We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Chapter 4

Microbial Degradation of Some Halogenated Compounds: Biochemical and Molecular Features

Yu-Huei Peng and Yang-hsin Shih

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/56306

1. Introduction

Due to the advance of organic and synthetic chemistry and many applications of man-made organic compounds, lots of xenobiotic chemicals are produced and benefit our life. However, some of them are persistent in the environment and their toxicities are accumulated through the food webs. Due to the potential hazard for human and the ecosystem, the regulations on the usage of these persistent organic pollutants (POPs) and the development of safe decomposition methods are now in great request.

Tetrachloroethene (PCE) and trichloroethene (TCE) (Table 1) have been widely used as dry cleaning solvents and degreasing agents. Due to poor disposal practices and accidental release, they are within the most-abundant groundwater contaminants. Exposure to PCE is injurious to epidermis, kidney and nervous system [1]. It has been classified as a probably carcinogen [2]. Exposure to TCE leads to acute effects on liver, kidney, central nervous, and endocrine systems. It is also associated with several types of cancers based upon epidemiological research [3]. PCE and TCE are regulated in U.S.A. to a maximum contaminant level of 5 ppb. The use of PCE and TCE in the food and pharmaceutical industries has been banned across much of the world since the 1970s. However, these chemicals are still used as a degreasing agent for other demand. Besides, the mono-chlorinated ethene, vinyl chloride (VC), is known as carcinogen that causes liver cancer [4] and the *cis*-1,2-dichloroethene (cDCE) is harmful to nervous system, liver and blood cells [5].

Polybrominated diphenyl ethers (PBDEs), composed by two phenol rings and linked by one oxygen atom (Table 1), allow maximum ten bromide atom incorporated on the phenol rings to form 209 possible congeners. They have been widely used as flame retardants in many products over more than three decades. Their usage has protected both human lives and their properties from fire damage. PBDEs disrupt the balance of thyroid hormone, lead to repro-

INTECH
open science | open minds

© 2013 Peng and Shih; licensee InTech. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

ductive toxicity, hepatic toxicity, immunotoxicity and developmental neurotoxicity in mammals [6, 7]. The toxicity of PBDEs and their metabolites are due to elevated free radicals, DNA damages, cell cycle blockage and apoptosis rate [8, 9]. Among the congeners of PBDEs, the usage of penta-BDEs and octa-BDEs has been banned in the European Union and several states of the USA; tetra-BDE to hepta-BDE have also been classified as POPs and the production of decabromodiphenyl ether (DBDE) will cease in 2013 in USA. However, the concentration of PBDEs in environment remains exponentially increasing because of the consequence of long-term usage [10]. Due to their ubiquitous distribution in the environment, potential toxicity, tendency for bioaccumulation, and the increased accumulation amount in the environment, the fates of PBDEs in the nature is serious concern for public health.

Hexabromocyclododecane (HBCD), a brominated aliphatic cyclic hydrocarbon (Table 1), is another widely used brominated flame retardant (BFR). It has animal thyroidal and developmental toxicity. The toxicity is due to altering the expression and function of metabolic enzymes, increasing hormone turn over and apoptosis [11, 12]. HBCD has been detected widely in biota and abiotic samples [11]. Due to its persistent, bioaccumulative, and toxic properties, HBCD has been proposed as a Substance of Very High Concern under the REACH regulations [13] and included on the USEPA's lists of Chemicals of Concern [14]; It is also under screening-level risk assessments to determine if it meets criteria of compounds in the Stockholm Convention and in the UN-ECE Protocol on POPs [15].

Most traditional remediation methods are not suitable for degrading chloroethenes, PBDEs and HBCD. For example, dehalogenation processes under oxidative, alkaline, or irradiation conditions are high cost of energy and treatment reagents [16, 17]. Pyrolysis is only limited for specific contaminated media with high heat conductance [18]. The generation of hazardous by-products is also a problem [19] It has been reported that PBDEs and HBCD could be photodegraded [20]; however, pollutants accumulated in the soils, sediments, water bodies are not easy approach to light. Recently, the permeable reactive barrier made by zerovalent iron offers a new direction for halogenated compounds remediation [21]. Electrons offered from iron can reduce the halogenated compounds through reductive dehalogenation.

Zerovalent iron is cheaper than above processing methods, and the shortage of low efficiency is compensated by newly developed nanotechnology. Nanoscale zerovalent metals can degrade chloroethenes, PBDEs, and other contaminants with a fast kinetics and high efficiency [22-26]. There are still some limitations by using nano-metals, such as: toxic by-products generation due to incomplete dehalogenation [23], potential hazardous effect from nanoparticles [27], and large requirement for metals. Therefore, it comes to be one of the recent trends in developing nanomaterials with high efficiency and low environmental impact, and combining with other treatment technologies.

Microbiological approaches produce less intervention to the environment and are less expensive than physical or chemical methods. Biodegradation of chloroethenes has been extensively investigated and reviewed [28-30]. Bioremediation for PBDEs and HBCD are just at the beginning. The main objective of this review is to summarize current knowledge of microbial degradation of chloroethene, PBDEs and HBCD, especially from the biochemical

http://dx.doi.org/10.5772/56306

and molecular point of view. We also attempt to compare the advantages and drawbacks of the combined approaches which may apply to field remediation.

2. Biodegradation of chloroethenes, PBDEs and HBCD

Biodegradation of chloroethenes, PBDEs and HBCD occurs in various environmental or living samples [31, 32]. In the environment, microorganisms play major roles in the degradation reactions; while intrinsic detoxicification systems in plants and animals bodies metabolize these compounds [31, 33]. In this article, the diverse and complex microbial degradation machineries are presented and compared.

Biotransformation of chloroethenes, PBDEs and HBCD in aerobic or anaerobic environments has been demonstrated (Fig. 1). In aerobic environment, chloroethenes and PBDEs are metabolized with the generation of energy or degraded cometabolically without energy-yield. In anaerobic condition, they are reduced through the energy yielded from the oxidation of electron donors, i.e. reductive dehalogenation or dehalorespiration. Biotransformation of HBCD might mediate through hydrolytic dehalogenation, which may occur either in aerobic or anaerobic conditions. Detail for the each type of reaction and the degraders will be described in the following sections.

2.1. Aerobic oxidative degradation

Under aerobic conditions, chloroethenes and PBDEs can be oxidized both cometabolically and metabolically (Fig. 1, left part). Metabolic degradation indicates the use of the above compounds as growth substrate. Chloroethenes and PBDEs more easily undergo aerobic transformation with less numbers of halogen substituent.

Metabolic degradation of cDCE and VC as sole carbon and energy source has been reported by many bacteria, such as the *Pseudomonas* sp. and *Bacillus* sp. [34, 35]. Using cDCE as auxiliary substrate for growth is much less [35] and not shown in TCE and PCE. After oxidative transformation, the auxiliary substrate may be mineralized or the carbon atoms may be incorporated into biomass. Microbial growth can be confirmed by monitoring the stable isotope fractionation and is suitable for field assessment [36].

On the contrary, cometabolic degradation of chloroethenes occurs fortuitously during the degradation of growth dependent substrates (auxiliary substrates), such as methane, ammonia, or aromatic hydrocarbons. Even cDCE can be cometabolized when VC is metabolic degraded [34]. Cometabolic degradation of TCE, DCE and VC is common [37, 38]. So far *Pseudomonas stutzeri* OX1 is the only one that could aerobically cometabolize PCE [39]. Therefore, without primary substrate supplement, intrinsic bioremediation with air or nutrients injection alone could not enhance the aerobic cometabolic mechanism and would not cause the microbial degradation of PCE and TCE contaminated sites.

PBDEs could be degraded into phenol or catechols by aerobic microbial through hydroxylation or bond cleavage [33] (Table 2). *Sphingomonas* sp. SS3 and SS33 can transform mono- or di-

halogenated DEs for growth [40, 41]. In addition, *Sphingomonas* sp. PH-07 could break down several lower-bronimated BDE congeners (up to tri-BDEs) [42]. Other PBDEs degradation bacteria are reported [43]: *Rhodococcus jostii* RHA1 and *Burkholderia xenovorans* LB400 transform several lower-brominated BDE congeners (up to penta- and hexa-BDEs); *Rhodococcus* sp. RR1 transforms di- and mono-BDEs and the *Pseudonocardia dioxanivorans* CB1190 only degrades mono-BDEs. The transformation by-products include phenol, catechol, halophenol and halocatehol, indicating nonspecific attractions. These degraders might transform PBDEs through cometabolic reactions because auxiliary substrates such as diphenyl ether are supplemented. The *Lysinibacillus fusiformis* strain DB-1, cometabolically debrominate DBDE with the metabolism of lactate, pyruvate and acetate, is isolated [44].

So far, there is only one degrader been reported can transform HBCD: *Pseudomonas* sp. HB01 [45]. Since bromide atom was not detected after degradation reaction, such transformation might not through haloelimination. In general, cometabolism requires supplement of auxiliary substrates and there is no energy yielded. Therefore, microorganisms do not favor proceed this kind of reaction. Besides, the dehalogenation reaction is usually incomplete, resulting accumulation of toxic intermediates. The contribution on the bioremediation from cometabolism is limited [30].

2.2. Anaerobic reductive dehalogenation

Reductive dehalogenation is an anaerobic respiration process. Electron donors are oxidized and the halogenated compounds are reduced through accepting the electrons. The free energy generated from this reaction supports the growth of microbial degraders. Hydrogen atom replaces the halogen atoms one after another resulting in the dehalogenation sequence from higher-numbered compounds to lower-numbered ones. Contrary to aerobic degradation, the potential for reductive dehalogenation increases with the number of halogenated substituent [29].

Hydrogen gas is generated primary by fermentative and acetogenic bacteria (Fig.1, right part). The dehalorespiration bacteria compete with hydrogenotrophs, such as sulphate-reducers, nitrate-reducers, methanogens, acetogens, and other reducers [46, 47]. Except hydrogen, other electron donors also can be used for reductive dehalogenation, ex. *Sulfurospirillum multivorans* can also use pyruvate and formate [48].

Several mixed cultures and pure strains are known to reductively transform chloroethenes. Mixed culture could cooperate and transform PCE to ethene. The pure strains belong to different genus, such as Bacillus, Dehalobacter, Dehalococcoides, Desulfitobacterium, Geobacter, and Sulfurospirillum (Table 2). Most of them only dechlorinate PCE and TCE to cDCE. Only *Dehalococcoides ethenogenes* strain 195 can reductively dechlorinate PCE to ethene. The accumulated hazardous DCE and VC is a major obstacle in bioremediation of chloroethene contaminated sites. *Dehalococcoides* sp. strain BAV1, dechlorinates DCE and VC and cometabolizes PCE and TCE; the accumulated toxic compounds can be transformed into benign ethene [49].

http://dx.doi.org/10.5772/56306

Reductive debromination of PBDEs has been reported through pure strains (Table 2) or mixed cultures. Most of the debromination processes require TCE to be co-substrate. 20 mixed microcosms can degrade octa-BDE mixture to hexa- to mono-BDEs within 2 months [50]. *Sulfurospirillum multivorans* could debrominate DBDE into hepta- and octa-BDEs after 2 months of incubation. *D. ethenogenes* strain 195 could debrominate the octa-BDE mixtures into hepta- to di-BDEs after 6 months of incubation [51]. *Dehalococcoides* sp. Strain DG could degrade octa-BDE mixture into terta- and penta-BDEs or transform penta-BDE mixture into terta-BDE [52]. Several dechlorinating bacteria, *Desulfitobacterium hafniense* PCP-1, *Dehalobacter restrictus* PER-K23, *Desulfitobacterium chlororespirans* Co23 and *Desulfitobacterium dehalogenans* JW/IU-DCI debrominate the octa-BDE mixture and the most frequently detected congeners, penta 99 and tetra 47 when PCP, PCE, 3-chloro-4-hydroxybenzoate, or 3-chloro-4-hydroxyphenylacetate are applied as co-substrates [53]. Some mixture cultures do not need halogenated compounds to stimulate PBDEs transformation [50]. Recently, a lactate-dependent bacterium, *Acetobacterium* sp. strain AG, was isolated and can transform penta-BDE mixtures without other halogenated electron acceptors [52]. We also found that the cometabolism with glucose facilitated the biodegradation of mono-BDE, in terms of kinetics and efficiency in one anaerobic sludge in Taiwan [54].

In a mix microcosm, anaerobic environment necessary for dehalorespiration could be established by other symbiotic microorganisms. In our previous study, the mono-BDE is transformed to diphenyl ether in an aerobic culture from sewage sediment, indicating an anaerobic debromination reaction occurred. The enriched *Clostridiales* specie shown in the denatured gradient gel electrophoresis (DGGE) may responsible for such reaction [55].

2.3. Degradation enzymes

The metabolic pathway of VC is much clearer than that of cDCE. Alkene monooxygenase (AkMO) involves in the initial epoxidation step. The encoded genes (*etnABCD*) and the structures have been identified. Downstream events of the transformation are mediated through coenzyme M transferase (encoded by *etnE* gene), alcohol/aldehyde dehydrogenase, CoA transferase and CoM reductase/carboxylase. The final product, acyl-CoA, is then metabolized through TCA cycle [56]. Proteomic and transcriptomic analyses have confirmed the roles of above enzymes in aerobic VC transformation process.

Aerobic cometabolic degradation of chloroethenes is supposed through several kinds of oxygenases: toluene monooxygenase, toluene dioxygenase, phenol monooxygenase and methane monooxygenase [57]. *P. stutzeri* OX1 depletes PCE and releases chloride irons when toluene is applied as an auxiliary substrate [39]. PCE, DCE, and VC could be transformed by the purified toluene-*o*-xylene monooxygenase (ToMO). ToMO is a four-component enzyme which consists a catalytic oxygen-bridged dinuclear center encoded by *touABE*, a NADH ferredoxin oxidoreductase (encoded by *touF*), a mediating protein (encoded by *touD*), and a Rieske-type ferredoxin (encoded by *touC*). The *touA~F* genes cloned into *E. Coli* could make it to be PCE-degradable.

Different dioxygenases are supposed to involve in aerobic degradation of lower numbered PBDEs. 1,2-dioxygenase is involving in the initial dihydroxylation step when mono-halogen-

ated DEs to be degraded [40]. Downstream degradation processes are supposed through phenol hydroxylases and catechol 1,2-dioxygenase. The transformation by-products range from phenol, catechol, halophenol and halocatechol, indicating nonspecific attack reactions [40, 41]. 2,3-dioxygenase is responsible to dihydroxylate lower numbered PBDEs and their similar chemicals such as DE in the close species *Sphingomonas sp.* PH-07 [42]. The range of PBDEs transformed by *R. jostii* RHA1 depends on the types of growth substrate. The enzymes responsible for degradation are inducible [43]. The expression of biphenyl dioxygenase (BPDO) and ethylbenzene dioxygenase (EBDO) are upregulated during PBDEs degradation. Ectopically expression of these enzymes in closed bacteria that bears no PBDEs degradation activity could transform PBDEs. EBDO depleted mono- through penta-BDEs and BPDO only depleted mono-, di- and one tetra-BDEs. [58].

The structures of HBCD and hexachlorocyclohexanes (HCHs) are quiet similar. Heeb et al. purified the HCH-converting haloalkane dehalogenase LinB, from *Sphigobium indicum* B90A and applied the enzyme for HBCD degradation. LinB transforms HBCD into pentabromocyclododecanols (PBCDOHs) and further tetrabromocyclododecadiols (TBCDDOHs) [59]. Whether LinB or other haloalkane dehalogenase are the de novo HBCD degradation enzyme is unknown. What enzyme responsible for HBCD degradation in *Pseudomonas* sp. HB01 is also waited to be uncovered.

Reductive dechlorination reactions are catalyzed by reductive dehalogenases (RDases) The purified PCE RDase, PceA, has proved to transform PCE and TCE to cDCE [60]. The function of TCE RDase, TceA, in transforming TCE to ethene has also been identified [61]. VcrA and BvcA catalyze the transformation of DCE to ethene [62, 63]. In addition to chloroethenes, RDases also could reduce other chlorinated compounds.

RDases which could debrominate PBDEs have not yet been identified. However, some PBDEs degradation bacteria also could transform chloroethene (Table 2), such as *Dehalococcoides* sp., *Desulfitobacterium* sp., and *Sulfurospirillum* sp. Whether these microorganisms use chloroethene RDases to transform PBDEs is unknown. It is also possible that enzymes with different degradation activities or substrate specificity within single degrader may cooperatively transform different PBDEs congeners.

2.4. The structure and function of reductive dehalogenase

Most RDases presented similar features and conserved motifs [28, 29]. In the N-terminus, RDases possess a putative signal sequence containing the twin-arginine translocation (Tat) motif. Such motif is presented in secretary proteins to be transported across the cytoplasmic membrane through the Tat export system. It is proposed that newly synthesized RDase proteins is folded with cofactors (corrinoid and iron-sulfur clusters) in the cytoplasm with the aids of chaperone proteins. The Tat sequence is then proteolytically cleaved during the maturation process. In the C-terminus, two iron-sulfur cluster binding motifs are presented. The Fe-S clusters cooperate with corrinoid, transfering electrons from upstream donors to chloroethenes and thus catalyze the dehalogenation reaction [28].

http://dx.doi.org/10.5772/56306

The localization of chloroethene RDases is supposed in the membrane, where they could accept electrons from proton producing hydrogenase via menaquinone. The membrane-bound characteristics of RDases has been proved, such as PceA of *D. ethenogenes*. The localization of constitutively expressed PceA in *S. multivorans* was initially found in the cytoplasmic fraction [65]. John et al. used freeze-fracture replica immunogold labeling technique and found it would be at the cytoplasmic membrane when cells grown on pyruvate or formate as electron donors [66].

2.5. Genomic structure and transcription regulation of reductive dehalogenases

The major catalysis reaction of RDases is directed by subunit A, encoded by reductive dehalogenase homologous A (*rdhA*) genes. Over 650 *rdhA* genes have been identified based upon genomic sequence annotation or homologous cloning [67]. However, most of them are not yet been functional characterized. It is common for one dehalorespiration bacterium baring multicopy of *rdhA* genes in the genome. Besides *tceA* and *pceA* genes, there is still 17 RDase genes with unknown function in the genome of *D. ethenogenes* strain 195 [68]. Whether the roles of these genes are relevant to dehalogenation remains unclear.

Most *rdhA* genes are organized with genes encoding for accessory proteins. The *pce*-gene cluster from *D. hafniense* strain Y51 constitutes *pceA* followed by *pceB*, *pceC* and *pceT* [69]. PceT is the trigger factor involving in folding newly synthesized polypeptides. It interacts with the Tat motif of PceA, thus solubilizing and stabilizing PceA polypeptide proceeding downstream maturation and transportation processes [62]. PceB protein contains three transmembrane domains and is assumed as a membrane anchor protein of PceA. PceC contains six-trans-membrane domains, an FMN binding domain, and a C-terminal polyferredoxin-like domain. It is similar to the membrane-binding transcription regulators [28, 60]. More examples for the organization of different RDase gene clusters are presented in [28]. *RdhA* and *rdhB* genes usually locate adjacent and are the basic components of the *rdh* gene cluster. They would co-express in order to perform dehalorespiration together.

The expression and silence of RDases during dechlorination reaction is dynamic and regulated. It could be monitored through the amount of RNA or protein. *Dehalococcoides* sp. strain MB bares 7 RDase genes. Only *dceA6* is highly expressed when PCE and TCE are transformed into tDCE. Transcription regulation protein binding site related to gene expression is detected in the upstream of *dceA6* gene [70]. A shotgun metagenome microarray is created to investigate gene transcription in a mixed culture. *rdhA14* and *rdhB14*, are the only two with higher transcript levels during VC degradation, while another 4 *rdhA* genes has higher transcript levels in the absence of VC [71]. The absolute quantification of RDase proteins during the dechlorination process is performed by using nano-liquid chromatography-tandem mass spectrometry in two PCE/TCE degradation consortia. Within 5 selected RDases, only the quantities of PceA and TceA are detectable [72]. The regulation on the expression of *rdh* genes during dechlorination reaction or steady state is not clear. How the physiological environments affect the gene expression is also unclear. Uncover these questions would be helpful for environmental monitoring and remediation.

2.6. The dynamic of degrading population and the evolution of degradation ability

The complete dehalogenation requires different microorganisms which bear various functions in degradation or growth support. Besides, it competes with methanogens and other reducers for H_2. The snapshot of the microbial composition stands for specific ecological condition. The dynamic of composition reveals the effect of various remediation treatments and the interaction between microorganisms. The microbial compositions when co-incubated with zerovalent iron (ZVI) are analyzed by DGGE. The enrichment of iron-reducing bacteria would support the reduction activity of iron for multiple rounds of reactions; the enrichment of nitrate-reducing bacteria also facilitates the cometabolic dehalogenation. These may due to the synergistic effect [54]. Terminal restriction fragment length polymorphism (TRFLP) analysis is also used to analyze the microbial compositions [73]. The resolution limitation of these techniques makes underestimating the complexity of a community. Therefore, new technique is needed to detect specific microbes that are responsible for a key biodegradation process while present in the communities in low numbers. The 16S rRNA genes within a community could be analyzed by recently evolved pyrosequencing or phylogenetic microarray (PhyloChip). PhyloChip composes ten thousands unique 16S rRNA genes. The microbial compositions in TCE contaminated groundwater that is biostimulated or bioaugmented are analyzed. The increase of methanogens at late treatment stage coincident with the increase in methane concentration [74].

There is no close phylogenic relation among diverse dehalorespration degraders. Horizontal gene transfer (HGT) though transposable elements, transmissible plasmids or phage infections is assumed for such convergent evolution. Phylogenetic analyses of the sequences of *rdh* operon and the adjacent genomic structures support HGT. The *pceABC* operon in *D. hafniense* strain TCE1 has been shown to be presented in a circularized transposable element, Tn-Dha1 [75].The single-copy transfer messenger RNA gene (*ssrA*) essential in bacteria is a common target for mobile element. Integration of mobile element results in the duplication of *ssrA* gene around transported gene cluster. Many strain-specific *rdhA* genes collocates within such structures and in a region of high genomic variability between *Dehalococcoides* strains [76]. According to the metagenomic sequence analysis, one prophage element is located adjacent to *tceAB* genes in the *Dehalococcoides*-containing consortium, KB-1. The failure in detecting *tceA* gene expression in virus and the more closed transposase genes indicating higher possibility for HGT through transposable elements [71]. It seems that dehalorespiration degraders do no acquire RDase genes through single way. This would increase the diversity of degraders and function of RDase, which is advantageous for remediation.

3. Integration of biodegradation with other remediation methods

The degradation rate of natural attenuation is slower than chemical or physical treatments. Biostimulation or bioaugement are common strategies for bioremediation of chloroethene [30] Chemical supplements such as potassium permanganate or oxygen injection which can increase oxygen concentration are benefit for microbial dechlorination [77]. Kuo and his

http://dx.doi.org/10.5772/56306

collages set biosparging wells for injection substrate and air into TCE contaminated area. Above 95% TCE was removed through cometabolic reactions because the elevated chemical oxygen demand (COD), microbial population, oxidation-reduction potential (ORP) and specific degrading genes after the supplement of substrate [78]. Shortage of auxiliary substrates or accumulation of toxic intermediates also decrease the dehalogenation effect, combined remediation methods may recover the above drawbacks.

Sequential anaerobic/aerobic biodegradation is one of the approaches to accelerate the degradation of recalcitrant halogenated compounds. Anaerobic degraders could target the higher-numbered halogenated compounds. Aerobic degraders only process lower-numbered halogenated compounds. They could transform the by-products produced from anaerobic degradation to antoxic compounds through metabolic or cometabolic reactions. Integration of these two systems makes it possible for complete mineralization.

Chloroethene and PBDEs can be depleted by microscale or nanoscale zerovalent metals [79, 80]. Preliminary dehalogenation of highly halogenated compounds by the reduced metal to generate less halogenated byproducts those are susceptible for microbial degradation. Therefore, the integration of zerovalent metals with biodegradation promotes the dehalogenation efficiency of each type of remediation methods. Reductive debromination of DBDE with nanoscale ZVI (nZVI) results various intermediates ranging from nona-BDEs to tri-BDEs. The known aerobic PBDEs degrader, *Sphingomonas* sp. strain PH-07, which is able to grow in the presence of nZVI, aerobically mineralizes the low brominated-DEs (tri-BDEs – mono-BDEs) from nZVI treatment [81]. The interactions between metals and microbes are complicated and delicate. H_2 generated from the oxidation of metals promotes the growth of some dehalorespiration microbes. Some microorganisms could reduce oxidized metals for multiple runs of reductive reactions or degrade target compounds through cometabolism. Co-incubation with ZVI, microbes in the DBDE-degrading anaerobic sludges hinders the accessibility of MZVI to DBDE and reduced the removal ability in initial stage. However, the synergistic effect in DBDE degradation appears later on. According to the analysis of the microbial community change, co-incubation with MZVI leads to the enrichment of heterotrophic microbial populations bearing nitrate- or iron-reducing activities. The interaction between MZVI and microbes contributed to the synergistic effect [54]. Not only is the growth of microbes affected by metals, but also the expression of functional RDases. Bare nZVI down-regulate the expression of *tceA* and *vcrA* genes while coated particles up-regulate their expression [82]. In addition to the reduced metals, combining the electro-fenton process in aerobic degradation is also a newly evolved and potential way in bioremediation. Application of electrolysis also stimulate the microbial reductive dechlorination and oxidative activities [83].

There are advantages by using each type of remediation approach, while there are also limitations and drawbacks. Combining biodegradation with other abiotic/biotic degradation approaches could overcome their weakness and accelerate the degradation efficiency. The recalcitrant halogenated compounds could be completely mineralized. The impact on environment might also be minimized. Integration of different approaches is a new direction for future investigation.

4. Conclusion

The current knowledge of microbial degradation of chloroethenes, PBDEs and HBCD, has been summarized and reviewed. The biodegradation of these halogenated compounds through aerobic oxidation, aerobic cometabolization, or reductive dehalogenation are introduced. The correspondent enzymes are discussed from the biochemical and molecular point of view. The structure and function of RDases, as well as gene expression regulation and genomic evolution are the major focus. Integration and sequential anaerobic/aerobic biodegradation or (electro)chemical/microbial degradation are suggested for overcoming the disadvantages of single type of treatment. It is possible to completely mineralize these halogenated pollutants by the combination of bio- and abiotic processes and shows promise for site remediation in natural settings and in engineered systems.

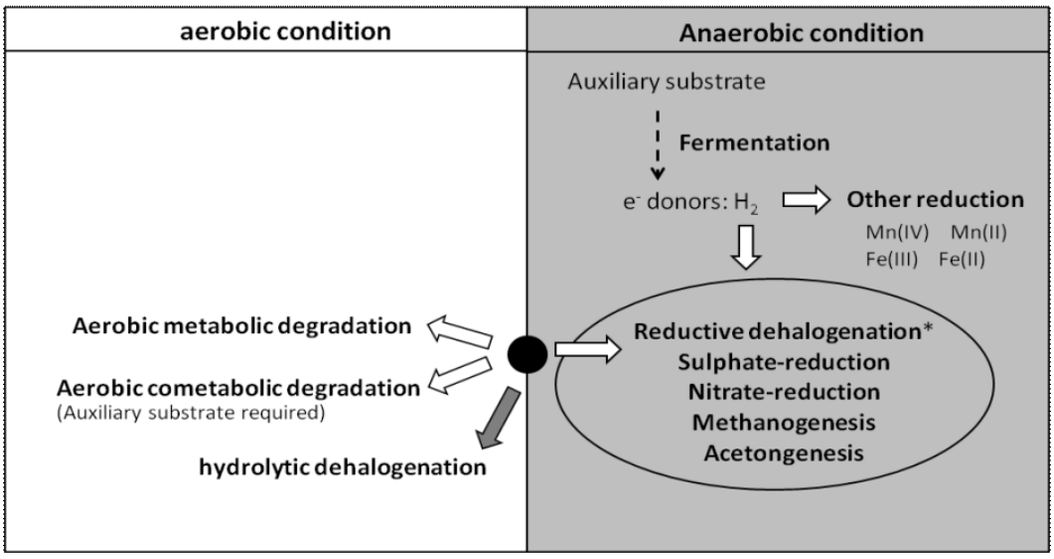
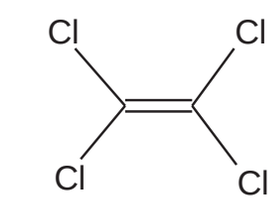
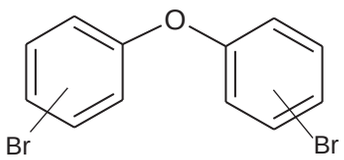
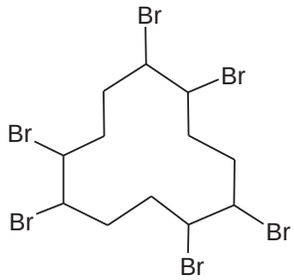


Figure 1. Aerobic degradation and anaerobic reductive dehalogenation reactions of chloeoethenes, PBDEs and HBCD. Circle dot indicates above compounds; star indicates reductive dehalogenation driven by the oxidation of electron donors or occurring cometabolically with other dehalorespiration process; gray arrow indicates the hydrolytic dehalogenation of HBCD.

PCE/TCE	PBDEs	HBCD
 or H		

http://dx.doi.org/10.5772/56306

Molecular weight	165/131	249.1 ~ 959.2	641.7
Water solubility (mg l^{-1})	150/1280	4.8 ~0.02	0.003
Toxicity[a]	Epidermis, liver and kidney damage, immune- and neuro-toxicity, reproductive and endocrine effects, probably cancer	Disrupt the balance of thyroid hormone, reproductive, hepatic, and immunotoxicity, developmental neurotoxicity	Thyroidal and developmental toxicity
Abiotic degradation	Chemical oxidation , irradiation, reduced metals (Fe, Fe/Pd)	Pyrolysis, photolysis, reduced metals (Fe, Fe/Pd),	Pyrolysis, photolysis
Biological degradation	Aerobic/anaerobic	Aerobic/anaerobic	Aerobic/anaerobic(?)

a: the toxicity of HBCD is based upon investigation in animal model.

Table 1. Physicochemical properties, biological impacts and degradation routes of chloroethenes, PBDEs and HBCD.

	Substrate	**End-products**	**Genes** [c]	**references**
Aerobic				
Burkholderia xenovorans LB400	Hexa-BDE to mono-BDE,	Hydroxylated-BDE		[43]
Lysinibacillus fusiformis strain DB-1	DBDE	ND		[44]
Pseudonocardia dioxanivorans CB1190	Mono-BDE	ND		[43]
Pseudomonas sp. HB01	HBCD	PBCDOHs, TBCDDOHs		[45]
Pseudomonas stutzeri OX1	PCE	Cl^-,	ToMO-*touABCDEF*	[39]
Rhodococcus jostii RHA1	Penta-BDE to mono-BDE	Hydroxylated-BDE	BPDO- *bphAa*, EBDO- *etbAa1*, *etbAc*	[43, 84]
Rhodococcus sp. RR1	Di-, and mono-BDE	ND		[43]
Sphingomonas sp. PH-07	Tri-, di-, and mono-BDE	Catechol, dibromophenol, dihydroxy mono- and dibromo-BDE		[42]
Sphingomonas sp. SS3	Fluoro-, chloro-, and bromo-DE	Phenol, catechol, Halophenol and Halocatehol		[40]

Sphingomonas sp. SS33	Di-, and mono-; fluoro-, chloro-, and bromo-DE	Phenol, catechol, di-, and mono-halophenol, di-, and mono-halocatehol		[41]
Anaerobic				
Acetobacterium sp. strain AG	Penta-BDE mixture[a]	Tetra-, tri-, and di-BDEs		[52]
Bacillus sp JSK1	PCE	*Cis*-DCE		[85]
Dehalobacter restrictus	PCE, TCE	*Cis*-DCE	*pceA,*	[67, 86]
Dehalococcoides sp. strain BAV1	DCE, VC	Ethene	*bvcA*	[49, 87]
Dehalococcoides sp. strain DG	TCE Octa-BDE mixture[b] Penta- BDE mixture[a]	Ethene Tetra- and penta-BDEs Tetra- BDE		[52]
Dehalococcoides ethenogenes strain 195	PCE, TCE, cis -DCE, and VC Octa-BDE mixture[b]	Ethene Tetra-, penta-, hexa- and Penta-BDEs	*pceA, tceA*	[51, 67, 89]
Dehalococcoides sp. strain MB	PCE, TCE	*Trans*-DCE		[90]
Desulfitobacterium chlororespirans strain Co23	Octa-BDE	ND		[53]
Desulfitobacterium dehalogenans strain JW/IU-DC1	PCE, TCE, Octa-BDE,	ND		[53, 67, 91]
Desulfitobacterium hafniense PCP-1	Octa-BDE	ND		[53]
Geobacter lovleyi strain SZ	PCE, TCE	cDCE		[92]
Sulfurospirillum multivorans	PCE, TCE DBDE	DCE Octa- and hepta-BDEs	*pceA*	[51, 67]

a penta- BDE mixture: hexa-, penta-, and tera-BDEs.

b Octa-BDE mixture: nona-, octa-, hepta-, and hexa-BDEs.

c Genes that only relevant to degradation of chloroethens, PBDEs and HBCD are listed.

ND: data not shown. BPDO: biphenyl dioxygenase. EBDO: ethylbenzene dioxygenase. PBCDOHs: pentabromocyclododecanols. TBCDDOHs: tetrabromocyclododecadiols.

Table 2. Selected bacteria which degrade chloroethens, PBDEs and HBCD.

http://dx.doi.org/10.5772/56306

Acknowledgements

The authors thank National Science Council (NSC), Taiwan, ROC for financial support.

Author details

Yu-Huei Peng and Yang-hsin Shih*

*Address all correspondence to: yhs@ntu.edu.tw

Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan

References

[1] Toxicological Profile for Tetrachloroethylene (Update). ATSDR 1997.

[2] Tetrachloroethylene. IARC monograph 2007.

[3] Toxicological Profile for Trichloroethylene (Update). ATSDR 1997.

[4] Toxicologica Review of Vinyl Chloride. USEPA 2000.

[5] 1,2-dichloroethene. ATSDR 1997.

[6] Costa L. G., Giordano G. Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. Neurotoxicology 2007; 28(6) 1047-1067.

[7] Tseng L. H. et al. Developmental exposure to decabromodiphenyl ether (PBDE 209): effects on thyroid hormone and hepatic enzyme activity in male mouse offspring. Chemosphere 2008; 70(4) 640-647.

[8] An J. et al. The cytotoxic effects of synthetic 6-hydroxylated and 6-methoxylated polybrominated diphenyl ether 47 (BDE47). Environmental Toxicology 2011; 26(6) 591-599.

[9] Yan C. et al. The involvement of ROS overproduction and mitochondrial dysfunction in PBDE-47-induced apoptosis on Jurkat cells. Experimental and Toxicologic Pathology 2011; 63(5) 413-417.

[10] Gauthier L. T. et al. Temporal trends and spatial distribution of non-polybrominated diphenyl ether flame retardants in the eggs of colonial populations of Great Lakes herring gulls. Environmental Science & Technology 2009; 43(2) 312-317.

[11] Marvin C. H. et al. Hexabromocyclododecane: Current Understanding of Chemistry, Environmental Fate and Toxicology and Implications for Global Management. Environmental Science & Technology 2011; 45(20) 8613-8623.

[12] Deng J. et al. Hexabromocyclododecane-induced developmental toxicity and apoptosis in zebrafish embryos. Aquat Toxicol 2009; 93(1) 29-36.

[13] HBCD Factsheet. BSEF 2009.

[14] Hexabromocyclododecane (HBCD) Action Plan. USEPA (2010).

[15] Summary of a Proposal to List Hexabromocyclododecane in Annex A to the Convention. POPRC 2009.

[16] Honning J. et al. Role of diffusion in chemical oxidation of PCE in a dual permeability system. Environmental Science & Technology 2007; 41(24) 8426-8432.

[17] Weber R. et al. PCB destruction in subcritical and supercritical water--evaluation of PCDF formation and initial steps of degradation mechanisms. Environmental Science & Technology 2002; 36(8) 1839-1844.

[18] Lin Y. M. et al. Emissions of Polybrominated Diphenyl Ethers during the Thermal Treatment for Electric Arc Furnace Fly Ash. Aerosol and Air Quality Research 2012; 12(2) 237-250.

[19] Barontini F. et al. Thermal stability and decomposition products of hexabromocyclododecane. Industrial & Engineering Chemistry Research 2001; 40(15) 3270-3280.

[20] Shih Y.-h., Wang C.-K. Photolytic degradation of polybromodiphenyl ethers under UV-lamp and solar irradiations. Journal of Hazardous Materials 2009; 165(1-3) 34-38.

[21] Beitinger E. Permeable treatment walls - Design, construction, and cost. in: NATO/CCMS Pilot Study: Evaluation of Demonstrated and Emerging Technologies for the Treatment of Contaminated land and Grooundwater (Phase III). 1998.

[22] He F., Zhao D. Y. Hydrodechlorination of trichloroethene using stabilized Fe-Pd nanoparticles: Reaction mechanism and effects of stabilizers, catalysts and reaction conditions. Applied Catalysis B-Environmental 2008; 84(3-4) 533-540.

[23] Shih Y. H., Tai Y. T. Reaction of decabrominated diphenyl ether by zerovalent iron nanoparticles. Chemosphere 2010; 78(10) 1200-1206.

[24] Su Y.-f. et al. Effects of various ions on the dechlorination kinetics of hexachlorobenzene by nanoscale zero-valent iron. Chemosphere 2012; 88(11) 1346-1352.

[25] Shih Y.-h. et al. Pentachlorophenol reduction by Pd/Fe bimetallic nanoparticles: Effects of copper, nickel, and ferric cations. Applied Catalysis B: Environmental 2011; 105(1-2) 24-29.

[26] Shih Y. H. et al. Reduction of hexachlorobenzene by nanoscale zero-valent iron: kinetics, pH effect, and degradation mechanism. Separation and purification technology (2011)., 76(3), 268-274.

[27] Lee C. et al. Bactericidal effect of zero-valent iron nanoparticles on Escherichia coli. Environmental Science & Technology 2008; 42(13) 4927-4933.

[28] Futagami T. et al. Biochemical and genetic bases of dehalorespiration. Chemical record 2008; 8(1) 1-12.

[29] Smidt H., de Vos W. M. Anaerobic microbial dehalogenation. Annual review of microbiology (2004)., 58, 43-73.

[30] Tiehm A., Schmidt K. R. Sequential anaerobic/aerobic biodegradation of chloroethenes - aspects of field application. Current Opinion in Biotechnology 2011; 22(3) 415-421.

[31] Huang H. et al. In vitro biotransformation of PBDEs by root crude enzyme extracts: Potential role of nitrate reductase (NaR) and glutathione S-transferase (GST) in their debromination. Chemosphere (2012)., 90(6), 1885-1892.

[32] Davis J. W. et al. The transformation of hexabromocyclododecane in aerobic and anaerobic soils and aquatic sediments. Water Research 2005; 39(6) 1075-1084.

[33] Hakk H., Letcher R. J. Metabolism in the toxicokinetics and fate of brominated flame retardants--a review. Environmental international 2003; 29(6) 801-828.

[34] Tiehm A. et al. Growth kinetics and stable carbon isotope fractionation during aerobic degradation of cis-1,2-dichloroethene and vinyl chloride. Water Research 2008; 42(10–11) 2431-2438.

[35] Olaniran A. O. et al. Aerobic biodegradation of dichloroethenes by indigenous bacteria isolated from contaminated sites in Africa. Chemosphere 2008; 73(1) 24-29.

[36] Schmidt K. R. et al. Aerobic biodegradation of cis-1,2-dichloroethene as sole carbon source: Stable carbon isotope fractionation and growth characteristics. Chemosphere 2010; 78(5) 527-532.

[37] Zhang Y., Tay J. H. Co-metabolic degradation activities of trichloroethylene by phenol-grown aerobic granules. Journal of Biotechnology 2012; 162(2-3) 274-282.

[38] Frascari D. et al. A kinetic study of chlorinated solvent cometabolic biodegradation by propane-grown Rhodococcus sp. PB1. Biochemical Engineering Journal 2008; 42(2) 139-147.

[39] Ryoo D. et al. Aerobic degradation of tetrachloroethylene by toluene-o-xylene monooxygenase of Pseudomonas stutzeri OX1. Nat Biotech 2000; 18(7) 775-778.

[40] Schmidt S. et al. Biodegradation of diphenyl ether and its monohalogenated derivatives by Sphingomonas sp. strain SS3. Applied and Environmental Microbiology 1992; 58(9) 2744-50.

[41] Schmidt S. et al. Biodegradation and Transformation of 4,4'-Dihalodiphenyl and 2,4-Dihalodiphenyl Ethers by Sphingomonas Sp Strain Ss33. Applied and Environmental Microbiology 1993; 59(11) 3931-3933.

[42] Kim Y. M. et al. Biodegradation of diphenyl ether and transformation of selected brominated congeners by Sphingomonas sp. PH-07. Applied microbiology and biotechnology 2007; 77(1) 187-194.

[43] Robrock K. R. et al. Aerobic biotransformation of polybrominated diphenyl ethers (PBDEs) by bacterial isolates. Environmental Science & Technology 2009; 43(15) 5705-5711.

[44] Deng D. et al. Aerobic debromination of deca-BDE: Isolation and characterization of an indigenous isolate from a PBDE contaminated sediment. International Biodeterioration & Biodegradation 2011; 65(3) 465-469.

[45] Yamada T. et al. Isolation of Pseudomonas sp. strain HB01 which degrades the persistent brominated flame retardant gamma-hexabromocyclododecane. Bioscience, Biotechnology, and Biochemistry 2009; 73(7) 1674-1678.

[46] Aulenta F. et al. Competition for H2 between sulfate reduction and dechlorination in butyrate-fed anaerobic cultures. Process Biochemistry (2008)., 43(2), 161-168.

[47] Conrad M. E. et al. Field evidence for co-metabolism of trichloroethene stimulated by addition of electron donor to groundwater. Environmental Science & Technology (2010)., 44(12), 4697-4704.

[48] John M. et al. Retentive Memory of Bacteria: Long-Term Regulation of Dehalorespiration in Sulfurospirillum multivorans. Journal of Bacteriology 2009; 191(5) 1650-1655.

[49] He J. Z. et al. Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature 2003; 424(6944) 62-65.

[50] Lee L. K., He J. Reductive Debromination of Polybrominated Diphenyl Ethers by Anaerobic Bacteria from Soils and Sediments. Applied and environmental microbiology 2010; 76(3) 794-802.

[51] He J. Z. et al. Microbial reductive debromination of polybrominated diphenyl ethers (PBDEs). Environmental Science & Technology 2006; 40(14) 4429-4434.

[52] Ding C. et al. Isolation of Acetobacterium sp. strain AG that reductively debrominates octa- and penta-brominated diphenyl ether technical mixtures. Applied and Environmental Microbiology (2012)., doi:10.1128/AEM.02919-12

[53] Robrock K. R. et al. Pathways for the anaerobic microbial debromination of polybrominated diphenyl ethers. Environmental Science & Technology 2008; 42(8) 2845-52.

[54] Shih Y. H. et al. Synergistic effect of microscale zerovalent iron particles combined with anaerobic sludges on the degradation of decabromodiphenyl ether. Bioresource Technology (2012)., 108, 14-20.

[55] Chen C. Y. et al. Microbial degradation of 4-monobrominated diphenyl ether in an aerobic sludge and the DGGE analysis of diversity. Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes 2010; 45(5) 379-385.

[56] Mattes T. E. et al. Aerobic biodegradation of the chloroethenes: pathways, enzymes, ecology, and evolution. FEMS Microbiology Reviews 2010; 34(4) 445-475.

[57] Furukawa K. Oxygenases and dehalogenases: molecular approaches to efficient degradation of Chlorinated environmental pollutants. Bioscience, Biotechnology, and Biochemistry 2006; 70(10) 2335-2348.

[58] Robrock K. R. et al. Biphenyl and Ethylbenzene Dioxygenases of Rhodococcus jostii RHA1 Transform PBDEs. biotechnology and bioengineering 2011; 108(2) 313-321.

[59] Heeb N. V. et al. Biotransformation of Hexabromocyclododecanes (HBCDs) with LinB—An HCH-Converting Bacterial Enzyme. Environmental Science & Technology 2012; 46(12) 6566-6574.

[60] Suyama A. et al. Molecular Characterization of the PceA Reductive Dehalogenase of Desulfitobacterium sp. Strain Y51. Journal of Bacteriology 2002; 184(13) 3419-3425.

[61] Magnuson J. K. et al. Trichloroethene reductive dehalogenase from Dehalococcoides ethenogenes: Sequence of tceA and substrate range characterization. Applied and Environmental Microbiology 2000; 66(12) 5141-5147.

[62] Maillard J. et al. Redundancy and specificity of multiple trigger factor chaperones in Desulfitobacteria. Microbiology-Sgm (2011)., 157, 2410-2421.

[63] Tang S. et al. Functional Characterization of Reductive Dehalogenases Using Blue Native Polyacrylamide Gel Electrophoresis. Applied and Environmental Microbiology (2012)., 79(3), 974-981.

[64] Maillard J. et al. Characterization of the corrinoid iron-sulfur protein tetrachloroethene reductive dehalogenase of Dehalobacter restrictus. Applied and Environmental Microbiology 2003; 69(8) 4628-4638.

[65] John M. et al. Growth substrate dependent localization of tetrachloroethene reductive dehalogenase in Sulfurospirillum multivorans. Archives of Microbiology 2006; 186(2) 99-106.

[66] Regeard C. et al. Development of degenerate and specific PCR primers for the detection and isolation of known and putative chloroethene reductive dehalogenase genes. J Microbiol Methods 2004; 56(1) 107-18.

[67] West K. A. et al. Comparative genomics of "Dehalococcoides ethenogenes" 195 and an enrichment culture containing unsequenced "Dehalococcoides" strains. Applied and Environmental Microbiology 2008; 74(11) 3533-3540.

[68] Furukawa K. et al. Biochemical and molecular characterization of a tetrachloroethene dechlorinating Desulfitobacterium sp strain Y51: a review. Journal of Industrial Microbiology & Biotechnology 2005; 32(11-12) 534-541.

[69] Chow W. L. et al. Identification and transcriptional analysis of trans-DCE-producing reductive dehalogenases in Dehalococcoides species. ISME journal 2010; 4(8) 1020-1030.

[70] Waller A. S. et al. Transcriptional Analysis of a Dehalococcoides-Containing Microbial Consortium Reveals Prophage Activation. Applied and Environmental Microbiology 2012; 78(4) 1178-1186.

[71] Werner J. J. et al. Absolute quantification of Dehalococcoides proteins: enzyme bioindicators of chlorinated ethene dehalorespiration. Environmental microbiology 2009; 11(10) 2687-2697.

[72] Révész S. et al. Bacterial community changes in TCE biodegradation detected in microcosm experiments. International Biodeterioration & Biodegradation 2006; 58(3–4) 239-247.

[73] Lee P. K. H. et al. Phylogenetic Microarray Analysis of a Microbial Community Performing Reductive Dechlorination at a TCE-Contaminated Site. Environmental Science & Technology 2011; 46(2) 1044-1054.

[74] Maillard J. et al. Isolation and characterization of Tn-Dha1, a transposon containing the tetrachloroethene reductive dehalogenase of Desulfitobacterium hafniense strain TCE1. Environmental microbiology 2005; 7(1) 107-117.

[75] Sahl J. W. et al. Coupling permanganate oxidation with microbial dechlorination of tetrachloroethene. Water Environ Res. 2007; 79(1) 5-12.

[76] Kuo Y. C. et al. Remediation of TCE-contaminated groundwater using integrated biosparging and enhanced bioremediation system. Research Journal of Chemistry and Environment 2012; 16(2) 37-47.

[77] Liu Y. Q. et al. TCE dechlorination rates, pathways, and efficiency of nanoscale iron particles with different properties. Environmental Science & Technology 2005; 39(5) 1338-1345.

[78] Keum Y. S., Li Q. X. Reductive debromination of polybrominated diphenyl ethers by zerovalent iron. Environmental Science & Technology 2005; 39(7) 2280-2286.

[79] Kim Y.-M. et al. Degradation of polybrominated diphenyl ethers by a sequential treatment with nanoscale zero valent iron and aerobic biodegradation. Journal of Chemical Technology & Biotechnology 2012; 87(2) 216-224.

[80] Xiu Z. M. et al. Effect of Bare and Coated Nanoscale Zerovalent Iron on tceA and vcrA Gene Expression in Dehalococcoides spp. Environmental Science & Technology 2010; 44(19) 7647-7651.

[81] Lohner S. T., Tiehm A. Application of Electrolysis to Stimulate Microbial Reductive PCE Dechlorination and Oxidative VC Biodegradation. Environmental Science & Technology 2009; 43(18) 7098-7104.

[82] Robrock K. R., Mohn, W.W., Eltis, L.D., Alvarez-Cohen, L. Biphenyl and ethylbenzene dioxygenases of Rhodococcus jostii RHA1 transform PBDEs. biotechnology and bioengineering 2010; 108(2) 313-321.

[83] Kalimuthu K. et al. Reductive dechlorination of perchloroethylene by bacillus sp JSK1 isolated from dry cleaning industrial sludge. Carpathian Journal of Earth and Environmental Sciences 2011; 6(1) 165-170.

[84] Holliger C. et al. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Applied and Environmental Microbiology 1993; 59(9) 2991-2997.

[85] Krajmalnik-Brown R. et al. Genetic identification of a putative vinyl chloride reductase in Dehalococcoides sp. strain BAV1. Applied and Environmental Microbiology 2004; 70(10) 6347-51.

[86] MaymoGatell X. et al. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 1997; 276(5318) 1568-1571.

[87] Cheng D., He J. Z. Isolation and Characterization of "Dehalococcoides" sp Strain MB, Which Dechlorinates Tetrachloroethene to trans-1,2-Dichloroethene. Applied and Environmental Microbiology 2009; 75(18) 5910-5918.

[88] Villemur R. et al. Occurrence of several genes encoding putative reductive dehalogenases in Desulfitobacterium hafniense/frappieri and Dehalococcoides ethenogenes. Canadian journal of microbiology 2002; 48(8) 697-706.

[89] Sung Y. et al. Geobacter lovleyi sp. nov. Strain SZ, a Novel Metal-Reducing and Tetrachloroethene-Dechlorinating Bacterium. Applied and Environmental Microbiology 2006; 72(4) 2775-2782.

IntechOpen

IntechOpen