Tomas Bata University in Zlín
Faculty of Technology

Doctoral Thesis

Microbial degradation of chlorinated ethenes and its potential application for in-situ bioremediation

Mikrobiální degradace chlorovaných ethylenů a její potenciální využití pro in-situ bioremediace

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Motto:

„Perfect motto needs time to emerge, time is something I do not have at this moment. “

[Michal Zálešák]

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ABSTRACT

The doctoral thesis deals with microbial degradation of chlorinated ethenes and its potential application for in-situ bioremediation. In a theoretical part of the thesis, the main reasons of soil and groundwater contamination by these compounds along with their transport and a fate in a subsurface environment are briefly outlined. The thesis then focuses on common procedures that must be undertaken prior to selecting a suitable remediation method at contaminated sites. Further, the thesis summarizes various microbial processes leading to a transformation and degradation of all chlorinated ethenes and outlines several options for the use of these processes within in-situ bioremediation of affected sites. Special attention is paid to aerobic cometabolic degradation of all three dichloroethenes by pure bacterial strains in a mineral salt medium. The theoretical part of the thesis ends with a chapter devoted to field demonstrations of in-situ bioremediation for clean-up of sites polluted by chlorinated ethenes.

A following experimental part of the thesis deals with degradation of selected chlorinated ethenes by *Comamonas testosteroni* strain RF2 and by several consortia in the mineral salt medium. At first, strain RF2 was tested to investigate its capacity for degrading 1,2-*cis*-dichloroethene (*c*DCE), 1,2-*trans*-dichloroethene (*t*DCE), and 1,1-dichloroethene (1,1DCE). Degradation assays were performed for single DCEs, as well as for a mixture of DCEs with TCE, which resembled contaminated plume in groundwater. Strain RF2 was capable of efficiently removing all three dichloroethenes (DCEs) at the initial aqueous concentrations of 6.01 mg L^{-1} for *c*DCE, 3.80 mg L^{-1} for *t*DCE and 0.65 mg L^{-1} for 1,1DCE, with a removal efficiency of 100 % for *c*DCE, 65.8 % for *t*DCE, and 46.8 % for 1,1DCE. Furthermore, complete removal of TCE, *c*DCE and 1,1DCE (122.5 μg L^{-1}, 84.3 μg L^{-1} and 51.4 μg L^{-1}, respectively) were observed in a mixture sample that also contained 72.33 μg L^{-1} of *t*DCE, which was removed to the amount of 72.3%. Moreover, degradation of *c*DCE (6.01 mg L^{-1}) led to a 92.2 % release of inorganic chloride, and 2,2-dichloroacetaldehyde was determined as the first intermediate of *c*DCE transformation. Further, a consortium composed of the strain RF2 and vinyl chloride (VC) utilizing *Mycobacterium aurum* DSM-6695 was tested to investigate its capacity for degrading TCE (115.7 μg L^{-1}), *c*DCE (662 μg L^{-1}), *t*DCE (42.01 μg L^{-1}), 1,1DCE (16 μg L^{-1}), and VC (7 mg L^{-1}; "all in a liquid phase") in mixed samples. The consortium was able to nearly completely remove all the compounds in the mixed sample within 21 days of the assay.

The findings of this thesis suggest that the consortium composed of the strain RF2 and *M. aurum* DSM-6695 exhibits the potential to remediate groundwater contaminated with chlorinated ethenes.

Keywords: Chlorinated ethenes, microbial degradation and transformation, in-situ bioremediation, *Comamonas testosteroni* RF2

ABSTRAKT

Dizertační práce se zabývá mikrobiální degradací chlorovaných ethylenů a jejím potenciálním využitím pro in-situ bioremediace. V teoretické části práce jsou stručně popsány hlavní důvody kontaminace půd a podzemních vod chlorovanými ethyleny i jejich následný transport v podzemním prostředí. Práce dále shrnuje běžné postupy při monitorování znečištěných lokalit, jež vedou k zvolení vhodné sanační metody. Následně jsou popsány různé mikrobiální procesy vedoucí k transformaci a degradaci chlorovaných ethylenů, načež jsou nastíněny známé způsoby využití těchto procesů pro in-situ bioremediace znečištěných lokalit. Zvláštní pozornost je věnována aerobní kometabolické degradaci všech tří dichloroethenů čistými bakteriálními kmeny v prostředí minerálního média. Teoretická část práce je zakončena kapitolou, která shrnuje uskutečněné in-situ bioremediační projekty, cílené na odstranění chlorovaných ethylenů z půd a podzemních vod.

Navazující experimentální část práce se zabývá degradací vybraných chlorovaných ethylenů kulturou *Comamonas testosteroni* RF2 a několika bakteriálními konsorcii v prostředí minerálního média. Kmen RF2 byl zvolen pro pokusy kometabolické degradace 1,2-cis-dichlorethylenu (*c*DCE), 1,2-trans-dichlorothylenu (*t*DCE), 1,1-dichlorethylenu (1,1DCE) a vinyl chloridu (VC). Degradační testy byly provedeny jednak pro jednotlivé dichlorethyleny (DCEs) a rovněž pro směs DCEs s TCE, simulující podzemní vodu znečištěnou těmito látkami. Kmen RF2 byl schopen odstraňovat všechny DCEs (dávkovány samostatně) při počátečních koncentracích v kapalné fázi: 6,01 mg L^{-1} *c*DCE, 3,80 mg L^{-1} *t*DCE a 0,65 mg L^{-1} 1,1DCE, s účinností odstranění 100% pro *c*DCE, 65,8 % pro *t*DCE a 46,8 % pro 1,1DCE. Úplné odstranění *c*DCE vedlo k uvolnění 92,2% anorganických chloridů. Dále bylo zjištěno úplné odstranění TCE, *c*DCE a 1,1DCE (122,5 µg L^{-1}, 84,3 µg L^{-1} a 51,4 µg L^{-1}) ve vzorcích obsahujících modelovou podzemní vodu. Ve stejných vzorcích rovněž došlo k odstranění 72,3 % *t*DCE o koncentraci 72,33 µg L^{-1}. Sledování kinetiky degradace *c*DCE ukázalo na existenci dvou metabolitů rozkladu, přičemž jako první meziprodukt transformace *c*DCE byl zjištěn 2,2-dichloracetaldehyd. V neposlední řadě bylo studováno konsorcium kmene RF2 s bakterií *Mycobacterium aurum* DSM-6695, jež metabolicky rozkládá VC. Toto konsorcium bylo podrobeno experimentu současné degradace TCE (115,7 µg L^{-1}), *c*DCE (662 µg L^{-1}), *t*DCE (42,01 µg L^{-1}), 1,1DCE (16 µg L^{-1}) a VC (7 mg L^{-1}, „vše v kapalné fázi“) a ukázalo schopnost téměř úplně odstranit všechny sloučeniny ve směsném vzorku do 21 dnů, čímž prokázalo značný potenciál pro jeho případné využití v rámci čištění podzemních vod znečištěných chlorovanými ethyleny.

Klíčová slova: Chlorované etheny, mikrobiální degradace a transformace, in-situ bioremediace, *Comamonas testosteroni* RF2

CONTENTS

INTRODUCTION

The extensive use of perchloroethene (PCE) and trichloroethene (TCE) as dry cleaning and degreasing solvents for industrial purposes has caused world-wide contamination of soils and groundwater. In particular, TCE has become a major groundwater contaminant on a global scale. Moreover, natural attenuation of PCE and TCE often leads to the formation of less-chlorinated *cis*-1,2-dichloroethene (*c*DCE) and vinyl chloride (VC), which may persist in soils and particularly groundwater plumes. Also, to a lesser extent, *trans*-1,2-dichloroethene (*t*DCE) and 1,1-dichloroethene (1,1DCE) may be formed as well. Therefore, contaminated groundwater with prevailing anaerobic conditions may contain a mixture of residual TCE, DCEs, and VC even after a period of several years or even decades.

Given the severity and extent of the environmental pollution caused by chlorinated ethenes, innovative remediation technologies ensuring efficient, low-cost, and environmentally friendly clean-up methods for their removal are essential. Bioremediation techniques are universally based on the use of microorganisms, which are capable of degrading the targeted compounds. A lot of such useful microbes naturally occur at polluted sites and can be directly used for a site clean-up when providing them with suitable conditions for their growth and metabolism. If the stimulation of indigenous microbial populations is not sufficient enough to achieve the site clean-up objectives, an application of strains with proven ability to degrade desired chlorinated ethenes might be an option.

In general, prior the actual use of selected microorganisms at polluted sites, detailed lab-scale studies on their capacities to degrade chlorinated ethenes CEs are necessary. Many studies from recent decades have focused on the bacterial degradation of particularly TCE, *c*DCE and VC in a mineral salt medium (MSM); however, aerobic cometabolic degradation of all three DCEs in pure bacterial strains is less known and therefore its investigation is desirable. Also, the same applies to the investigation of pure bacterial strains capable of degrading a broad range of chlorinated ethenes. Hence, a study summarizing microbial degradation of chlorinated ethenes and its potential application for in-situ bioremediation is highly needed.

Main aims of this thesis are to summarize the current understanding regarding the behaviour of chloroethenes in a subsurface environment; outline microbial processes that lead to the degradation and transformation of all chloroethenes; examine the ability of selected bacterial strains and consortia to degrade the compounds in MSM; evaluate the potential of selected strains and consortia for in-situ bioremediation of contaminated sites.

1. CURRENT STATE OF THE ISSUES DEALT WITH

1.1 Structure and nomenclature of CEs

Chlorinated ethenes (CEs) are represented by tetrachloroethene, commonly referred as perchloroethene (PCE); trichloroethene (TCE); *cis*-1,2-dichloroethene (*c*DCE); *trans*-1,2-dichloroethene (*t*DCE); 1,1-dichloroethene (1,1-DCE); and vinyl chloride (VC). CEs possess two carbon centres joined by a carbon-carbon double bond known as a π-bond system, which does not allow covalently bonded chlorine substituents to rotate freely in the plane perpendicular to the direction of the π –bond (Cwiertny and Scherer, 2010). Also, due to the double-bonded carbon centres of CEs, they can accommodate at most two chlorine substituents. Thus, PCE with its four chlorine substituents is the most chlorinated ethene. The nomenclature of CEs follows a simple convention that is used to express a number and a position of the substituted chlorine atoms in the ethene molecule, e.g. 1,1,2-trichloroethene contains overall three chlorine substituents from which two of them are located at one carbon centre and the third one at the other carbon centre. Further, additional nomenclature is needed to distinguish the possible isomers of dichloroethenes (DCEs). DCEs can exist as either of two structural isomers (1,1-DCE or 1,2-DCE) with both chlorine substituents positioned at one carbon centre (1,1-DCE) or with a single chlorine substituent at each of the carbon centres (1,2-DCE). Moreover, 1,2-DCE has two conformational isomers named *c*DCE and *t*DCE, which differ in the spatial arrangement of their chlorine substituents (Vollhardt and Schore, 1994). Whilst chlorine atoms in *c*DCE are bonded on the same side of the carbon-carbon double bond, each of the chlorine atoms in *t*DCE is bonded on the opposite side, which results in their different physical, chemical, and biological properties (Cwiertny and Scherer, 2010; Galizia, 2010). Notably, the lone exception of the convention in the nomenclature of CEs is vinyl chloride, which is a trivial name of chloroethene. Vinyl chloride is abbreviated as VC and commonly replaces the use of chloroethene in the nomenclature of CEs. Finally, chemical structures of all CEs are depicted in Fig. 1.1.

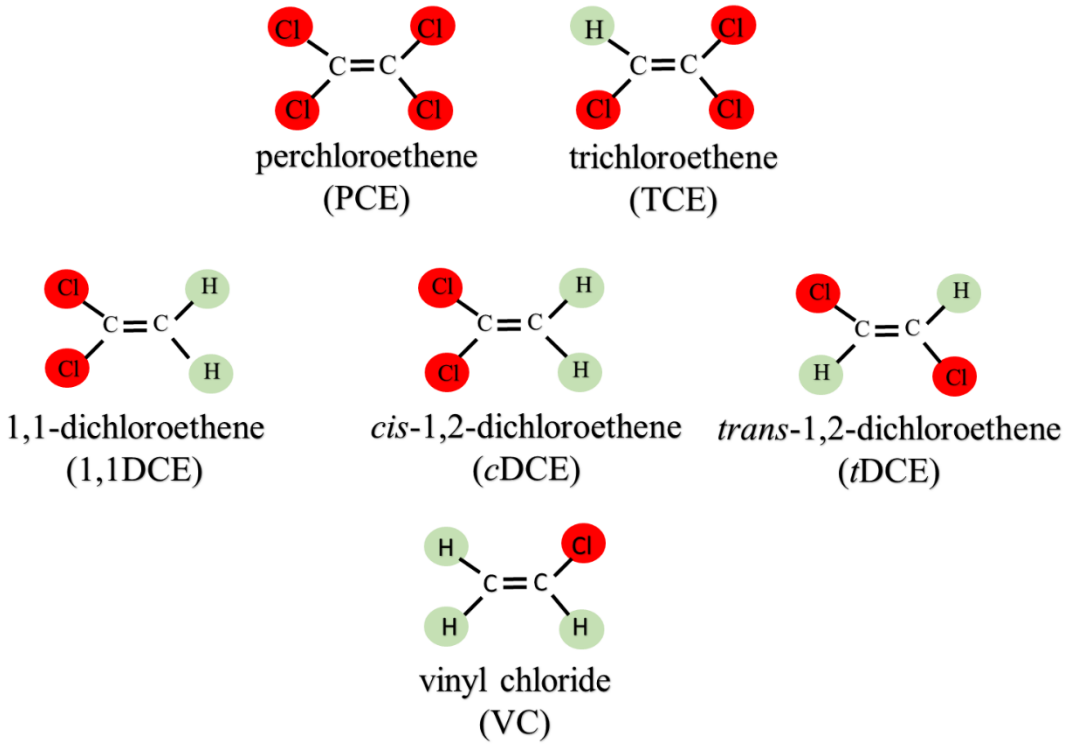

Fig. 1.1 Chemical structure of chlorinated ethenes

1.2 Industrial uses and toxicity of CEs

Generally, CEs are volatile organic compounds that belong to a class of chlorinated organic solvents, which are used for a variety of commercial and industrial purposes, especially as degreasers, cleaning solutions, and paint thinners (Cwiertny and Scherer, 2010; Cloelle et al., 2010; Nishino et al., 2013). Because of their chlorine-containing chemical structure, CEs can efficiently dissolve organic materials like fats and greases and to serve as raw materials or intermediates in the production of other chemicals (NSW, 2011). Besides, CEs have several undesirable properties that include high toxicity, environmental resistance, and ability to accumulate in living and non-living components of an environment, including humans. PCE and TCE are the suspected human carcinogens, DCEs are highly toxic compounds, and VC is well known as the human carcinogen (Binbin et al., 2014).

PCE is a non-flammable colourless liquid at room temperature, which has been produced commercially since the early 1900s primarily for dry cleaning and textile processing (NSW, 2011). The use pattern of PCE represents 55% as a chemical intermediate, 25% for metal cleaning and vapour degreasing, 15% for dry cleaning and textile processing, and 5% for other unspecified uses (US EPA, 2012a; ATSDR, 2014). Importantly, spills of PCE are a significant source of groundwater contamination.

TCE is a non-flammable colourless liquid at room temperature, which is mainly used as a solvent to remove grease from metal parts (NSW, 2011). TCE is also used as an extraction solvent for oils, fats, waxes, and tars as well as the chemical intermediate of other chemicals, and as a refrigerant (ATSDR, 1997). Furthermore, TCE can be found in some household products, such as paint removers, adhesives and spot removers (NSW, 2011). It is considered a primary pollutant with 5 $\mu g\ L^{-1}$ maximum contamination level allowed in drinking water (ATSDR, 1997; US EPA, 2011).

1,1-DCE is a highly flammable colourless liquid used to make certain plastics, such as flexible films like food wrap, and in packaging materials (ATSDR, 1995). Also, 1,1DCE is used to make flame retardant coatings for fibre and carpet backings, and in piping, coating for steel pipes, and in adhesive applications (ATSDR, 1995). Although the industrial production of 1,1DCE is not as frequent and severe source of soils and groundwater pollution as in the case of PCE and TCE, certain amount of this compound might be formed in an anaerobic subsurface as a result of PCE and TCE reduction (He-Ping et al., 2010; Tiehm and Schmidt, 2011).

1,2-DCE is highly flammable, colourless liquid, which has historically been used as a solvent for waxes and resins as well as in the extraction of rubber, and as a coolant in refrigeration plants (US EPA, 2010). Currently, only the *trans*-isomer is commercially available in the United States (ATSDR, 1996). Nowadays, *t*DCE is mainly used as a degreasing agent and for cleaning of electronic components. (ATSDR 1996; US EPA, 2010). Oddly enough, it is predominantly *c*DCE isomer that is frequently detected in subsurface due to the anaerobic reduction of PCE and TCE (He-Ping et al., 2010; Tiehm and Schmidt, 2011).

VC is colourless, highly flammable gas at room temperature, however, it may exist in a liquid form if kept under high pressure or at low temperatures (ATSDR, 2006). Most of the produced VC is used to make a polyvinyl chloride polymer (PVC), an ideal material for electrical wire insulation and production of various types of cables and pipelines. Also, it is commonly used in numerous products in building construction, automotive industry etc. (ATSDR, 2006). As in the case of DCEs, the occurrence of VC in the subsurface is mainly caused by the anaerobic reduction of PCE and TCE though production of PVC might contribute to the environmental pollution as well (He-Ping et al., 2010; Dolinova et al., 2017).

1.3 Fate and transport of CEs in a subsurface environment

The fate and transport of CEs in a subsurface environment along with their degradability by both biotic and abiotic processes are all closely related to their physicochemical parameters as well as to the geochemical and hydrological conditions of contaminated sites, such as organic matter content of the subsurface, soil porosity, hydraulic conductivity, and hydraulic gradient (Huling and Weaver,

1991; Pant and Pant, 2010; Kret et al., 2015). Appendix 1.1 summarizes some of the most important physicochemical parameters of CEs, which influence their distribution in an unsaturated zone (vadose zone) and groundwater in aquifers, i.e. their partitioning between soil, water, air, and non-aqueous phase liquids (NAPLs), occurring as dense (DNAPLs) and light (LNAPLs) (Cwiertny and Scherer, 2010). Typically, the resulting distribution of CEs in the contaminated subsurface is a highly complex process that results in their non-uniform occurrence in soils and groundwater, which is caused by limited water solubility, high volatility, moderate hydrophobicity, and relative density of these compounds. Fig. 1.2 depicts the major phases in the subsurface and the properties of CEs that govern the partitioning between these phases. While their water solubility along with an actual amount of contaminant(s) determine the maximum concentration of chloroethene(s) in water at the given temperature, thus controlling the extent of CEs transfer from a vadose zone into groundwater, partitioning of CEs into soil air and onto a soil phase (soil particles and aquifer material) depends on their vapour pressure and hydrophobicity.

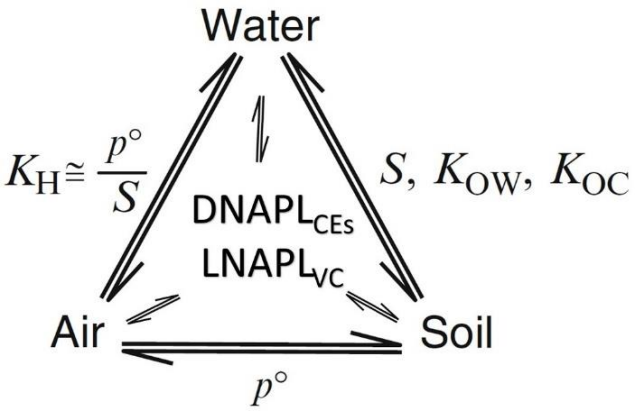

Fig. 1.2 Major phases occurring in the subsurface and the properties of CEs that govern the partitioning between these phases[1]

Vapour pressure represents the maximum attainable concentration of CEs in soil air and strongly influences the partitioning of CEs from dry soils into soil air. CEs with high values of vapour pressure such as VC and 1,1DCE tend to partition more readily from dry soils into soil air than PCE or TCE with lower vapour pressure (Schwarzenbach et al., 2003). Further, partitioning of dissolved CEs in groundwater into soil air on a boundary of a water table and a vadose zone

[1] *Taken from the work of Cwiertny and Scherer (2010) and revised*

Koc: soil organic carbon/water; Kow: octanol/water partitioning coefficient; KH: Henry's law constant; p°: vapour pressure; S: water solubility

$DNAPL_{CEs}$: PCE, TCE, and DCEs as Dense Non-aqueous Phase Liquids

$LNAPL_{VC}$: VC as Light Non-aqueous Phase Liquid

can be described by Henry's law constant (K_H), according to which, CEs with a greater K_H value are more prone to partition from water into soil air in a vadose zone. For instance, data in Appendix 1.1 show that *c*DCE and *t*DCE, compounds with high vapour pressure and low K_H values, have higher tendency to partition from dry soils into soil air than from water into soil air, which is a result of relatively good water solubility of these compounds. In comparison, PCE has much lower vapour pressure than *c*DCE and *t*DCE; however, its low water solubility results in more significant partitioning from water into soil air than in the case of *c*DCE and *t*DCE. Clearly, VC with the extremely high vapour pressure and slight water solubility has a great tendency to partition into soil air from both, dry soils and groundwater, which makes this compound highly mobile in a subsurface environment. Furthermore, gaseous vapours of all CEs have greater relative density compared to air and may sink through a vadose zone into groundwater causing its contamination (US EPA, 1991).

Hydrophobicity of CEs influences their partitioning (sorption) onto aquifer materials in a saturated zone and can be expressed by an octanol-water partitioning coefficient (K_{ow}), which is defined as the equilibrium concentration of CEs in octanol relative to its equilibrium concentration in water (Cwiertny and Scherer, 2010). Even more precise values describing the sorption of CEs on an organic matter offers a soil organic carbon-water partitioning coefficient (K_{oc}), which represents a measure of a compound's equilibrium partitioning between water and the aquifer solids (US EPA, 1990; Cwiertny and Scherer, 2010). In general, values of both, (K_{ow}) and (K_{oc}), increase with the number of substituted chlorine atoms in the chloroethene molecule, which means that PCE has the greatest sorption tendency towards the aquifer material while VC has the lowest one. Although the affinity of CEs for this process is not very high due to their moderate hydrophobicity, as it can be seen from relatively low values of Kow and Koc in Appendix 1.1, it affects a transport rate of CEs in the subsurface and also reduces their bioavailability for potential degrading bacterial strains (Lu et al., 2011).

Relative densities of CEs are responsible for their partitioning into NAPLs in a subsurface environment and play a crucial role in the contamination of aquifers (US EPA, 1992; Binbin et al., 2014). In general, PCE and TCE densities are significantly greater than the relative density of water and therefore these solvents tend to migrate through a vadose zone into an underlying aquifer where typically form a contamination plume of DNAPLs. In such a case, not only PCE and TCE are present in the plume, but also their reductive intermediates, mainly *c*DCE, VC, and to some extent, *t*DCE and 1,1DCE are present within the plume after a prolonged time of natural attenuation (Bradley, 2000; Major et al., 2002; Aulenta et al., 2006). Also, a certain portion of CEs as DNAPLs ($DNAPL_{CEs}$) can diffuse into a matrix of low permeable deposits formed in the vadose zone, such as silt and clay layers (Scheutz et al., 2010). As a consequence, this residual $DNAPL_{CEs}$ become a significant long-term secondary source

of contamination of underlying aquifers due to their gradual back diffusion from the matrix (Chambon et al., 2010). Moreover, low hydraulic conductivity and capillarity of soils are also responsible for the lateral migration of $DNAPL_{CEs}$ and in some cases might prevent this phase of CEs from reaching a water table (US EPA, 1991). This scenario might occur in soils with a thick layer of clay, which has very low hydraulic conductivity and contain no natural fractures through which the contaminants can migrate into the aquifer. On the other hand, retardation of $DNAPL_{CEs}$ in soils with higher hydraulic conductivity, such as those composed of mainly silty sands or fine sand, seems negligible. Typical values of hydraulic conductivities for selected soil types are listed in Table 1.1

Table 1.1 Typical values of hydraulic conductivities for soils[2]

Soil type	Hydraulic conductivity range ($cm\ s^{-1}$)
Clay	10^{-9} to 10^{-6}
Silts, sandy silts, clay silts, tills	10^{-6} to 10^{-4}
Silty sand and fined sands	10^{-5} to 10^{-3}
Well-sorted sands	10^{-3} to 10^{-1}
Well-sorted gravels	10^{-2} to 10^{0}

In any case, when the amount of CEs is sufficient to overcome the fraction depleted by residual saturation in a vadose zone, mobile $DNAPL_{CEs}$ sink under the force of gravity through permeable groundwater aquifers until a non-permeable zone is reached (US EPA, 1991; 2012; Atlas and Philp, 2005; Matteucci et al., 2015). Further distribution of $DNAPL_{CEs}$ in groundwater, i.e. direction, velocity, and the amount of transported contaminants is affected by a hydraulic gradient of groundwater, sorption of $DNAPL_{CEs}$ onto aquifer sediments, their dispersion and diffusion in the aquifer as well as by topography of an impermeable zone (US EPA, 1995; Pant and Pant, 2010; WDNR, 2014). On the contrary to the $DNAPL_{CEs}$, VC has a lower density than that of water and therefore can occur at the interface of a vadose zone and a groundwater table as LNAPL (Vallero, 2004). Moreover, VC can well diffuse into a soil gas in a vadose zone where forms an extensive contamination plume as well as it can form a significant contamination plume in an upper part of an aquifer due to its partial water solubility. Oddly enough, despite the physicochemical parameters of VC, this compound can be found in deeper parts of aquifers contaminated by PCE and TCE where anaerobic reductive dechlorination of the latter mentioned CEs may result in the formation of VC (Witt et al., 2002).

[2] *Taken from the work of Cookson (1995)*

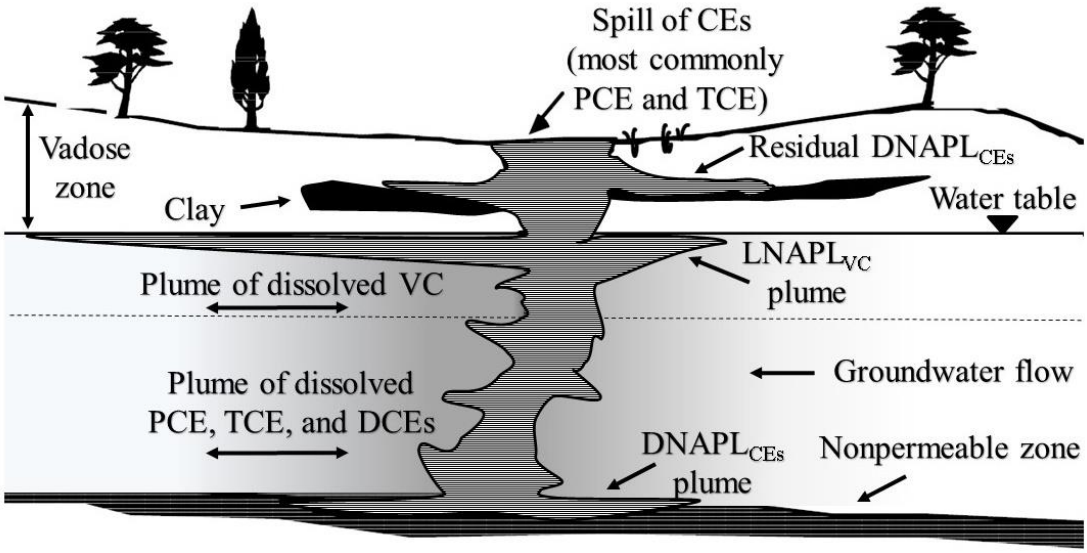

Fig. 1.3 Likely transport of CEs in the subsurface[3]

1.4 Site investigation and monitoring

A performance of in-situ bioremediation of CEs depends on the suitability of environmental conditions for microbial strain or consortia capable of degrading targeted CEs as well as on bioavailability of CEs in soils and groundwater. Factors influencing the bioavailability of CEs and suitability of the environmental conditions for microbial activities are highly site-specific and must be understood prior initiating an actual site clean-up.

A site investigation is an integral part of a whole in-situ bioremediation process and plays an irreplaceable role in the selection of an appropriate clean-up method. The principal objectives of the site investigation should be as follows: (1) gather information about the extent of soil and groundwater contamination including horizontal and vertical subsurface distribution of CEs in all phases, i.e. groundwater, soil gas, DNAPL, LNAPL and portions adsorbed on organic matter; (2) examine prevailing environmental conditions and likely degradative processes of CEs; (3) a determination of a cost-effective clean-up strategy that ensures sufficient removal of contaminants within an acceptable time frame (EPA Victoria, 2006; WDNR, 2014; Kret et al., 2015).

1.4.1 Investigation of a subsurface distribution of CEs

To determine the extent of soil and groundwater contamination, it is important to know the history of the site contamination, i.e. the source area, amount and type of released CEs, when the spill occurred, whether it was a one-time event or episodic release, and to know the risk of the possible future release(s) and how to prevent them (WDNR, 2014). Also, it is necessary to understand the

[3] *Inspired by US EPA, 1991*

physicochemical parameters of CEs reviewed in section 1.3, site hydrogeology, and physical properties of a soil along with its composition (Johnson et al., 2006). Therefore, to get a complete picture of the CEs distribution throughout a contaminated zone, the investigation must lead to the characterization of soil properties and hydrogeology of a site that both significantly control the extent of the contamination. Whereas various techniques, such as direct push technologies, core samplers, geophysics etc., are typically used to investigate a grain size and hydraulic conductivity of a soil, investigation of the site hydrological conditions is usually done by using a number of observation wells, and at least one piezometer (Naudet et al., 2004; WDNR, 2014). Typically, the observation wells serve to determine an ambient water table, and the piezometers are used to determine the hydrostatic pressure at particular depths (USDA, 2010). After determination of the soil properties and hydrological conditions of the site, a proper well network can be installed and subsequently used for collecting subsurface sampling data, which makes possible to identify the primary flow path of contamination plume in a subsurface environment (Johnson et al., 2006; WDNR, 2014). For instance, soil gas samples are commonly used to screen the concentrations of CEs, oxygen, carbon dioxide and the presence of methane in the soil (WDNR, 2014). Groundwater should be sampled and analysed for the presence of CEs, physical and geochemical parameters (US EPA, 1990; Ohio EPA, 2014; Ben Maamar et al., 2015). Hence, the monitoring well network needs to be extensive enough to detect vertical and horizontal movements of the CEs-plume, which are usually caused by horizontal changes in the water table and shifting flow direction of groundwater in response to seasonal weather changes, such as rainy seasons, snowmelt or dry seasons (WDNR, 2014). Also, it is necessary to consider other possible factors that might influence the plume movements, such as the use of adjacent drinking wells and activities on nearby water bodies (WDNR, 2014). Further, the overall risk of the potential CEs transport into a regional flow system, which might serve as a source of well-drinking water, should be examined along with the migration of CEs vapours in soil gas within a vadose zone. Especially VC vapours can travel long distances through a vadose zone(s) and should be of significant concern when buildings are located near a spill source(s) or at the water table of groundwater containing CEs (Vallero, 2004). Additionally, when monitoring the plume migration, geochemical data, such as redox state, presence of electron acceptor(s), CH_4, and total organic carbon (TOC) should be collected to determine different geochemical settings across the soils and groundwater, e.g. anaerobic conditions nearby the source of the PCE and TCE spill, and aerobic conditions down-gradient of the pollution source (Johnson et al., 2006; Ohio EPA, 2014). Such data can be used to establish the footprint of the plume and so identify where the plume is and where it is not (WDNR, 2014). In general, the data collected from the site should be a basis for a selection of a clean-up approach, which ensures that all exposure pathway are addressed.

1.4.2 Investigation of prevailing environmental conditions

A hydrological regime of a site along with its geochemistry and physical parameters determine the actual environmental conditions in the subsurface under which indigenous or applied microorganisms capable of degrading CEs operate. Generally, microbial activities depend on the availability of dissolved oxygen (aerobic processes); electron acceptors and donors (both aerobic and anaerobic processes); nutrient availability (N, P); pH and alkalinity; temperature; water content in the soils; salinity; and toxicity versus biodegradability of CEs (Atlas and Philp, 2005; Azubuike et al., 2016). These physical and nutritional requirements are influenced by a pore structure of soils, which plays an important role in determining the availability of water, oxygen and CEs to microbes (Atlas and Philp, 2005; Kuppusamy et al., 2016). Moreover, it strongly influences pH, heat transfer, and the transport of CEs from a vadose zone into aquifers (DePaoli, 1996; Atlas and Philp, 2005; Kuppusamy et al., 2016). Generally, soils with low porosity are accompanied with a low value of hydraulic conductivity, which makes bioremediation difficult or impossible, unless the soil permeability is increased by engineering technologies, such as by hydraulic and pneumatic fracturing (Scheutz et al., 2010).

1.4.3 Monitoring the loss of CEs

The loss of CEs at a site is typically a result of a combination of various abiotic and biotic processes. Therefore, to understand the predominant microbial process(es) that lead to the removal of CEs as well as to assess a ratio of the microbial and abiotic removals of the contaminants, appropriate field data must be regularly collected over a prolonged period of time. Hence, the data collected at the site should not only document the loss of CEs but also provide further evidence about microbial degradation of CEs, such as biogeochemical indicators, the formation of CEs-daughter compounds, and direct microbial evidence (Witt et al., 2002). Of special concern must be redox state at different locations and at different depths of the site. The redox state is the end result of a set of electron transfer reactions driven by variety of microorganisms (and *vice versa*) that control transfer of electrons between electrons donors (organic carbon in the subsurface) and electron acceptors, such as dissolved O_2, NO^{3-} or Fe^{3+} (Ohio EPA, 2014; WDNR, 2014; Badin et al., 2016). Basically, different parts and depths of the site reveal the following sequence of electron acceptors (not considering CEs as electron acceptors) from more oxidizing to more reducing conditions: O_2 > NO^{3-} > Mn^{4+} > Fe^{3+}> SO_4^{2-} > CO_2 (Bradley, 2000; Ohio EPA, 2014; Badin et al., 2016). The usefulness of the redox reactions lies in the reliable determination of the likely prevailing degradative processes and conditions under which these processes are occurring, e.g. anaerobic reductive dechlorination of CEs under methanogenic conditions or their oxidation (direct or cometabolic) in presence of dissolved oxygen. (Bradley, 2000; Badin et al., 2016). For instance, suitable

conditions for anaerobic reductive dechlorination of PCE and TCE are typically accompanied with higher concentration of naturally produced electron donors, e.g. CH_4, H_2S or Fe^{2+}, and conversely, more oxidized electron acceptors, such as NO^{3-}, Fe^{3+} or SO_4^{2-} are not present or their concentrations are low (Ohio EPA, 2014; WDNR, 2014).

Besides monitoring the redox state at the site, it is particularly important to search for specific microbial strains known for their ability to degrade CEs, such as those of genus *Dehalococcoides*, responsible for anaerobic reductive dechlorination of all CEs, or some microbial strains of genus *Mycobacterium*, which can aerobically degrade VC (Le and Coleman, 2011 Florey et al., 2017). Generally, the 16S rRNA sequencing is a diagnostic tool commonly used to determine whether the microorganisms with proven ability to degrade CEs are present at the site or not (Hendrickson et al., 2002; Rahm et al., 2006). Moreover, more detailed information about the degradation capacities of microbial indigenous populations is provided by new molecular tools, which are designed to detect specific site biomarkers, such as RDase genes involved in final reduction steps of *c*DCE and VC to ethene (Regeard et al., 2003; Muller et al, 2004) as well as to detect mono- and dioxygenases involved in cometabolic degradation of all CEs (Ryoo et al., 2000).

In fact, monitoring the loss of CEs, redox conditions, and presence and distribution of specific microbial strains along with their functional genes serves as a basis of Monitored Natural Attenuation (MNA). MNA is a clean-up approach, which might be, under certain conditions, selected to attain desirable removals of contaminants at sites. This approach is described in more detail in sections 1.7 and 1.8.1.

1.5 Bacterial strategies for degradation and transformation of CEs

Bacteria have evolved several strategies for enzyme-catalysed dechlorination and degradation of CEs; specifically, anaerobic metabolic and cometabolic degradation (reductive dechlorination), and aerobic metabolic and cometabolic degradation are the most prevailing degradation processes in a subsurface, which may be applied for in-situ bioremediation.

Despite the fact that the aim of this section is to summarize the above mentioned bacterial strategies for degradation and transformation of CEs, including degradation pathways and enzymes involved in these processes (if possible), it is worth noting that other biotic and abiotic processes may influence both degradation and formation of some CEs, especially at the sites polluted by a mixture of chlorinated solvents. For instance, biotic vicinal reduction or dichloroelimination of 1,1,2,2-perchloroethane (1,1,2,2-PCA) may result in the production of *t*DCE and *c*DCE (Chen et al., 1996; Arnold et al., 2002). Also, VC can be formed by either dichloroelimination of 1,1,2-trichloroethane

(1,1,2-TCA) or during abiotic dehydrochlorination of 1,2-dichloroethane (1,2-DCE) (Jeffers et al., 1989; Chen et al., 1996). Similarly, dehydrochlorination of 1,1,2-TCA often leads to the production of 1,1-DCE (Pagan et al., 1998). Besides the formation of CEs from another category of chlorinated solvents, Koudelkova et al. (2013), described substitutive dechlorination of VC to ethene as a hydrolytic process catalyzed by halidohydrolases of some bacterial genera, such as *Rhodococcus*, *Pseudomonas*, *Sphingomonas*, *Agrobacterium*, *Ralstonia*, *Nocardia*, and *Mycobacterium.* On the basis of these facts, any planned bioremediation project should consider all possible aspects of CEs occurrence at the site so that it is possible to distinguish their origin as well as to determine the main degradation pathways of these contaminants.

1.5.1 Anaerobic metabolic dechlorination (organohalide respiration)

Organohalide respiration, also known under the term metabolic reductive dehalogenation, is a process in which CEs serve as the only electron acceptors that accept electrons from other compounds, e.g. propionate, butyrate, lactate, methanol, or ethanol, which are fermented in a subsurface to H_2 or acetate and serve as the actual electron donors utilized by dechlorination bacteria (Aulenta et al., 2006; Frascari et al., 2015). In other words, organohalide respiration of CEs is a replacement of the chlorine substituent in the chloroethene molecule with a hydrogen atom; each dechlorination step consumes two electrons and two protons and releases H^+ and Cl^- (Loffler et al., 2013); the mechanism of the process along with some microbial representatives capable of the organohalide respiration of CEs are depicted in Fig. 1.4. Further, reductive dehalogenase enzymes (RDases) are the key enzymes responsible for catalysing a cleavage of the carbon-chlorine bond in CEs during organohalide respiration (Futamata et al., 2009). Each bacterial strain can possess several of RDases, including PCE reductive dehalogenase (PCE-RDase) catalysing dechlorination of PCE and/or TCE to *c*DCE (Neumann et al., 1996), TCE-RDase, which dechlorinates TCE via *c*DCE to VC (Magnuson et al., 1998), or VC-RDase controlling dechlorination of *c*DCE and VC to ethene (Müller et al., 2004). These enzymes are encoded by a variety of genes that are expressed under different environmental conditions as well as in presence of different CEs, yet the exact relationship between the enzyme activity and the expression of the genes is not fully understood (Chow et al. 2010).

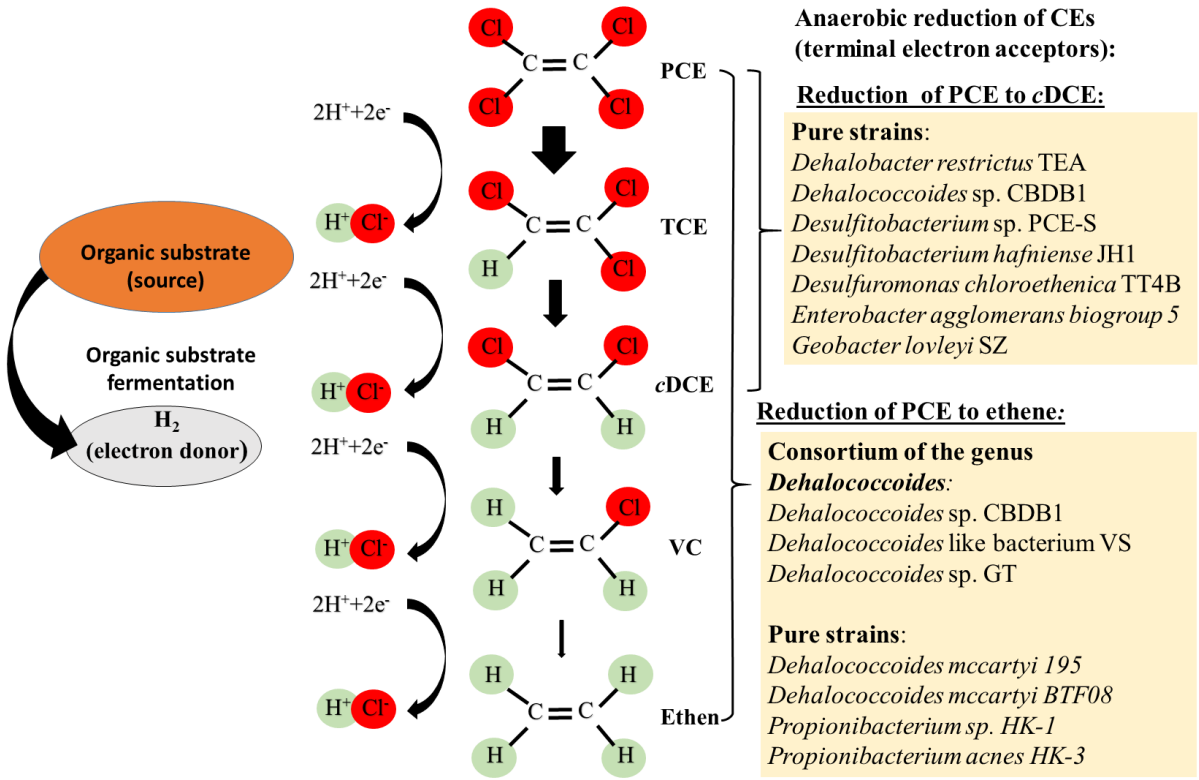

Fig. 1.4 Organohalide respiration of CEs

In general, organohalide respiration of CEs often leads to the accumulation of less chlorinated ethenes, particularly *c*DCE and VC, that may accumulate at sites where PCE and TCE are degraded through this process; however, anaerobic metabolic reduction of an entire range of CEs to ethene has been observed (Bourg et al., 1992; Fennell et al., 2001; Aeppli et al., 2010; Imfeld et al., 2011; Frascari et al., 2015). *Dehalobacter restrictus* was the first organism, which has been described to be able to utilize PCE as the electron acceptor while using H_2 as the elector donor (Holliger and Schraa, 1994; Hollinger et al., 1998). In comparison, PCE dechlorinating *Desulfuromonas* isolates were found to prefer several reduced organic compounds, including acetate, as the electron donors instead of H_2 (Sung et al., 2003). Further, *Geobacter lovleyi* strain SZ could utilize both acetate and H_2 as the electron donors (Sung et al., 2006). While several strains of the *Desulfitobacterium* and *Propionibacterium* genera, namely *Desulfitobacterium* sp. strain Y-51, *D. frappieri* strain TCE1, *D. hafniense* strain JH1, *Propionibacterium* sp. strain HK-1 and *P. acnes* strain HK-3 have been described to dechlorinate PCE to *c*DCE as the dechlorination end product, bacteria of genus *Dehalococcoides* are known to be able to perform the complete reductive dechlorination of PCE to ethene (Gerritse et al., 1999; Suyama et al., 2001; Fletcher et al., 2008; Cloelle et al., 2010). Typically, a number of bacterial strains of the *Dehalococcoides* genus are involved in the complete dechlorination of CEs (He et al., 2005; Lee et al., 2008). However, reduction of DCEs and VC has been found to be very limited not only because of the lower potential of these compounds for accepting electron(s) but also due to the fact that *Dehalococcoides*

are very sensitive to oxygen, and generally less robust towards changes in environmental conditions than other organohalide-respiring bacteria (Smidt and de Vos, 2004; Tiehm and Schmidt, 2011). Moreover, organohalide respiration rates might be influenced through interaction with biogeochemical processes and through competition for H_2 in diverse microbial populations (Aulenta et al., 2007; Azizian et al., 2010; Chambon et al., 2013).

1.5.2 Anaerobic cometabolic reductive dechlorination

In cometabolic reductive dechlorination, the flow of electrons is mainly accepted by primary electron acceptor(s), such as sulfates or ferric ions, and only a small fraction of the electrons flow is fortuitously used on dechlorination of CEs (Frascari et al., 2015). Similarly, as in the organohalide respiration, the efficiency of this process decreases by an order of magnitude with each removed chlorine substituent, which often leads to the accumulation of less chlorinated ethenes, typically *c*DCE and VC (Tiehm and Schmidt, 2011). Moreover, this process is very slow compared to metabolic reductive dechlorination as the CEs are not the electron acceptors (Futagami et al., 2008).

Anaerobic cometabolic degradation was first observed in methanogenic cultures that utilize abundant reduced transition-metal cofactors and incidentally dechlorinate PCE and TCE (Bouwer and McCarty, 1983; Fathepure et al., 1987; Vogel and McCarty, 1985). Further, acetogenic, sulfate-reducing, and iron-reducing bacteria have been found to perform this kind of PCE and TCE reduction as well (Holliger and Schraa, 1994). In contrast, studies describing the cometabolic reduction of less chlorinated ethenes are rare; Maymo-Gatell et al. (2001) observed cometabolic reductive dechlorination of VC to ethene by *Dehalococcoides ethenogenes* in groundwater. Finally, not much research has been done to elucidate transformation pathways and enzymes involved in catalysing the cleavage of the carbon-chlorine bond in the molecules of CEs. This is presumably due to the low efficiency of this process, which makes of it less suitable option for bioremediation of contaminated sites in comparison with organohalide respiration, and therefore, not that attractive field of research.

1.5.3 Aerobic metabolic degradation (direct aerobic oxidation)

Aerobic metabolic degradation, also known as direct aerobic oxidation, is a process in which bacteria utilize CEs as their growth substrate, i.e. CEs serve as the sole sources of organic carbon and energy. Oxidative dechlorination is a result of mono and/or dioxygenases that catalyse the incorporation of one or two oxygen atom(s) into the molecule of chloroethene (Frascari et al., 2015). In most cases, bacteria use epoxidation catalysed by monooxygenases, which results in the formation of chloroethene epoxides that are highly unstable in aqueous systems and react very quickly, often causing cell damage (Dolinova et al., 2017). As an evolutionary defence tool, microorganisms produce variety of

epoxide-transforming enzymes detoxifying harmful epoxides, such as the epoxyalkane coenzyme M transferase (EaCoMT) (Coleman and Spain, 2003), epoxide carboxylases (Allen et al., 1999), epoxide hydrolases (van Loo et al., 2006) and the glutathione S-transferases (van Hylckama Vlieg et al., 1977; 1998). Another possible degradation pathway may include a carbon-chlorine cleavage catalysed by the glutathione S-transferase as a first step (Jennings et al., 2009).

Generally, several bacteria have been identified as capable of aerobic metabolic degradation, mostly of VC (Dolinova et al., 2017). Indeed, VC has been found to be directly oxidized by a variety of bacterial strains, such as *Mycobacterium aurum* strain L1 (Hartmans et al., 1985), *Mycobacterium* sp. strains JS60, JS61, JS616, and JS617 (Coleman et al., 2002) or *Pseudomonas putida* strain AJ (Danko et al., 2004). On the contrary, direct aerobic TCE and DCEs oxidation occur rarely, but some studies on their direct oxidation exist (Schmidt and Tiehm 2008; Dey and Roy 2009; Kim et al., 2010). While metabolic oxidation of TCE in *Bacillus* sp. strain 2479 has been described by two studies (Dey and Roy 2009; Kim et al., 2010), the same process has been confirmed and thoroughly investigated in *Polaromonas* sp. strain JS666 capable to degrade *c*DCE (Jennings et al., 2009; Nishino et al., 2013). Other studies that focused on degradation of DCEs described metabolic oxidation of a binary mixture of *c*DCE and *t*DCE in a consortium consisting of *Achromobacter xylosoxidans*, *Acinetobacter* sp., and *Bacillus* sp. (Olaniran at al., 2006) as well as direct oxidation of 1,1DCE in an unknown microbial consortium (Wang and Yang, 2016). Besides, PCE, the compound generally considered as non-biodegradable in this process, has been found to be directly oxidized in *Pseudomonas* sp. (Deckard et al., 1994) and *Pseudomonas aeruginosa* ATCC 9027 (Jebakumar and Legge, 2008); however, the results of these studies are quite speculative and should be confirmed by further investigation and more reliable data.

Nevertheless, direct oxidation may be characterized by significant advantages over cometabolic processes as there is no need for a growth substrate and the use of supplied oxygen is very efficient because it is not consumed during the utilization of the growth substrate by bacterial cultures (Frascari et al., 2015). Also, in some cases, groundwater quality is not deteriorated by oxidized by-products of the growth substrate(s), such as nitrite in the case of ammonia oxidizers (Tiehm and Schmidt, 2011). Furthermore, the formation of toxic intermediates resulting from CEs direct oxidation seems to be not significant (Mattes et al., 2010). However, this process is significantly hampered by the limited number of strains capable of performing direct CEs oxidation, especially in the case of highly chlorinated ethenes. Another drawback is that CEs, which are not biodegradable via direct oxidation, might potentially cause an inhibition on rates of direct metabolism of other CEs, such as *c*DCE, and VC (Zhao et al., 2010).

1.5.4 Aerobic cometabolic degradation (cometabolic oxidation)

Aerobic cometabolism occurs when the enzymes originally produced for degradation of bacterial growth substrates (auxiliary/primary substrates) fortuitously catalyses oxidation of a non-growth substrate (CEs) (Semprini, 1997); the processing mechanism of the cometabolic oxidation of CEs along with some key enzymes involved in their transformation and degradation are shown in Fig. 1.5. Key enzymes involved in the cometabolic oxidation of CEs are different mono and/or dioxygenases; their production is initiated by variety of suitable growth substrates, such as isoprene (van Hylckama Vlieg et al., 1998), methane (Kim et al., 2008), o-xylene (Li et al., 2014), propene (Ensign et al., 1992; Kim et al., 2008), toluene (Azizian et al., 2007), phenol (Hopkins and McCarty, 1995) and many others (Findlay et al., 2016). Among the commonly produced cometabolic enzymes belongs methane monooxygenase (Fox et al., 1990), ammonia monooxygenase (Arciero et al., 1989), phenol monooxygenase (Fries et al., 1997), toluene monooxygenase, toluene-2,3-dioxygenase (Byrne et al., 1995; Johnson and Olsen, 1995), and alkene monooxygenase (Ensign et al., 1992), all of which have relatively broad substrate specificity and are produced by a variety of bacteria. Furthermore, it is assumed that monooxygenases and dioxygenases primarily epoxidate the carbon-carbon double bond in the chloroethene molecule, which results in the formation of highly reactive epoxides (Hartmans et al., 1989). As in the direct oxidation, CEs epoxides commonly produce harmful degradation intermediates that either interact with bacterial cells, often causing their damage and eventually death, or spontaneously hydrolyse into variety of products, such as CO_2, formic acid, dichloroacetic acid, and other products (Nelson et al., 1986; Oldenhuis et al., 1989; Tsien et al., 1989).

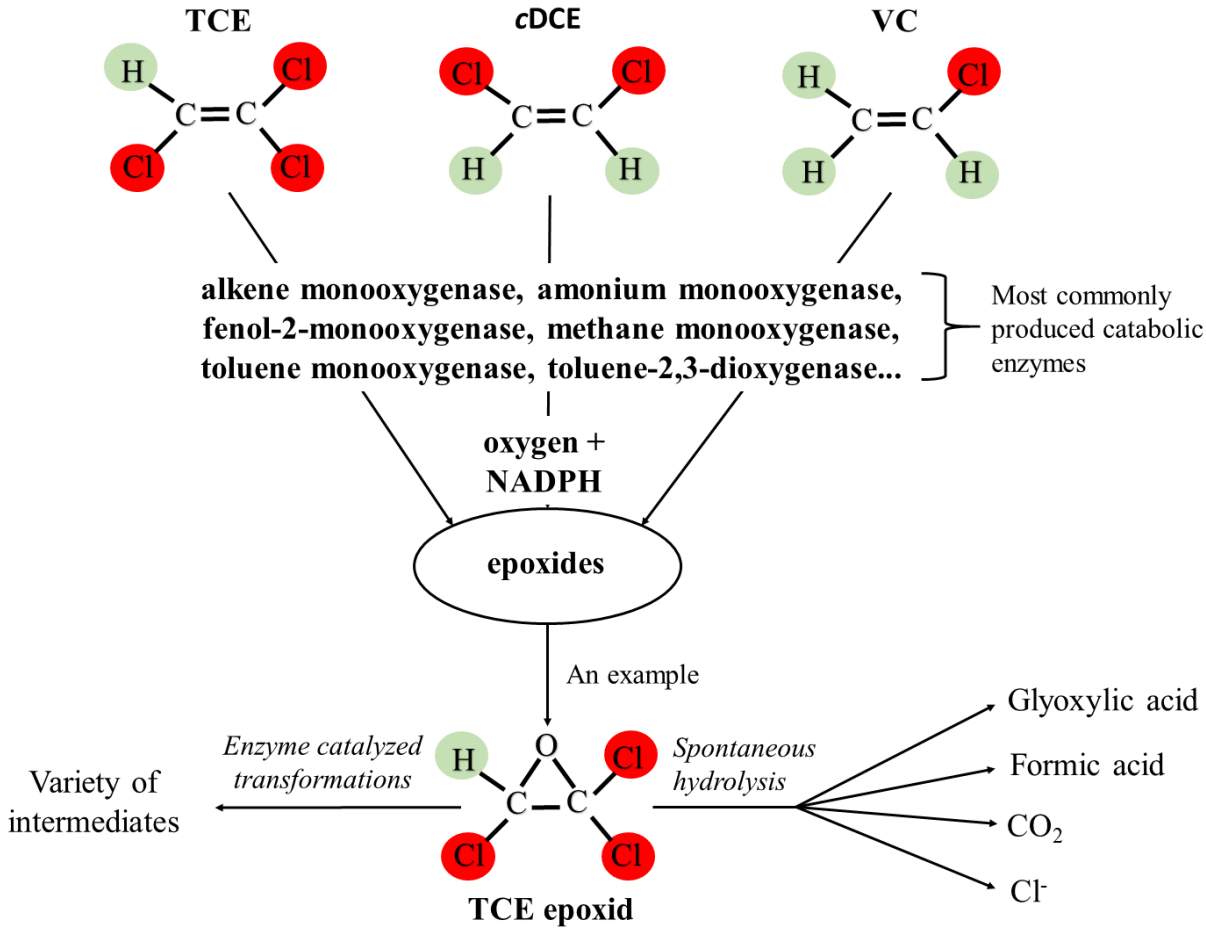

Fig. 1.5 Cometabolic oxidation of CEs

Many bacteria are capable of cometabolic oxidation of TCE, *c*DCE and VC, though fewer strains are capable of the same process in the case of *t*DCE and 1,1DCE, and only *Pseudomonas stutzeri* OX1 has been reported to be able to degrade PCE cometabolically (Ryoo et al., 2000). Up to date list of bacteria capable of degrading TCE, *c*DCE and VC via cometabolic oxidation can be found in a study published by Dolinova et al. (2017). In most cases, *t*DCE and 1,1DCE are not the major contaminants in a subsurface compare to PCE, TCE, *c*DCE and VC; which might be a reason why less research has been devoted to the degradation of 1,1DCE, *t*DCE, or mixtures of all DCEs and their potential mutual interactions. Indeed, the presence of *t*DCE and 1,1DCE in a subsurface might influence a rate of the degradation of other CEs, especially *c*DCE and VC (Verce et al., 2002).

Generally, the cometabolic oxidation is slower (and usually time-limited) than the direct oxidation of CEs, however, it has a potential to degrade a broader spectrum of CEs, specifically TCE, and all DCEs. Also, this process is not restricted to the degradation of single CEs and the degradation rates of less chlorinated ethenes are usually higher than those in organohalide respiration, and typically results in the formation of less toxic end-products (Frascari et al., 2013). Hence, more attention should be paid to the degradation of all DCEs. Several

studies have studied cometabolic oxidation of all DCEs in mineral salt medium (MSM) and the results obtained as well as the bacterial strains involved in this process are reviewed in the next section.

1.6 Bacterial cometabolic degradation of DCEs in mineral salt medium (MSM)

Several studies have described cometabolic oxidation of all DCEs in MSM by pure cultures and obtained various results (Ewers et al., 1990; Ensign et al., 1992; Hartmans and De-Bont, 1992; Chang and Alvarez-Cohen, 1996; Vardar and Wood, 2005). Therein, it was found that two isoprene (2-methyl-1,3-butadiene) utilizing bacteria, *Alcaligenes denitrificans* ssp. *xylosoxidans* JE 75 and *Rhodococcus erythropolis* JE 77, could oxidize all the given DCEs. Primarily, a high initial specific rate of conversion was discerned for 1,1DCE. However, degradation of the compound caused an 80% reduction in cell activity within the first 20 min of the process (Ewers et al., 1990). As distinct from it, *Xanthobacter* cells (strain Py2) utilizing propene as a growth substrate were able to degrade *c*DCE and *t*DCE, although degradation of 1,1DCE proved less efficient (Ensign et al., 1992). Similarly, *Mycobacterium aurum* L1, growing on VC, removed 1,1DCE less efficiently; this showed initial oxidation rates for *c*DCE > *t*DCE > 1,1DCE (30, 25 and 10 nmol min^{-1} (mg of dry weight)$^{-1}$, respectively) (Hartmans and De-Bont, 1992). Also, methane-oxidizing *Methylosinus trichosporium* OB3b expressing the particulate methane monooxygenase showed a transformation capacity (Tc) for all DCEs; the highest Tc was observed for *t*DCE, while the levels of Tc for 1,1DCE and *c*DCE were significantly lower (Chang and Alvarez-Cohen, 1996). Finally, *Pseudomonas stutzeri* OX1, possessing toluene-o-xylene monooxygenase, degraded all DCEs individually as well as in different mixtures when it was grown either on toluene or o-xylene (Vardar and Wood, 2005).

Surprisingly, although the previously mentioned studies researched the cometabolic degradation of all DCEs, none of these works has described a phenol-utilizing pure strain for degrading all DCEs. Unlike the other commonly used primary substrates, mentioned above in section 1.5.4, using phenol for inducing production of catabolic enzymes has several advantages. It is well soluble, a non-volatile substance that is easily available for a variety of bacterial cells. Despite the fact that phenolic compounds are abundant natural agents, commonly present in different environmental spheres, some contradictions against its use for bioremediation purposes might exist. Thus, further research verifying the suitability of this substrate for in-situ bioremediation should be done.

1.7 Main approaches for in-situ clean-up of CEs-polluted sites

In-situ clean-up of soils and groundwater do not require any excavation of polluted soils or groundwater. Also, in comparison with ex-situ techniques,

in-situ treatment can be applied for a deep subsurface as well as on contaminated sites in inhabited areas, and even under buildings (Azubuike et al., 2016). On the other hand, controlling in-situ processes is much harder than in the case of ex-situ clean-up methods, especially in the deep subsurface where altering of temperature and pH might be a difficult issue (Atlas and Philp, 2005; Kuppusamy et al., 2016). In any case, constantly improving scientific knowledge along with the acquisition of new practical experience with in-situ bioremediation technologies, the popularity of these treatment methods is increasing (US EPA, 2001).

Monitored natural attenuation (MNA), biostimulation, and bioaugmentation belong among the three main clean-up approaches based on the ability of microorganisms to degrade CEs at polluted sites. While MNA relies on monitoring of spontaneous natural processes that reduce concentrations of CEs, biostimulation and bioaugmentation, both bioremediation techniques, are based on enhanced degradation process(es) using microorganisms (indigenous or introduced) along with other manipulations to remove CEs within a delimited and controlled subsurface environment. Therefore, for the purpose of this work, MNA relying on natural processes is not considered as a bioremediation technique for sites clean-up; although MNA is commonly referred as a passive bioremediation approach in a scientific community (US EPA, 2000; Kao et al., 2006). Hence, in view of the above-mentioned, MNA is seen as a stand-alone clean-up strategy that is based on monitoring of a contamination plume and its surrounding area in terms to determine whether the natural attenuation of CEs is occurring at sufficient rates to attain site-specific treatment goals (US EPA, 1998).

At sites, where MNA is not sufficient enough to meet treatment goals, biostimulation and/or bioaugmentation must be applied (Lacinova et al., 2013; Dolinova et al., 2016). Firstly, biostimulation includes modification of existing conditions in a subsurface environment in a manner favouring specific degradation process controlled by indigenous microbial populations (Mihopoulos et al., 2002). For instance, to enhance organohalide respiration of PCE and TCE, injection of suitable electron donor(s) is crucial. On the other hand, to support/initiate aerobic degradation processes of less chlorinated ethenes, a supply of electron acceptors (predominantly O_2) is essential and may be followed by the addition of carbon source(s) and nutrients (N, P) if necessary. Whereas enhancing the direct oxidation of CEs requires a supply of only oxygen and nutrients, aerobic cometabolism must include additional injection of the primary substrate(s) that induce production of a catabolic enzyme(s). Secondly, for in-situ bioremediation of sites that do not harbour suitable indigenous microbial population(s) capable to degrade CEs, bioaugmentation is the only option. This approach involves all the aspects of the biostimulation along with an addition of a pre-cultured exogenous bacterial strain or consortium with proven degradation activity towards CEs (Steffan et al., 1999; Ellis et al., 2000).

1.8 Field demonstration of in-situ clean-up of CEs-polluted sites

Several field studies (pilot and full-scale) have been devoted to in-situ MNA and bioremediation of CEs-polluted sites applying biostimulation or bioaugmentation techniques. Studies that have achieved promising results are divided into three subchapters based on a chosen clean-up approach (MNA, biostimulation, and bioaugmentation), and their important technical aspects along with removal efficiency of targeted CEs are further discussed. The discussion is supplemented by Appendixes 1.2 – 1.6, which include basic information about each reviewed study, such as technical designs, attained contaminant removals, means of biostimulation/bioaugmentation, prevailing degradation processes, and where possible, study durations. Finally, the overall feasibility of all three clean-up approaches for removal of CEs at polluted sites is critically evaluated.

1.8.1 In-situ MNA of CEs-polluted sites

MNA generally employs a network of monitoring wells that are installed at a site in a manner to cover the largest possible plume area with respect to the likely migration paths of the plume in the future. Typically, at least one monitoring well is placed directly in a source zone and several other wells are placed downstream and upstream of the zone with respect to regional hydrological conditions as shown in Fig. 1.6.

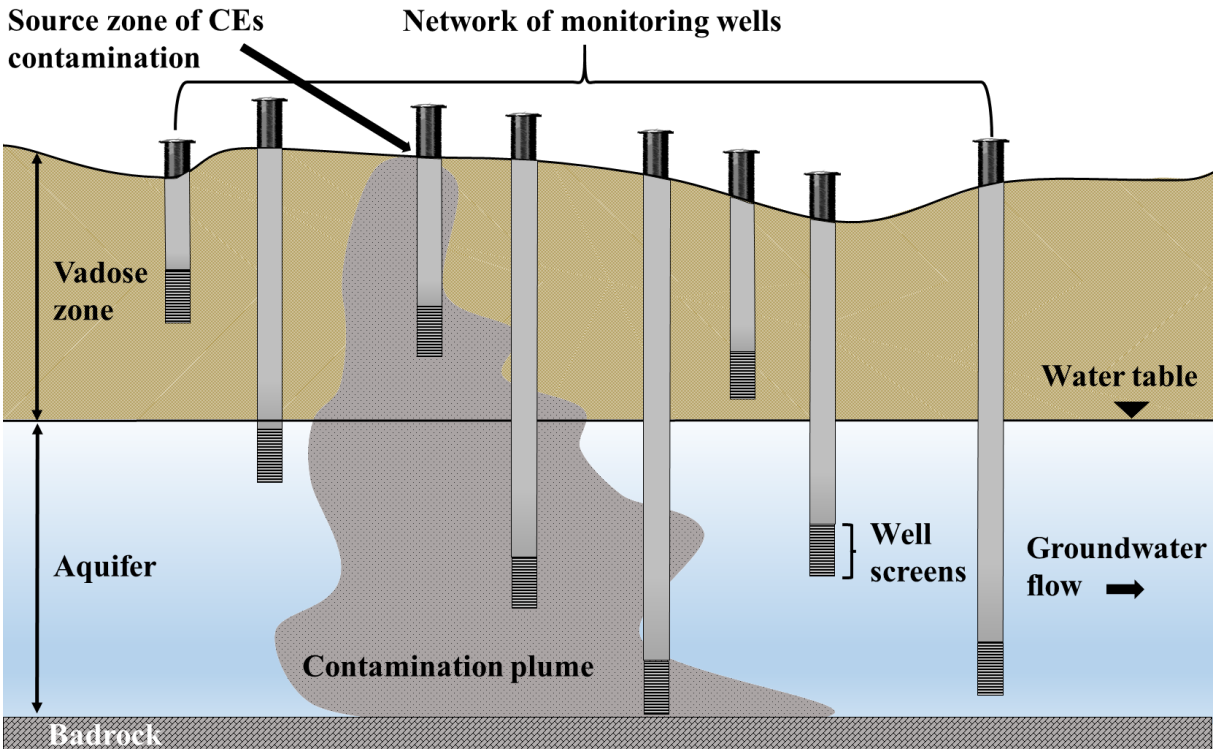

Fig. 1.6 Schematic of a site monitoring using a network of monitoring wells

Such network of monitoring wells ensures collection of field data, which are ideally used to document the loss of CEs and formation of their daughter

compounds, presence and distribution of biogeochemical indicators of natural attenuation, and direct microbiological evidence (Witt et al., 2002). Only the collection of these heterogeneous data ensures sufficient evidence about ongoing natural attenuation of CEs at the site. The loss of CEs and formation of their daughter compounds are predominantly monitored by analysing collected samples of soil, soil gases, groundwater, and alternatively from samples of tree cores; however, three core sampling is restricted to only shallow subsurface layers containing tree roots (Larsen et al., 2008). While the loss of CEs and formation of their daughter compounds is a primary line of evidence about ongoing natural attenuation at the site, monitoring of biogeochemical indicators, such as redox potential (Eh), redox state, specific conductance, pH, dissolved oxygen (DO), total organic carbon (TOC), presence of hydrogen, methane, and dissolved chloride can be used to determine under what conditions natural attenuation of CEs is occurring (Holmes et al., 1998; Witt et al., 2002). Further, identification of microbial species and/or their functional genes involved in CEs degradation serves as a third line of evidence about ongoing natural attenuation, which allows assessing the biodegradation potential (capacity) of indigenous microbial populations. Besides these three crucial lines of evidence, an approximate time needed to achieve attenuation goals at sites may be estimated by using Natural Attenuation Software that models variety of attenuation processes, e.g. contaminant concentration changes, redox processes, advection, dispersion, sorption, and diffusion and dissolution of $NAPL_{CEs}$ (Mendez et al., 2004).

Natural attenuation of CEs-contaminated sites has been repeatedly monitored under strictly anaerobic conditions, which are essential for reductive dechlorination of targeted contaminants (Nemecek et al., 2017). While several studies have described partial dechlorination of PCE and TCE along with formation of their daughter products *c*DCE and VC (Weigand et al., 1998; Larsen et al., 2008), other studies reported dechlorination of *c*DCE and VC to ethene (Lehmicke et al., 2000; Ruttinger et al., 2006). Unfortunately, only limited information about biogeochemical indicators and direct microbial evidence is available to evaluate these studies thoroughly. Nevertheless, even in the case that CEs dechlorination led to the formation of ethene, *c*DCE and VC were still present suggesting a very slow rate of their reduction. Interestingly, only a few studies evaluated the role of combined anaerobic and aerobic processes leading to the natural attenuation of CEs-contaminated groundwater. Holmes et al. (1998) described anaerobic reduction of PCE and TCE to *c*DCE and VC under methanogenic conditions nearby a source zone, followed by oxidative destruction of the aforementioned daughter products to carbon dioxide outside of an anaerobic region of a groundwater plume; yet more detailed information on the type of aerobic processes as well as involved indigenous microorganisms is missing. Another study described the sequential anaerobic/aerobic microbial degradation of CEs in a contaminated aquifer and provided several lines of evidence for the ongoing natural attenuation (Witt et al., 2002). Whereas reductive dechlorination

of PCE and TCE was observed in an anaerobic part of the aquifer with elevated concentrations of dissolved methane and hydrogen, concentrations of *c*DCE and VC decreased below a detectable level nearby an anaerobic/aerobic interface, probably as a result of aerobic transformation and degradation processes (Witt et al., 2002). Furthermore, genes for mono- and dioxygenases were detected in a zone with measurable DO suggesting removal of *c*DCE and VC by aerobic cometabolic degradation (Witt et al., 2002). Among others, the study detected elevated chloride concentration in groundwater, which is an additional line of evidence supporting the microbial degradation of CEs. Thus, it can be strongly suggested that natural attenuation of CEs was a result of sequential anaerobic/aerobic processes, which can potentially remove an entire range of CEs in a shorter period of time compare to anaerobic natural attenuation alone.

On the other hand, MNA generally requires much longer time frames to achieve clean-up objectives in comparison with bioremediation approaches, biostimulation and bioaugmentation. Moreover, MNA is not appropriate where imminent site risks are present, i.e. humans or environmental receptors exposure (US EPA, 2012b). Also, if possible, groundwater clean-up should ensure drinking water standards within a reasonable timeframe, which is not applicable in the case of MNA (US EPA, 2012b).

1.8.2 Biostimulation of CEs-polluted sites

Biostimulation allows considerable flexibility in technical design of clean-up systems, which depends on a part of subsurface that is treated, i.e. vadose zone or aquifer as well as on the method selected to establish a bioreactive zone, the part of a subsurface environment in which microbial degradation of CEs is stimulated (Das and Dash, 2014). The remedy systems for biostimulation commonly contain a variety of injection wells used to deliver biostimulative agents (electron acceptors, electron donors, primary substrates or nutrients) into a subsurface environment along with a network of monitoring wells. In general, the systems should be designed in a manner ensuring the formation of an extensive bioreactive zone in the subsurface and sufficient retention of CEs within the zone (Semprini, 1997, Frascari et al., 2015). In the last few decades, many studies have examined a variety of systems for biostimulation of either anaerobic reductive dechlorination or cometabolic aerobic degradation of CEs in a vadose zone and groundwater. (Hopkins et al., 1993; Bennett et al., 2007; Kim et al., 2008; Dugat-Bony et al., 2012). This effort has led to the development of three main strategies for establishment of the bioreactive zone; (1) direct injection of biostimulative agents into the treatment zone; (2) groundwater extraction amendment and reinjection in a closed loop system; (3) recirculation of amended groundwater using recirculation wells (Semprini, 1997; Frascari et al., 2015).

Systems for a direct injection of biostimulative agents

This strategy uses injection wells for direct introduction of biostimulative agents into a delimited zone of the contaminated subsurface and thus allowing to establish a bioreactive zone through which contamination plume gradually migrates. Simplified wells that are commonly used to stimulate the microbial degradation of CEs in a subsurface environment are shown in Fig. 1.7.

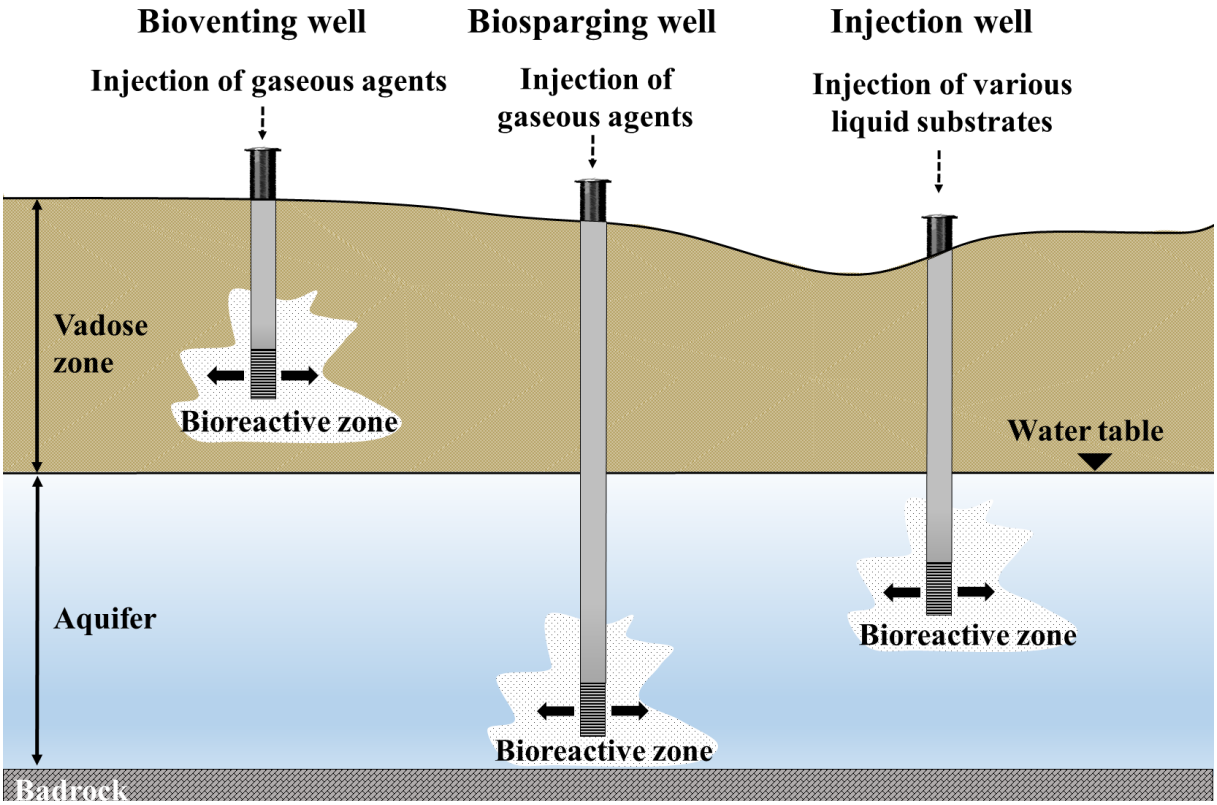

Fig. 1.7 Wells for biostimulation of microbial degradation of CEs in a subsurface environment[4]

This strategy appears as the only option to stimulate microbial degradation of CEs in a vadose zone by using bioventing wells for direct injection of gaseous substrates and oxygen into the bioreactive zone (Frascari et al., 2015). A few pilot-scale studies confirmed microbial degradation of CEs in a vadose zone applying bioventing technologies. First, anaerobic bioventing using a mixture of oxygen-free gases (1% H_2, 1% CO_2, 5% He, balance N_2) resulted in rapid dechlorination of PCE, mostly to *t*DCE and VC under methanogenic conditions (Sayles et al., 1997). The accumulation of PCE dechlorination products in the study indicated that indigenous populations capable of reducing PCE to ethene were not present at the site or surpassed by other populations. Another study reported mineralisation of 1,2DCE (the isomer was not specified) and VC to CO_2 within a bioreactive zone established by a system for cometabolic bioventing that utilized

[4] *The position of the monitoring boreholes is not shown for simplicity*

continuous injection of methane and oxygen (Cox et al, 1998). Finally, a PCE-polluted soil was treated through a system for sequential anaerobic/aerobic bioventing, which was based on delivering a hydrogen gas (reducing agent) and oxygen into the soil (Mihopoulos et al., 2001). While the hydrogen gas was injected into an anaerobic zone of the soil, oxygen was injected in an aerobic zone. This system setting resulted in a complete removal of PCE vapours. Besides the observed removal of PCE vapours, the study monitored the temporal accumulation of *t*DCE and VC under anaerobic conditions, and subsequently rapid oxidation of these two compounds under aerobic conditions that were coupled with the formation of CO_2. Since the study provided no information about prevailing aerobic degradation processes, it can only be assumed that *t*DCE and VC were removed by a combination of cometabolic aerobic degradation and direct oxidation. Despite the fact that all the bioventing studies have observed promising removals of CEs, further investigation should be focused on collecting more information about biogeochemical indicators and direct microbial evidence so that the prevailing degradation conditions, as well as microorganisms degrading CEs, are better understood.

Also, the systems for direct injection of biostimulative agents (both liquid and gaseous) can be used for bioremediation of CEs-polluted aquifers. Whilst it is a common practice that a variety of injection wells are used to introduce liquid agents into aquifers, gaseous agents are delivered through biosparging wells.

A full-scale study operating with a system consisting of four injection wells for the introduction of lactate solution into a TCE-contaminated aquifer documented its complete reduction to ethene by stimulated dehalorespiring populations in less than 400 days (Dugat-Bony et al., 2012). Besides the observed loss of TCE and temporal formation of DCEs and VC, the study regularly collected data about biogeochemical indicators and direct microbiological evidence. Repeated injections of lactate into aerobic groundwater underlying the pollution source area led to the establishment of reducing conditions mainly characterized by oxidation-reduction potential (ORP) that ranged from -79.9 to -268.3 mV and production of methane. Further, molecular tools were used to detect the presence of dehalorespiring populations and specific biomarkers at the site. Although the wide diversity of dehalorespiring populations including *Sulfurospirillum*, *Dehalobacter*, *Desulfitobacterium*, *Geobacter*, and *Dehalococcoides* genera was found at the site, the detection of two reductive dehalogenase genes, bvcA and vcrA, allows to suggest that the reduction of DCEs and VC to ethene could probably be mediated by bacteria from populations of *Dehalococcoides mccartyi*, which are known to express both bvcA and vcrA genes (Mayer-Blackwell et al., 2017). Importantly, Dugat-Bony et al. (2012) observed accumulation of *c*DCE in parts of the site where bvcA and vcrA genes were not detected. These findings showed that the established reducing conditions in the subsurface environment could control the spatial and temporal formation of key microbial populations involved in the degradation of CEs.

Further, several pilot-scale studies have focused on the biostimulation of microbial populations capable of aerobic cometabolism. A study utilizing a horizontal well for biosparging of a shallow aquifer with dissolved methane and oxygen successfully demonstrated partial biotransformation of TCE, *c*DCE, *t*DCE, and VC by stimulated methanotrophic population in a two-metre bioreactive zone (Semprini et al., 1991) Likewise, another study reported significant biotransformation of TCE and *c*DCE by enhanced methanotrophic population using a vertical biosparging well for delivery of methane and air to the aquifer along with a soil vapour extraction unit for physical treatment of CEs vapours in a vadose zone (Sutfin and Ramey, 1997). Although the study concluded that 60 to 80 % of CEs was removed from the aquifer within three months of operation, the rates of their biotransformation and volatilization cannot be determined due to the lack of data provided. The further study documented enhanced biotransformation of TCE and *c*DCE mediated by microbial populations that were stimulated by propane and oxygen through a vertical biosparging well (Kim et al., 2008). Moreover, a presumption that TCE and *c*DCE were transformed by propane utilizing populations was supported by the detection of a propane monooxygenase in collected groundwater samples within a bioreactive zone. The enzyme is known to be able to catalyse a transformation of both contaminants (Kim et al., 2008). On the contrary, the same study reported that no biotransformation of TCE was observed when propane was replaced with methane despite the stimulated population expressed a methane monooxygenase. This result may be due to the expression of a particulate methane monooxygenase (pMMO), which is known to be less efficient in catalysing the cometabolic degradation of CEs compare to soluble MMO (sMMO) (Oldenhuis et al. 1991).

In general, although all the pilot-scale studies utilizing a single-well system for biostimulation of CEs-polluted sites have achieved substantial removals of targeted contaminants, this system allows establishing only a narrow bioreactive zone that is typically formed in the immediate vicinity of each injection well. Hence, further migration of contaminants beyond the treatment zone is highly likely, which appears as the main obstacle for implementation of the system for a full-scale bioremediation of heavily polluted sites where contamination plumes might extend several kilometres from its original source (Hoelen et al., 2006).

Closed loop systems

These systems are utilized for bioremediation of aquifers and generally consist of a pair of injection and extraction wells connected to a surface gas-tight tank for amendment of groundwater extracted from an aquifer prior to its re-injection into the aquifer. A simplified scheme of a typical closed loop system is shown in Fig. 1.8.

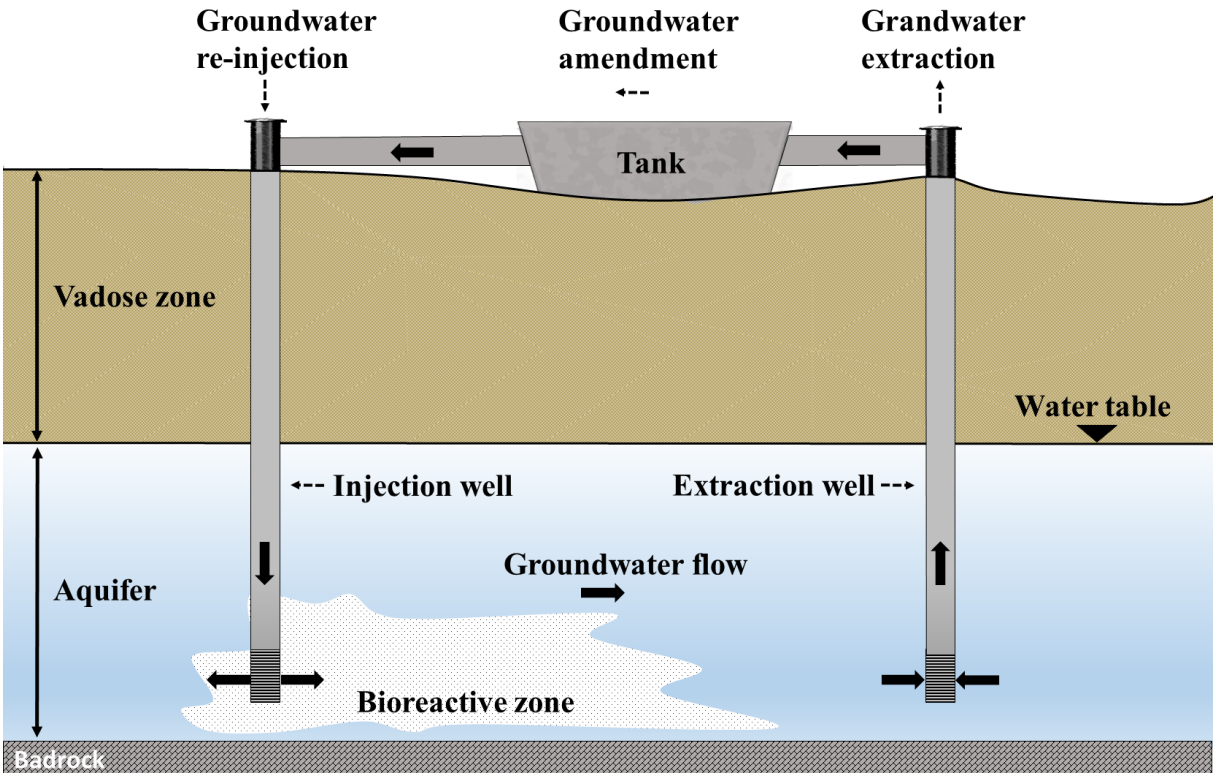

Fig. 1.8 Closed loop system[5]

Closed loop systems promote recirculation of polluted groundwater through an established bioreactive zone within a delineated part of an aquifer that usually extends several meters between the injection and extraction wells. The feasibility of these systems for stimulation of aerobic cometabolic degradation and anaerobic reductive dechlorination of CEs has been tested by a wide range of pilot-scale studies.

Several studies have stimulated aerobic cometabolic degradation of CEs in polluted aquifers by using either soluble phenol and toluene or gaseous methane as primary substrates along with different sources of oxygen, e.g. air, molecular oxygen, or hydrogen peroxide (Semprini, 1997). It was found that while stimulated phenol and toluene utilizers have appeared to be very efficient towards removal of the most CEs except PCE and *t*DCE (Hopkins et al., 1993; Hopkins and McCarty, 1995), methane utilizers proved to be efficient degraders of *t*DCE and VC but their ability to remove TCE and *c*DCE was shown to be significantly lower compared to the phenol and toluene utilizers (Semprini et al., 1990). If compared the efficiency of the tested primary substrates towards removal of CEs, it is obvious that both phenol and toluene appear to be more suitable substrates for bioremediation of sites relying on aerobic cometabolism. Furthermore, a filed investigation has revealed that phenol and toluene were reduced to very low levels (less than 1 μg L^{-1}) after completing the remedy process

[5] *The position of the monitoring boreholes is not shown for simplicity*

in all the studies (Hopkins et al., 1993; Hopkins and McCarty, 1995), suggesting rapid utilization of these substrates by indigenous populations. This finding may serve as a strong argument in terms to obtain regulatory approval for in-situ application of these regulated chemicals. Besides these promising findings, Hopkins and McCarty (1995) observed that a removal of TCE in phenol and toluene-oxidizing microorganisms was significantly inhibited in presence of 1,1DCE, which was artificially added into a tested zone of the treated aquifer. The authors stated that the inhibition was due to a highly toxic intermediate of a 1,1DCE transformation. Also, a similar issue was previously found in laboratory studies with methane as a primary substrate (Dolan and McCarty, 1994). Nevertheless, 1,1DCE is usually not found at sites polluted by PCE and TCE, but rather the compound can be formed in a process of 1,1,2-TCA dehydrochlorination (Pagan et al., 1998). Therefore, utilization of phenol and toluene for bioremediation of sites polluted by a mixture of chlorinated ethenes and ethanes might not be a suitable strategy to achieve treatment goals. Also, further field studies should focus on identification of phenol and toluene utilizers capable of the cometabolic degradation of CEs.

Other studies have described the significant reduction of TCE, *c*DCE and VC by applying enhanced reductive dechlorination as a remedy approach (Bennett et al., 2007; Nemecek et al., 2018). The first study used sucrose and a dextrose-fructose mixture as a source of electron donors along with yeast extract to stimulate dechlorination of the contaminants in an unconfined aquifer (Bennett et al., 2007). The addition of electron donors into the aquifer resulted in a sequential reduction of electron acceptors, namely nitrate, sulfate, ferrous iron, and eventually in a production of methane in conjunction with reductive dechlorination of TCE to ethene. These findings suggest that supplied electron donors were utilized not only by dehalorespiring populations but also nitrate reducers, sulfate reducers and methanogens. Even though no dehalorespiring populations involved in the dechlorination of CEs were determined in this study, it can be assumed that observed reduction of TCE to ethene was mediated by some of the *Dehalococcoides* populations. Further, the second study investigated thermally enhanced reductive dechlorination of mainly *c*DCE and VC in two underlying aquifers enriched with cheese whey (Nemecek et al., 2018). The study used a closed loop system supplemented with a heating unit consisting of solar absorbers and an electrical heater for increasing temperature in the aquifers. The addition of cheese whey along with adjustments of the temperature in the aquifers (20 – 30 ºC) rapidly accelerated reductive dechlorination of *c*DCE and VC to ethene. Furthermore, the study reported that the most favourable reducing conditions for dehalorespiring microorganisms were established after a reduction of ferrous iron and sulfate. Additionally, a close relation was found between the efficiency of TCE dechlorination to ethene and an increase in the abundance of *Dehalococcoides mccartyi* and *Dehalobacter* sp., along with detected functional genes vcrA and bvcA. Importantly, these results showed that appropriate heating

of aquifers accelerated anaerobic reductive dechlorination of CEs but only under suitable reducing conditions, which were established by the addition of cheese whey. Thus, not only the system seems to offer a promising solution to accelerate bioremediation of CEs-polluted aquifers, but it might also be a potential bioremediation approach for a clean-up of aquifers in colder climate zones.

Even though all the described pilot-scale studies have accomplished significant removals of targeted CEs by applying closed loop systems, a full-scale application of these systems as the only remedy strategy is unlikely due to two main reasons. First of all, only a limited bioreactive zone can be established within these systems (1 to 2 meters from the injection well) and second, pumping of groundwater to the surface is costly.

Systems employing recirculation wells

Another strategy for in-situ bioremediation of aquifers is an application of systems employing subsurface recirculation wells equipped with a submersible pump installed between two screens, one of which serves for extraction of groundwater and second for its discharge (Semprini, 1997). Biostimulative agents are introduced into the wells through feed lines and mixed with CEs-contaminated groundwater using mixers placed inside the wells; each well ensures the formation of a bioreactive zone around the discharge screen as well as recirculation of groundwater across the zone allowing more time for biological reactions to occur (McCarty et al., 1998). In general, utilization of systems employing recirculation wells ensures a treatment of aquifers without a need to pump groundwater on the surface, hence the complete process occurs in-situ, which is less expensive compared to the previously reviewed closed loop systems (Frascari et al., 2015).

Two field studies have employed a remedy system consisted of a couple of recirculation wells each spanning two aquifers to ensure vertical circulation of groundwater between the upper and lower aquifers as well as its horizontal circulation between the two wells. A simplified scheme of a system employing two recirculation wells for treatment of two underlying aquifers is illustrated in Fig. 1.9.

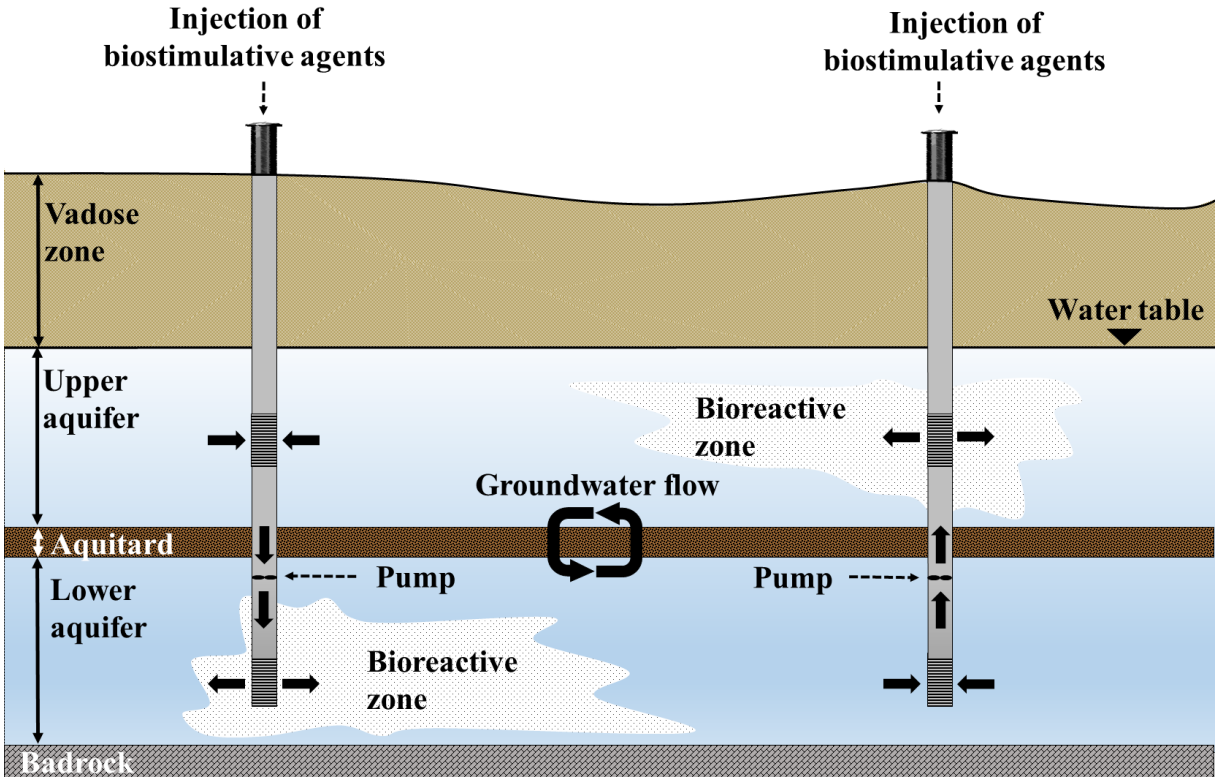

Fig. 1.9 System employing two recirculation wells[6]

First, McCarty et al. (1998) demonstrated significant removal of TCE in two bioreactive zones (one in each aquifer) created by the injection of toluene, oxygen, and hydrogen peroxide through the discharge screens of the two recirculation wells located 10 meters apart. Besides, the study repeatedly observed a high utilization rate of the injected toluene, which was directly linked to its very low residual concentrations in aquifers (approximately 1.1 µg L^{-1}). Moreover, the introduction of toluene into the aquifers initiated production of ortho-monooxygenase in microbial populations. These findings may be attributed to the favourable conditions for growth of indigenous microorganisms during the bioremediation process, such as the sufficient concentrations of oxygen, nitrogen phosphorus, and other nutrients. Although the study did not identify any particular bacteria involved in the cometabolic degradation of TCE, it is highly likely that the compound was degraded by toluene utilizing populations.

Another study investigated anaerobic reductive dechlorination of *c*DCE and VC at sulfidogenic groundwater site (Hoelen et al., 2006). The study chosen a bioremediation strategy based on the introduction of sodium propionate solution into both aquifers using two recirculation wells located 7.2 meters apart. Despite the fact that the system settings allowed the formation of a bioreactive zone in

[6] *Inspired by the work of Hoelent et al. (2006), and Bennett et al. (2007); the position of the monitoring boreholes is not shown for simplicity*

each of the aquifers, the paper exclusively focused on the removal of the contaminants in the lower aquifer. The study observed a nearly stoichiometric reduction of *c*DCE (~1000 μg L^{-1}) to ethene with only low residual concentrations of VC in the bioreactive zone established between the two wells by the end of the two months of the system operation. Interestingly, the reduction of the contaminants occurred under sulfate-reducing conditions, which prevailed until the end of the study so that no methanogenesis was monitored. Such observation is inconsistent with the findings of the other studies that have described enhanced reductive dechlorination of CEs under methanogenic conditions (Sayles et al., 1997; Holmes et al., 1998). Thus, it can be inferred that propionate was slowly utilized by indigenous microbial populations, which in turn resulted in the release of hydrogen at concentrations that stimulated sulfate reduction along with anaerobic dechlorination of *c*DCE and VC, yet the concentrations of hydrogen were not sufficient for inducing methanogenic conditions. Indeed, previously performed laboratory studies also confirmed that propionate served as a hydrogen precursor that stimulated dechlorination without promoting methanogenesis (Hoelen, 2005). Among others, the presence of sulfate in groundwater did not appear to inhibit dechlorination of the contaminants even though previous studies have observed that CEs dechlorination can be inhibited or might occur very slowly when sulfate is present (Mazur and Jones 2001; Hoelen and Reinhard, 2004). On the other hand, unlike the study conducted by Hoelen et al. (2006), none of the two studies stimulated the dechlorination of CEs by propionate or any other substrates. Hence, propionate seems like a suitable substrate sustaining anaerobic reductive dechlorination of CEs under sulfate-reducing conditions. Furthermore, it also appears as a generally suitable hydrogen precursor for enhancing dechlorination of CEs in dehalorespiring bacteria, such as those of a genus Dehalococcoides that require hydrogen as an electron donor to reduce CEs (Loffler et al., 2013). Nevertheless, it is not clear from the study of Hoelen et al. (2006), which organohalide-respiring bacteria were involved in the dechlorination of *c*DCE and VC. Therefore, further research should be done to identify specific dehalorespiring bacteria responsible for CEs dechlorination under sulfate-reducing conditions stimulated by propionate.

In general, systems employing two recirculation wells have shown to be effective for removal of CEs either by stimulating their reductive dechlorination or aerobic cometabolic degradation. The efficiency of CEs removal is given by the establishment of the zone of recirculation between the two wells, which serves to mix biostimulative agents and groundwater in-situ. Moreover, these systems create two bioreactive zones each extending several meters, which allows the treatment of larger parts of aquifers in comparison with the closed loop systems. On the other hand, recirculation wells may be prone to biofouling due to an excessive growth of microorganisms in the near vicinity of the well-screens, which can result in clogging of wells (Semprini, 1997). Nonetheless, the risk of microbial clogging at well-screens can be substantially reduced by a pulsing

addition of biostimulative agents. For instance, aerobic cometabolic degradation can by stimulated by alternating pulses of oxygen and a primary substrate, which may or may not be separated by a pause (Semprini et al., 1991). Applying this strategy promotes more uniform distribution of the agents within a treated zone, and thus reduces the risk of well clogging (Frascari et al., 2015). Further, hydrogen peroxide can be used as an alternative source of oxygen, which might also prevent the well-screens from clogging if dosed properly; however, its application is more expensive than using air or pure oxygen (Hopkins and McCarty, 1995). Finally, although the main specificity of these systems is a possibility to remediate two underlying aquifers at polluted sites, the same systems can be used for the treatment of single aquifers.

1.8.3 Bioaugmentation of CEs-polluted sites

Two different bioaugmentation approaches have been developed for in-situ bioremediation of CEs-polluted sites. While in the first approach, large amounts of microbial suspensions are injected to the subsurface with the aim to achieve rapid removal of contaminants before the injected cells are inactivated or perished (Duba et al., 1996), the second approach aims to achieve prolonged survival and growth of the added microorganisms along with long-term degradation of targeted contaminants within an established bioreactive zone in the subsurface (Semprini et al., 2007). Whereas the rapid clean-up approach can only be applied for treatment of aquifers, the second approach, relying on the establishment of the bioreactive zone, allows treating not only aquifers but also low permeable matrices, such as clay layers.

For the purpose of this doctoral thesis, the two bioaugmentation approaches are further referred to as a rapid bioaugmentation approach and a long-term bioaugmentation approach.

Rapid bioaugmentation approach

Suitability of this approach has been investigated by two studies utilizing systems containing various wells for a direct injection of large amounts of microbial suspensions into polluted aquifers. First of them injected 1800 L of a suspension comprising resting cells of *Methylosinus trichosporium* OB3b (5.4 x 109 cells/mL) into a TCE-contaminated aquifer using a single well system. (Duba et al., 1996). The *M. trichosporium* OB3b was selected for its ability to sustain soluble methane monooxygenase (sMMO) as well as due to the high resting-cell biotransformation capacity for TCE (0.25 mg of TCE/mg of dry cell weight) (Taylor et al., 1993). The application of its resting cells resulted in nearly 98% removal of TCE within a 1.1-meter radius from the injection well in the first two days. Moreover, no addition of oxygen was needed for resting-state metabolic activity of *M. trichosporium* OB3b as the concentration of DO in the aquifer

remained sufficiently high (above 1 mg L^{-1}) to oxidize present concentration of TCE (Duba et al., 1996).

In a second study, nine wells were utilized to rapidly distribute 550 L of a suspension containing cells of *Burkholderia cepacia* PR1301 possessing constitutive toluene ortho-monooxygenase and oxygen throughout the entire test zone that measured 4.6 m wide by 12 m long and contained TCE, *c*DCE, and VC (Steffan et al., 1999). The strain was used for its ability to grow to high cell density (~100 g L^{-1}) in a fermentor as well as due to its ability to degrade TCE, which was earlier found by Munakata-Marr et. al (1996). The injection of a high-density cell suspension led up to 78% decrease of the total mass of TCE, *c*DCE, and VC within approximately a 4.5-metre radius from the centre of the treatment zone during first 2 days.

Both of the studies have proven that the rapid bioaugmentation of aquifers is an efficient clean-up strategy ensuring significant removal of CEs within a few days of operation. In comparison, biostimulation may require several months of operation to achieve the same removal efficiency. However, the high efficiency of the fast bioaugmentation approach is time-limited and its application seems to be restricted to only highly contaminated parts of aquifers underlying the source of contamination.

Long-term bioaugmentation approach

This approach has been investigated by several studies using different remedy systems. Whereas closed loop systems have been used for both anaerobic (Major et al., 2002) and aerobic bioaugmentation of aquifers (Semprini et al., 2007), systems employing wells for direct injection have been used for anaerobic bioaugmentation of low permeable matrices (Scheutz et al., 2010; Verce et al., 2015).

Two successive pilot-scale studies have used a butane-grown suspension, primarily comprised of a *Rhodococcus* sp. for bioaugmentation of an aquifer intentionally contaminated by 1,1DCE and two chlorinated ethanes, 1,2DCE and 1,1,1TCA. (Semprini et al., 2007; 2009). These three contaminants often form contamination plumes in groundwater polluted by 1,1,1TCE due to the abiotic and abiotic transformation of the latterly mentioned compound (Vogel and McCarty, 1987). Both studies utilized a closed loop system for repeated groundwater amendment with the contaminants, butane, oxygen, and *Rhodococcus* sp. (4 mg L^{-1} of biomass) into the 7-metre treatment zona. Repeated injection of the contaminants along with biostimulative agents and the microbial suspension resulted in transformations of 1,1DCE (~97%), 1,1-DCA (77%), and 1,1,1-TCA (36%) within a one metre distance from the injection well during the early stages of testing (first 20 days); however, little or no transformation of contaminants was observed beyond the one metre zone. Furthermore, the removals of contaminants were significantly decreased after the first 20 days of the testing, which might

have been associated with 1,1-DCE transformation toxicity combined with the limited dosage of butane (Semprini et al., 2007; 2009).

Studies devoted to anaerobic bioaugmentation of CEs-polluted sites have used enrichment KB-1 consortium consisting of phylogenetic relatives of *Dehalococcoides ethenogenes* (Major et al., 2002; Scheutz et al., 2010; Verce et al., 2015).

First, bioaugmentation of an aquifer containing PCE, TCE and *c*DCE was explored by a pilot-scale study using a closed loop system (Major et al., 2002). The system first supplied the aquifer with methanol and acetate in terms to promote reducing conditions prior to the injection of 13 L of the KB-1 consortium. The study observed a significant reduction of PCE and TCE as well as reduction of *c*DCE to ethene via temporarily produced VC within 200 days. Although the study detected *Dehalococcoides* sequences as far as 7 meters downgradient of the injection well, the estimated rates of CEs dechlorination were fastest in the first 2.4 meters of the bioreactive zone, where the highest concentrations of acetate and biomass were found (Major et al., 2002).

Further, two long-term pilot-scale studies have used the KB-1 consortium for bioaugmentation of low permeable subsurface matrices. First of the studies investigated bioaugmentation of clayey till containing mainly *c*DCE and VC, and trace amounts of TCE (Scheutz et al., 2010). The study employed a single injection well for the addition of a dilute groundwater solution containing emulsified soybean oil and the KB-1 consortium (208 L) into a sand-filled hydraulic fracture. The injection of the solution led to the establishment of a methanogenic dechlorinating bioreactive zone in the fracture within a month, which then extended approximately 5 to 6 cm into the adjacent clayey till matrix in next four months. As a result, not only *c*DCE and VC were completely reduced to ethene within the induced fracture in a five-month period, but also reductive dechlorination of these contaminants was observed in the bioreactive zone extending the clay matrix. Moreover, model predictions made by (Chambon et al., 2010) and empirical observations published be Scheutz et al. (2010) indicated that a bioreactive zone from a 1 to 2 cm thick sand-filled fracture might have extended into clay matrix as far as 34 cm on either side in 1.5 years. Hence, applying the KB-1 consortium for bioaugmentation of clay layers through several sand-filled hydraulic fractures spaced 10 to 40 cm apart might potentially be a remedy strategy ensuring dechlorination of *c*DCE and VC in a clay matrix on a larger scale within a reasonable timeframe (Scheutz et al., 2010).

Another study examined the bioaugmentation of groundwater bearing cemented conglomerate contaminated by TCE (Verce et al., 2015). The formation was characterized as low permeable subsurface containing friable sand lenses and opened fractures capable of transmitting flow and so allowing to deliver electron donor(s) and KB-1 consortium into highly indurated (i.e., hard) and recharge limited (i.e., contains little water) conglomerate. The study utilized a system composed of two injection wells spaced approximately 9 meters apart. Well-1 was

used for an injection of a large amount of tracer water (35,297 L) along with periodic lactate injections of lactate (696 L) to hydraulically characterize the site and to establish reducing conditions within the formation, respectively.

Although the large volume of the injected tracer water increased the local hydraulic gradient and so accelerated the transport of lactate in a low permeable subsurface formation, it also led to the dilution of some TCE out of the treatment zone. As a consequence, the well-1 was not used for a bioaugmentation test because TCE concentrations were low in its vicinity. Thus, the KB-1 consortium (10 L) was injected into the formation by using a well-2. Besides, the well-2 was used for two single additions of lactate just prior to bioaugmentation and shortly after it. The study found that prior the addition of lactate and the KB-1 consortium into the formation, TCE and *c*DCE were only contaminants present at the site. However, after periodic injection of lactate (during the tracer test), the amount of *c*DCE increased suggesting that TCE was reduced by indigenous populations stimulated by lactate. Moreover, VC and ethene appeared only after bioaugmentation under methanogenic conditions, which clearly showed the importance of the KB-1 consortium for dechlorination of *c*DCE and VC at the site. Additionally, dissolved mass of total CEs (TCE, *c*DCE, VC) was reduced by about 80% within the treatment zone (15.2 m x 15.2 m) by the end of the study (day 2,931). These results serve as a proof that using KB-1 consortium for in-situ bioaugmentation can reduce *c*DCE and VC to ethene in fractured conglomerate, yet the displacement of contaminants caused by a large amount of the injected tracer water seems to be inevitable and was estimated by the study to be responsible for about 25% share in a total 80% removal.

Overall, the long-term bioremediation approach has shown to be a promising clean-up strategy for CEs-polluted aquifers and low permeable matrices. Especially bioaugmentation of clay deposits showed a great promise for reducing contamination within the matrix and so diminishing the risk of secondary contamination of aquifers. On the other hand, the efficiency of the site bioaugmentation containing 1,1DCE showed to be only temporal and so not suitable as the long-term clean-up strategy.

2. OBJECTIVES OF THE THESIS

A theoretical part of the doctoral thesis aimed to write a comprehensive summary dealing with an influence of the physicochemical properties of CEs on their transport and a fate in a subsurface environment, and to outline common procedures of a site investigation as well as to review different bacterial strategies for degradation and transformation of CEs, and the possible use of these strategies for in-situ bioremediation of contaminated soils and groundwater. Within this aim was also to summarize field studies that have achieved significant removal of chloroethenes at polluted sites. Such a review should be later published in an appropriate scientific journal with an impact factor.

In an experimental part of the thesis, the main aim was to investigate the microbial degradation of all three dichloroethene isomers (DCEs), trichloroethene (TCE), and vinyl chloride (VC) in a mineral salt medium (MSM) by *Comamonas testosteroni* RF2. Within that objective were performed studies focusing on the degradation of single compounds as well as on degradation of the compounds in mixed samples. Degradation studies investigating the ability of *C. testosteroni* RF2 to degrade various mixtures of the chloroethenes were performed in terms to imitate model conditions in contaminated groundwater. In addition, kinetics of single DCEs in *C. testosteroni* RF2 along with tracking transformation intermediates of the compounds were studied too. Also, in order to find a suitable consortium capable of degrading VC and other chloroethenes in mixed samples, several bacterial consortia containing *C. testosteroni* RF2 and one of the following strains capable of degrading VC, *Mycobacterium aurum* DSM-6695, *Pseudomonas putida* DSM-7189, and *Rhodococcus ruber* DSM-7511, were studied for their degradation potential.

3. METHODOLOGY OUTLINE

3.1 Chemicals

TCE (99%), *c*DCE (99.1%), *t*DCE (99.7%), 1,1DCE (99.9%), VC (99.5%), chloroacetyl chloride (min. 99.0%), Mercury(II) thiocyanate, and sodium lactate were obtained from Sigma-Aldrich. Ordinary chemicals were purchased from local suppliers.

The composition of a mineral salt medium (MSM) (in g L^{-1} if not otherwise stated): Na_2HPO_4 1.91, KH_2PO_4 0.18, NH_4Cl 0.3, $MgSO_4.7H_2O$ 0.1, $Fe(NH_4)_2.(SO_4).6H_2O$ 0.03, $CaCl_2.2H_2O$ 0.01, NaCl 0.5 and trace elements solution 1 mL (Muchova et al., 2009); pH 7,4.

The composition of a chloride-free mineral salt medium was the same as for the MSM, except for the complete lack of NaCl and NH_4Cl being substituted for $(NH_4)_2SO_4$ instead; pH 7,4.

Tryptone Yeast extract Agar (TYA), Reasoner's 2A agar (R2A), and MIDDLEBROOK MEDIUM were used as solid growth media for the cultivation of microorganisms. While TYA and R2A agars were purchased from HiMEDIA, MIDDLEBROOK MEDIUM was prepared according to a guideline published by German Collection of Microorganisms and Cell Cultures (DSMZ) that is available online (DSMZ, 2008).

3.2 Description of strains

Comamonas testosteroni RF2 was isolated earlier from activated sludge fed by phenol; the sludge was acclimated for 2 weeks by daily phenol addition (300 mg L^{-1}). The strain is able to grow on phenol concentrations up to 300 mg L^{-1}; it is deposited in the Czech Collection of Microorganisms under the catalogue number CCM 7350 (Ruzicka et al., 2002; 2011).

Mycobacterium aurum DSM-6695 was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). *M. aurum* DSM-6695 was isolated from contaminated soil in Arnhem, Netherlands, and designated as *M. aurum L1* (Hartmans et al., 1985). It is an aerobic strain with an optimum growth temperature of 28 ºC, which utilizes ethene and vinyl chloride as its only source of energy and organic carbon.

Pseudomonas putida DSM-7189 was purchased from DSMZ. *P. putida* DSM-7189 was isolated from a sample of soil collected in California, USA, and designated as *Pseudomonas putida* PS (Castro and Belser, 1990). It is an aerobic strain with an optimum growth temperature of 28 ºC, which utilizes 3-chloropropan-1-ol and chloroallylalcohols as its growth substrates (Castro et al., 1992). Further, the resting cells of the strain previously grown on the above-listed growth substrates are capable of vinyl chloride mineralization (Castro et al., 1992).

Rhodococcus ruber DSM-7511 was purchased from DSMZ. *R. ruber* DSM-7511 was isolated from contaminated subsurface sediments at the Savannah River Site in the USA, and designated as *Rhodococcus ruber* Sm-1 (Malachowsky et al., 1994). It is an aerobic strain with an optimum growth temperature of 25 ºC, which utilizes a variety of alkanes, benzene, toluene, phenol, benzoate, naphthalene or biphenyl as its sole source of energy and organic carbon (Phelps et al., 1991). Further, it cometabolically mineralize vinyl chloride, trichloroethene, and other chlorinated aliphatic compounds (Phelps et al., 1991; Malachowsky et al., 1994).

3.3 Rehydration of dried microbial strains

Lyophilised strains of *M. aurum* DSM-6695, *P. putida* DSM-7189, and *R. ruber* DSM-7511 were obtained in glass ampules. The tip of each ampule was heated in a flame and subsequently, a few drops of water were applied onto the hot tips, which resulted in cracking of the glass. Then, the glass tips were carefully cut off using a sterile tweezer. The same tweezer was then used for removing the insulation material, and the inner vials sealed with cotton plugs were taken out. The cotton plugs were lifted using the tweezer (all kept under sterile conditions) and the tops of the inner vials were flamed. Subsequently, 0.5 mL of broth medium was added into the inner vials containing dry bacterial pellets. Then, the vials were sealed with the previously removed plugs and the dry pellets were rehydrated for up to 30 minutes. After that, the rehydrated content of each vial was mixed with an inoculation loop and transferred onto selected solid growth media.

3.4 Growth conditions

The RF2 strain was routinely cultivated on Tryptone Yeast Extract Agar plates (HiMedia) enriched with phenol (200 mg L^{-1}) at 25 ºC for three days. The cells that grew were harvested and dispersed in sterile physiological saline (NaCl 8.5 g L^{-1}) to obtain the cell density 10^8 mL^{-1}, and finally utilized in degradation assays.

M. aurum DSM-6695 was cultivated either onto the MIDDLEBROOK MEDIA or R2A agar; *P. putida* DSM-7189 and *R. ruber* DSM-7511 were both cultivated onto R2A agar (all strains were cultivated at 25 ºC). While the cells of *P. putida* DSM-7189 and *R. ruber* DSM-7511 were grown for three days prior their utilization for degradation assays with CEs, *M. aurum* DSM-6695 required the prolonged time of cultivation (ideally two weeks) prior its utilization for the degradation assays.

3.5 Growth tests of microbial strains in presence of different organic substrates

Microbial strains purchased from DSMZ as well as *C. testosteroni*. RF2 were examined for their ability to utilize different organic substrates as their only source of energy and organic carbon. The growth tests were performed in 40 mL sterile glass vials sealed with sterile (UV irradiated) gas-tight septum caps (WHEATON). Each vial contained 10 mL of MSM, a cell suspension (10 µL), and some of the selected organic substrates, namely sodium benzoate (200 mg L^{-1}), phenol (200 mg L^{-1}), 3-chloropropan-1-ol (50 – 100 mg L^{-1}). Prepared samples were incubated for several days as described in section 3.7, and the growth of microbial strains was evaluated according to the observable turbidity in the vials.

3.6 Preparation of methanolic solutions of chloroethenes

Each of the purchased DCEs was used for the preparation of methanolic stock solutions at a concentration of 20 g L^{-1}. The procedure was as follows: 2 mL of methanol was added into 2 mL vials equipped with special ON / OFF caps and gastight septa (SUPELCO). Then, the added portion of methanol was weighed on analytical scales (Sartorius) before the subsequent addition of chloroethene (32 µL) by using a gas-tight syringe (HAMILTON). Afterwards, the vials containing methanol and chloroethene were weighed and actual concentrations of DCEs in the stock solutions were calculated from the obtained data.

Further, standard methanolic solutions at concentrations of 2000 mg L^{-1} and 200 mg L^{-1} of *c*DCE, *t*DCE, and 1,1DCE were obtained by diluting the stock DCEs solutions (10x; 100x). Specifically, standard methanolic solutions of *c*DCE, *t*DCE, and 1,1DCE were obtained by transferring either 0.2 mL of each dichloroethene stock solution (20 g L^{-1}) into 1.80 mL of methanol (2000 mg L^{-1}), or by transferring 0.02 mL of the same into 1.98 mL of methanol (200 mg L^{-1}).

The prepared standard solutions of DCEs along with a purchased methanolic solution of VC (2000 mg L^{-1}) (Sigma-Aldrich) and an aqueous solution of TCE (1.1 g L^{-1}), obtained from the previous work (Ruzicka et al., 2011), were used for preparation of degradation samples as described in section 3.7.

Furthermore, dichloroethene solutions (200 mg L^{-1}; 2000 mg L^{-1}), as well as the TCE (1.1 g L^{-1}) and VC (2000 mg L^{-1}) solutions, were used for the preparation of calibration solutions.

3.7 Degradation assays

The assays were performed in 40 mL sterile glass vials sealed with sterile (UV irradiated) gas-tight septum caps (WHEATON). Experiments were always conducted at least in two duplicates, in addition to which abiotic blanks were always carried out. Each vial contained 10 mL of MSM, a cell suspension (10

μL), phenol (100 mg L^{-1}), lactate sodium (100 mg L^{-1}) and the chloroethenes, the latter being added as methanolic solutions via a gastight syringe (HAMILTON). The initial chloroethene concentrations utilized were arrived at through an experiment conducted (described in the Results); all the concentrations listed are intended as actual concentrations in a liquid phase unless otherwise stated. Test vials were incubated in darkness on a shaker (150 rpm) set to semi-continuous mode at 25 ºC for 7- 23 days, according to the objective of the research.

3.8 Analysis of samples containing chloroethenes

The chloroethenes were first extracted by the Purge and Trap method in the concentrator Teckmar LSC 2000 and monitored on a Hewlett Packard 5890 Series II GC device equipped with a Quadrex capillary column (Methyl phenyl cyanopropyl silicone) at 29.87 m length, 0.53 mm (inner diameter) and 3 μm in film thickness, supplemented with an electron-capture detector (ECD), as previously described (Ruzicka et al., 2011). The oven temperature was set at 30 ºC min-1, up to a maximum of 150 ºC, and maintained until all the compounds had eluted.

Setting parameters of the TECKMAR LSC 2000 concentrator

The concentrator was set as follows: Standby 35 ºC; Purge 11 min; Dry purge 4 min; Desorb preheat 245 ºC; Desorb 4 min 250 ºC; Bake 4 min 260 ºC; Auto drain ON; Valve 150 ºC; Line 150 ºC.

The concentrator was connected to a pressure bottle filled with an inert gas (nitrogen), which was simultaneously a stripping and carrier gas. Further, the concentrator was connected to a gas chromatographer (Hewlett Packard 5890 Series II GC) by means of a gas chromatograph column heating tube.

Setting parameters of the HEWLETT PACKARD 5890 SERIES II gas chromatograph

Oven temperature 35 ºC; Initial value 35 ºC; Initial time 10 min; Rate 4 DEG/min; Final temperature 150 ºC; Final value 150; Final time 5,00; Detector B (ECD) temperature 250 ºC.

Setting parameters of the HP 3396 SERIES II Integrator

[ZERO] 5 – defining the chromatographic baseline on the paper. [ATT2] 7 – defining the sensitivity of recording. [CHT SP] 0.3 - defining the paper speed. [AR REJ] 80000 - defining the minimum peak area. [THRSH] 2 - defining the minimum width for the peak detection. [PKWD] 0.04 – defining the expected peak width that is optimal for quantification.

3.9 Assembly of calibration curves for individual chlorinated ethenes

To determine initial concentrations of CEs in prepared samples as well as to determine achieved removals of the compounds, several calibration curves showing the dependence of the peak area on the concentration of the chloroethene were assembled for each of the compound, except of VC for which only one curve was assembled due to the problematic determination and monitoring of the compound using the GC-ECD system. Thus, a different analytical procedure was used to monitor the loss of VC as described in section 3.10. Data for plotting the curves were obtained from series of standard solutions (5 mL) containing various volumes of distilled water and CEs so that the concentration range of CEs differed from 0.02 mg L^{-1} up to 25 mg L^{-1}. All standard solutions were processed as described in section 3.8.

In general, a large number of calibration curves was required due to the variety of factors, mainly due to the need to cover broad ranges of CEs concentrations. Indeed, this was the main reason for the high number of calibration curves present in this thesis as the dependence of the peak area on different concentrations of each chloroethene generally shifts from initial linear into a non-linear dependence with increasing concentrations of CEs (analysed by GC-ECD). Among other factors influencing the high number of the assembled calibration curves were a long-term investigation (several years) along with the high volatility of the compounds, which was associated with the need to prepare new methanolic solutions with verified concentrations of CEs. Finally, a replacement of a trap of the concentrator Teckmar LSC 2000 was an additional factor that led to the assembly of additional calibration curves. Hence, due to a large number of the calibration curves, only one for each chloroethene, except VC, is shown as a figure (Figs 3.1; 3.2; 3.3; 3.4), while other curves are given in a form of equations and R^2 values that are listed in Table 3.1.

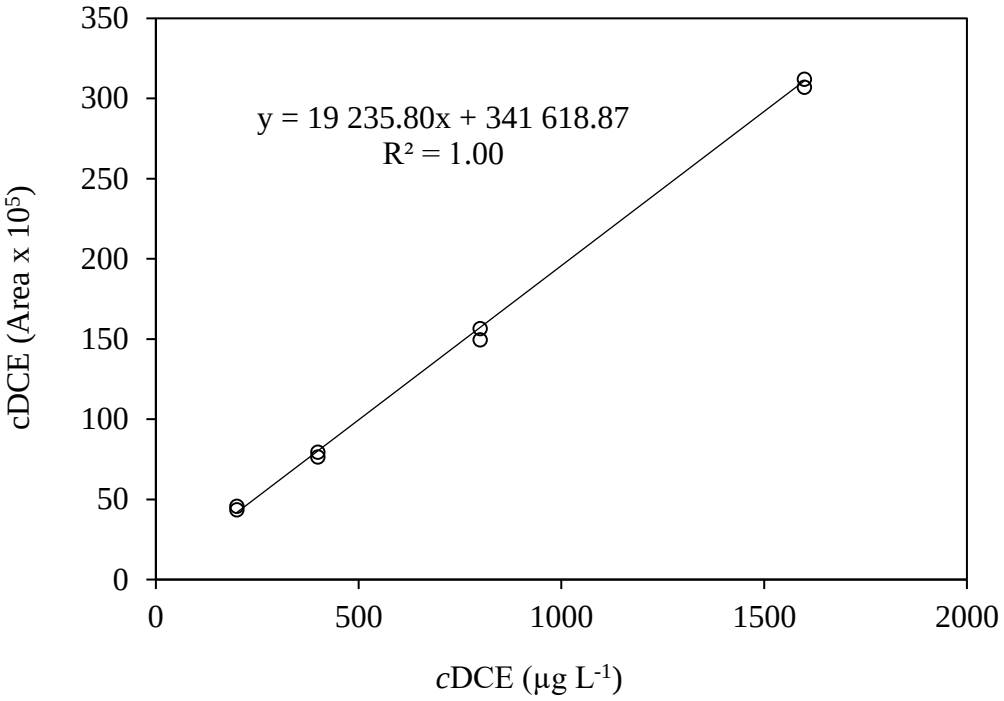

Fig. 3.1 Calibration curve of cDCE (concentration range from 200 µg L^{-1} to 1600 µg L^{-1})

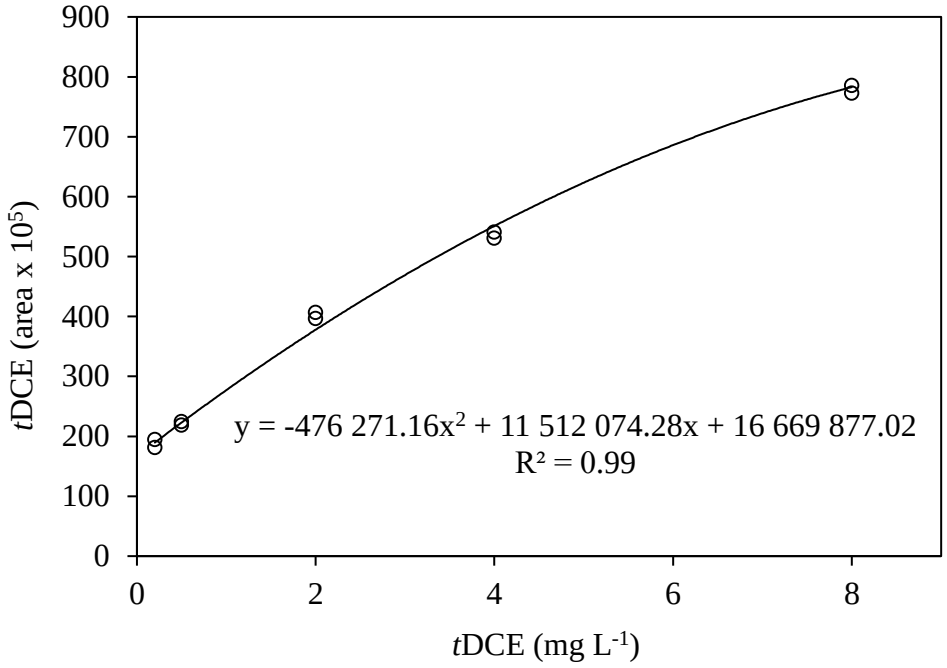

Fig. 3.2 Calibration curve of tDCE (concentration range from 200 µg L^{-1} to 8000 µg L^{-1})

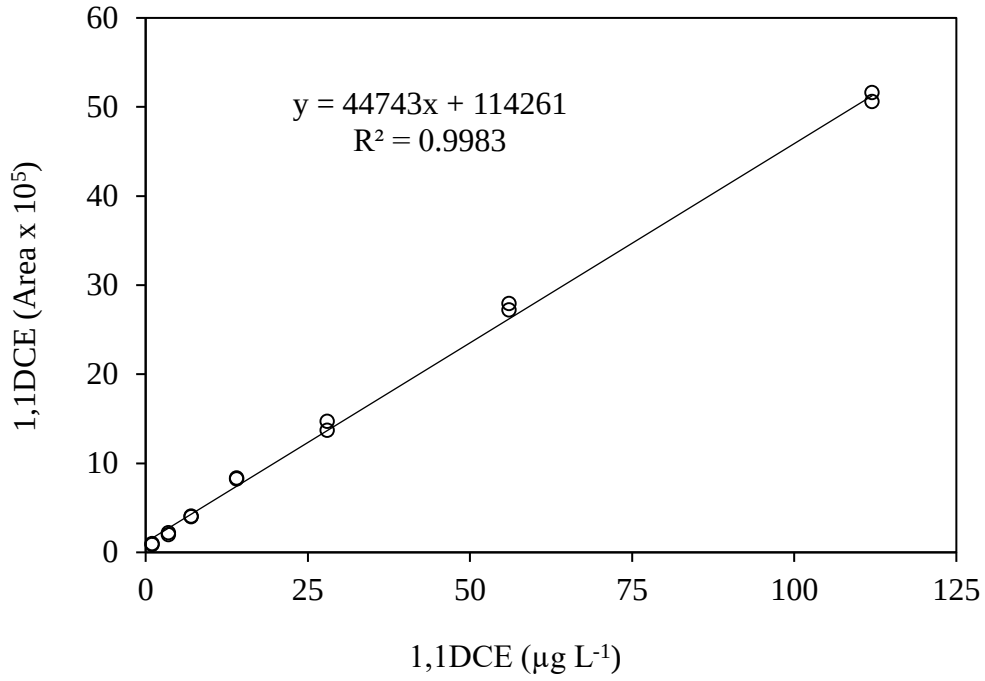

Fig. 3.3 Calibration curve of 1,1DCE (concentration range from 1 µg L^{-1} to 112 µg L^{-1})

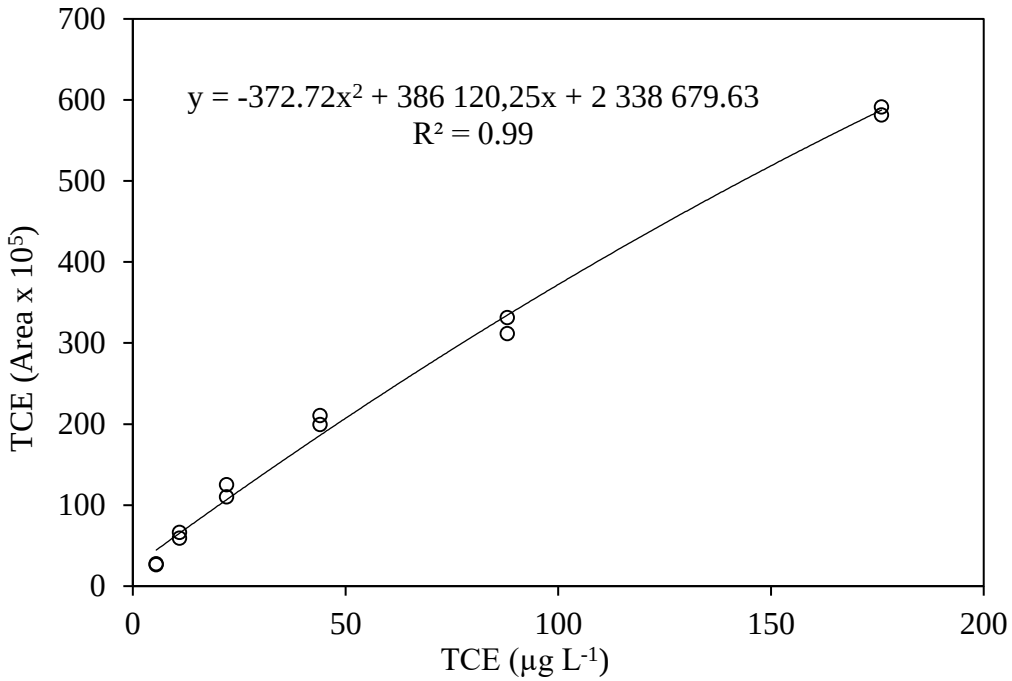

Fig. 3.4 Calibration curve of TCE (concentration range from 5.5 µg L^{-1} to 176 µg L^{-1})

Table 3.1 Equations and R^2 values of assembled calibration curves of CEs

Compound	Concentration range	Equation	R^2
*c*DCE	20 – 164 μg L^{-1}	y = 17671x + 13902	0.99
*c*DCE	0.2 – 1.6 mg L^{-1}	y = 18193x - 255263	0.99
*c*DCE	1.0 – 25 mg L^{-1}	y = -77 788.13x^2 + 4 536 730.68x + 25 368 920.07	0.90
*t*DCE	20 – 187 μg L^{-1}	y = 20219x + 120603	0.99
*t*DCE	0.2 – 8 mg L^{-1}	y = -476 271.16x2 + 11 512 074.28x + 16 669 877.02	0.99
1,1DCE	19 – 154 μg L^{-1}	y = -877.88x^2 + 323216x - 3000000	0.99
1,1DCE	0.5 – 8.0 mg L^{-1}	y = -942 630.89x2 + 21 884 344.40x + 33 377 207.75	1.00
TCE	2.5 – 154 μg L^{-1}	y = -53943.67x^2 + 4062922.24x	0.98
VC	0.2 – 1.6 mg L^{-1}	y = 403.64x + 37511	0.99

3.10 Analysis of samples containing vinyl chloride

Samples containing VC were prepared and cultivated identically as described in section 3.7. The analysis of samples was carried out at the Institute of Chemistry at the Tomas Bata University in Zlín, and the procedure was as follows. The samples were taken from the incubation room at different times and subsequently enriched with 10 μL of propane-2-ol (IPA) using a gas-tight syringe (HAMILTON); IPA was used as an internal standard. Then, each sample was incubated for 2 hours at room temperature before 250 μL of a gas phase of each sample was taken and injected into GC with a split ratio of 1/100.

Samples analysis was performed by using GC-MS Shimadzu QP-2010 equipped with the Equity-1 (30 m, 0.32 mm, 1μm) column, and He as carrier gas at constant linear velocity (58.8 $cm \cdot s^{-1}$) was used; GC method: 40 °C/10 min, 20 °C/min to 250 °C, hold for 14.5 min, IS 200 °C/70 eV; MS method: acquisition was started at 0.41 min and two mass-selected ions were detected (SIM mode). As shown in Fig. 3.5, ion m/z 62 and 45 were selected as characteristic ions for VC and IPA, respectively. This approach enabled unambiguous observation of two peaks with no interference with other compounds, which were present in the sample (other chloroethenes and degradation intermediates). The SIM mode was stopped at 4.99 min and full-scan mode was applied for the residual time of the analysis (m/z 40–450).

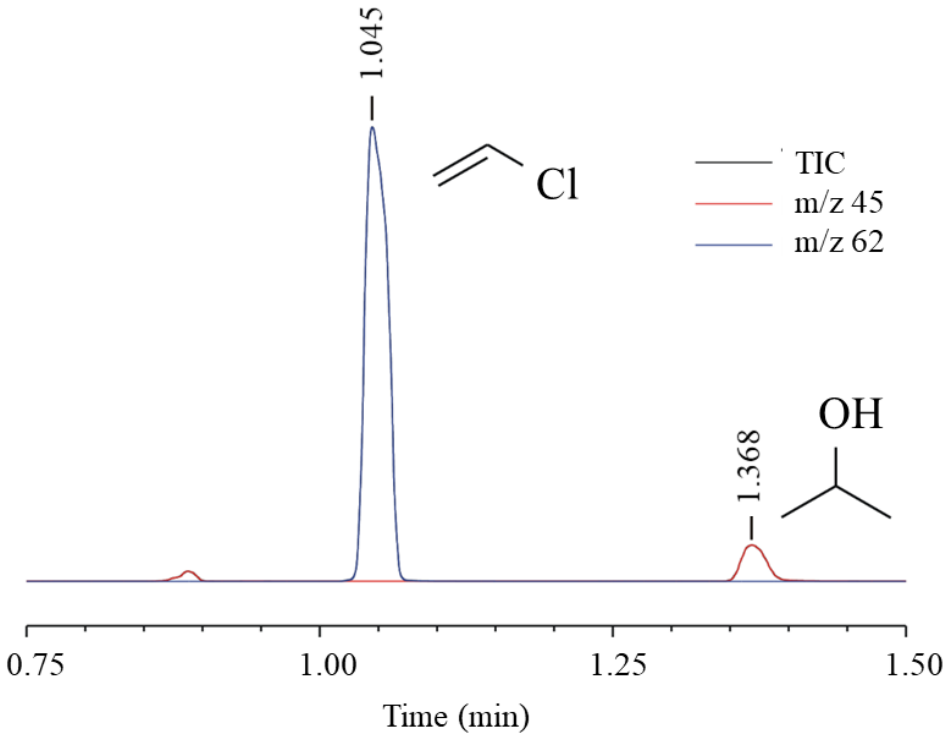

Fig. 3.5 Characteristic ions and retention times for VC and IPA on a portion of the typical chromatogram[7]

3.11 Cell survival after dichloroethene degradation

The experimental procedure was identical to the degradation assays described above. Samples were prepared in duplicates for each dichloroethene tested, and after seven days of incubation, the liquid phases of the samples under investigation were diluted in physiological saline and subsequently inoculated on Tryptone Yeast Extract Agar plates. Colony-forming units (CFUs) were counted after two days of incubation at 25 ºC and compared with the CFUs determined in parallel samples without the given dichloroethene(s).

3.12 Determination of intermediates of chloroethene degradation

Two different methods were applied to determine the intermediates of *c*DCE and *t*DCE transformations. All samples were measured after 4 days of degradation, which was characterized by the high values of peak areas of both intermediates detected by GC-ECD.

[7] TIC line is a sum of the two mass-selected lines.

Solid phase microextraction-GC-MS

Degradation samples were prepared as described in section 3.7 and sent to the Institute for testing and certification (ITC) in Zlín, Czech Republic, for the determination of intermediates produced during cometabolic degradation of *c*DCE and *t*DCE in *C. testosteroni* RF2.

The degradation mixtures (2 mL) were transferred into 20 mL crimp vials, along with one teaspoon of sodium chloride. The samples were subjected to headspace analysis. Each vial was incubated at 70 ºC, with sorption of volatile compounds taking place on CAR-PDMS df 85 mm for 20 min. The substances were thermally released (235 ºC) and analysed by GC, the device having been equipped with an Rtx 502.2 column, at 105 m, a Splitless Injection Mechanism and MS detector (detection mass range m/z 40-200). The final identification of the intermediates was performed by comparing the mass spectra for the released substances with the mass spectra for substances in the library via GC MS Solution software (Shimadzu).

Purge and Trap-GC-MS

Degradation samples were prepared as described in section 3.7 and sent to the Regional Hygiene Station of the Olomouc Region based in Olomouc, Czech Republic, for the determination of intermediates produced during cometabolic degradation of *c*DCE and *t*DCE in *C. testosteroni* RF2.

The degradation mixture (10 mL) was extracted by the Purge and Trap method, in a Teledyne Tekmar Dohrmann Aquatek 70 Liquid Autosampler, and analysed by GC; the latter involving a VOCOL™ column, with the specifications 60 m, 0.32 mm ID, 1.8 mm film, and an MS detector.

3.13 Determination of chloride release

Conditions during dichloroethene degradation resembled those in the degradation assays described above; with the exception of MSM that was replaced by the chloride-free mineral medium. After a certain period of degradation, the liquid phases of the samples were analysed for the presence of inorganic chlorides. Prior the determination of chloride concentration in the samples according to the Iwasaki method (Iwasaki et al., 1952), bacterial cells were removed by filtration through a 0.22 μm pore-sized syringe filter (Millex). Also, blank tests without the appropriate dichloroethene as well as tests without cells and with the dichloroethene were performed in parallel; obtained results were subtracted.

Determination of inorganic chlorides according to Iwasaki (1952)

A filtrate of each degradation sample (2 ml) was transferred into a test tube using automatic pipette equipped with replaceable tips. Then, the filtrates were enriched with 0.2 mL of Mercury(II) thiocyanate solution (3 g of $Hg(SCN)_2$ in 1 L of 95% ethanol) and 1 mL of Ammonium iron (III) sulfate solution

(Lachema), which was prepared by dissolving 13.38 g of $H_4NFeO_8S_2.12\ H_2O$ in 96 ml of distilled water and 58 ml of 65% HNO_3. Then the filtrates were stirred and allowed to stand for approximately 10 minutes, and subsequently transferred into cuvettes prior their colour intensity was measured at 460 nm using spectrophotometer (Helios E). The chloride content was calculated from the equation of the assembled calibration curve shown below in Fig. 3.6. The curve was obtained by processing a series of standard solutions containing 0.5; 1.0; 2.0; 3.0; 4.0; 5.0; 8.0 and 10.0 mg L^{-1} of Cl^-, which were prepared by the dilution of a standard solution of NaCl (32.97 mg L^{-1}) that is equal to 20 mg L^{-1} of Cl^-. All standard solutions of Cl^- were processed identically as described above for the degradation samples.

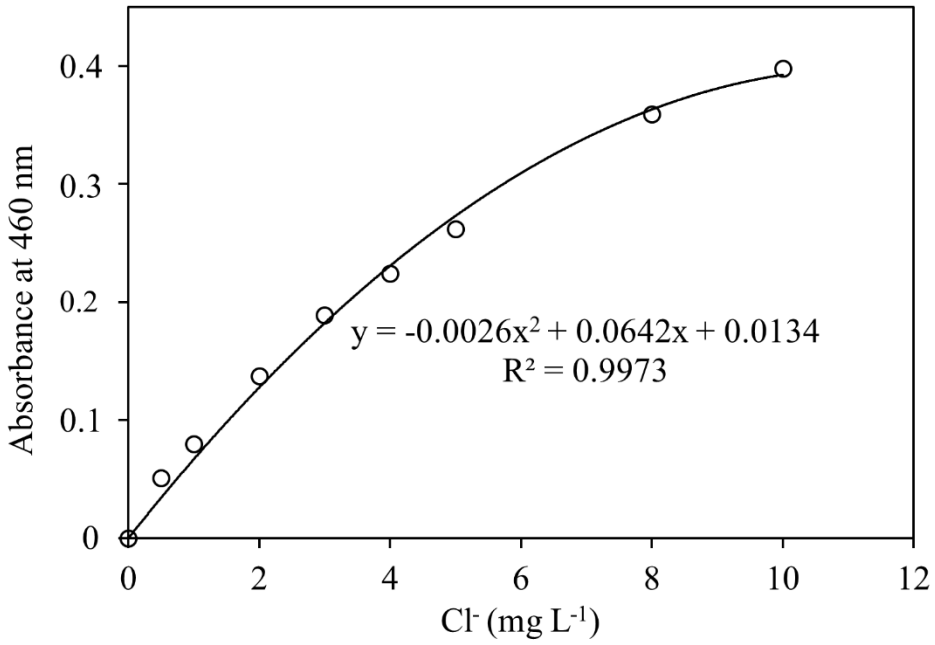

Fig. 3.6 Calibration curve for determination of inorganic Cl- in degradation samples containing chloroethenes[8]

[8] The data shown in the Fig. 3.6 are average values of three measurements

4. RESULTS AND DISCUSSIONS

4.1 Use of two substrates for aerobic cometabolic degradation of *c*DCE

Results of previous studies showed that resting cells of RF2 grown on phenol (200 mg L^{-1}) were able to cometabolically degrade TCE (1 - 1.5 mg L^{-1}; 60 - 90 %) until monooxygenase was actively involved in the process (approximately first 24 hours) (Ruzicka, 2004; Ruzicka et al., 2011). The obtained results served as a reason for further research on the ability of RF2 to cometabolically degrade other chloroethenes, particularly dichloroethenes (DCEs). However, the previously mentioned results showed that the resting cells could degrade only relatively low concentrations of TCE (up to 1.5 mg L^{-1}) within a limited time, whilst in the state of growing cells, the bacterium was able to partially degrade substantially higher TCE concentrations (up to 10 mg L^{-1}) (Ruzicka, 2004) Therefore, in terms to improve the degradation ability of RF2 towards chloroethenes as well as to prolong the degradation time, a combination of phenol and sodium lactate (each 100 mg L^{-1}), was tested in a preliminary assay for the aerobic cometabolic degradation of *c*DCE, which is the most prevailing isomer of all DCEs in a subsurface polluted by PCE and TCE. The course of *c*DCE degradation under such conditions was compared with the process in which phenol was the only carbon source. The entire assay had been carried out under growing conditions for 9 days, and the obtained data are shown in Fig. 4.1 that is supported by Table 4.1.

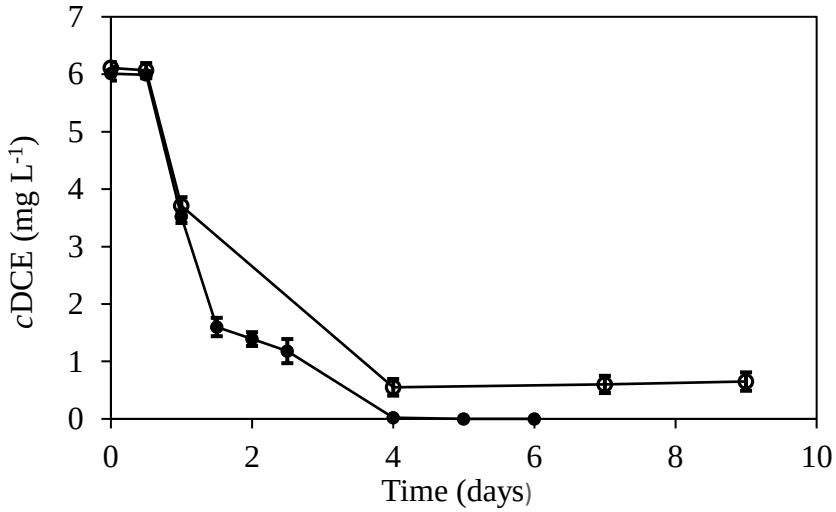

Fig. 4.1 Course over time of cDCE degradation under the use of different substrates[9]

[9] (●) phenol and sodium lactate; (o) phenol

Table 4.1 The influence of the addition of lactate sodium on removal efficiency of *c*DCE by *C. testosteroni* (means ± SD)[10]

Time (days)	Series of samples A *c*DCE (mg L^{-1})	Series of samples B *c*DCE (mg L^{-1})
0	6.11 ± 0.10	6.01 ± 0.12
0.5	6.07 ± 0.13	5.99 ± 0.06
1	3.71 ± 0.15	3.52 ± 0.11
1.5	N	1.60 ± 0.16
2	N	1.39 ± 0.12
2.5	N	1.18 ± 0.21
4	0.53 ± 0.14	0.02 ± 0.01
5	N	0.00
6	N	0.00
7	0.6 ± 0.15	N
9	0.65 ± 0.16	N

The use of both phenol and lactate proved useful to the *c*DCE degradation process, as a complete *c*DCE removal was observed in this case. In contrast to this result, incomplete *c*DCE elimination was observed when phenol served as the only substrate. Therein, the lack of lactate slightly slowed the rate of *c*DCE degradation in the course of the fourth day of the assay, and residual *c*DCE corresponding to 8.67 ± 2.30 % of its initial concentration remained non-degraded at the end of the assay. Hence, utilization of both phenol and lactate was applied throughout the whole thesis for sufficient multiplication of bacteria as well as induction of the desired enzyme production that sustained the degradation of *c*DCE until its complete removal on the fourth day. Finally, the use of lactate (100 mg L^{-1}) allowed to reduce the concentration of phenol from 200 mg L^{-1} to 100 mg L^{-1}, which might play a key role in approving its application for in situ bioremediation purposes. Despite the fact that phenolic compounds are abundant natural agents, commonly present in different environmental spheres, some contradictions against its use for bioremediation purposes might exist. However, this should not be of concern to the thesis herein, as RF2 can easily utilize phenol by up to 200 mg L^{-1}, in addition to which merely 100 mg L^{-1} of this primary substrate along with the same concentration of lactate sodium was applied in the degradation assays. Above all, unlike other commonly used primary substrates, such as isoprene (van Hylckama Vlieg et al., 1998), methane (Kim et al., 2008), oxylene (Li et al., 2014), propene (Ensign et al.,1992; Kim et al., 2008) or toluene (Azizian et al., 2007), using phenol for inducing catabolic enzymes has several

[10] Series of samples A contained only phenol (100 mg L^{-1}); series of samples B contained phenol (100 mg L^{-1}) and lactate (100 mg L^{-1}); *c*DCE concentrations are actual concentrations of the compound in a liquid phase at given times; N: not tested

advantages. It is a well soluble, non-volatile substance that is easily available for a variety of bacterial cells.

4.2 Cometabolic aerobic degradation of single DCEs

C. testosteroni RF2 was subjected to degradation assays of *c*DCE, *t*DCE and 1,1DCE separately. Each degradation test lasted for seven days, and at least two different concentrations of each isomer were applied. The data obtained on removing the DCEs and subtracted numbers of CFUs at the end of the chosen tests are given in Table 4.2.

Table 4.2 Cometabolic degradation of single DCEs by *Comamonas testosteroni* RF2 (means ± SD), including abiotic blanks[12]

CE	Initial concentration "all in liquid phase" (mg L^{-1})	Actual initial concentra-tion (mg L^{-1})	Compound removal (%)	Cell number (10^7 CFU mL^{-1})	
				DCE degradation	Blank test without dichloro-ethene
*c*DCE	1.93	1.72	100	11 ± 1.5	11 ± 2.5
*c*DCE	7.06	6.01	100	N	N
*c*DCE[a]	7.06	6.01	<0.5	N	N
*t*DCE	1.35	0.97	72.2 ± 3.0	8 ± 0.5	5 ± 0.5
*t*DCE	6.92	3.80	65.8 ± 1.0	N	N
*t*DCE[a]	6.92	3.80	<0.5	N	N
1,1DCE	0.91	0.25	100	N	N
1,1DCE	1.33	0.37	65.5 ± 7.9	3 ± 1.0	28 ± 1.0
1,1DCE	6.91	1.77	1.6 ± 0.9	0.001	28 ± 1.0
1,1DCE[a]	6.91	1.77	1.6 ± 0.9	N	N

The results listed in Table 4.2 showed that the strain is able to degrade all three DCE isomers with prominent activity towards *c*DCE, as the entire 6.01 mg L^{-1} of this compound was removed at the end of the assay. In comparison to *c*DCE degradation, efficiencies for removing 1,1DCE and *tDCE* were significantly lower, yet still interesting for potential strain utilization in bioremediation processes. Despite *t*DCE did not influence RF2 growth, this compound was not

[12] [a] Abiotic blank (without cells); N: not tested; * Initial concentration "all in a liquid phase": calculated and injected dosage of chloroethenes into test vials; Actual initial concentration: measured concentrations of chloroethenes in a liquid phase after equilibrium partitioning using GC-ECD system

fully degraded in any of the assays. Therefore, such incomplete degradation of *t*DCE could be explained by deficient molecular "lock and key" conformation between the molecules of phenol-2-monooxygenase of the strain RF2 and *t*DCE, rather than by the toxic effects of *t*DCE itself or its degradation intermediates. In contrast, 1,1DCE appeared to be the only dichloroethene with a toxic effect against RF2 cells under higher tested concentrations (Table 4.2). Indeed, 1,1DCE turned out to be the most troublesome isomer, as only the low concentration of 0.25 mg L^{-1} was degraded completely and just partial removal of 0.37 mg L^{-1} was observed. Furthermore, no or almost negligible removal was found when 1.77 mg L^{-1} was applied. All the acquired results for DCE degradation were well supported by counting the cells at the end of the processes; the results are listed in Table 4.2. While *c*DCE and *t*DCE degradation led to the same or slightly reduced cell counts as the blank tests without *c*DCE and *t*DCE enrichments, samples with higher 1,1DCE concentrations showed significantly reduced numbers of living cells. This reduction was especially dramatic if 1.77 mg L^{-1} of 1,1DCE was applied; under such a condition, bacterial growth was completely inhibited. The results of 1,1DCE degradation (1.77 mg L^{-1}) highlighted a key importance of RF2 growing cells for the degradation and this fact was fostered by abiotic blanks in which no or negligible reductions in DCEs concentrations were observed.

Amongst others, during the test series of *c*DCE and *t*DCE degradations, a concomitant phenomenon was observed in all assays when two unknown peaks were detected in all GC-ECD chromatograms, including the case of complete *c*DCE removal; however, neither of these peaks appeared after degradation of 1,1DCE. The peaks were believed to be degradation intermediates of both isomers and were monitored in a further part of the thesis (refer to section 4.5).

4.3 Cometabolic degradation of a quaternary mixture containing TCE and all three DCEs

The RF2 strain was examined for its ability to degrade all DCEs along with TCE in a prepared mixture that included 1,1DCE, *c*DCE, *t*DCE and TCE (51.4 µg L^{-1}; 144.3 µg L^{-1}; 91.6 µg L^{-1}; 122.5 µg L^{-1}, respectively). The composition of the mixture and the chloroethene concentrations were chosen to resemble groundwater pollution at a site affected by chlorinated ethenes. The results for removing the chloroethenes after seven days of the assay are given in Table 4.3

Table 4.3 Cometabolic degradation of a quaternary mixture of chloroethenes by *Comamonas testosteroni* RF2 (means ± SD)[13]

Compound	Initial concentration "all in a liquid phase" (μg L^{-1}) *	Actual initial concentration (μg L^{-1}) *	Compound removal (%)
TCE	154.2	122.5 ±1.70	100
*c*DCE	170.7	144.3 ± 3.46	100
*t*DCE	151.4	91.6 ± 5.86	79.0 ± 3.7
1,1DCE	158.6	51.4 ± 4.21	100

When all the chloroethenes were used at concentrations corresponding to approx. 150 μg L^{-1} "all in liquid phase", *C. testosteroni* RF2 was able to completely remove 1,1DCE, *c*DCE and TCE and a significant portion of *t*DCE (79.0 ± 3.7 %). The results obtained can be considered valuable proof that RF2 bacterium is able to degrade not only single DCEs but also all three isomers along with TCE in the given mixture of CEs. Furthermore, no abiotic reductions in TCE and DCEs concentrations were observed during the assay (data not shown).

Comparing the results of the present study with findings from previous lab-scale studies, *Xanthobacter* cells (strain Py2) partially degraded 24.24 mg L^{-1} of *c*DCE, *t*DCE and 1,1DCE (Ensign et al., 1992). After 30 min of incubation, the resting cells of this bacterium removed 84% of *t*DCE and 69% of *c*DCE, but only 6% of 1,1DCE. This reveals the highly favourable potential of Py2 to degrade high concentrations of both *c*DCE and *t*DCE. Nevertheless, utilizing resting cells does not reflect actual conditions that exist during groundwater bioremediation, under which bacteria have to multiply and produce an appropriate enzyme for degrading chloroethenes. Similarly, the resting cells of *Mycobacterium aurum* L1 that were tested for their ability to degrade all the DCEs may also remove 1,1DCE less efficiently (Hartmans and De-Bont, 1992). In another study, *Methylosinus trichosporium* OB3b, grown under batch conditions, degraded all DCEs and demonstrated its highest transformation capacity for tDCE (8.0 mmol mg^{-1}), yet Tc for *c*DCE - and particularly 1,1DCE - were significantly lower (2.6 and 0.36 mmol mg^{-1}, resp.) (Chang and Alvarez-Cohen, 1996).

In addition to the lab-scale studies, interesting results were observed by Hopkins and McCarty (1995) during a field study in which phenol was applied as the primary substrate to initiate cometabolic degradation of chloroethenes by an unknown consortium at a site contaminated with a mixture of chloroethenes. This consortium degraded TCE (250 μg L^{-1}) and *c*DCE (125 μg L^{-1}) with removal efficiencies greater than 90%, as well as 74% of *t*DCE (125 μg L^{-1}). However,

[13] * Initial concentration "all in a liquid phase": calculated and injected dosage of chloroethenes into test vials; Actual initial concentration: measured concentrations of chloroethenes in a liquid phase after equilibrium partitioning using GC-ECD system

only 50% of 1,1DCE (65 μg L^{-1}) was degraded. The results of the aforementioned study are similar to those obtained in the present thesis, although herein RF2 was seen to completely degrade 51.4 μg L^{-1} of 1,1DCE in the given mixture of chloroethenes (Table 4.3). Furthermore, in the assays containing single dichloroethenes, RF2 degraded much higher concentrations of DCEs than observed in the field study referenced with the unknown consortium.

4.4 Chloride production during *c*DCE degradation

Chloride release after degradation of chloroethenes is an important indicator of DCE dechlorination. *c*DCE was chosen for this assay as it is one of the most significant pollutants occurring in groundwater. Moreover, this compound was readily degraded by RF2 in previous experiments. In order to investigate the dechlorination of potentially formed chlorinated intermediates, the assay lasted 22 days and obtained data are listed in Table 4.4. Also, the course of the assay over time is shown in Fig. 4.2.

Table 4.4 Production of chlorides during *c*DCE degradation in RF2 (means ± SD)[14]

Time (days)	*c*DCE (mg L^{-1})	*c*DCE removal (%)	Cl^- absorbance*	Cl^- production (mg L^{-1})	Cl^- production (%)
0	6.04 ± 0.08	-	0.00	0.00	0.00
0.5	6.08 ± 0.11	0.00	0.00	0.00	0.00
1	3.62 ± 0.23	39.80 ± 3.81	0.00	0.00	0.00
4	0.53 ± 0.13	91.18 ± 2.15	0.065 ± 0.003	0.83 ± 0.04	18.78 ± 0.90
7	0.00	100	0.163 ± 0.008	2.60 ± 0.70	58.82 ± 15.8
9	0.00	100	0.216 ± 0.004	3.72 ± 0.09	84.16 ± 2.04
22	0.00	100	0.232 ± 0.004	4.08 ± 0.10	92.31 ± 2.26

[14] *c*DCE concentrations are actual concentrations of the compound in a liquid phase at given times; * Listed data for Cl^- absorbance were obtained by deduction of abiotic blanks (samples without a dosage of *c*DCE)

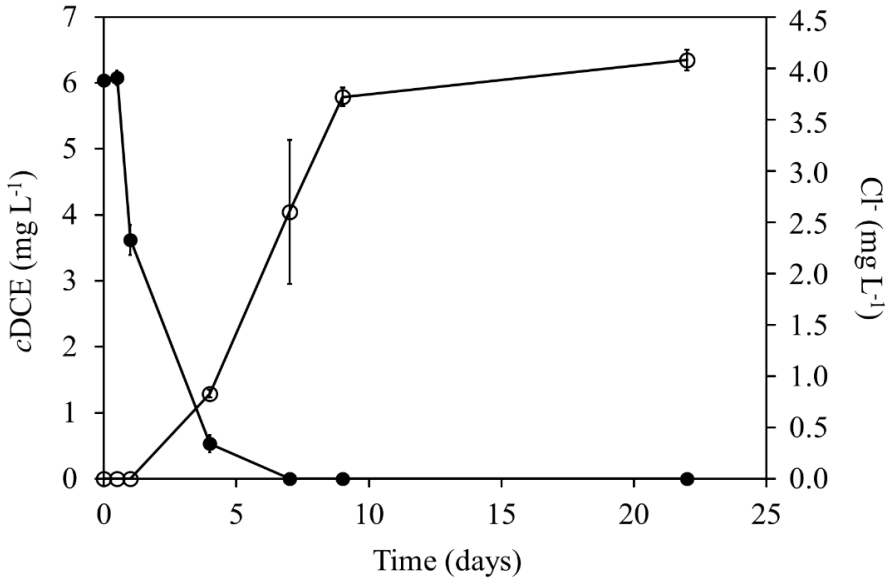

Fig. 4.2 Release of inorganic chlorides during cDCE degradation[15]

Generally, a decrease in *c*DCE concentration was clearly accompanied by the production of chlorides. However, although *c*DCE was completely removed in 7 days, a further increase in chloride concentration occurred in the next few days. Based on these results, the formation of the two unstable chlorinated intermediates mentioned in section 4.2 is the most probable explanation for the chloride release observed in the later stage of the experiment. Finally, over 92 % mineralization of the chlorine originally bound in *c*DCE was discerned after 22 days of the assay (Fig. 4.2).

4.5 DCEs degradation kinetics and metabolites monitoring

So as to monitor the formation of *c*DCE and *t*DCE intermediates, further assays on the degradation kinetics of these DCEs were performed. Monitoring the *c*DCE and *t*DCE intermediates lasted until no intermediate was detected by GC-ECD. The resultant unknown intermediates were named I1 and I2 respectively; their formation and dissipation along with *c*DCE and *t*DCE degradation are given in Fig. 4.3. Furthermore, the percentage removals of *c*DCE and *t*DCE over time are listed in Table 4.5.

[15] *(●) cDCE; (o) inorganic chlorides (abiotic blanks subtracted)*

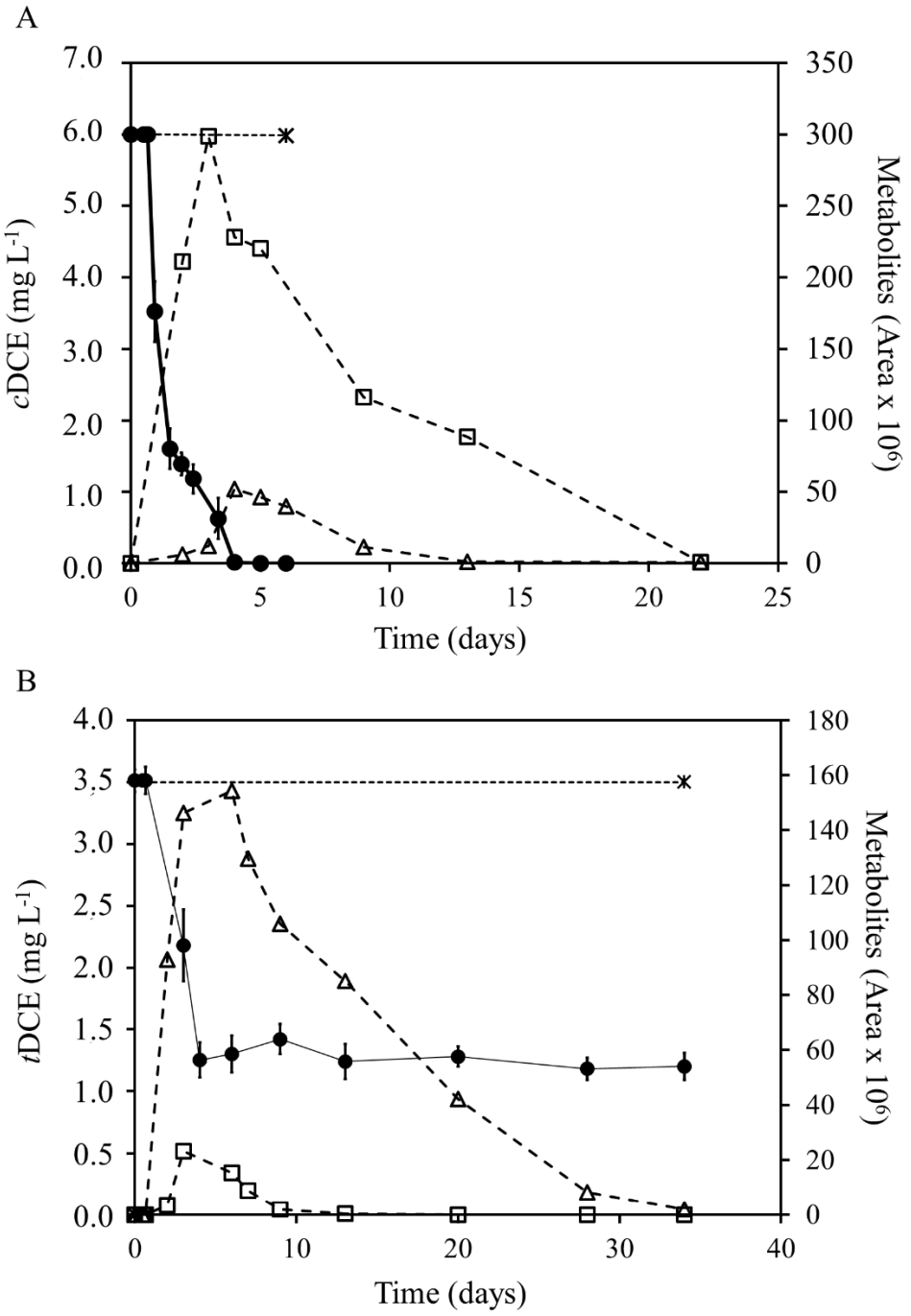

Fig. 4.3 cDCE (A) and tDCE (B) degradation kinetics and formation of intermediates[16]

Fig. 4.3 shows that during individual degradation of both *c*DCE and *t*DCE, certain amounts of the two unknown intermediates (I1 and I2) transiently accumulated during the first few days of the assays but subsequently disappeared later. In the course of *c*DCE degradation, I1 had a major response on GC-ECD,

[16] *(●) cDCE and tDCE; (□) intermediate I1; (Δ) intermediate I2; both intermediates are expressed as peak areas; (x) abiotic tests.*

followed by a considerably reduced response of I2, whereas degradation of *t*DCE resulted in exactly the reverse formation of peaks, with the main response for I2 and a significantly lesser response for I1.

Besides, the removal of both DCEs was most efficient between the first and fourth day of the assays when practically whole *c*DCE (99.67 ± 0.17 %) and 64.18 ± 4.01 % of *t*DCE were removed as seen in Table 4.5.

Table 4.5 Percentage removal of *c*DCE and *t*DCE over time (means ± SD)[17]

Time (days)	*c*DCE (mg L^{-1})	*c*DCE removal (%)	*t*DCE (mg L^{-1})	*t*DCE removal (%)
0	6.01 ± 0.03	-	3.49 ± 0.09	-
0.5	6.03 ± 0.09	0.00	3.51 ± 0.01	0.00
0.7	5.98 ± 0.05	0.09 ± 1.50	3.49 ± 0.11	0.00
1	3.52 ± 0.42	41.34 ± 0.70	N	-
1.5	1.60 ± 0.28	73.34 ± 4.70	N	-
2	1.39 ± 0.16	76.84 ± 2.67	N	-
2.5	1.18 ± 0.20	80.34 ± 3.34	N	-
3	N	-	2.18 ± 0.29	37.54 ± 8.31
3.4	0.63 ± 0.29	89.50 ± 4.83	N	-
4	0.02 ± 0.01	99.67 ± 0.17	1.25 ± 0.14	64.18 ± 4.01
5	0.00	100	N	-
6	0.00	100	1.30 ± 0.15	62.75 ± 4.30
9	N	-	1.42 ± 0.12	59.31 ± 3.44
13	N	-	1.24 ± 0.14	64.47 ± 4.01
20	N	-	1.28 ± 0.08	63.32 ± 2.29
28	N	-	1.18 ± 0.09	66.19 ± 2.58
34	N	-	1.20 ± 0.11	65.62 ± 3.15

Moreover, interestingly, whereas degradation of *c*DCE and *t*DCE resulted in the formation of the two intermediates, degradation of 1,1DCE (under the identical conditions) did not result in any of the two intermediates produced during degradation of *c*DCE and *t*DCE even though 1,1DCE was completely degraded as shown in Table 4.6 and Fig. 4.4.

[17] Listed *c*DCE and *t*DCE concentrations are actual concentrations of the compounds in a liquid phase at given times; N: not tested

Table 4.6 Percentage removal of 1,1DCE over time (means ± SD)[18]

Time (days)	1,1DCE (μg L^{-1})	1,1DCE removal (%)
0	256 ± 7.00	-
0.5	249 ± 3.00	2.73 ± 1.17
1	255 ± 1.53	0.39 ± 0.60
2	92.84 ± 16.1	63.37 ± 6.29
3	55.65 ± 14.4	78.26 ± 5.63
4	49.72 ±11.2	80.58 ± 4.38
5	0.38 ± 0.11	99.85 ± 0.04
6	0.00	100
7	0.00	100

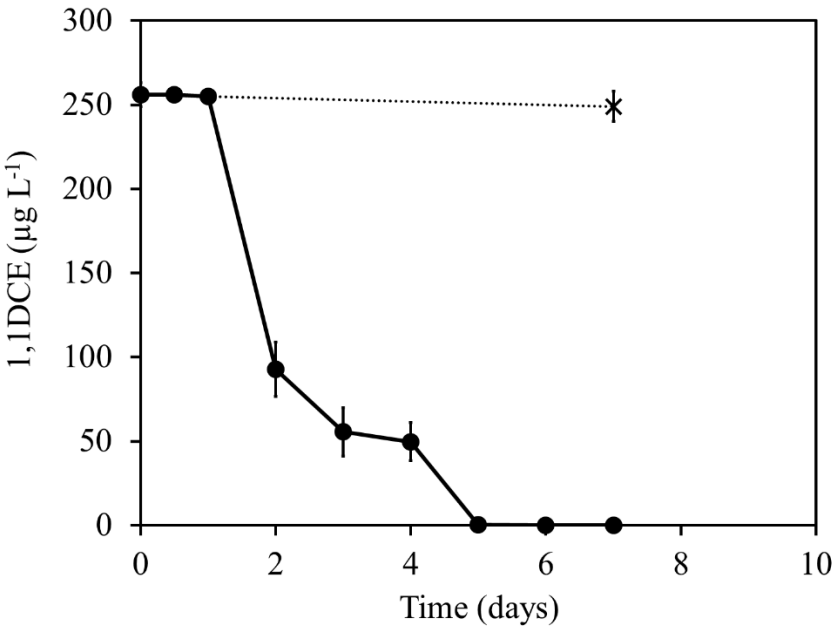

Fig. 4.4 Degradation kinetics of 1,1DCE[19]

Regarding the degradation kinetics of single DCEs, RF2 was able to efficiently degrade 1,1DCE (256 ± 7.00 μg L^{-1}), cDCE (6.01 ± 0.03 mg L^{-1}) and tDCE (3.49 ± 0.09 mg L^{-1}) during the first 5 days of the assays, after approximately one-day lasting lag phase. Based on the results obtained from the degradation kinetics of *t*DCE, it can be concluded that under the given conditions RF2 is unable to perform the degradation process longer than approximately 4 - 5 days, due to

[18] 1,1DCE concentrations are actual concentrations of the compound in a liquid phase at given times

[19] *(●) 1,1DCE; (x) abiotic tests.*

probable consumption of enzyme inducer or/and a lack of oxygen or/and NADH consumed during the first 4 - 5 days of the process.

4.6 Determination of *c*DCE and *t*DCE intermediates in the RF2 strain

A great effort was made to identify the I1 and I2 intermediates. To this end, I1, which showed a major peak in GC-ECD chromatograms during *c*DCE degradation, was detected by both SPME-GC-MS and Purge and Trap-GC-MS as 2,2-dichloroacetaldehyde; however, I2 was not detected by these methods. Interestingly, I2 displayed a significantly greater response on GC-ECD if the desorption temperature was reduced from 250 ºC to 100 ºC.

Furthermore, chloroacetyl chloride was taken into account as a potential candidate for the intermediate I2 (Benson, 2003) and several GC-ECD trials were performed; however, retention times for chloroacetyl chloride and the intermediate I2 significantly differed (Table 4.7).

Table 4.7 Retention times for tested chlorinated compounds and intermediates detected in GC-ECD chromatograms

Detected compound	Retention time (minutes)
1,1DCE	2.665
*t*DCE	3.465
*c*DCE	4.911
Unknown intermediate (I2)	6.969
2,2-dichloroacetaldehyde (I1)	14.67
Chloroacetyl chloride	16.32

Hence, unfortunately, I2 was not identified by any of the described methods in this thesis; a similar issue with an unknown intermediate produced during degradation of *c*DCE in *Polaromonas* sp. strain JS666, which grows on *c*DCE as the sole carbon and energy source under aerobic conditions, was observed by Nishino et al. (2013). The reason for testing 2-chloroacetyl chloride as a possible candidate for I2 came from the fact that this compound, along with 2,2-dichloroacetaldehyde and DCE-epoxide, was described as one of the three primary intermediates of 1,1DCE degradation in mice and rat tissues by monooxygenase action (Benson, 2003).

Among others, 2-chloroacetyl chloride was not found in any case of DCEs degradation by RF2, i.e. no peak in the retention time of the compound (16.32 minutes) was observed on GC-ECD chromatograms. Indeed, considering the possible reactions for the initial steps of *c*DCE degradation controlled by monooxygenase would mean that various amounts of an aldehyde, such as 2,2-Dichloroacetaldehyde and *c*DCE epoxide, could be produced (Nishino et al.,

2013); the proposed pathways for *c*DCE transformations in RF2 strain are described in Fig. 4.5.

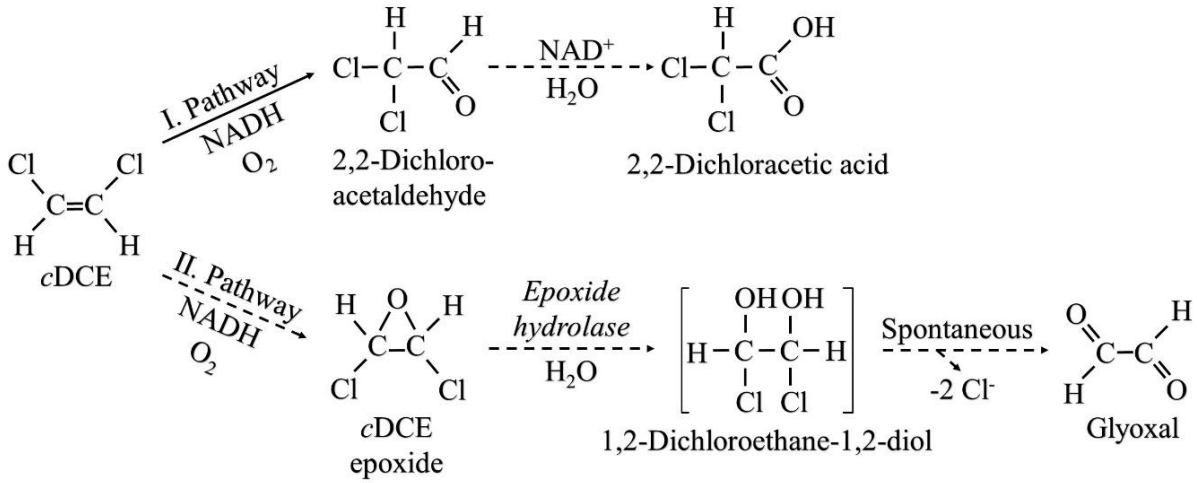

Fig. 4.5 Potential degradation pathways for cDCE degradation in RF2[20]

Consequently, it can be hypothesized that I2 might be 1,2-dichloroethane-1,2-diol, which was described as a product of hydrolysis of the *c*DCE epoxide (Nishino et al., 2013). However, this could not be clearly proven due to the fact that the compound is not commercially available, hence was not tested in this study. On the basis of the results obtained, it is not possible to state which of the two pathways, as described in Fig. 4.5, prevails during *c*DCE degradation. This is because the responses of both intermediates in the ECD chromatograms are significantly influenced by their molecular structure. Nevertheless, the previously reported 92.31 ± 2.26 % release of chlorides clearly demonstrates the high level of desired mineralization of organically bound chlorine.

Further, it may be supposed that *t*DCE degradation pathways proceed similarly as in the case of *c*DCE, except the *t*DCE epoxide formation instead of the *c*DCE epoxide in the II. pathway as shown in Fig. 4.6.

[20] *Solid lines: degradation steps based on the results of the present study; Dashed lines: degradation steps proposed by Nishino et al. (2013)*

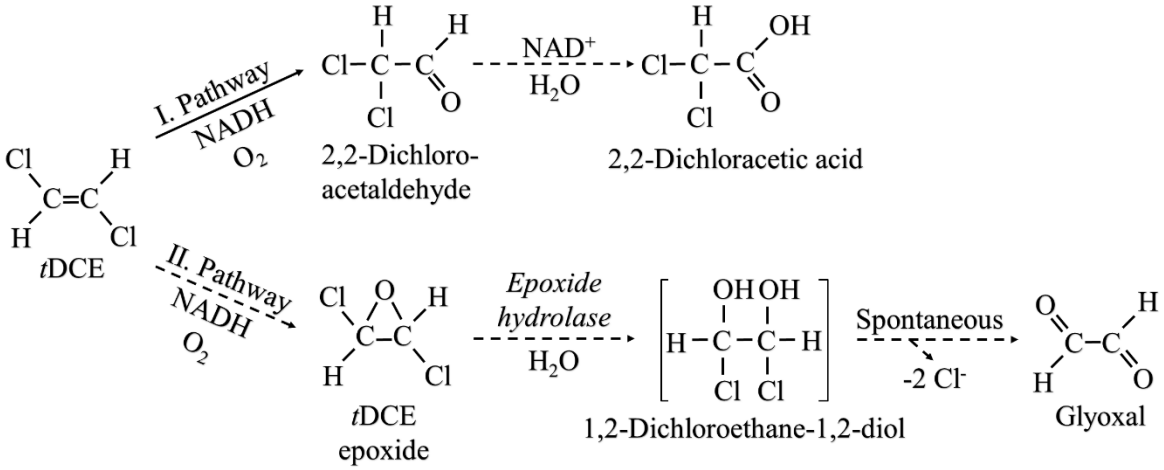

Fig. 4.6 Potential degradation pathways for tDCE degradation in RF2[21]

However, even though the removal of *t*DCE (6.92 mg L^{-1}; 65.8 ± 1.0 %) was lower than the removal of *c*DCE (7.06 mg L^{-1}; 100 %) under same conditions, its transformation resulted in the more significant production of the unknown intermediate (I2) than in the case of *c*DCE transformation. Hence, it might be speculated that other degradation pathway, such as the proposed II. pathway in Fig. 4.6, is more preferred than the I. pathway with 2,2-dichloracetaldehyde as the main transformation intermediate. Therefore, it can be stated that a position of chlorine atoms in the DCE molecules not only plays a crucial role in the removal efficiency of the compounds but it may also be a key factor in determining a distribution of degradation pathways. Nevertheless, to the best knowledge of the author of the thesis, the exact determination of *t*DCE degradation pathways in aerobic bacterial strains has yet not been done by any study.

In contrast to these results, degradation kinetics of 1,1DCE (256 ± 7.00 μg L^{-1}) by RF2 did not result in the formation of any of the two intermediates seen in the case of *c*DCE and *t*DCE degradation, although 2,2-dichloracetaldehyde was previously reported as one of the primary intermediates of 1,1DCE degradation in mice and rat tissues by monooxygenase action (Benson, 2003); these primary intermediates are shown in Fig. 4.7.

[21] *Solid lines: degradation steps based on the results of the present study; Dashed lines: possible degradation steps derived from the work of Nishino et al. (2013)*

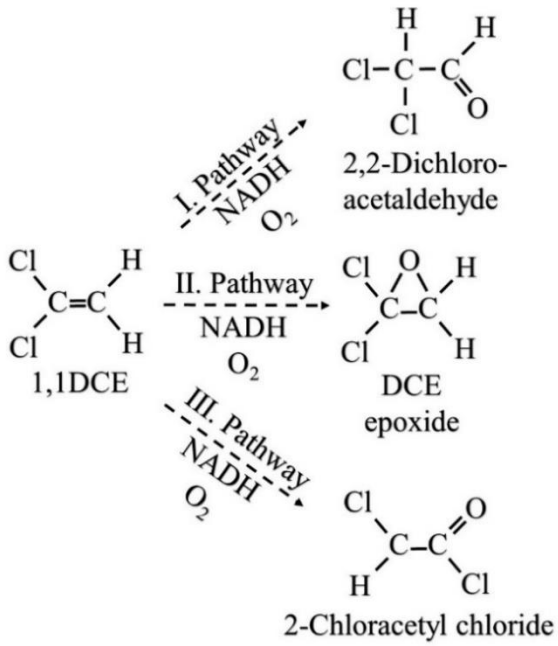

Fig. 4.7 Three primary intermediates of 1,1DCE in mice and rat tissues by monooxygenase action[22]

The results of 1,1DCE transformation in the strain RF2 showed that only the II. pathway is possible for the process, as neither 2,2-dichloroacetaldehyde nor 1,2-dichloroethane-1,2-diol were detected by any of the methods used in the thesis. Therefore, it is very probable that transformation process involves a formation of 1,1DCE epoxide, which was described as a compound with inhibitory influence on mice and rat cells (Forkert, 1999; Benson, 2003). Indeed, the toxic effect of 1,1DCE was observed in the present study when 1.77 mg L^{-1} of the compound was applied (Table 4.2). This observation was supported by an additional assay that showed complete inhibition of *c*DCE (6.01 mg L^{-1}) degradation in presence of 1,1DCE (1.82 mg L^{-1}) as shown in Fig. 4.8.

[22] *Dashed lines: the pathways described by Benson (2003)*

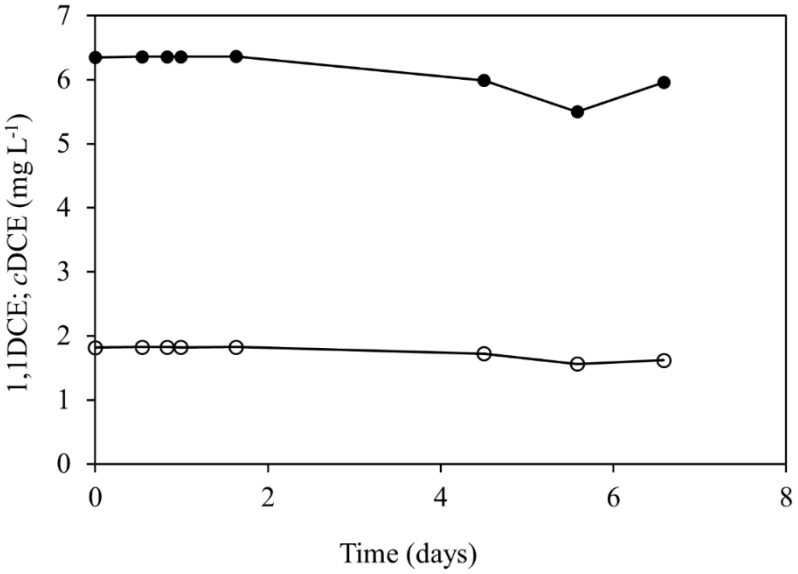

Fig. 4.8 Complete inhibition of cDCE degradation by 1,1DCE[23]

Also, several previous studies have investigated the toxicity of 1,1DCE. A paper that looked into DCE degradation by isoprene-grown cells of *Alcaligenes denitrificans* ssp. *xylosoxidans* JE 75 and *Rhodococcus erythropolis* JE 77 described that the 1,1DCE epoxide is a probable intermediate of 1,1DCE degradation, which is extremely reactive and decomposes under physiological conditions with a half-life time of 2 seconds (Ewers et al., 1990). Another study also concluded that the toxic nature of 1,1DCE was unlikely to be caused by the compound itself but by the formation of strongly reactive intermediate (believed to be a DCE epoxide) that covalently bind to microsomal proteins (Forkert, 1999). Despite the toxic nature of the DCE epoxide, previous findings have proven that some bacteria such as *Pseudomonas stutzeri* OX1, possessing the ToMO enzyme, can break down a certain proportion of this compound without any fatal effect on bacterial cells (Verce et al., 2001). Nevertheless, herein it was shown that 1,1DCE also exerted a toxic effect itself on RF2, which was neither able to grow at an elevated concentration of the same (1.77 mg L^{-1}, Table 4.2), nor to degrade *c*DCE (6.01 mg L^{-1}) when applying 1.82 mg L^{-1} of 1,1DCE as shown in Fig. 4.8. Thus, reasons may exist, other than the DCE epoxide alone, for the toxicity of 1,1DCE.

[23] *(●) cDCE; (o) 1,1DCE*

4.7 Cometabolic degradation of binary mixtures containing VC and *c*DCE

VC and *c*DCE are commonly formed at the edges of contamination plumes in groundwater originally polluted by PCE and TCE. Thus, bacterial strains capable of degrading both compounds are of interest for bioremediation purposes.

Several assays were performed in terms to evaluate the ability of RF2 to cometabolically degrade VC and *c*DCE in binary mixtures. While the applied concentration of *c*DCE (1.18 mg L^{-1}) was the same throughout all the assays, the concentration of VC (0.67 mg L^{-1}; 0.93 mg L^{-1}), as well as the duration of the assays, differed. Table 4.8 shows results obtained in the series of samples containing 0.67 mg L^{-1} of VC; the results of the series of samples containing 0.93 mg L^{-1} of VC were almost identical and therefore are not shown.

Table 4.8 Cometabolic degradation of *c*DCE and VC in a binary mixture by RF2 (means + SD)[24]

Time (days)	VC (mg L^{-1})	Abiotic blanks	VC removal (%)	*c*DCE (mg L^{-1})	*c*DCE removal (%)
0	0.67 ± 0.08	-	-	1.18 ± 0.6	-
7	0.71± 0.02	0.69 ± 0.01	0.00	0.00	100
51	0.65 ± 0.06	0.61 ± 0.04	2.99 ± 8.96	0.00	100
96	0.60 ± 0.03	0.63 ± 0.01	10.45 ± 4.48	0.00	100

Despite the variety of the assays, only negligible removal of VC was found, most probably due to abiotic leaks (Table 4.8). Nevertheless, none of the applied concentrations of VC in binary mixtures with cDCE (1.18 mg L^{-1}) resulted in neither an inhibitory nor a toxic effect against bacterial cells. Most importantly, it exerted no influence on *c*DCE degradation, which was completely removed. The inability of RF2 to cometabolically degrade VC might be caused by the lack of an appropriate enzyme capable of VC degradation. In contrast, many aerobic bacteria utilizing VC are commonly present in the environment. Some of them, such as *Pseudomonas aeruginosa* DL1, cometabolically degrade VC if grown on ethene (Verce et al., 2001), while other bacteria like *Mycobacterium aurum* L1 or *Pseudomonas aeruginosa* MF1 utilize VC as the sole carbon and energy source and cometabolize *c*DCE (Hartmans and De-Bont, 1992; Verce et al., 2002).

[24] *c*DCE and VC concentrations are actual concentrations of the compound in a liquid phase at given times

4.8 Utilization of different organic substrates by selected bacterial strains degrading VC

Given to the fact that the strain RF2 did not degrade any of the applied VC concentrations in the previous assays, further work was focused on a selection of a suitable bacterial consortium composed of the strain RF2 and a strain degrading VC. Ideally, such consortium would be able to degrade a mixture of TCE, all DCEs, and VC.

Firstly, *P. putida* DSM-7189, *R. ruber* DSM-7511, and *M. aurum* DSM-6695, the strains that have been reported to be able to degrade VC, were purchased from DSMZ and examined for their ability to utilize different organic substrates. Whereas *R. ruber* DSM-7511 and *P. putida* DSM-7189 were found to cometabolize VC in presence of benzoate sodium (Phelps et al., 1991) and 3-chloropropan-1-ol, respectively (Castro et al., 1992), *M. aurum* DSM-6695 was described to be able to utilize VC as its only source of organic carbon (Hartmans et al., 1985). Regarding the above mentioned, benzoate sodium and 3-chloropropan-1-ol were used in the tests to verify their suitability for the growth of the *R. ruber* DSM-7511 and *P. putida* DSM-7189, respectively. Further, in terms to find out a possible substrate competition between the two DSMZ strains and RF2, both compounds were used as growth substrates for RF2. Also, other possible substrate competition between the RF2 and the three DSMZ strains was examined by applying phenol, the primary substrate that initiates production of monooxygenase in RF2, as a growth substrate for all DSMZ strains. The tests were performed as described in methods and the results are listed in Table 4.9

Table 4.9 Growth of bacterial strains on different organic substrates –part 1

Strain	Organic substrate (growth substrate)			
	Benzoate sodium 200 mg L^{-1}	3-chloropropan-1-ol 50 mg L^{-1}	3-chloropropan-1-ol 200 mg L^{-1}	Phenol 200 mg L^{-1}
	Observed turbidity			
Comamonas testosteroni RF2	++	+	+	++
Rhodococcus ruber DSM-7511	++	N	-	++
Pseudomonas putida DSM-7189	N	++	++	+

Table 4.9 Growth of bacterial strains on different organic substrates – part 2[25]

Strain	Organic substrate (growth substrate)			
	Benzoate sodium 200 mg L^{-1}	3-chloropropan-1-ol 50 mg L^{-1}	3-chloropropan-1-ol 200 mg L^{-1}	Phenol 200 mg L^{-1}
	Observed turbidity			
Mycobacterium aurum DSM-6695	N	N	N	-

The results in Table 4.9 confirmed the suitability of benzoate sodium and 3-chloropropan-1-ol for the growth of *R. ruber* DSM-7511 and *P. putida* DSM-7189, respectively. Also, RF2 could utilize both compounds, particularly benzoate sodium was found as a suitable growth substrate. Moreover, interestingly, *R. ruber* DSM-7511 was able to efficiently utilize phenol although the previous study concluded that the strain cannot utilize this compound (Phelps et al., 1991). Finally, while *P. putida* DSM-7189 could only slightly utilize phenol, *M. aurum* DSM-6695 did not grow at all in presence of the compound.

4.9 *c*DCE and VC degradation kinetics by different bacterial consortia

Degradation kinetics of *c*DCE (6.09 mg L^{-1}) and VC (10 mg L^{-1}; "all in a liquid phase") in binary mixtures were examined by three consortia, each of which contained the strain RF2 and one of the DSMZ strains. Growth substrates for the consortia were selected based on the results of the previous tests and are listed in Table 4.10 along with consortia designations.

Table 4.10 Bacterial consortia used for degradation of *c*DCE and VC in a binary mixture

Consortium designation	Composition of a bacterial consortium	Used organic substrates
RF2-Rr	RF2 + R. ruber DSM-7511	phenol (200 mg L^{-1})
RF2-Pp	RF2 + P. putida DSM-7189	phenol (150 mg L^{-1}) + 3-chloropropan-1-ol (50 mg L^{-1})
RF2-Ma	RF2 + M. aurum DSM-6695	phenol (100 mg L^{-1}) + lactate (100 mg L^{-1})

[25] *(++) strongly positive; (+) slightly positive; (-) negative; N: not tested*

Unfortunately, due to the technical issue with GC-ECD, monitoring the loss of VC using the system appeared to be tricky and no VC removal could be confirmed as no peak in a retention time for VC appeared on chromatograms, including the initial concentrations of the compound (10 mg L^{-1}; "all in a liquid phase"). In contrast, degradation kinetics of *c*DCE (6.09 mg L^{-1}) was well monitored and the results obtained for each consortium are shown in Table 4.11 as well as in Fig. 4.9.

Table 4.11 Degradation kinetics and percentage removals of *c*DCE in a binary mixture with VC by different bacterial consortia (means ± SD)[26]

	RF2-Rr		RF2-Pp		RF2-Ma	
Time (hours)	*c*DCE (mg L^{-1})	*c*DCE removal (%)	*c*DCE (mg L^{-1})	*c*DCE removal (%)	*c*DCE (mg L^{-1})	*c*DCE removal (%)
0	6.08 ± 0.02	-	5.94 ± 0.04	-	6.10 ± 0.09	-
2	6.03 ± 0.08	0.82 ± 1.32	2.44 ± 0.22	58.9 ± 3.70	1.57 ± 0.22	74.3 ± 3.61
4	0.80 ± 0.33	86.8 ± 5.43	0.61 ± 0.32	89.7 ± 5.39	0.00	100
6	0.00	100	0.00	100	0.00	100
8	0.00	100	0.00	100	0.00	100

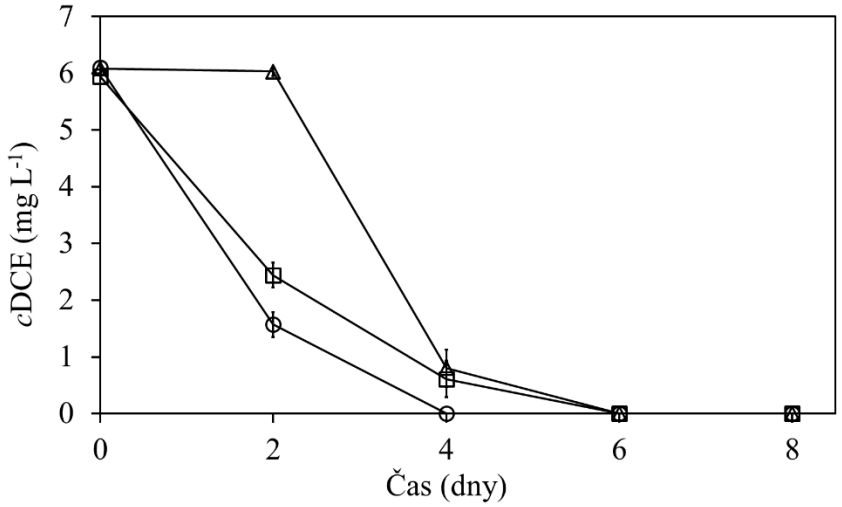

Fig. 4.9 Degradation kinetics of cDCE in a binary mixture with VC by different bacterial consortia[27]

[26] *c*DCE concentrations are actual concentrations of the compound in a liquid phase at given times

[27] *(○) C. testosteroni RF2 and M. aurum DSM-6695; (□) C. testosteroni RF2 and P. putida DSM-7189; (Δ) C. testosteroni RF2 and R. ruber DSM-7511*

Although the results showed that *c*DCE in a binary mixture with VC was efficiently degraded by the strain RF2 in all three consortia, the degradation kinetics of the compound by each consortium differed as seen in Fig. 4.9. Whereas *c*DCE was completely removed within the first four days of the assay by a consortium composed of RF2 and *M. aurum* DSM-6695, the removal of the compound by other two consortia, composed of RF2 and *R. ruber* DSM-7511 or *P. putida* DSM-7189, took more time. The faster removal of *c*DCE may be attributed to the partial degradation of the compound by the *M. aurum* DSM-6695. Indeed, the strain was described to be able to partially degrade all three dichloroethenes when grown on VC (Hartmans and De-Bont, 1992). Also, there is no substrate competition between the strain RF2 and *M. aurum* DSM-6695, which cannot utilize phenol (Table 4.9). In comparison, *P. putida* DSM-7189 and especially *R. ruber DSM*-7511 can compete with RF2 for phenol as well as RF2 can compete with *P. putida* DSM-7189 for 3-chloropropan-1-ol. Further, a significantly different trend of *c*DCE degradation kinetics was observed in a case of the consortium containing *R. ruber* DSM-7511, which showed an extended lag phase that was followed by rapid degradation of the compound between the second and fourth days of the assay.

In all cases, the formation of *c*DCE intermediates by all three consortia resembled the previous finding, so that 2,2-dichloroacetaldehyde had a major response on GC-ECD, followed by a considerably reduced response of unknown intermediate (I2) as shown in Fig 4.10 for degradation kinetics of *c*DCE by RF2-Rr consortium.

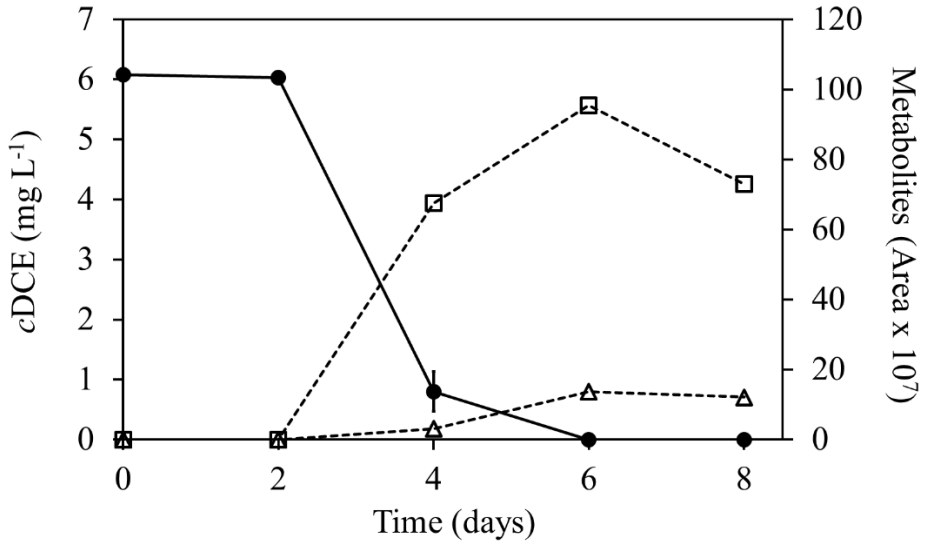

Fig. 4.10 Degradation kinetics of cDCE and formation of intermediates in a binary mixture with VC by an RF2-Rr consortium[28]

[28] *(●) cDCE; (Δ) unknown intermediate I1; (□) 2,2-dichloracetaldehyde*

4.10 Degradation kinetics of TCE, all DCEs and VC in mixed samples

Degradation kinetics of TCE (115.7 μg L^{-1}), all DCEs (662 μg L^{-1} of *c*DCE; 42.01 μg L^{-1} of *t*DCE; 16.02 μg L^{-1} of 1,1DCE), and VC (7 mg L^{-1}; "all in a liquid phase") in mixed samples by a consortium composed of the strain RF2 and *M. aurum* DSM-6695 was examined with the aim to ideally achieve a complete removal of all chloroethenes. The consortium was selected based on the previous results obtained from the degradation kinetics of *c*DCE in a binary mixture with VC, which are listed in section 4.9. While removals of TCE and DCEs were monitored by GC-ECD, the system that has already been proven to be a reliable analytical method for monitoring of these compounds, VC removals were monitored by GC-MS as described in methods. Results of all measurements are given in Tables 4.12 and 4.13 as well as in Fig. 4.11.

Table 4.12 Percentage removals of TCE and all DCEs by a consortium composed of RF2 and *M. aurum* DSM-6695 (means ± SD) – part 1

Compound	Time (days)						
	0	0.5	1	2	3	5	6
TCE (μg L^{-1})	115.7 ± 6.94	114.8 ± 4.59	93.32 ± 6.53	29.18 ± 1.93	0.09 ± 0.04	0.04 ± 0.01	0.05 ± 0.02
TCE removal (%)	-	0.78 ± 3.97	19.3 ± 5.64	74.8 ± 1.67	99.9 ± 00.3	~ 100	~ 100
*c*DCE (μg L^{-1})	662 ± 13.5	657 ± 18.1	138 ± 29.2	0.00	0.00	0.00	0.00
*c*DCE removal (%)	-	0.76 ± 2.04	79.2 ± 4.41	100	100	100	100
*t*DCE (μg L^{-1})	42.01 ± 2.52	40.53 ± 1.92	39.71 ± 2.07	41.09 ± 3.39	2.55 ± 1.66	0.39 ± 0.21	0.15 ± 0.05
*t*DCE removal (%)	-	3.52 ± 4.57	5.50 ± 4.93	2.19 ± 8.07	93.9 ± 3.95	99.1 ± 0.50	99.6 ± 0.12

Table 4.12 Percentage removals of TCE and all DCEs by a consortium composed of RF2 and *M. aurum* DSM-6695 (means ± SD) – part 2[29]

Compound	Time (days)						
	0	0.5	1	2	3	5	6
1,1DCE ($\mu g\ L^{-1}$)	16.02 ± 0.91	16.52 ± 1.58	16.86 ± 3.16	0.16 ± 0.07	0.00	0.00	0.00
1,1DCE removal (%)	-	-	-	99.0 ± 0.44	100	100	100

Table 4.13 VC removal over tine (means ± SD)[30]

Time (days)	I(VC)/I(IPA)	Remaining VC (%)
0	13.91 ± 0.235	100
1	13.89 ± 0.164	99.42 ± 1.18
2	5.199 ± 0.414	37.22 ± 2.98
3	4.705 ± 0.244	33.68 ± 1.75
7	4.596 ± 0.240	32.90 ± 1.73
21	0.059 ± 0.036	0.395 ± 0.26

[29] Chloroethene concentrations are actual concentrations of the compounds in a liquid phase at given times

[30] I(VC)/I(IPA): ratios of integral surfaces of VC and IPA measured by GC-MS

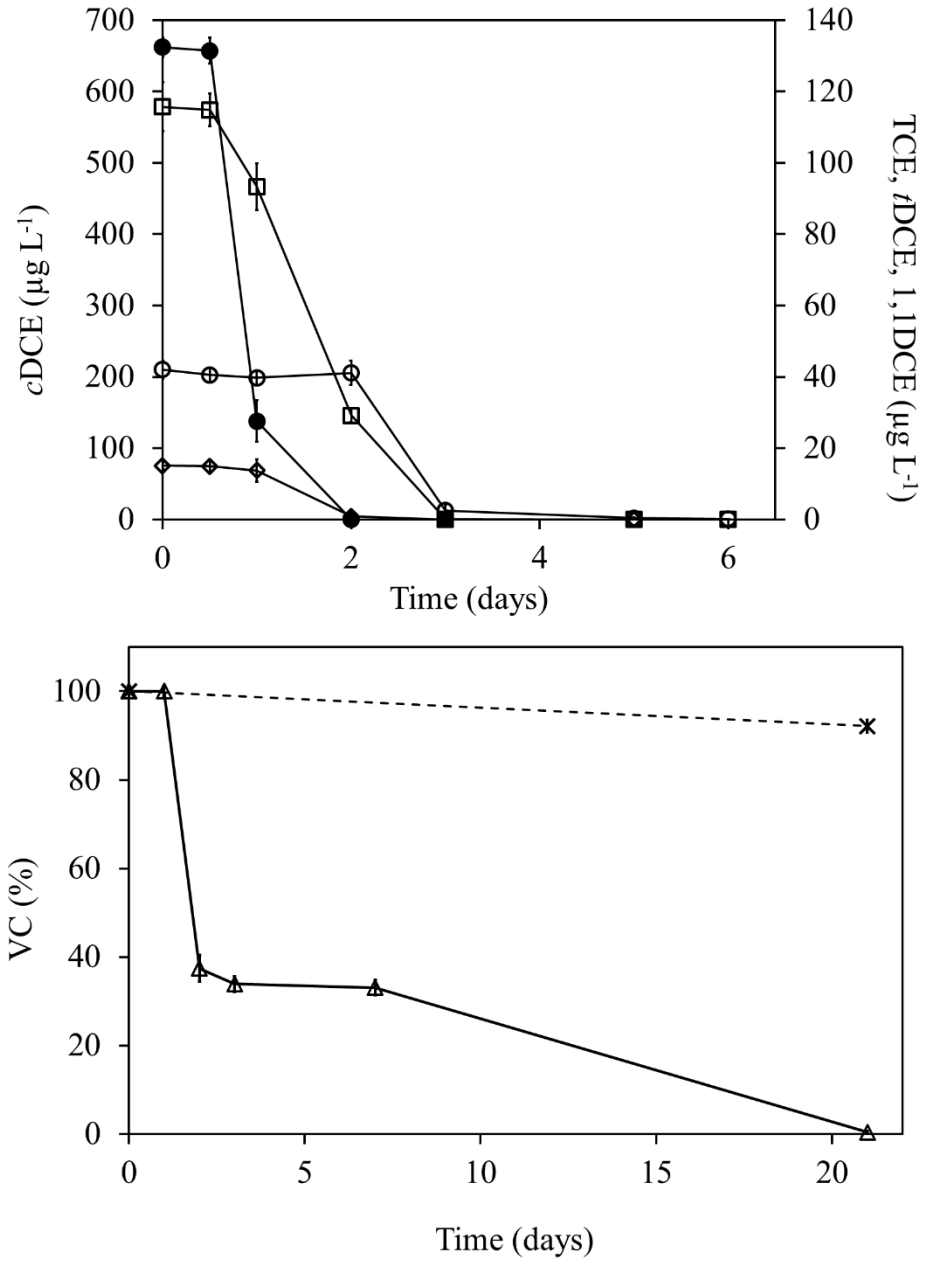

Fig. 4.11 Degradation kinetics of TCE, all DCEs, and VC in mixed samples by a bacterial consortium composed of C. testosteroni RF2 and M. aurum DSM-6695[31]

Fig. 4.11 shows that all chloroethenes were practically fully removed after 21 days of the assay. Whereas TCE and all DCEs were removed during first 6 days, complete removal of VC lasted longer. However, a real degradation time required for the complete removal of VC could have been shorter than 21 days (Fig. 4.11; Table 4.13) as no sample analyses were made between days 7 and 21. The decision to not analyse the samples during the mentioned period was made

[31] *(□)* TCE; *(●) cDCE; (○) tDCE; (◊) 1,1DCE; (Δ) VC; (x) abiotic tests*

intentionally as only negligible removal of VC was observed between days 2 and 7 (4.32 %). It may be speculated that inefficient removal of VC within the mentioned period could have been caused by the temporary presence of transformation intermediates of other chloroethenes or even by a transformation intermediate of VC. Among others, 99.6 % of *t*DCE (42 μg L^{-1}), the compound that was not fully degraded in previous assays, was removed that could be explained either by its lower concentration applied in the assay or due to its partial cometabolic degradation by *M. aurum* DSM-6695. Furthermore, Fig. 4.11 shows that degradation of *t*DCE (42.01 μg L^{-1}) was initiated only after the complete removal of *c*DCE (662 μg L^{-1}) and 1,1DCE (16.02 μg L^{-1}) and significant removal of TCE (115.7 μg L^{-1}; 74.8 ± %) and VC (7 mg L^{-1}; 62.8 ± 2.98). Thus, the results suggest that cells of the strain RF2 and, perhaps to a lesser extent, *M. aurum* DSM-6695 have a lower affinity towards *t*DCE than *c*DCE, 1,1DCE, and TCE.

Further, with regard to investigate a possible influence of VC degradation by *M. aurum* DSM-6695 on degradation efficiency of other chloroethenes by RF2, the previous assay was repeated but no VC nor *M. aurum* DSM-6695 was added into degradation samples containing TCE (117.2 μg L^{-1}), *c*DCE (671 μg L^{-1}), *t*DCE (44.09 μg L^{-1}), 1,1DCE (16.13 μg L^{-1}), and cells of RF2; results are shown in Fig. 4.12. Also, calculated removals for each compound are given in Table 4.14.

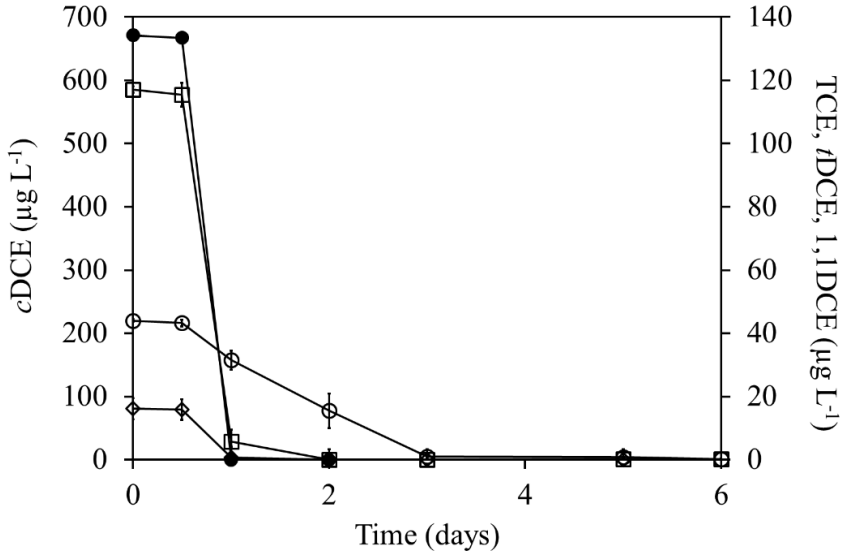

Fig. 4.12 Degradation kinetics of TCE and all DCEs by RF2[32]

Degradation kinetics of chloroethenes in Fig. 4.12 shows that RF2 could alone remove all the compounds. Furthermore, all chloroethenes were removed in

[32] *(□)* TCE; *(●) cDCE; (○) tDCE; (◊) 1,1DCE*

significantly shorter times if compared with results, where VC and *M. aurum* DSM-6695 were applied. Especially efficient degradation was observed for *c*DCE (671 μg L^{-1}) and 1,1DCE (16.13 μg L^{-1}), which were both completely removed during the first day. Also, TCE (117.2 μg L^{-1}) was nearly fully removed after 2 days of the assay, which is 1 day less than its degradation in the previous case. Finally, *t*DCE (44.09 μg L^{-1}) degradation was initiated during the first day when 28.7 ± 4.69 % of the compound was removed (Table 4.14).

Table 4.11 Percentage removals of TCE and all DCEs by strain RF2 (means ± SD)[33]

Compound	Time (days)						
	0	0.5	1	2	3	5	6
TCE (μg L^{-1})	117.2 ± 1.00	115.4 ± 0.70	5.74 ± 1.81	0.1 ± 0.04	0.1 ± 0.02	0.2 ± 0.08	0.2 ± 0.09
TCE removal (%)	-	1.54 ± 0.60	95.1 ± 1.54	99.9 ± 0.03	99.9 ± 0.02	99.8 ± 0.07	99.8 ± 0.08
*c*DCE (μg L^{-1})	671 ± 5.03	667 ± 7.13	0.00	0.00	0.00	0.00	0.00
*c*DCE removal (%)	-	0.60 ± 1.06	100	100	100	100	100
*t*DCE (μg L^{-1})	44.09 ± 2.52	43.22 ± 1.92	31.45 ± 2.07	15.46 ± 3.39	0.968 ± 1.66	0.820 ± 0.21	0.260 ± 0.05
*t*DCE removal (%)	-	1.97 ± 4.35	28.7 ± 4.69	64.9 ± 7.69	97.8 ± 3.77	98.1 ± 0.48	99.4 ± 0.11
1,1DCE (μg L^{-1})	16.13 ± 0.04	15.87 ± 0.3	0.70 ± 0.32	0.04 ± 0.02	0.00	0.00	0.00
1,1DCE removal (%)	-	1.61 ± 1.86	95.7 ± 1.98	99.0 ± 0.12	100	100	100

Based on these findings, it could be suggested, though unexpectedly, that RF2 itself can completely degrade low concentrations of *t*DCE. Moreover, it seems

[33] Chloroethene concentrations are actual concentrations of the compounds in a liquid phase at given times

that rather than *M. aurum* DSM-6695 itself, but transformation intermediates of VC might have a temporary adverse effect on degradation kinetics of chloroethenes by RF2, i.e. reduction of degradation rates of the compounds. The most probable transformation intermediate with such an impact on RF2 cells could be chlorooxirane, which was described to be formed during the initial step in VC metabolism in *M. aurum* L1 that is catalysed by alkene monooxygenase as shown in Fig. 4.13 (Hartmans and De-Bont, 1992).

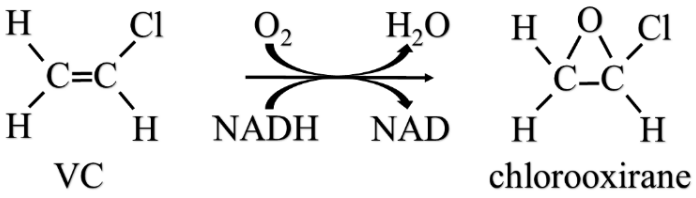

Fig. 4.13 Initial step in vinyl chloride metabolism of M. aurum L1, catalysed by alkene monooxygenase[34]

Nevertheless, even though the reactive epoxide chlorooxirane could be formed during degradation of VC in *M. aurum* DSM-6695, and thus might also adversely affect degradation rates of chloroethenes by RF2, its effect was not significant enough to prevent the complete degradation of chloroethenes in this thesis. Therefore, the consortium composed of RF2 and *M. aurum* DSM-6695 displays potential for in situ bioremediation of CEs-polluted subsurface.

[34] Taken and redrawn from the work of Hartmans and De-Bont, (1992)

5. CONCLUSION

The frequent presence of CEs in soils and groundwater poses a serious risk to humans. Particularly, contamination of groundwater, the worldwide source of drinking water, is potentially a significant exposure source of CEs for humans. Therefore, the development of suitable cleanup methods ensuring the removal of these compounds is necessary to protect human health and the environment. Nowadays, in-situ bioremediation approaches using microorganisms capable of CEs degradation are considered as possible cleanup alternatives offering efficient, low-cost, and environmentally friendly solutions for the removal of these contaminants from a subsurface environment.

As a consequence of the above, this thesis (mainly) investigated the potential of *Comamonas testosteroni* strain RF2 to degrade all three dichloroethene isomers (DCEs) as well as the potential of a bacterial consortium, composed of the strain RF2 and *Mycobacterium aurum* DSM-6695, to degrade TCE, all DCEs, and VC in mixed samples that resembled contaminated groundwater, and thus, the suitability of the train RF2 and the consortium for in-situ bioremediation of contaminated sites was examined.

The results of this thesis proved that phenol growing cells of the strain RF2 could degrade all DCEs along with TCE either as single compounds or in a mixture of these chloroethenes. The strain showed prominent activity towards *c*DCE, as the entire 6.01 mg L^{-1} of this compound was removed at the end of the assay. Further, despite *t*DCE (3.80 mg L^{-1}) did not influence RF2 growth, this compound was not fully degraded, which was most probably due to deficient molecular "lock and key" conformation between the molecules of phenol-2-monooxygenase of the strain RF2 and *t*DCE. Nevertheless, very low concentrations of *t*DCE (44.09 ± 2.52) in samples with other chloroethenes were almost completely degraded. Next, 1,1DCE turned out to be the most troublesome isomer, as only the low concentration of 0.25 mg L^{-1} was degraded completely and just partial removal of 0.37 mg L^{-1}) was observed. Furthermore, higher concentrations of 1,1DCE (1.77 mg L^{-1}) proven to be toxic towards RF2 cells and completely inhibited the degradation of *c*DCE (6.01 mg L^{-1}) in mixed samples with 1,1DCE.

Among others, the strain RF2 could not degrade any of the applied VC concentrations. Nevertheless, the ability of strain RF2 to degrade other chlorinated ethenes remained unaffected in presence of VC and this fact enabled construction of several consortia composed of strain RF2 and one of the purchased DSMZ strains (*P. putida* DSM-7189, *R. ruber DSM*-7511, and *M. aurum* DSM-6695). The consortium composed of growing cells of RF2 and *M. aurum* DSM-6695 showed the best results and efficiently removed TCE, all DCEs and VC in a subsequent assay; although degradation of VC in *M. aurum* DSM-6695 temporarily affected degradation rates of other chloroethenes in strain RF2, nearly

complete removal of all these compounds was observed within three weeks of the assay.

The importance of these results can be supported by the fact that the application of phenol as the primary substrate for a field remediation has already been successfully tested (Hopkins and McCarty, 1995). Furthermore, it should be noted that all the assays performed herein resembled natural conditions and that *c*DCE, which is the most common intermediate produced during PCE and TCE anaerobic dehalogenation in groundwater, was efficiently removed both by strain RF2 and its consortium with *M. aurum* DSM-6695. In addition, the ability of the strain RF2 to degrade only low 1,1DCE concentrations should not pose a serious issue, as the production of this compound during natural attenuation of higher chlorinated ethenes was rarely observed (Futagami et al., 2007; Schmidt and Tiehm, 2008; Tiehm and Schmidt, 2011). Also, *M. aurum* DSM-6695 could degrade VC in consortium with strain RF2 in a mixed sample containing TCE and all DCEs including toxic 1,1DCE. Therefore, the degradation of dichloroethenes and TCE in strain RF2 and the degradation of their mixture with VC in a consortium of the above-mentioned strains appeared to be a practical process for sufficient remediation of contaminated groundwater.

6. CONTRIBUTION OF THE THESIS TO SCIENCE AND PRACTICE

This thesis focused on the bacterial degradation of chlorinated ethenes (CEs) in mineral salt medium (MSM); the compounds, which are a significant source of soil and groundwater pollution worldwide. Although many studies have investigated the bacterial degradation of the compounds in MSM, the number of strains and consortia capable to degrade a broader range of chloroethenes is very limited. Hence, it is obvious that the research aiming to isolate new strains or consortia and further examine their degradation ability towards chloroethenes is desirable, and it might potentially lead to the application in practice for in-situ bioremediation of polluted sites by chloroethenes.

The contribution of the thesis to science and practice is as follow:

- It is the very first study describing the cometabolic degradation of all three dichloroethenes by a pure bacterial strain, *Comamonas testosteroni* RF2, utilizing phenol.
- Degradation of *c*DCE, the most common isomer found in polluted groundwater by perchloroethene and trichloroethene, in the RF2 lead to the high production of inorganic chlorides (92.2 %).
- 2,2-dichloroacetaldehyde was determined as one of the main transformation intermediates in RF2, and one of the degradation steps in *c*DCE degradation catalysed by monooxygenase was shown.
- Phenol-growing cells of the strain RF2 could degrade all three dichloroethenes along with trichloroethene, which is not usual for a pure bacterial strain, and it indicates its potential suitability for in-situ bioremediation applications.
- Most importantly, the consortium composed of the strain RF2 and *M. aurum* DSM-6695 could remove trichloroethene, all three dichloroethenes and vinyl chloride in mixed samples.
- The result shown in the thesis could be used for a further study focusing on improving the degradation efficiency of vinyl chloride by the consortium used in the present study.
- The degradation ability of the consortium towards chloroethenes could potentially be examined in a pilot-scale study.

7. LIST OF PUBLICATIONS

Articles published in journals with impact factor:

ZALESAK, M., J. RUZICKA, R. VICHA and M. DVORACKOVA, 2017. Cometabolic degradation of dichloroethenes by *Comamonas testosteroni* RF2. *Chemosphere*. November 2017, vol. 186, p. 919-927. ISSN 0045-6535. Available from: doi: 10.1016/j.chemosphere.2017.07.156

Journal Impact factor: 4.427 (2017); 4.551 (5 years)
Quartile in Category: Q1

WITCZAK, A., H. ABDEL-GAWAD, **M. ZALESAK** and A. POHORYLO, 2017. Tracking residual organochlorine pesticides (OCPs) in green, herbal, and black tea leaves and infusions of commercially available tea products marketed in Poland. *Food Additives and Contaminants Part A – Chemistry Analysis Control Exposure and Risk Assessment*. 2018, vol. 35, iss. 3, p. 479-486. ISSN 1944-0049. Available from: doi: 10.1080/19440049.2017.1411614

Journal Impact factor: 2.129 (2017); 2.23 (5 years)
Quartile in Category: Q2/Q3

MERKOVA, M., **M. ZALESAK**, E. RINGLOVA, M. JULINOVA and J. RUZICKA, 2018. Degradation of the surfactant Cocamidopropyl betaine by two bacterial strains isolated from activated sludge. *International Bioremediation and Biodegradation*. February 2018, vol. 127, p. 236-240. ISSN 0964-8305. Available from: doi: 10.1016/j.ibiod.2017.12.006

Journal Impact factor: 3.562 (2017); 3.631 (5 years)
Quartile in Category: Q1/Q2

Articles that are being prepared:

ZALESAK, M. and J. RUZICKA, 2019. Evaluation of the potential techniques for in situ bioremediation of chlorinated ethenes: A review.

Attended conferences:

ZALESAK, M., 2016. Human Activities as a Cause of Introducing of Chlorinated Ethenes into the Environment: Their Fate, Potential Risks and Biological Methods of Removal. II. International Conference Human Ecology, Szczecin, Poland. ISBN 978-83-7663-214-8

ZALESAK, M. and J. Ruzicka, 2018. Chlorinated ethenes in groundwater: Significant factors affecting their transport in a subsurface environment and the use of microorganisms for their controlled removal. I. Conference “Mlada Voda Brehy Mele 2018”, Brno, Czech Republic. ISBN 978-80-270-3802-2

Attended workshops:

CYBERWARER 2018 - NATO ARW Workshop - Physical and Cyber Safety in Water Critical Infrastructure. October 2018, Oslo, Norway – Kiel, Germany.

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9. LIST OF SYMBOLS AND ABBREVIATIONS

1,1DCE	1,1-dichloroethene
CAHs	chlorinated aliphatic compounds
CAR-PDMS	Carboxen/Polydimethylsiloxane
*c*DCE	*cis*-1,2-dichloroethene
CEs	chlorinated ethenes
CFUs	colony forming units
DCEs	dichloroethenes
df	film thickness
$DNAPL_{CEs}$	chlorinated ethenes as dense non-aqueous phase liquids
DNAPLs	dense non-aqueous phase liquids
EaCoMT	epoxyalkane coenzyme M transferase
ECD	electron capture detector
GC	gas chromatography
I1	intermediate 1
I2	intermediate 2
K_H	Henry's law constant
Koc	carbon-water partitioning coefficient
Kow	octanol-water partitioning coefficient
LNAPLs	light non-aqueous phase liquids
$LNAPL_{VC}$	vinyl chloride as light non-aqueous phase liquids
MNA	monitored natural attenuation
MS	mass spectroscopy
MSM	mineral salt medium
NAPLs	aqueous phase liquids
PCE	perchloroethene
PCE-RDase	perchloroethene reductive dehalogenase enzyme
P^o	vapour pressure
RDase	reductive dehalogenase enzyme
S	water solubility
Tc	transformation capacity
TCE	trichloroethene
TCE-RDase	trichloroethene reductive dehalogenase enzyme
*t*DCE	*trans*-1,2-dichloroethene
TOC	total organic carbon
VC	vinyl chloride
VC-RDase	vinyl chloride reductive dehalogenase enzyme

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11. CURRICULUM VITAE

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Profile

An inquisitive, reliable, and easily adaptive PhD student with a broad background in Environmental Science who is interested in biodegradation of organic pollutants and the fate of artificial materials in the environment. Furthermore, a person with a passionate interest in ecology and environmental sustainability on a global scale.

Employment history

Water treatment technologist in Aquarex WATERPROFIT
November 2018 up to now

National Exchange Coordinator at the IAESTE Czech Republic
November 2015 – February 2018

Internships

Molecular microbiology of fungal strains isolated from the environment at Tokyo University of Science, Japan
September 2017 – November 2017

Tracking residual organochlorine pesticides (OCPs) in tea leaves and tea infusions at West Pomeranian University of Technology, Szczecin, Poland
March 2016 – June 2016

Removal of nitrogen and phosphorus from wastewater by Chlorella vulgaris at Osaka Institute of Technology, Osaka, Japan
August 2015 – September 2015

Monitoring the quality of drinking water at Water Board of Lemesos (state enterprise), Limassol, Cyprus
June 2014 – August 2014

Observation and biometric measurement of agricultural plants at West Pomeranian University of Technology, Szczecin, Poland
June 2013 – January 2014

Education

PhD, Tomas Bata University in Zlín, Czech Republic
September 2014 until now

BC/MS in Environmental Protection Engineering, Tomas Bata University in Zlín, Czech Republic
September 2008 – June 2014

Chalmers University of Technology, Gothenburg, Sweden
September 2012 – January 2013

Volunteering activities

Reintroduction of Grey Seals into the Baltic Sea, Hel, Poland
March 2014, 2015

Protection of Minke whales within the project „Meet Us Don't Eat Us“ in Reykjavik, Iceland
July 2016 – August 2016

Participation in grant projects

IGA/FT/2015/012
IGA/FT/2016/012
IGA/FT/2017/003
IGA/FT/2018/009
The Water Harmony Erasmus +

- Funded by EU Erasmus + Programme - Key Action 2 - Capacity building in the field of higher education
- Official website: https://www.waterh.eu/en/water-harmony-erasmus/
- Facebook: https://www.facebook.com/WHEplus/

12. APPENDIXES

Appendix 1.1 Summary of some important physicochemical properties of CEs at 25 ºC[35]

Compound	Molar weight (g mol^{-1})	Carbon oxidation state	Density (g cm^{-3})	Solubility in water (g L^{-1})	Log (Kow)	Log (Koc)	Vapor pressure (kPa)	Henry's Law Constant $K_{(H)}$ (x 10^{-3} atm.m^3 mol^{-1})	Relative vapour density	Boiling point (°C)
PCE	165.8	+ II	1.63	0.15	2.9	2.42	2.41	26.3	1.12	121.1
TCE	131.4	+ I	1.46	1.10	2.42	1.81	9.87	11.7	1.35	87.2
*c*DCE	96.9	0	1.28	3.50	2.0	1.77	27	7.40	1.63	60.2
*t*DCE	96.9	0	1.26	6.26	2.09	-	44.3	9.38	1.97	48.5
1,1DCE	96.9	0	1.22	3.34	1.32	-	80.3	23.0	2.86	32.0
VC	62.5	- I	0.91	2.76	1.38	0.39	353.8	79.2	2.2	-13.4

[35] *Data describing physicochemical properties of all CEs were provided from the works of Cwiertny and Scherer (2010) and Binbin et al. (2014)*

Appendix 1.2 An overview of the field studies – part 1[36]

Clean-up approach	Technical design	Biostimulative agents	Removals of CEs + Main degradation process	Contaminated media	Study duration	References
MNA^{F}	Several monitoring wells	Non	95% removal of a total mass of CEs: PCE (2,5 mg L^{-1}), TCE (82 μg L^{-1}), *c*DCE (6,5 mg L^{-1}), VC (430 μg L^{-1}) • Sequential aerobic/anaerobic processes)	unconfined aquifer	2 years	Witt et al., 2001
BS^{P}	Horizontal biosparging well	Methane + O2 (anaerobic dechlorination)	VC (0.045 mg L^{-1}, 90 – 95%); *t*DCE (0.11 mg L^{-1}, 80 – 90%); *c*DCE (0.14 mg L^{-1}, 45 – 55%); TCE (0.1 mg L^{-1}, 20 – 30%) •AC	shallow, confined aquifer	N	Semprini et al., 1991
BS^{F}	Vertical sparging well + soil vapour extraction equipment	Methane + air	TCE (30 mg L^{-1}, 60 – 80%); *c*DCE (5 mg L^{-1}, 60 – 80%); VC (0.025 mg L^{-1}, 60 – 80%) •AC	aquifer and vadose zone	3 months	Sutfin and Ramey, 1997
BS^{P}	Bioventing well	Mixture of: 1% H_2, 1% CO_2, He in N_2 as a balance gas)	PCE (10 mg L^{-1}) was rapidly reduced to *t*DCE and VC • RD	vadose zone	N	Sayles et al., 1997

[36] *F: full-scale study; P: pilot-scale study; AC: aerobic cometabolism; MNA: monitored natural attenuation RD: reductive dechlorination; BS: biostimulation; N – Not specified time of remedy*

Appendix 1.3 An overview of the field studies – part 2[37]

Clean-up approach	Technical design	Biostimulative agents	Removals of CEs + Main degradation process	Contaminated media	Study duration	References
BS^P	Bioventing well	Methane, O_2	Significant degradation of *c*DCE, VC – concentrations not specified •AC (*c*DCE); Direct oxidation VC)	vadose zone	N	Cox et al., 1998
BS^P	Bioventing well	H_2/O_2	PCE, *t*DCE, VC (concentrations not specified) • Sequential anaerobic/aerobic processes	vadose zone	N	Mihopoulos et al., 2001
BS^F	Direct single sparging well	Propane or methane + O_2	TCE (100 – 400 μg L^{-1}) and *c*DCE (20 – 60 μg L^{-1}) rapid removal • AC	unconfined aquifer	N	Kim et al., 2008
BS^F	Four injection wells	Lactate solution	TCE (502 μmol L^{-1}) was reduced to *c*DCE (18 – 279 μmol L^{-1}), VC (384 – 624 μmol L^{-1}) and ethene (39 – 111 μmol L^{-1}) •RD	aquifer	~ 1year	Dugat-Bony et al., 2012
BS^P	Closed loop system	Methane + O_2	VC (0.045 mg L^{-1}, 90 – 95%); *t*DCE (0.11 mg L^{-1}, 80 – 90%); *c*DCE (0.14 mg L^{-1}, 45 – 55%); TCE (0.1 mg L^{-1}, 20 – 30%) •AC	semiconfined aquifer	N	Semprini et al., 1990

[37] *F: full-scale study; P: pilot-scale study; AC: aerobic cometabolism; RD: reductive dechlorination; BS: biostimulation; N – Not specified time of remedy*

Appendix 1.4 An overview of the field studies – part 3[38]

Clean-up approach	Technical design	Biostimulative agents	Removals of CEs + Main degradation process	Contaminated media	Study duration	References
BS^P	Closed loop system	Phenol or toluene + O_2	TCE (0,045 mg L^{-1}, 92%); *c*DCE (0,045 mg L^{-1}, > 90%); *t*DCE (0,045 mg L^{-1}) was only slightly degraded (data not provided) • AC	shallow confined aquifer	35 days	Hopkins et al., 1993
BS^P	Closed loop system	Phenol or toluene +O_2 or 10% H_2O_2	TCE (250 μg L^{-1}, > 90%); *c*DCE (125 μg L^{-1}, > 90%); *t*DCE (125 μg L^{-1}, 74%); 1,1DCE (65 μg L^{-1}, 50%); VC (60 μg L^{-1}, > 98%). • AC	confined aquifer	25 - 83 days	Hopkins and McCarty, 1995
BS^P	Closed loop system	Sucrose or dextrose fructose mixture + yeast extract	TCE, cDCE, VC (all 500 μg L^{-1}) were reduced to >10 μg L^{-1} • RD	unconfined aquifer	~ 1.8 years	Bennett et al., 2007
BS^P	Closed loop system	Cheese whey + thermal enhancement	The total concentration of CEs (5.5 mg L^{-1}) including TCE, *c*DCE, and VC was reduced bellow 1 μg L^{-1} •RD	Unconfined and confined aquifers	3 months	Nemecek et al., 2018

[38] *P pilot-scale study; AC: aerobic cometabolism; RD: reductive dechlorination; BS: biostimulation*

Appendix 1.5 An overview of the field studies – part 4[39]

Clean-up approach	Technical design	Biostimulative agents	Removals of CEs + Main degradation process	Contaminated media	Study duration	References
BS^F	A pair of recirculation wells	Toluene + O_2 or H_2O_2	TCE (1000 μg L^{-1}, 97 – 98%) •AC	unconfined and confined aquifers	~ 1.2 months	McCarty et al., 1998
BS^P	A pair of recirculation wells	sodium propionate	*c*DCE (1 mg L^{-1}) was nearly stoichiometrically reduced to ethene via VC •RD	unconfined and confined aquifers	2 months	Hoelen et al., 2006
BA^P	Closed loop system	Methanol + acetate + KB-1 consortium	PCE (1 mg L^{-1}) and lower amounts of TCE and *c*DCE were below 5 μg L^{-1} •RD	Aquifer	~ 6.5 months	Major et al, 2002
BA^P	Closed loop system	Butane + O_2 butane + *Rhodococcus* sp BP183	1,1DCE (65 μg L^{-1}, ~ 97%); 1,1DCA (100 – 200 μg L^{-1}, ~ 77%); 1,1,1TCA (140 – 195 μg L^{-1} ~36%) •RD	shallow confined aquifer	20 days	Semprini et al., 2007
BA^P	Vertical well for direct injection	Emulsified soybean oil + KB1 consortium	*c*DCE, VC (complete reduction to ethene) •RD	Clayey till	~ 5 months	Scheutz et al., 2010

[39] *F: full-scale study; P: pilot-scale study; AC: aerobic cometabolism; RD: reductive dechlorination; BS: biostimulation; BA: bioaugmentation*

Appendix 1.6 An overview of the field studies – part 4[40]

Clean-up approach	Technical design	Biostimulative agents	Removals of CEs + Main degradation process	Contaminated media	Study duration	References
BA^P	Vertical well for direct injection	O_2 + *Burkholderia cepacia* ENV435 producing Toluene-ortho monooxygenase	The total mass of CEs (TCE + *c*DCE 1000 – 2500 μg L^{-1}) was reduced up to 78% •AC	semi-confined silty-sand aquifer	2 days	Steffan et al., 1999
BA^P	Two vertical wells for direct injection	Sodium lactate (60% w/w solution) + KB1 consortium	TCE (20 mg L^{-1}) reduction via *c*DCE to ethene (~ 80 %) •RD	Groundwater bearing fractured conglomerate	5.7 years	Verce et al, 2015

[40] *P pilot-scale study; AC: aerobic cometabolism; RD: reductive dechlorination; BA: bioaugmentation*

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