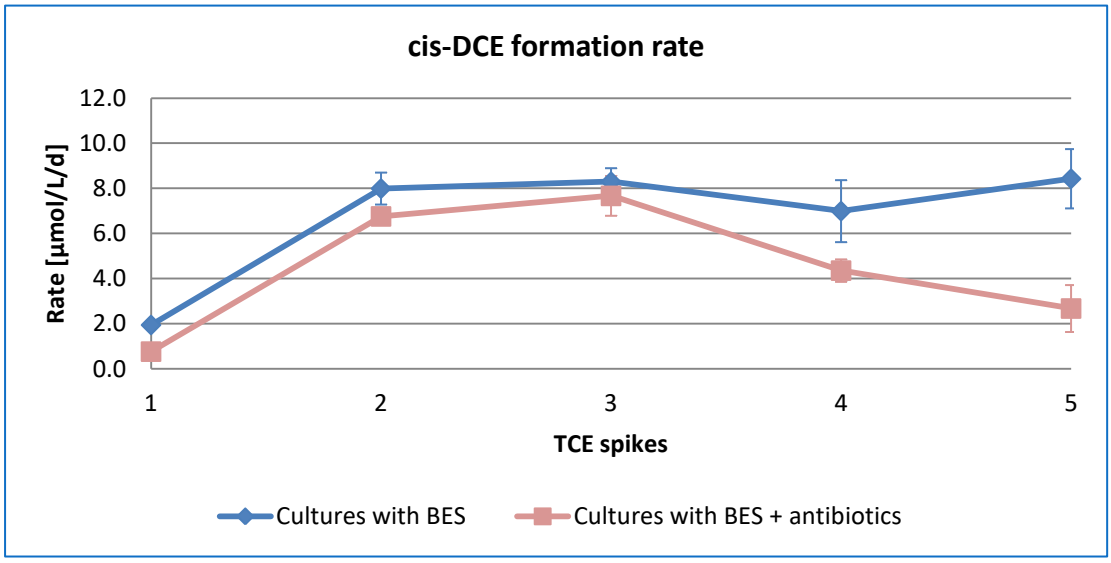

Figure 24: Rate of cis-DCE production in cultures with antibiotics in defined mineral media for five consecutive TCE spikes. Data shown include possible abiotic losses and are the average of three replicates. Standard error bars shown.

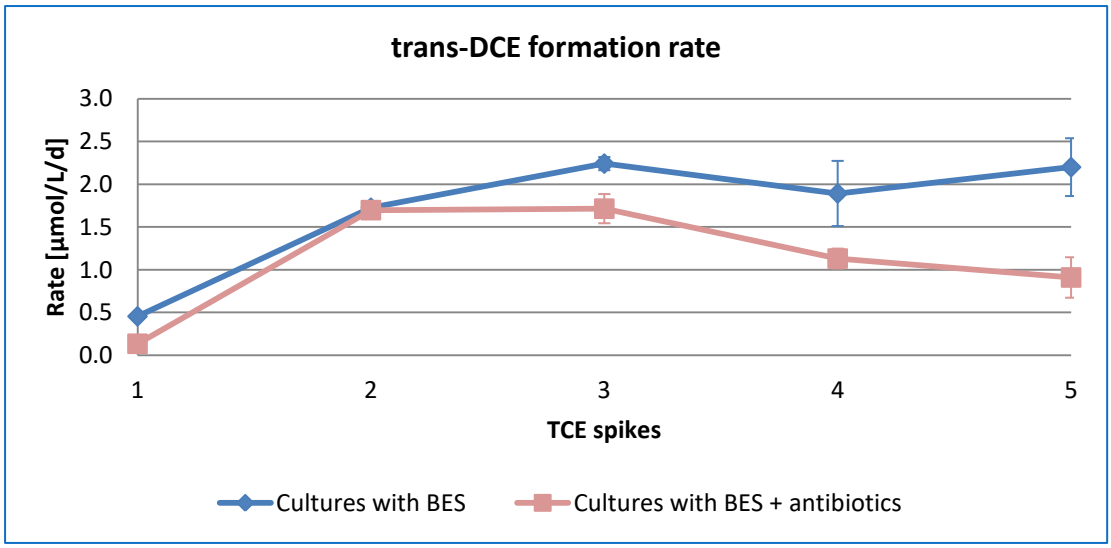

Figure 25: Rate of trans-DCE production in cultures with antibiotics in defined mineral media for five consecutive TCE spikes. Data shown include possible abiotic losses and are the average of three replicates. Standard error bars shown.

The results on the production rate of cis- and trans-DCE in cultures with BES are not differentiate much from the ones in the other set. Formation of cis- and trans-DCE is higher in cultures with BES as expected from the pattern in TCE depletion rate. Microorganisms seem to behave in similar manner in both of the cultures. To be able to examine the formation of products in each of the cultures, the following figures were formed. They show the distribution of dehalogenation products at each TCE spike.

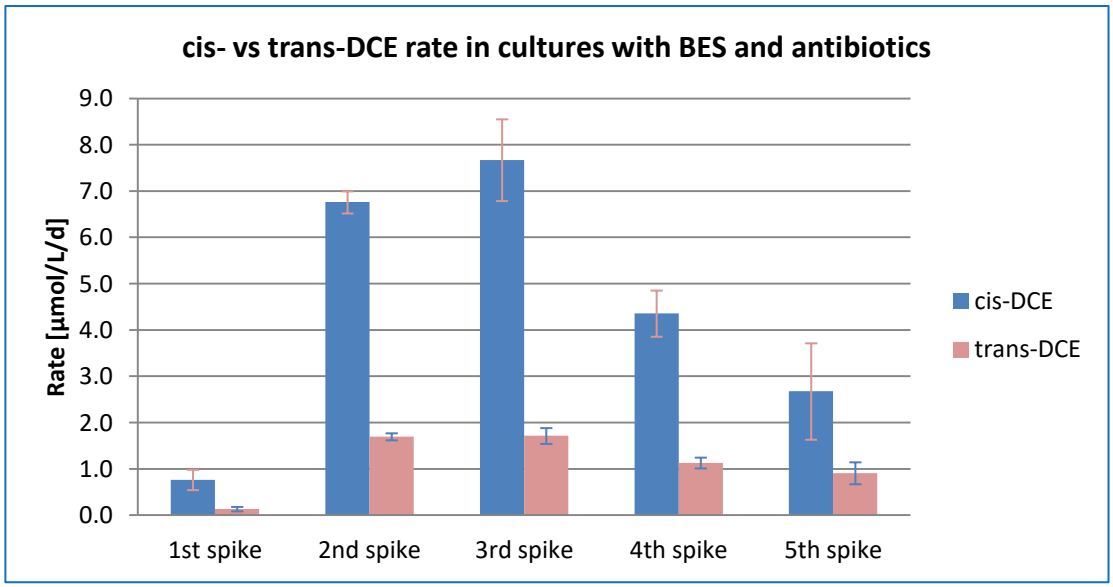

Figure 26: Comparison of cis- and trans-DCE formation rates in cultures with ampicillin, vancomycin and BES in defined mineral media for five consecutive TCE spikes. Data shown include possible abiotic losses and are the average of three replicates. Standard error bars shown.

In cultures with antibiotics and BES, as shown in **Figure 26**, the formation rates of dechlorination products were getting higher until the third spike but then they began to decrease. Microbes showed resistance in the presence of the antibiotics and until the third spike they seemed like they started to adapt in their environment but eventually antibiotics affected their dechlorination action keeping it in low levels. Nevertheless, even though formation rates were varying throughout the analyses, average ratio of cis- to trans-DCE was quite stable at 4.2±0.8.

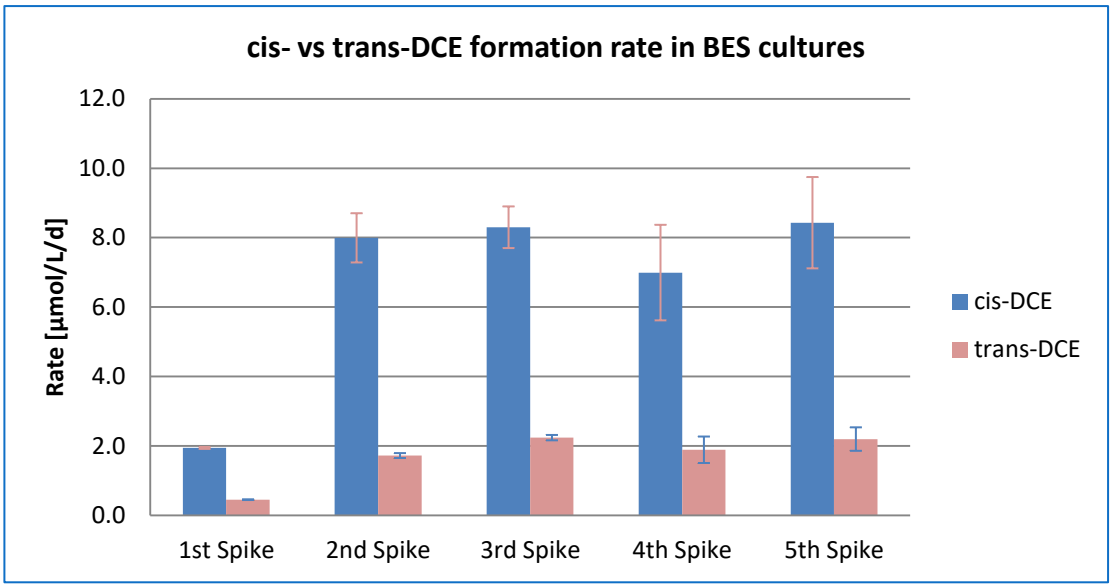

Figure 27: Comparison of cis- and trans-DCE formation rates in cultures with BES in defined mineral media for five consecutive TCE spikes. Data shown include possible abiotic losses and are the average of three replicates. Standard error bars shown.

Although the production of cis-DCE was dominant in both cultures, the ones with BES seem to preserve a stability in its formation rate from the second TCE spike till the fifth one. The average ratio of cis- to trans-DCE was 4.0±0.4, which was a little lower than the one in cultures with antibiotics. So even though cultures with only BES dechlorinated TCE in higher rate and displayed more stable production rates, the ones with BES, ampicillin and vancomycin demonstrated an equivalent ratio between the two intermediate TCE products.

Furthermore, the presence of antibiotics did not totally inhibit dechlorination as there were microorganisms inside the cultures that exhibited resistance and kept up with dechlorination. Since several members of Dehalococcoidia have been found to be resistant to bacterial peptidoglycan synthesis inhibitors like ampicillin and vancomycin, it is expected *non-Dehalococcoides* VLD-1 and VLD-2, which they were considered as the main participants in reductive dehalogenation in the former sediment cultures, to be enriched in this set of cultures. Thus, molecular analysis was performed on DNA samples obtained from microcosms amended with antibiotics. The 16S rDNA Illumina sequencing verified the enrichment of Dehalococcoidia members. However, as depicted below, only *non-Dehalococcoides* VLD-1 of the class Dehalococcoidia was found present within the cultures. In comparison with **Figure 11** in **section 2.2.4.**, the

following figure shows an increase of Dehalococcoidia from 17% to 69% of the total microbial community.

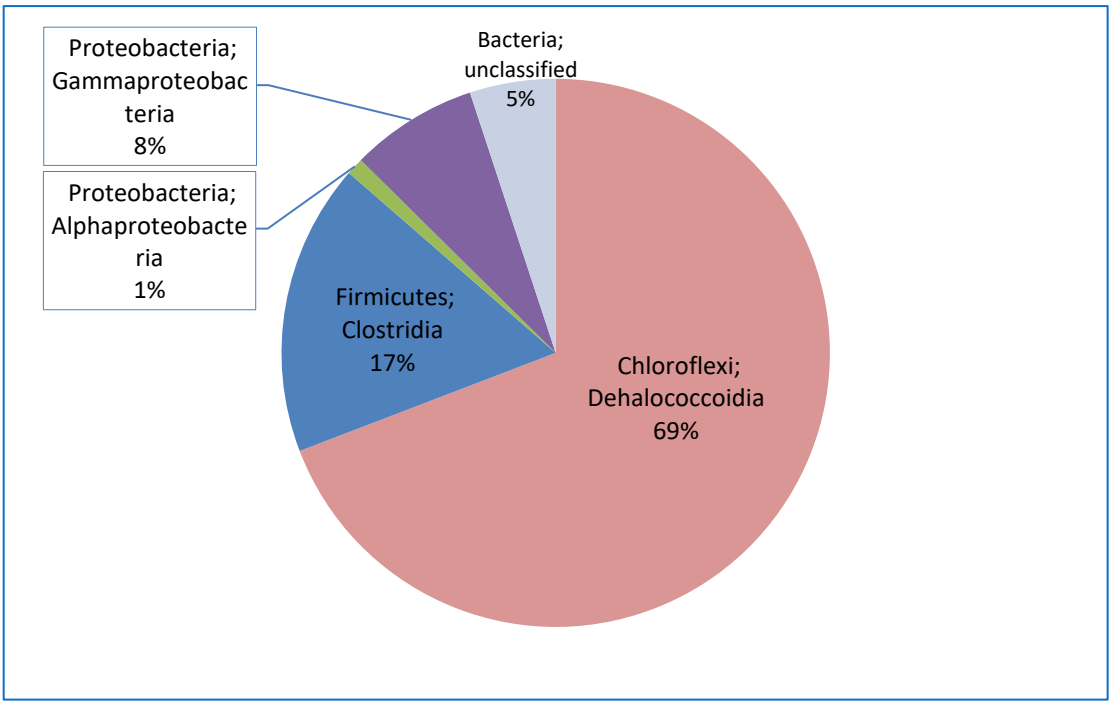

Figure 28: Pie chart of percent mean relative abundance of microbial composition at Class level in cultures amended with BES, ampicillin, vancomycin as determined by 16S rDNA Illumina Sequencing.

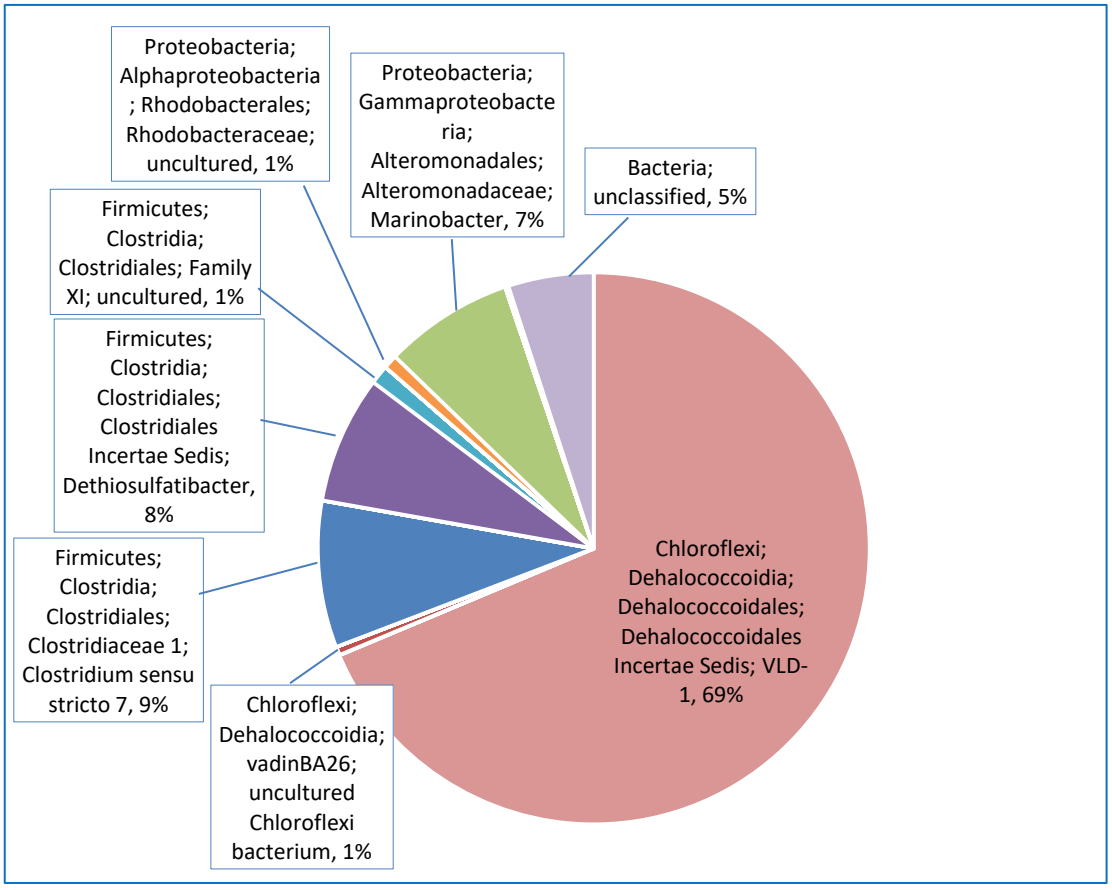

Figure 29: Pie chart of percent mean relative abundance of microbial composition at Genus level in cultures amended with BES, ampicillin, vancomycin as determined by 16S rDNA Illumina Sequencing.

In the previous section, a change in dechlorination products, where the formation rate of trans-DCE became higher than the one of cis-DCE, was observed initially in cultures with pyruvate and after several TCE spikes in cultures with lactate. A hypothesis was formed that VLD-1 might be the responsible phylotype having this effect as it has 98.8% sequence identity the dechlorinating Chloroflexi bacterium *Dehalobium chlorocoercia* DF-1 that possess the ability to produce higher amounts of *trans*-DCE than *cis*-DCE. Nevertheless, cultures amended with antibiotics did not exhibit higher trans- than cis-DCE accumulation. Yet, chemical monitoring of dechlorination products in microcosms with BES, ampicillin and vancomycin was continued for the possibility of change. After four more consecutive TCE spikes, the following results, shown in Figure 30, regarding the formation of cis- and trans-DCE, were obtained.

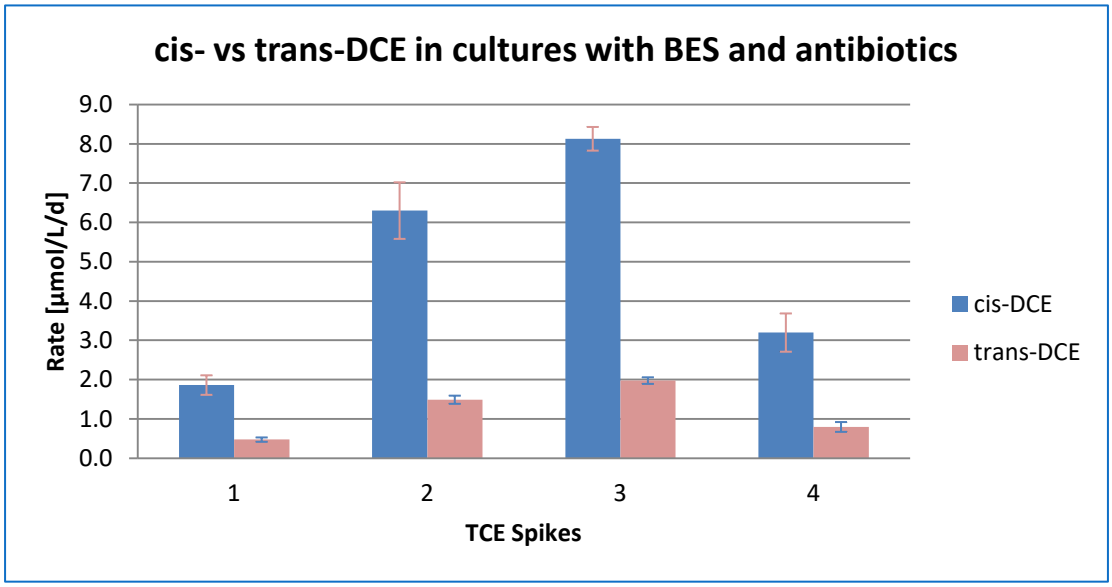

Figure 30: Comparison of cis- and trans-DCE formation rates in cultures amended with antibiotics and BES in defined mineral media for another four consecutive TCE spikes. Data shown include possible abiotic losses and are the average of three replicates. Standard error bars shown.

Cultures demonstrated a similar pattern as in the former spikes, in which formation rates are fluctuating but the cis- to trans-DCE ratio remained almost the same at 4.1±0.1. As VLD-1 is the dominant phylotype for the reductive dechlorination in this set of cultures which was subjected to 8 consecutive TCE spikes and cis-DCE remained the major product of dechlorination, the hypothesis either should be rejected because there might be another microorganism able to

produce more trans- than cis- isomers or should be accepted since there might be an unknown biochemical mechanism favoring trans-isomer over cis-isomer formation and vice versa.

4.2. Microcosms amended with different organohalides

Dechlorination was evaluated in microcosms spiked with different organohalides. As it was mentioned above, microcosms amended with lactate were selected for subculturing and each of the microcosms formed was spiked with a different contaminant (PCB, 1,2,3,4-TeCDD, HCBe, PCP and TCE). Unfortunately, none of the microcosms except of TCE-amended ones exhibited dehalogenation. That could mean microorganisms, once able to dehalogenate all organohalides tested (see **section 2.2.4.**), have confined through enrichments their dehalogenation ability to degradation of trichloroethene only. However, in order to see if microbial communities were still alive and maintained dehalogenation action, trichloroethene was added to all of the microcosms. They underwent chemical analysis for 8 weeks to monitor TCE dechlorination.

Spiked TCE (initial concentration at 104.7±3.8 μmol/L), shown in **Figure 31**, was completely dechlorinated within 3 and a half weeks. The production of cis-DCE and trans-DCE but no 1,1-DCE accumulation was observed. Rate of TCE dechlorination was 4.2±0.1 μmol/L/d, while rate of products formation was 3.1±0.3 μmol/L/d for cis-DCE and 1.2±0.0 μmol/L/d for trans-DCE.

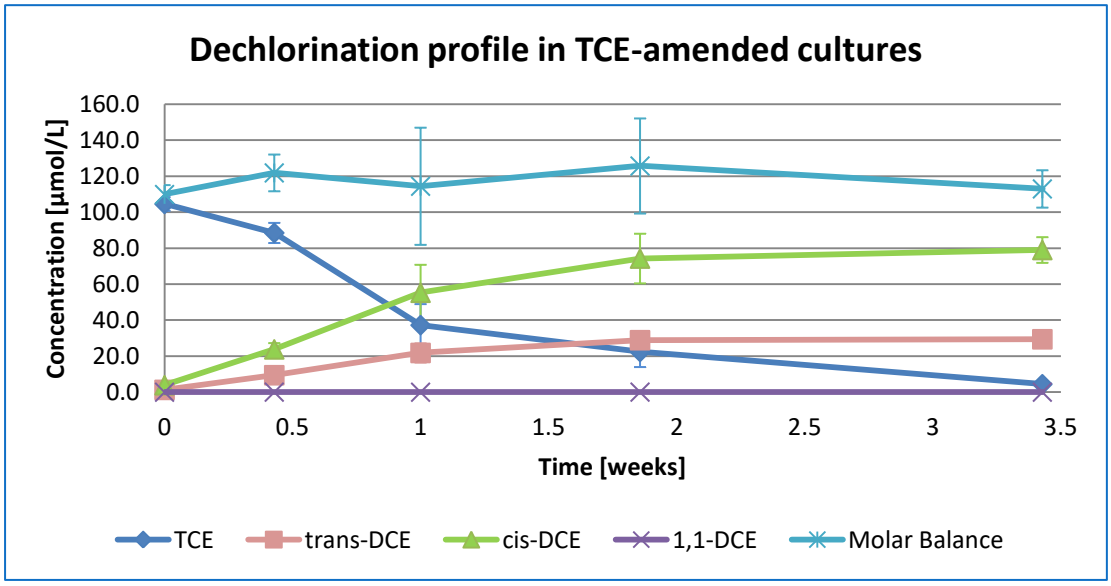

Figure 31: TCE dechlorination profile in cultures in defined mineral media. Data are the average of three replicates. Standard error bars shown.

Microcosms amended with PCBs at an initial TCE concentration of 106.8±13.4 μmol/L did not show any dechlorination action in the time interval of 8 weeks that they were monitored. Trichloroethene's concentration was reducing due to abiotic losses only as no TCE intermediates were formed. Similar results obtained from PCP- and TeCDD-amended microcosms with initial TCE concentrations of 141.3±25.5 μmol/L and 106.3±15.2 μmol/L, respectively.

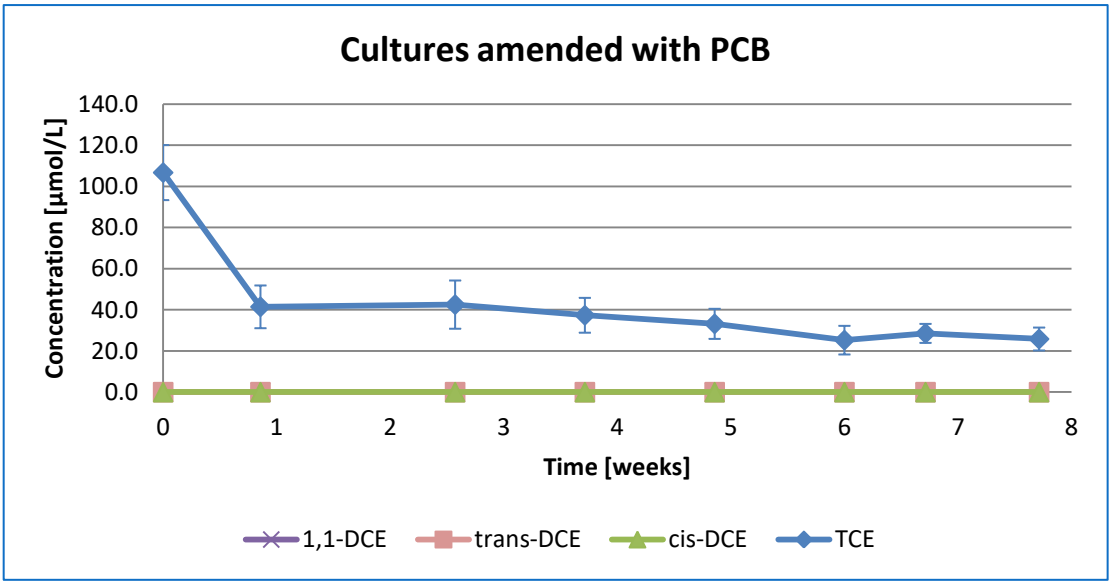

Figure 32: TCE depletion trends in cultures amended with PCB in defined mineral media. Data are the average of three replicates. Standard error bars shown.

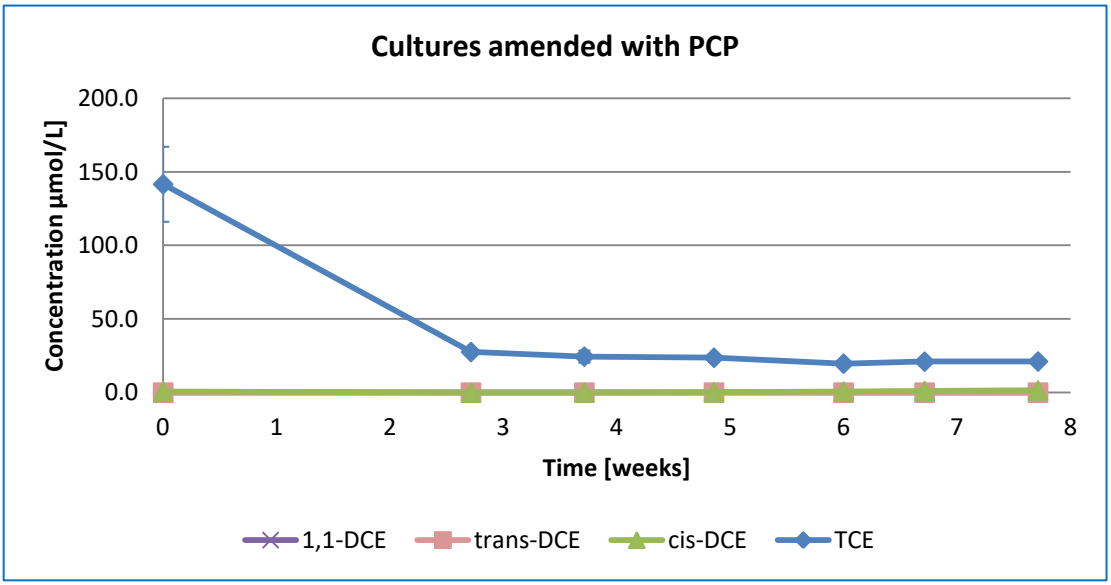

Figure 33: TCE depletion trends in cultures amended with PCP in defined mineral media. Data are the average of three replicates. Standard error bars shown.

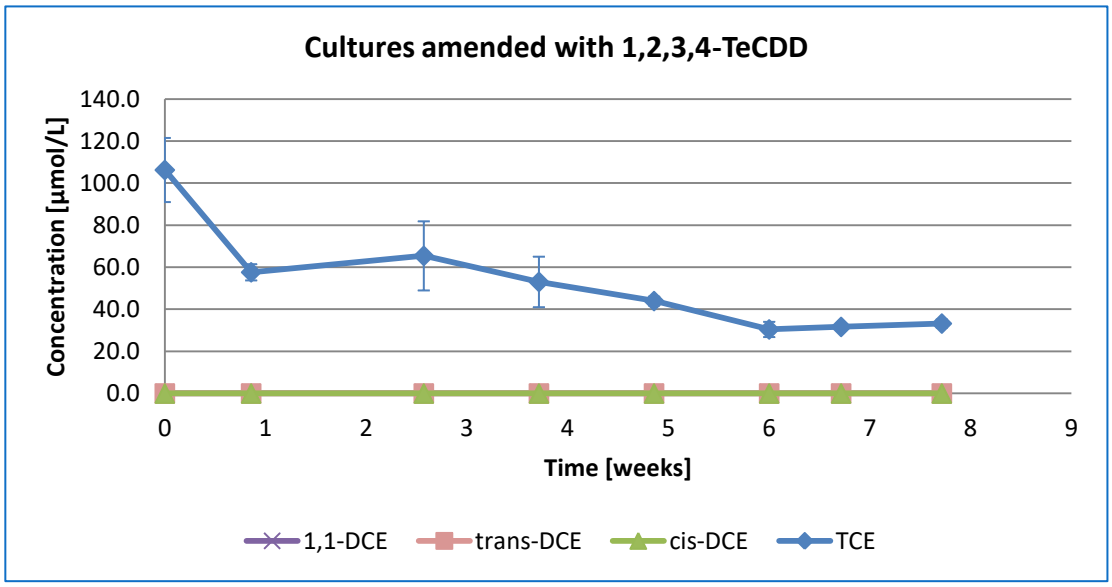

Figure 34: TCE depletion trends in cultures amended with 1,2,3,4-TeCDD in defined mineral media. Data are the average of three replicates. Standard error bars shown.

Results obtained from hexachlorobenzene-amended microcosms were controversial. Only one of three HCBe microcosms exhibited TCE dechlorination as it is shown it **Figure 35**. Bacteria were able to convert TCE mostly to cis-DCE but also a small accumulation of trans-DCE was observed. The rest of the microcosms were monitored for 8 weeks but did not show any activity except for TCE depletion, while the active one set apart for further research.

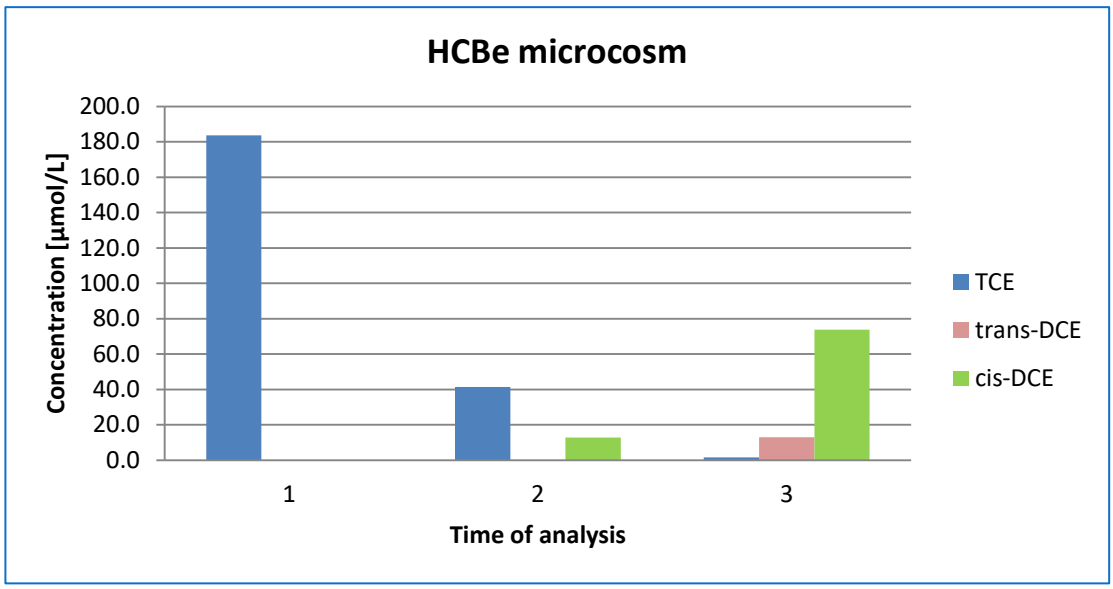

Figure 35: TCE dechlorination and products formation pattern in one culture amended with HCB in defined mineral media.

Although, organohalide-respiring microbes from the previous enriched PCB-dechlorinating sediment cultures could dehalogenate all the organohalides tested in this section, they lost this ability while subculturing in defined mineral media as they seem to dechlorinate only trichloroethene. Chloroflexi phylotype VLD-1, which was the one strongly enriched through subculturing in defined mineral media, and thus it is considered as the one microbe responsible for TCE biotransformation, was previously correlated with dechlorination of other organohalides (Nuzzo et al., 2017). Potentially, the presence of other synergistic microbes is required as they maybe produce a necessary molecule to activate the production of reductive dehalogenases responsible for dehalogenation of other organohalides. Therefore, because of the enrichment of the cultures that caused the loss of these microbes, VLD-1 could only dechlorinate trichloroethene. Moreover, there is the possibility that bacteria in microcosms amended with PCB, HCBe, PCP and 1,2,3,4-TeCDD were killed because of high solvent concentration. Thus, with the addition of TCE no dechlorination was observed except for one microcosm amended with hexachlorobenzene. Initially, in the former enriched sediment cultures these concentrations weren't harmful to the microorganisms. This can be mean that the presence of sediment in the microcosms might protect bacteria from possible high concentration of solvents.

With that being said, for subsequent studies, lower solvent concentrations should be added in cultures in defined mineral media to test if that is the reason for the loss of bacteria in cultures amended with different organohalides. The active microcosm with hexachlorobenzene should also be tested in order to check potential dechlorination activity of HCBe from Chloroflexi VLD-1 as it may prove if it has indeed lost its ability to dechlorinate other compounds.

CHAPTER 5

CONCLUSIONS

Enrichment cultures in defined mineral media developed from sediment samples recovered from Brentella Canal at Porto Marghera area of Venice Lagoon (Italy) were amended with carbon sources, growth inhibitors and different electron acceptors to test reductive dehalogenation activity of OHRB and to observe further enrichment of them among other bacteria in the microbial community.

Since dechlorinating microbes can use a range of electron donors such as the H_2 molecule and H_2-releasing fermentable organic substrates to reductively dechlorinate the electron acceptors given in the study, both hydrogen and organic acid lactate and pyruvate were provided to the cultures. Time courses for the dechlorination of TCE showed the simultaneous production of both trans- and cis-DCE. The formation of 1,1-DCE was never observed. The cis-DCE is frequently produced as dechlorination end product in laboratory-based microcosm studies. In addition, most of TCE dechlorinating bacteria produce the cis-DCE isomer as the major intermediate or end product. This observation appeared to be true in these set of cultures at least at the beginning of chemical monitoring. Yet, after a few weeks, trans-DCE constituted the major DCE isomer detected initially in pyruvate microcosms and afterwards in lactate ones. Apparent accumulation of one DCE isomer could be due to its preferential production or to isomer-specific degradation. Since no cis-DCE consumption occurred, there a microbial process favoring trans-isomer over cis-isomer formation. Former studies where cultures were in sediment slurry media, revealed that two Chloroflexi phylotypes of the class Dehalococcoidia, VLD-1 and VLD-2 were considered as the main phylotypes for the dehalogenation activity. VLD-1 is suggested to be the one responsible for this change in products formation as it has 98.8% sequence identity with the dechlorinating Chloroflexi bacterium *Dehalobium chlorocoercia* DF-1 which is known to produce more trans- than cis-DCE.

Further, the involvement of these Dehalococcoidia populations is supported by the fact that dechlorination of TCE to trans- and cis-DCE also occurred in the presence of ampicillin, vancomycin and 2-bromoethanesupfonate (BES) since several species of Dehalococcoidia class are known to be resistant to them. Dechlorination activity was very slow at the beginning indicating that most of TCE depletion was due to abiotic losses but afterwards it started to accelerate. This was ascribed as the growth of VLD-1 since it was the phylotype massively enriched as molecular analysis of the microbial community revealed. In contrast to the results in cultures with lactate and pyruvate, no change in accumulation of DCE isomers was observed, indicating that there might be an unknown biochemical mechanism favoring the trans- or the cis-isomer and maybe it is connected to the presence of other OHRB.

Finally, enrichment cultures were amended with different organohalides as electron acceptors but no reductive dehalogenation was observed. Dehalogenation ability is limited to TCE only either because of bacteria's enrichment that lacked production of dehalogenases capable of other contaminants' dechlorination or because of high concentration of contaminants turning to be harmful to OHRB. Additional microbiological analyses are needed to be done based on expression studies to acquire more information about the biochemical mechanisms responsible for organohalide-respiring bacteria's behavior that will lead to more certain conclusions and deeper understanding.

To be concluded, most of the known dechlorinators grow slowly (e.g. OHRB of the class Dehalococcoidia) and they do not tend to be the dominant populations even in an enrichment culture. This study demonstrated that certain bacterial species of the *non-Dehalococcoides* genus can be enriched in the presence of antibiotics. OHRB cultures can be enriched without necessary isolation by continuous amendment of the organohalide substrate and by providing the proper conditions for their growth. Apart from the microorganisms involved, comparison between the dehalogenation patterns and ratios showed highlights and also some differences attributable to other factors affecting microbial dechlorination such as the influence of organic acids provided as electron donors, the presence of growth inhibitors, the organohalide supplied as electron

acceptor and its concentration as it may be inhibitory for the process. Given the great biochemical potential of anaerobic reductive dehalogenation, additional development is needed to convert the many microbiological studies of OHRB into practical methods for bioremediation. Their capacity to grow by using contaminants and specific-stimulating carbon sources allows them to proliferate in contaminated soil, sediment or groundwater offering promising *in situ* decontamination efforts.

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