Bioremediation of Tetrachloroethene Contaminated Groundwater by Dechlorinating Microbial Communities

Sayali Surendra Patil

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UNIVERSITY

Adelaide

South Australia

Principal Supervisor: Associate Professor Ian Menz

Co-supervisors: Professor Andrew Ball, Professor Jim Mitchell

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Abstract

Improper disposal of chlorinated compounds widely used as industrial solvents, intermediates in chemical industries, pesticides and pharmaceuticals has led to severe subsurface contamination. Common chlorinated compounds include tetrachloroethene or perchloroethylene (PCE), trichloroethene (TCE), dichloroethenes (DCE) and vinyl chloride (VC). Enhanced reductive dechlorination (ERD) represents a promising approach for the complete degradation of these compounds. Successful microbial-mediated remediation has to date been associated with major dechlorinating species such as *Dehalococcoides (Dhc), Desulfitobacterium, Desulfuromonas, Dehaloginomonas, Geobacteriaceae* and *Sulfurospirillum.* This research explored the degradation potential of microbial communities other than these well studied groups within groundwater collected from a PCE-contaminated site in Australia. Laboratory based enrichment cultures using groundwater samples with high PCE levels (146 μg L^{-1}) showed the dominance of *Proteobacteria*, *Spirochaetes, Firmicutes, Bacteroidetes, Methanomicrobiaceae, Methanosaetaceae* and *Methanosarcinaceae*groups. The indigenous groundwater community was found capable of the complete dechlorination of PCE to the environmentally safe end product ethene over 24 weeks, with the sequential degradation of PCE via intermediate products. The molecular culture-independent microbial profiling techniques like polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) along with novel statistical Pareto-Lorenz and moving windows analyses were used to assess changes in the indigenous microbial community during PCE removal. A comparison of the effects of using either biostimulation only (BS) with biostimulation plus bioaugmentation (BS-BA) for PCE remediation in a laboratory based system showed that both remediation regimes were successful, with complete PCE degradation occurring over 17 and 21 weeks for BS only and BS-BA, respectively compared to controls which had only 30% PCE degradation.

Furthermore, quantitative real time PCR and live-dead cell count (LDCC) analyses showed a 2-3 fold increase in microbial cell abundance with approximately 70–80% viability in both treatments indicating active growth of PCE dechlorinators. We further employed BS, BS-BA and monitored natural attenuation (MNA) strategies for commercial bioremediation at TCE contaminated site in Victoria, Australia. Over the period of nine months of BS, MNA and BS-BA treatments TCE concentration was reduced from 40, 79 and 150 μg $L^{-1}$to below maximum concentration level of 5μg L^{-1},respectively. Although, this work highlighted ERD as an effective way of PCE remediation, this technology has a few disadvantages. Hence, an alternative microbial electric system (MES) was established where bioenergy was generated through the catalytic actions of microorganisms during PCE dechlorination. Multiple lab-scale MESs fed with acetate and carbon electrode/PCE as electron donors and acceptors, respectively under BS only and BS-BA regimes further highlighted the bio-electrochemical potential of indigenous non-*Dhc* community against previously well studied *Dhc* and *Geobacteriaceae* species. The indigenous non-*Dhc* community was found to contribute significantly to electron transfer with ~61% of the current generated. Microbial colonization and biostimulation resulted in 100% dechlorination in both treatments with complete dechlorination occurring 4 weeks earlier in BS-BA samples and up to 11.5 μA of current being generated than BS only MES. Overall, this study contributes to better understanding of the dechlorinating potential of indigenous non-*Dhc* microorganisms; their structure, dynamics and functional organization in response to PCE dechlorination that will assist to advance the bioremediation field in a rational manner. In addition, evidence of advances in the current bioremediation practices in terms of methodology (LDCC) and techniques (MES) are presented.

Declaration

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the ward of any other degree or diploma at Flinders University of South Australia or any other educational institution, except where due acknowledgments is made in the thesis. Any contribution made to the research by others, with whom I have worked at Flinders University or elsewhere, is explicitly acknowledged in this thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

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Patil, S.S., Adetutu E.M., Aburto–Medina, A., Menz I.R., Ball A.S. 2014. Biostimulation of indigenous communities for the successful dechlorination of tetrachloroethene (perchloroethylene) - contaminated groundwater. *Biotechnology Letters* 36, 75-83 doi:10.1007/s10529-013-1369-1

Patil, S.S., Adetutu E.M., Sheppard P.J., Morrison P., Menz I.R., Ball, A.S. 2014. Site-specific pre-evaluation of bioremediation technologies for chloroethene degradation. *International Journal of Environmental Science and Technology* 11 (7), 1869 – 1880.

Patil, S.S., Adetutu E.M., Rochow, J., Mitchell, J.G., Ball, A.S. 2013. Sustainable remediation: electrochemically assisted microbial dechlorination of tetrachloroethene contaminated groundwater. *Microbial Biotechnology* 7 (1), 54-63 doi: 10.1111/1751-7915.12089

Gundry, T.D., **Patil, S.S.**, Ball, A.S. 2014. Application of molecular biological tools for the assessment of the *in situ* bioremediation potential of TCE. Submitted to *Groundwater Monitoring and Remediation* (Under review)

During the candidature, I attended two conferences, one international and other national which presented an opportunity to present my work. The conferences included:

- Proffered paper presentation on 'Sustainable remediation: bioenergy generation during the bioremediation of tetrachloroethene contaminated groundwater' at 54th 'Annual Scientific Meeting and Exhibition of the Australian Society for Microbiology' held between 7-10 July 2013, Adelaide, South Australia.

- Poster presentation on 'Indigenous microbial community dynamics during reductive dechlorination of groundwater at a chloroethene contaminated site in Victoria, Australia' at 14th 'International Symposium on Microbial Ecology' held between 19–24 August, 2012, Copenhagen, Denmark (Appendix 1, page 116).

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Finally, I would like to thank my parents, my beloved father and mother for being a constant source of inspiration, courage and the greatest teacher in my life: this is for both of you!

Structure of the Thesis

This thesis is subdivided into 8 main Chapters. An introduction to the project and a review of the current literature is provided in Chapter 1. Chapter 2 contains details of the general experimental materials and methods used in this study throughout the research chapters (3 to 6). Details of the methodologies used in specific research chapters are given in the relevant result chapters.

Chapter 3 represents the first results chapter and describes how the biostimulation strategy was implemented to enhance indigenous communities for successful dechlorination of tetrachloroethene contaminated groundwater.

Based on the Chapter 3 results, Chapter 4 further describes work on the site-specific pre-evaluation of biostimulation and biostimulation plus bioaugmentation based bioremediation strategies for chloroethene degradation. This chapter also demonstrates the applicability of quantitative microbiological tools like real – time PCR and Live/dead Cell Count analyses for preliminary site assessments.

Chapter 5 extends the laboratory based work described in Chapter 3 and 4 to *in situ* commercial application.

Chapter 6 explores the avenue of Microbial Electric System (MES) as a ground-breaking alternative to current remediation technology. This chapter highlights the potential of indigenous non-*Dehalococcoides* bacterial community in bio-electrochemically reducing tetrachloroethene to enhance MES efficiency for successful bioremediation.

Chapter 7 presents an overall discussion of the research carried out in the thesis and draws final conclusions.

Chapter 8 contains the references cited in Chapter 1, Chapter 2 and Chapter 7. Appendices and Corrigenda have also been included in Chapter 8.

The Results Chapters 3, 4 and 6 represents peer-reviewed articles in international journals and has been reproduced in their published format. Chapter 5 has been submitted to a refereed academic journal and is reproduced in the submitted format.

List of Abbreviations and Terminologies

bgs	Below ground surface
BS	Biostimulation
BA	Bioaugmentation
cDCE	*cis*-Dichloroethene
DCA	Dichloroethane
Dhc	*Dehalococcoides*
DGGE	Denaturing Gradient Gel Electrophoresis
DNAPL	Dense Non-aqueous Phase Liquid
GW	Groundwater
16S rRNA	16S sub-unit of ribosomal DNA gene
MBTs	Molecular Biological Tools
MCL	Maximum Contaminant Level
MES	Microbial Electric Systems
MNA	Monitored Natural Attenuation
MWs	Monitoring Wells
NA	Natural Attenuation
LNAPL	Light Non-aqueous Phase Liquid
PCR	Polymerase Chain Reaction
PCE	Tetrachloroethene (Perchloroethene)
RDase	Reductive Dehalogenase
RNA	Ribonucleic Acid
TCE	Trichloroethene
TCA	Trichloroethane
VC	Vinyl Chloride
VOCs	Volatile Organic Compounds

Terminologies used in this study

Biodegradation: Biologically mediated conversion of one compound to another.

Bioremediation: Use of microorganisms to control, transform and/or destroy contaminants

Biotransformation: Microbiologically catalyzed transformation of a chemical to some other product.

Biostimulation: The addition of nutrients, electron acceptors (or electron donors), and sometimes auxiliary substrates to stimulate growth and activity of specific indigenous microbial populations.

Bioaugmentation: The addition of exogenous, specialized microorganisms with enhanced capabilities to degrade the target pollutant.

Chlorinated Solvent: A hydrocarbon in which chlorine atoms substitute for one or morehydrogen atoms in the compounds structure. Chlorinated solvents commonly are used for grease removal in manufacturing, dry cleaning, and other operations.

Co-metabolism: A reaction in which microbes transform a contaminant even though the contaminant cannot serve as an energy source for the organisms. To degrade the contaminant, the microbes require the presence of other compounds (primary substrates) that can support their growth.

Dechlorination: The removal of chlorine atoms from a compound.

Dehydrohalogenation: Elimination of a hydrogen ion and a halide ion resulting in the formation of an alkene.

Dihaloelimination: Reductive elimination of two halide substituents resulting in formation of an alkene.

***Ex Situ* Bioremediation:** The use of aboveground bioreactors to treat contaminated soil or groundwater that has been extracted from the contaminated site.

***In Situ* Bioremediation**: Bioremediation process that occur below the ground surface, where the contaminated zone becomes the bioreactor.

Electron Acceptor: Compound that gains electrons (and therefore is reduced) in oxidation-reduction reactions that are essential for the growth of microorganisms. Common electron acceptors are oxygen, nitrate, sulfate, iron and carbon dioxide. Highly chlorinated solvents (e.g. TCE) can act as electron acceptors.

Electron Donor: Compound that loses electrons (and therefore is oxidized) in oxidation-reduction reactions that are essential for the growth of microorganisms. In bioremediation organic compounds serve as electron donors. Less chlorinated solvents (e.g., VC) can act as electron donors.

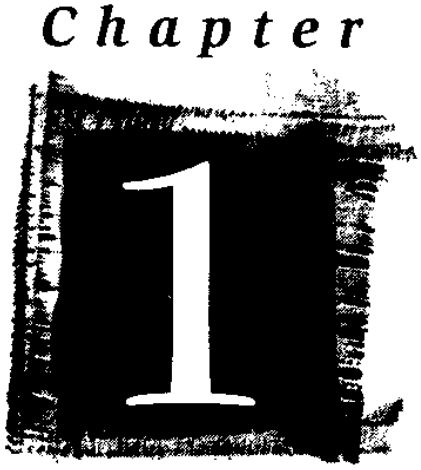
Enhanced Anaerobic Bioremediation: Addition of carbon sources (electron donors) and/or nutrients to the subsurface in order to stimulate bacteria which can destroy chlorinated solvents by using them as an electron acceptor in the process of reductive dechlorination.

Intrinsic Remediation or Natural Attenuation: *In situ* remediation that uses naturally occurring processes to degrade or remove contaminants without using engineering steps to enhance the process.

Reduction: Transfer of electrons to a compound such as oxygen. It occurs when anothercompound is oxidized.

Reductive Dechlorination: The removal of chlorine atoms from an organic compound and their replacement with hydrogen atoms (same as reductive dehalogenation).

Reductive Dehalogenation: A variation on biodegradation in which microbially-catalyzed reactions cause the replacement of a halogen atom (e.g. chlorine) on an organic compound with a hydrogen atom. The reactions result in the net addition of two electrons to the organic compound.



Introduction

CHAPTER 1: Introduction

1.1 Bioremediation and Its Place In the World

The past century witnessed a vast increase in global pollution. Industrial development, population growth, urbanization and a disregard for the environmental consequences of releasing chemicals into the environment all contributed to the pollution situation. There was a large increase in the diversity of organic compounds that were industrially produced and which were carelessly released into the environment. Consequently, many natural resources show some degree of anthropogenic impact, including the widespread contamination of groundwater aquifers by hazardous wastes (Atlas and Philp, 2005). This is particularly significant because groundwater represents about 98% of the available freshwater of the planet (Fig 1.1). Table 1.1 summarizes the main sources of groundwater contamination. The fact that we are already using about 50% of readily available fresh water makes groundwater protection and clean-up of paramount importance.

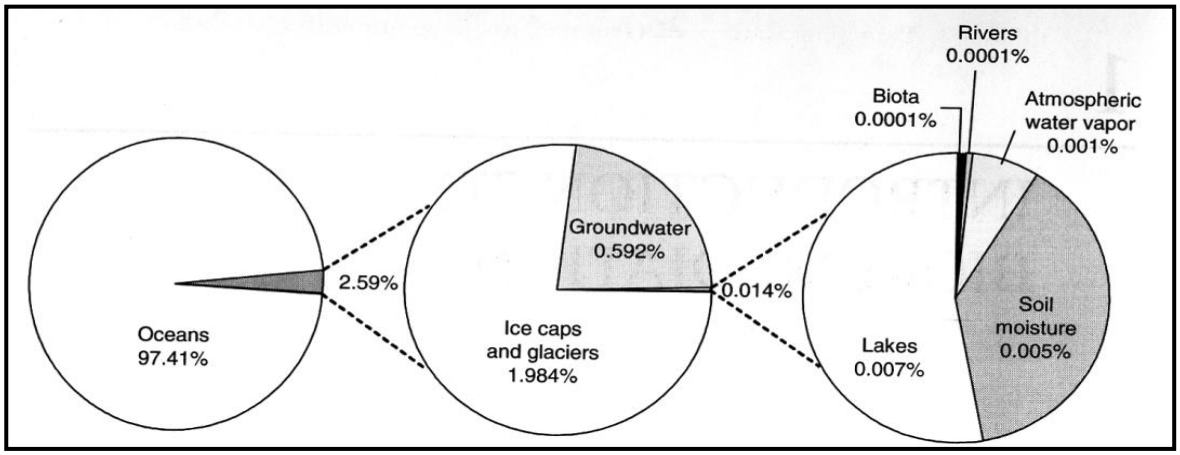


Figure 1.1: Distribution of the World's water (adapted from Speidel and Agnew, 1998)

Table 1.1: Principal sources of groundwater contamination

- Leaking underground storage tanks
- Municipal solids and hazardous waste landfills
- Hazardous waste management sites
- Unlined pits, ponds and lagoons
- Household septic systems
- Pesticide application areas
- Abandoned petroleum wells
- Saltwater intrusion along the coastline
- Surface spills

Sources: LaGrega *et al*., 1994; NRC (1994, 1997)

1.1.1. The Need for Bioremediation

Bioremediation – which is broadly defined as a managed or spontaneous process in which biological catalysis acts on pollutants, thereby remedying environmental contamination present in water, wastewater, sludge, soil, aquifer material, or gas streams– hold great potential as a practical and cost - effective approach to solve a wide variety of contamination problems (Alvarej and Illman, 2006). Therefore, it is expected that bioremediation will play an increasingly important role in the clean-up of soils, sediments and groundwater contaminated with hazardous organic chemicals.

The major reasons for the control of water pollution and the consideration of bioremediation are first and foremost, public health concerns; second, environmental conservation; and finally, the cost of decontamination. Although environmental contaminants can pose acute health risks, the concerns normally associated with contaminated land and water are with long term effects. The main motivation to use biotechnology for environmental clean-up is also economics and bioremediation is an emerging technology that holds great promise for the cost effective removal of a wide variety of environmental pollutants (Table 1.2). Remediation costs for sites contaminated with hazardous wastes in Europe were expected to exceed $ 1.5 trillion in the near future (ENTEC, 1993). In the United States, the Office of Technological

Assessment (OTA) of the U.S Congress estimated that the cost of cleaning up more than 300,000 highly contaminated sites will exceed $500 billion (U.S. Environmental Protection Agency (USEPA, 2003). This does not include leaking underground storage tanks or about 19,000 landfill sites used for disposal of municipal and industrial wastes. Thus there is an urgent need for cost-effective treatment approaches. Recognising the economic and environmental benefits of bioremediation, the Organisation for Economic Co-operation and Development (OECD) estimated that the growth of bioremediation would be from $40 billion per year in 1990 to over $75 billion at the current time (OECD, 1998). Estimated Australian Government contribution in the bioremediation industry for the year 2006-07 was AUD 10-20 million (SoE, 2006). Others have also estimated significant growth in bioremediation market in North America and Europe.

Table 1.2: **Economics of remediation treatments**

Method	**Range of Cost of Remediation ($US/ton of soil)**
Incineration	400-1,200
Washing	200-300
Bioremediation	20-200

Source: Tata Research Energy Institute

(http://www.cleantechindia.com/eicnew/successstories/oil.htm)

The successful application of bioremediation is well documented for many sites contaminated with three major classes of hazardous wastes that are amenable to bioremediation: petroleum hydrocarbons (33% of all applications), creosotes (22%), and chlorinated solvents (9%). Bioremediation offers several advantages over traditional site remediation approaches such as pump and treat or soil excavation followed by incineration. The advantages and limitations of bioremediation are listed in Table 1.3 (Alvarej and Illman, 2006). According to a report from the McIlvaine Company (News Release, 1998; http://www.mcilvainecompany.com/news), the popularity of soil incineration and groundwater pump and treat techniques is wanning while that of bioremediation is increasing.

Table 1.3: Advantages and limitations of bioremediation

Advantages • **Organic hazardous wastes can be destroyed (e.g., converted to H_2O, CO_2, and mineral salts) rather than transfer from one phase to another, thus eliminating long term liability.** • **Relies on natural biodegradation processes that can be faster and cheaper (at least 10 x less expensive than pump and treat).** • **Minimum land and environmental disturbance** • **Can attack hard to withdraw hydrophobic pollutants** • **Environmentally sound with public acceptance** • **Does not dewater the aquifer due to pumping** • **Can be used in conjunction with (or as a follow up to) other treatment technologies.**
Disadvantages • **It may require extensive monitoring** • **Requirements for success and removal efficiency may vary considerably from one site to other** • **Some contaminants can be present at high concentrations that inhibit microorganisms** • **Can be a scientifically intensive technique** • **There is a risk for accumulation of toxic biodegradation products**

1.2 Chlorinated Compounds in the Environment: Causes for Concern

1.2.1. Presence, Properties and Health Effects of Chlorinated Compounds:

Groundwater contamination by hazardous substances is commonly the result of accidental spills that occur during production, storage or transportation activities. Table 1.4 lists the top 25 hazardous groundwater contaminants.

The most common classes of organic groundwater pollutants include aromatic hydrocarbons, chlorinated solvents and pesticides. Common inorganic groundwater pollutants include nitrate (NO_3^-), arsenic (As), selenium (Se) and toxic heavy metals such as lead (Pb), cadmium (Cd) and chromium (Cr^{6+}). Hexachlorobenzene and

pentachlorophenol (which are common fungicides used as wood preservers) or polychlorinated biphenyls (PCBs, which are common dielectric fluids in transformer oil) are similar to polyaromatic hydrocarbons (PAHs) in terms of their potential carcinogenicity and lipophilic nature (i.e. high affinity for fatty tissues) which is conducive to bioaccumulation. These compounds also have a strong tendency to get absorbed into soil and aquifer sediments and their dispersal is often due to co-transport with sorbents such as colloidal matter or eroded sediments. The following discussion will focus on the main topic of this study, chlorinated pollutants in the groundwater that are treated using bioremediation techniques.

Table 1.4: The 25 most frequently detected priority pollutants at hazardous waste sites

1. Trichloroethylene (TCE)	14. Cadmium (Cd)
2. Lead (Pb)	15. Magnesium (Mg)
3. Tetrachloroethylene (PCE)	16. Copper (Cu)
4. Benzene	17. 1,1-dichloroethane (1,1-DCA)
5. Toluene	18. Vinyl Chloride (VC)
6. Chromium (Cr)	19. Barium (Ba)
7. Dichloromethane (DCM)	20. 1,2-dichloroethane (1,2-DCA)
8. Zinc (Zn)	21. Ethylbenzene (EB)
9. 1,1,1- trichloroethane (TCA)	22. Nickel (Ni)
10. Arsenic (As)	23. Di (ethylhexyl)phthalate
11. Chloroform (CF)	24. Xylenes
12. 1,1-dichloroethene (1,1-DCE)	25. Phenol
13. 1,2-dichloroethene (1,2-DCE)	

Source: NRC, 1994

Chlorinated aliphatic and aromatic compounds make up an important group of organic pollutants that are both ubiquitous and relatively persistent in aquifers. Chlorinated ethenes fall into a class of chemically stable compounds commonly known as “safety

solvents". Because they are resistant to combustion and explosion, these compounds were widely used as industrial solvents, lubricants, degreasers, intermediates in chemical industries, pesticides and pharmaceuticals for most of the twentieth century (Olaniran *et al.,* 2004). The combination of extensive use, volatility and chemical stability has led to the widespread contamination of groundwater and soil by such ubiquitous and recalcitrant pollutants (SCRD, 2007). Common volatile organic compounds (VOCs) in the chlorinated solvents group include tetrachloroethylene (perchloroethylene, PCE), trichloroethylene (TCE), dichloroethylene (DCE) and vinyl chloride or chloroethylene (VC) (Fig 1.2). All of these VOCs are potential carcinogens (ASTDR 2007a, 2007b). Groundwater contamination by 1, 1, 1- trichloroethane (TCA) and chlorinated methanes, such as carbon tetrachloride (CCl_4) and chloroform ($CHCl_3$) is also common. Table 1.5shows the chemical and physical properties of chloroethenes, which is important for their ultimate fate and transport in the environment. VC is considered the greatest threat to human health because of its carcinogenic property and has a drinking water maximum contamination level (MCL) of 2µg L^{-1} (USEPA, 2006).

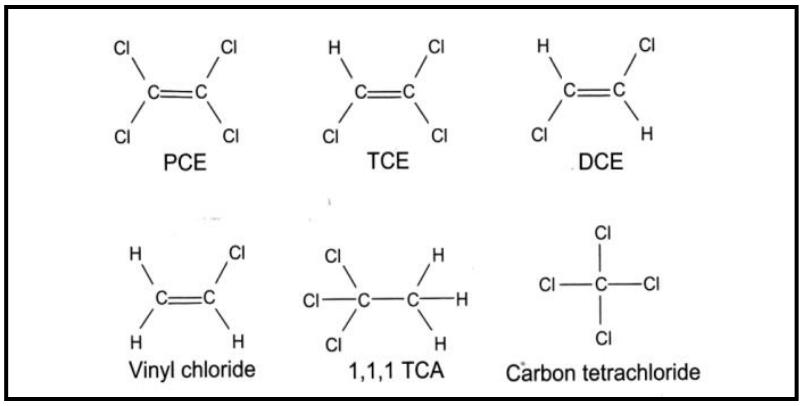


Figure 1.2: Common chlorinated solvents found in contaminated groundwater aquifers (adapted from Alvarej and Illman, 2006)

Table 1.5: Physical and chemical properties of chloroethene compounds and their toxicity effects on human health (Olarinan *et al*., 2004)

Comp-ound	Melting Point (°C)	Density (g/cc)	Log K_{ow}	Log K_{oc}	Water Solubility (mg/L)	Vapour Pressure (mm Hg)	Henry's Law Constant (atm-m^3 mol)	MCL µg/L	Toxicity effects on Human Health
PCE	-22.7	1.625	3.14	2.82	1.5E+02	1.40E+01	2.27E-02	5	Liver, Kidney problem; increased risk of cancer
TCE	-87	1.462	2.42	2.10	1.0E+03	5.87E+01	8.92E-03	5	
***cis*-DCE**	-81	1.284	1.86	1.50	3.5E+03	2.0E+02	7.5E-03	70	
***trans*-DCE**	-50	1.257	2.09	1.77	6.3E+03	2.65E+02	6.6E-03	100	
1,1-DCE	-97.4	1.175	1.79	1.48	5.5E+03	1.82E+02	5.7E-03	7	
VC	-157	0.9121	0.60	0.91	1.1E+03	2.30E+03	6.95E-01	2	

K_{ow} :**Octano-water partition coefficient** is a measure of the tendency of the compounds to partition into lipids and used to estimate the bio-concentration of the chlorinated organic; **K_{oc}: Adsorption coefficient** of a compound in a particular environment compartment and is a function of the organic content and other properties of the environment compartment as well as properties of the organic compound.

Chlorinated solvents generally have a higher specific gravity than water and tend to sink to the bottom of the aquifer if present in a separate organic phase – the so called dense non-aqueous phase liquid (DNAPL) (Fig 1.3). These DNAPLs represent a major challenge to site remediation due to their persistence and relative inaccessibility.

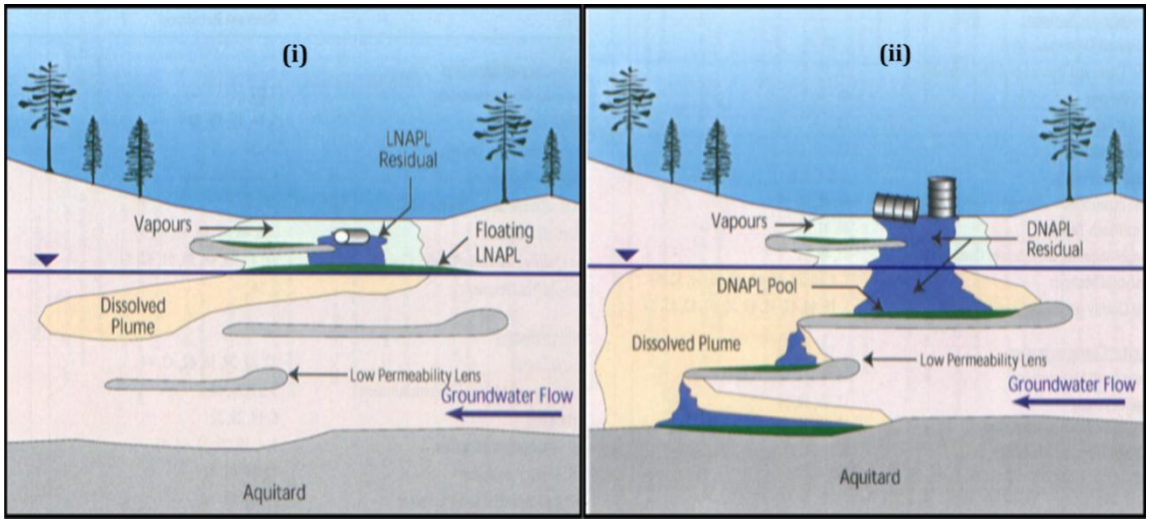


Figure 1.3: Schematic showing two types of non-aqueous phase liquid (NAPL) spills in an aquifer (adapted from ITRC, 1999)**; (i)** Oil has a lower specific gravity than water and floats on the water table, forming a light non-aqueous phase liquid (LNAPL); **(ii)**PCE on the other hand, is heavier than water and sinks, forming a dense non-aqueous phase liquid (DNAPL). The dissolved phase travels with flowing groundwater.

1.2.2. State of Practice: *In situ* and *Ex situ* Chloroethene Bioremediation

A common approach to treating chlorinated solvents in groundwater is 'pump and treat', an *ex situ* process whereby contaminated water is pumped to the surface for treatment by a number of processes including carbon filtration, air-stripping or chemical oxidation. Pump and treat is often an energy intensive process requiring large capital costs and longer time frames in order to bring the contaminant levels down to drinking water standards (Olaniran *et al*., 2004). The U.S. EPA (2001) studied the average operating costs for pump-and-treat systems at 32 Superfund-financed sites and found the annual cost to be approximately $767,000/site. Because of the high cost and lengthy operating periods for pump-and-treat remedies, use of *in situ* treatment technologies is increasing. Microorganisms that naturally live in the subsurface may also degrade, detoxify or immobilize contaminants, a process called *in situ* bioremediation. An *in situ* approach which has gained popularity is placing permeable

reactive barriers (PRBs) in the subsurface which use zero-valent iron as a reactive substance to chemically reduce chlorinated ethenes to non-toxic byproducts. However, this is only feasible and cost effective for treating shallow, restricted areas of contamination (Lovley, 2001).

Until recently, practical applications of *in situ* bioremediation have focused mostly on aerobic microorganisms (Alexander, 1999) which gain energy by oxidizing organic compounds to carbon dioxide with oxygen serving as the electron acceptor. The most important haloalkenes are the chlorinated ethenes. Halogenation of organic molecules generally makes them more resistant to aerobic biodegradation (Atlas and Philp, 2005). Hence, aerobic microorganisms do not degrade common chloroethenes (PCE, TCE) under the conditions typically found in aquifers (Lee *et al*., 1998). The scarcity of oxygen in many contaminated subsurface environments and inability of aerobes to degrade chloroethene contaminants has raised interest in the *in situ* bioremediation potential of anaerobes which grow in the absence of oxygen.

1.2.3. Enhanced *In Situ* Anaerobic Bioremediation: A Promising Technology for Chloroethene Bioremediation

'Enhanced *in situ* anaerobic bioremediation involves the delivery of an organic substrate into the subsurface for the purpose of stimulating microbial growth and development (biostimulation) creating an anaerobic groundwater treatment zone and generating hydrogen through fermentation reactions' (ESTCP, 2004). This creates conditions conducive to anaerobic biodegradation of chlorinated solvents dissolved in groundwater. In some cases, organisms may need to be added (bioaugmentation), but only if the natural microbial population is incapable of performing the required transformations. This technique has emerged in recent years and advantages include complete mineralization of the contaminants *in situ* with little impact on infrastructure and relatively low cost compared to traditional methods (Löffler and Edwards, 2006).

The addition of organic substrate(s) increases the flux of acetate and hydrogen, which are the relevant direct electron donors for many reductive detoxification processes. To stimulate the desired microbial activity, adjustments of pH and redox conditions are feasible through addition of a base (e.g., bicarbonate or NaOH) or easily oxidizable organic carbon substrates, respectively (Löffler and Edwards, 2006). There are many organic substrates which can be naturally degraded and fermented in the subsurface that result in the generation of hydrogen. Examples of easily fermentable

organic substrates include alcohols, low-molecular-weight fatty acids (e.g., lactate), carbohydrates (e.g., sugars), vegetable oils, and plant debris (e.g., mulch). The substrates most commonly added for enhanced anaerobic bioremediation include lactate, molasses, hydrogen release compound (HRC®), and vegetable oils (Ernst 2009; Lee *et al*., 2000). Substrates used less frequently include ethanol, methanol, benzoate, butyrate, high - fructose corn syrup (HFCS), whey, bark mulch and compost, chitin, and gaseous hydrogen. Pilot-scale bioremediation field demonstration studies often rely on closed loop recirculation systems (Fig 1.4a).

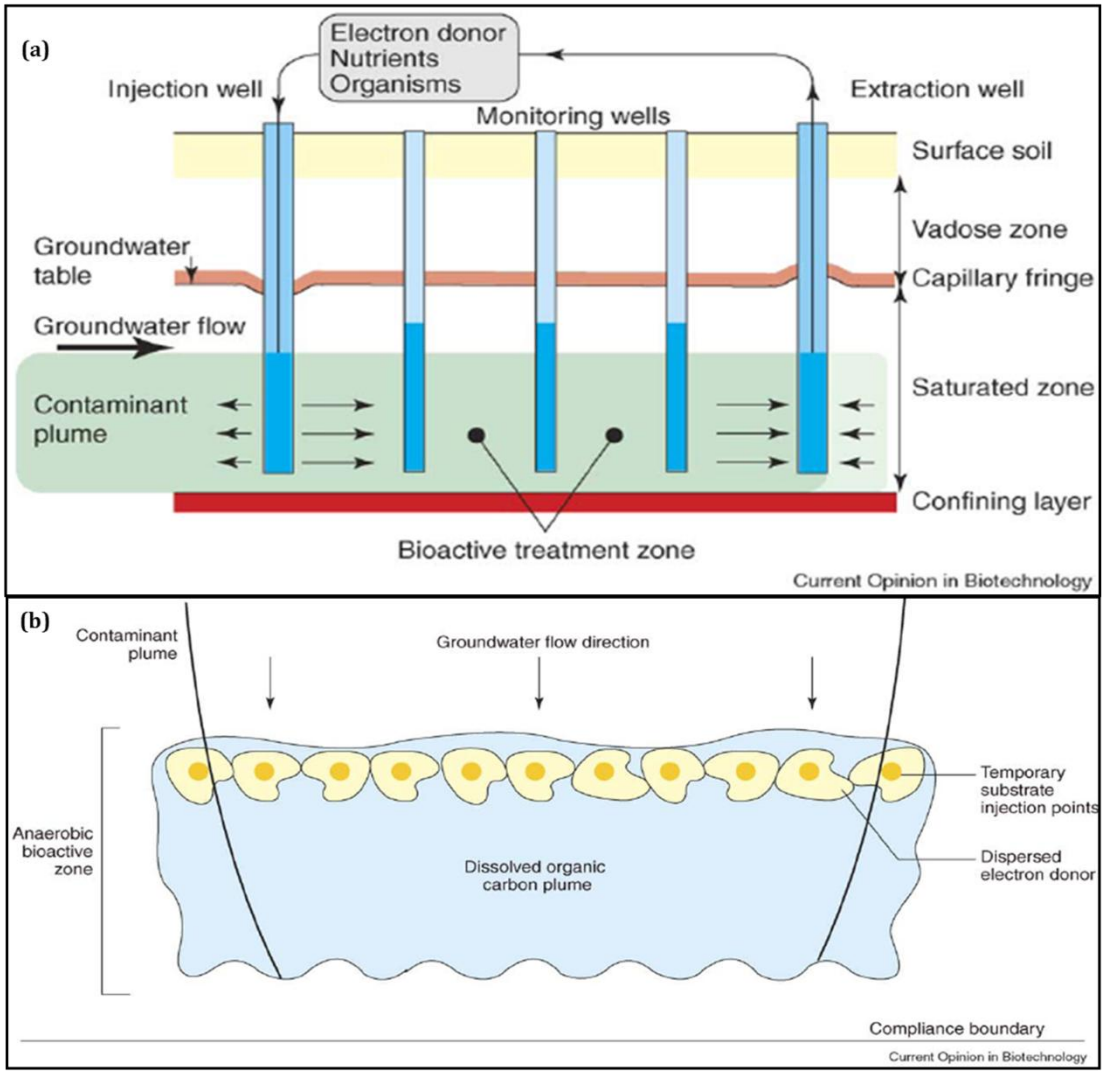


Figure 1.4: Delivery of electron donors via (a) closed loop recirculation system and; (b) biobarrier (adapted from Löffler and Edwards, 2006)

In a passive biobarrier (Fig 1.4b) a slowly dissolving and slowly fermentable substrates (e.g., vegetable oil, HRC®) are injected at numerous temporary injection

points in a line perpendicular to the direction of flow to intercept the plume. As the substrates slowly dissolves over several months and its fermentation increases the flux of electron donors (e.g., acetate and H_2) that support the reductive processes. In an active biobarrier, injection and extraction wells are used continuously or periodically which amend and recirculate readily fermentable substrates such as lactate (Löffler and Edwards, 2006).

1.2.4. Microbial Electric System: The Promising Future of Bioremediation

The extensive use of fossil fuels in recent years has triggered a global energy crisis. Major efforts are devoted to renewable bioenergy, and alternative electricity production methods. It has been known for many years that it is possible to generate electricity directly using bacteria to breakdown organic substrates (Allen and Bennetto, 1993; Bennetto, 1984). The recent energy crisis has reinvigorated interest in microbial electrical systems (MES) among academic researchers as a way to generate electric power or hydrogen from biomass without a net carbon emission into the ecosystem. A MES is a bioreactor that converts the chemical energy stored in the chemical bonds of organic compounds to electrical energy through catalytic reactions of microorganisms under anaerobic conditions (Du *et al*., 2007). MES can be used in wastewater treatment facilities to breakdown organic matters (Feng *et al*., 2008; Min *et al*., 2005; Oh and Logan, 2005). They have also been studied for applications as biosensors for pollutant analysis and*in situ* process monitoring and control (Chang *et al*., 2004, 2005).

One of the most promising applications of MES is bioremediation of chloroethene contaminated groundwater. At present, current engineered approaches for the bioremediation of chlorinated contaminants (both *in situ* or in on–site bioreactors) typically involve the addition of H_2 or H_2 generating organic substrates to stimulate the metabolism of reductive dechlorinating microorganisms (Löffler and Edwards, 2006). Some problems often associated with this approach are the extensive competition for the carbon source and H_2 between dechlorinators and other microorganisms (e.g. sulphate reducers, methanogens, homoacetogens), the accumulation in the subsurface of large amounts of fermentation products with the resulting deterioration of groundwater quality, possible aquifer clogging due to excessive biomass growth and even explosion hazards through excessive methane production (Aulenta *et al*., 2009a). A groundbreaking alternative to this approach is to

use insoluble electrodes to directly and selectively deliver electrons (instead of chemicals) via MES to dechlorinating communities growing as biofilms at the electrode surface (Aulenta *et al*., 2007, 2008, 2009a, 2009b; Lohner *et al*., 2009; Lovley, 2011). The main advantages resulting from the use of electrodes to stimulate biological reduction in the subsurface are (i) the delivery of electrons to microorganisms can be continuously monitored in terms of current and potential; and (ii) no chemicals need to be injected, which eliminates the need for transport, storage, dosing and post-treatment (Aulenta *et al*., 2009b). This would probably represent a clean, versatile and efficient way of *in situ* bioremediation.

MES can be designed in various ways depending upon the specific requirements. Most commonly used systems are single or double chamber MES. Single chamber MES offer simpler designs and cost savings. They typically possess only an anodic chamber without the requirement of aeration in the cathodic chamber (Park and Zeikus, 2003). On the other hand, a typical two chamber MES (Fig 1.5) has an anodic and cathodic chamber connected by a permeable electron membrane (PEM) or sometimes a salt bridge, to allow protons to move across to the cathode while blocking the diffusion of oxygen into the anode. They are currently used only in laboratories and contain a more controlled environment than single chamber MES (Du *et al*., 2007). In this study, two chamber MES were used.

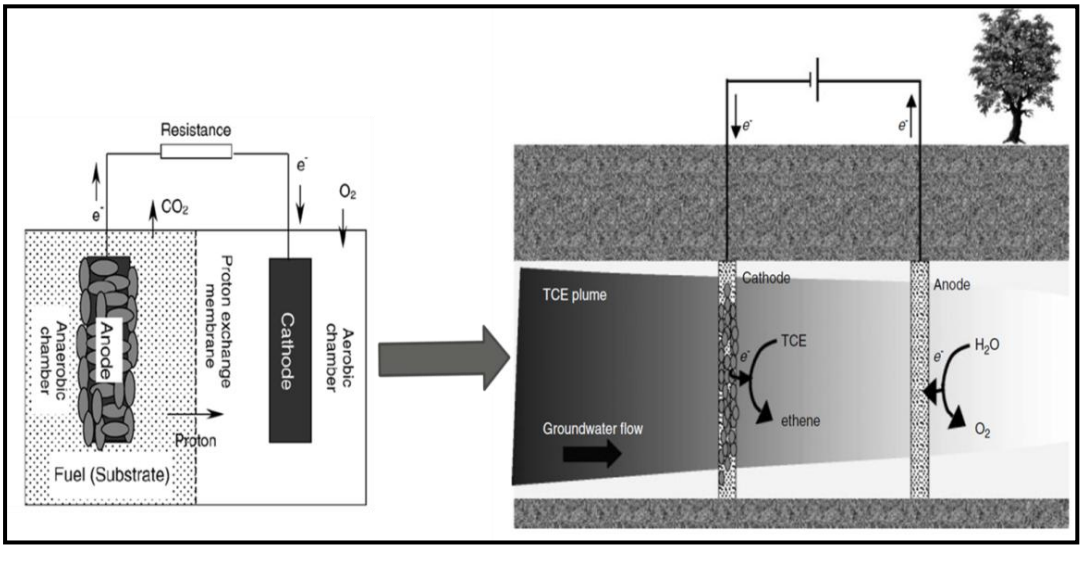


Figure 1.5: Schematic of typical two chamber MES and its potential for *in situ* treatment of PCE contaminated groundwater (adapted from Aulenta *et al*., 2009a; Du *et al*., 2007)

Microbes in the anodic compartment of two chamber MES oxidize added substrates and generate electrons and protons. Carbon dioxide is produced as an oxidized product; however there is no net carbon emission because carbon dioxide in the renewable biomass originally comes from the atmosphere in the photosynthesis process (Du *et al*., 2007). Unlike in a direct combustion process, the electrons are absorbed by the anode and are transported to the cathode through an external circuit. After crossing a PEM, the protons entre the cathodic chamber where they combine with oxygen to form water. Microbes in the anodic chamber extract electrons and protons in the dissimilative process of oxidizing organic substrates (Rabaey and Verstraete, 2005). Electric current generation is made possible by keeping microbes separated from oxygen or other end terminal acceptor other than the anode and this requires an anaerobic anodic chamber. Typical electrode reactions are shown below using acetate as an example substrate.

Anodic reaction: $CH_3COO^- + 2H_2O \rightarrow 2CO_2 + 7H^+ + 8e^-$ (1)

Cathodic reaction: $O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$ (2)

The overall reaction is the breakdown of the substrate to carbon dioxide and water with a concomitant production of electricity as a by-product. Many microorganisms from various sources like marine sediments, soil, wastewater, fresh water sediment and activated sludge possess the ability to transfer the electrons from the metabolism of organic matters to the anode (Bond and Lovley, 2003; Du *et al*., 2007; Liu *et al*., 2005; Niessen *et al*., 2006; Zhang *et al*., 2006). Pronounced enrichment of microorganisms from *Proteobacteria, Geobacteraceae, Desulfuromonas, Desulfitobacteriacea* and *Dehalococcoides* groups have been observed to possess the ability to bio-electrochemically convert PCE to ethane (Bond *et al*., 2002; Aulenta *et al*., 2008).

1.3 Chloroethene Contaminant Detoxification: The Microbiology

Bioremediation based on metabolic processes, in which the organisms benefit and derive energy for growth from contaminant transformation are generally preferable over fortuitous, co-metabolic processes (Rittmann and McCarty, 2001; Rittmann *et al.,* 2006). The discovery of microorganisms in the mid-1990s which gain energy from the process called, reductive dechlorination of chloroethene led to a turning point from a predominantly co-metabolic view of chloroethene biodegradation to the concept of chloroethenes serving as primary substrates for microbial metabolism (Sharma and

McCarty, 1996; Maymó-Gatell, 1997; Holliger *et al.,* 1998). Most of the chlorinated compounds have a synthetic origin and have not been in contact with microorganisms through evolutionary periods of time (Alvarej and Illman, 2006). As a result, chlorinated solvents are not frequently metabolized by indigenous organisms which are more labile in the environment. Nevertheless, several biotransformation mechanisms have been identified that could be exploited for site remediation. The main biotransformation pathways (Fig 1.6a) for chlorinated ethenes are explained below:

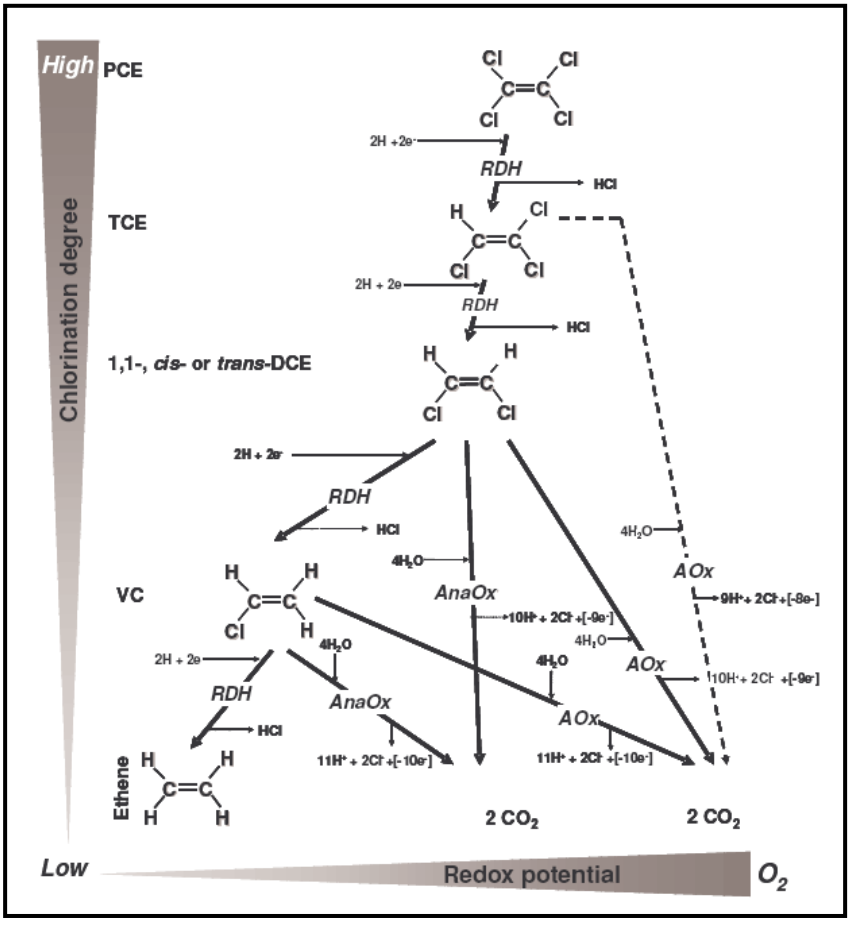


Figure 1.6a: Pathways for the degradation of chlorinated ethane (adapted from Imfeld, 2009). The different pathways are indicated as follows: RDH-reductive dechlorination; AnaOx-anaerobic oxidation; AOx: aerobic oxidation.

1] ***Aerobic Oxidation*****:**In this pathway, the pollutant serves as the primary substrate for growth. Oxygen (O_2) serves as the electron acceptor and is supplied by air sparging, bioventing, H_2O_2 or oxygen-releasing compounds. Since chlorinated compounds are volatile, some volatilization losses may occur with air sparging or bioventing. Aerobic

metabolism is limited to the less chlorinated compounds such as chloromethane, dichloromethane, chloroethane, 1, 2-DCA and VC.

2] *Aerobic Cometabolism*: In addition to providing oxygen and nutrients, this approach requires that an electron donor also be added. In general, the fewer the number of Cl atoms, the better the cometabolic process will work. Toluene, methane, propane, butane and phenol have been used as primary substrates to support such cometabolic transformation.

3] *Anaerobic Oxidation*: In this mechanism, the chlorinated organic serves as an electron donor for growth. Only a few chlorinated aliphatics are amenable to this treatment (i.e., dichloromethane; 1, 2-dichloroethene; *cis*- and *trans*-DCE and VC). Nitrate and sulfate can serve as electron acceptors in such cases and dichloromethane can also be fermented. Nevertheless, degradation rates are relatively slow and this process has not yet been demonstrated or exploited for site remediation.

4] *Anaerobic Reductive Dechlorination*: In this process, the compound serves as an electron acceptor (Fig 1.6b). All chlorinated aliphatics are susceptible to anaerobic, *cometabolic*, reductive dechlorination. This requires a suitable electron donor and it works mainly under sulfate-reducing or methanogenic conditions. An exception is carbon tetrachloride, which can also be dechlorinated under denitrifying conditions.

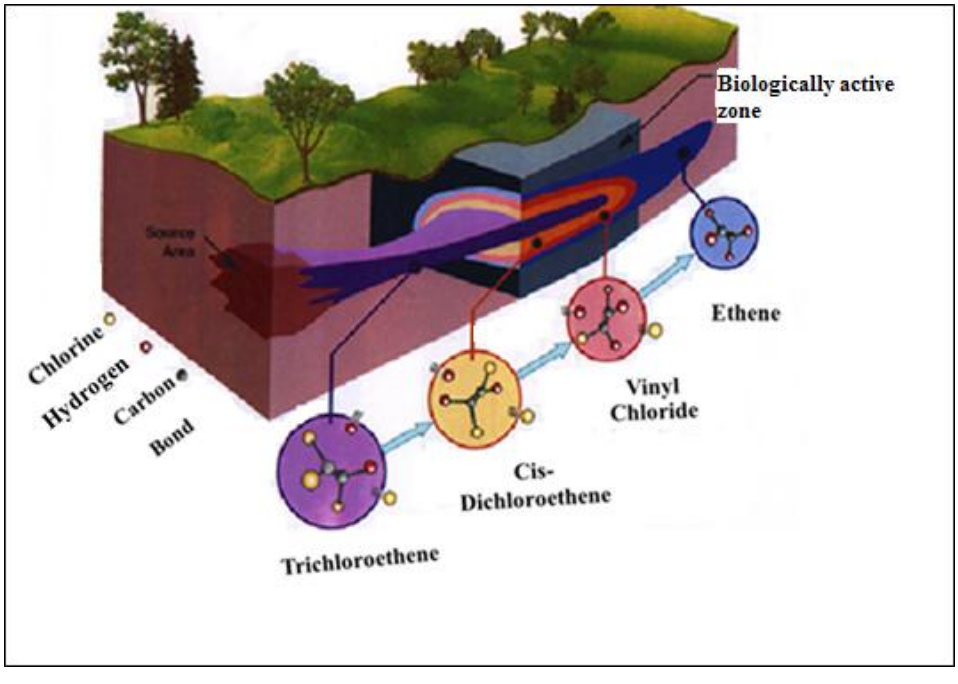


Figure 1.6b: Anaerobic reductive dechlorination of chloroethene plume (adapted from ITRC, 1999)

Much current research activity is focused on dehalorespiration, where PCE, TCE, DCE and VC serve as terminal electron acceptors in support of microorganism growth. There are two reductive dehalogenation mechanisms. The first is hydrogenolysis or hydrodehalogenation, which involves replacing halogen atoms such as Cl, Br and F by a hydrogen atom. This is illustrated in Figure 1.6b for the stepwise reduction of TCE via DCE to VC and ultimately to ethene. The other reductive dehalogenation mechanisms are dihaloelimination, which involves the simultaneous removal of two halogen atoms after two electrons are transferred. Reductive dechlorination generally decreases the toxicity and enhances the solubility (bioavailability) of the pollutant, but there are exceptions where the toxicity can be accentuated (e.g. TCE reduction to VC). Reductive dechlorination is often a cometabolic reaction since the microorganisms that catalyze it cannot harvest the energy released by the redox process. Recently however, many strains have been found that can utilize PCE and TCE as a terminal electron acceptor during respiration using H_2, formate, acetate and pyruvate as electron donor. This process is known as halorespiration and it can be mediated by species such as *Desulfomonile tiedjei*, *Dehalobacter restrictus*, *Desulfitobacterium* and *Dehalococcoides ethenogenes* (Maymó-Gatell, 1997; Holliger *et al.,* 1998).

PCE and TCE readily undergo reductive dechlorination but the efficiency of the reaction decreases with decreasing chlorination degree. Some dechlorinators sequentially dechlorinate PCE to TCE, some preferentially to cis-DCE and some to VC. However, the conversion of DCE and VC as electron acceptor to non-toxic ethene is principally mediated by *Dehalococcoides* species-affiliated bacteria. Conversely, the tendency for aerobic oxidation of chlorinated ethenes increases with decreasing number of chlorine atoms of the molecule. Both metabolic and cometabolic oxidation of lower chlorinated ethenes have been reported. However, mineralization of DCE and VC tends to increase with higher reduction potential.

1.3.1. Dehalorespiring Bacteria

The evolutionary history of dehalorespiring organisms is of considerable interest. Many dehalorespirers are gram-positive bacteria that cluster with the *Clostridium-Bacillus* subphylum, while the others lie in the ε and γ branches of Proteobacteria (Holliger *et al*., 1999). On the other hand, *D. ethenogenes* is more phylogenetically distant from the other dehalorespiring bacteria. *D. ethenogenes* is a bacterium possessing a unique archeaeon-like cell wall and its precise relationship to other bacteria is uncertain, though a phylogenetic analysis by Magnuson *et al*. (2000)

suggests that it lies within the green non-sulfur division of bacteria. They are Gram-positive, coccoid cells closely related to a member of the *Chloreflexi* phylum (green non-sulfur bacteria) possess diverse dehalogenation ability, grow robustly in mixed cultures and are present globally in microbial populations (Ernst, 2009). The phylogenetic affiliation between different dehalorespiring bacteria is illustrated in Figure 1.7.

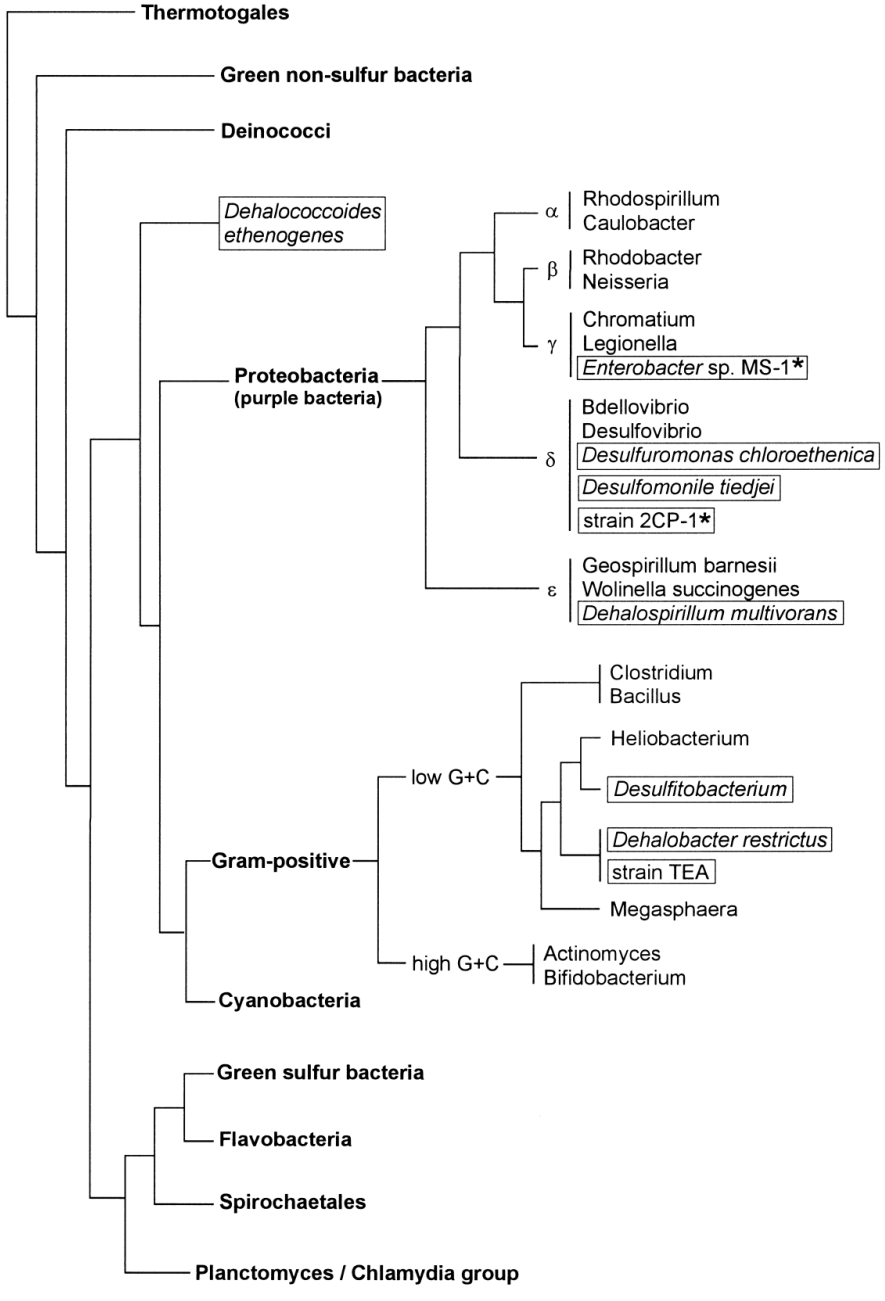


Figure 1.7: Phylogenetic affiliations, based on analysis of 16S rRNA gene sequences of reductive dechlorinating bacteria (framed). The facultative anaerobes are marked with an asterisk.

All of the known dehalorespiring microorganisms are bacteria and their dehalogenation capacities are highly strain-dependent (Futagami *et al.,* 2008). Anaerobic bacteria that grow with chloroethenes as final electron acceptors include *Dehalobacter, Dehalococcoides, Desulfitobacterium, Desulfuromonas, Geaobacter* and *Sulfurospirillum*. The well-studied organisms *Sulfurospirillum multivorans* and *Dehalobacter restrictus* PER-K23 dechlorinate PCE to *cis*-DCE (Holliger *et al.,* 1998). *S. multivorans* is a Gram-negative anaerobic spirilum, which belongs to ε-subdivision of proteobacteria. The *Dehalobacter* genus belongs to *Firmicutes* and is allied with the genus *Desulfitobacterium;* however dehalorespiration is the sole system of energy production in the genus *Dehalobacter.* Although the above mentioned strains can utilize PCE or TCE as electron acceptors, they cannot completely dechlorinate cis-DCE or VC to ethene.

One genus of particular interest for such bioremediation is '*Dehalococcoides*' *(Dhc),* obligate anaerobes that cannot use oxygen, nitrate or sulfate as electron acceptors. *Dhc* species is of particular interest as members of the genus are the only known bacteria to date capable of the complete reduction of chlorinated ethenes (PCE and TCE) to ethene (Fig 1.8). *D. ethenogenes* 195 and *Dhc* sp. FL2 respectively dechlorinate PCE and TCE to ethene (Maymó-Gatell, 1997; Löffler *et al.,* 2000; He *et al.,* 2005). However, these two strains are unable to use VC as an electron acceptor. Thus, the slow dechlorination of VC to ethene is considered to proceed in a cometabolic fashion uncoupled to energy production (Maymó-Gatell, 1999). On the other hand, till to date four other *Dhc* strains BAV1, VS, GT and KB1/VC have been reported to use VC as the electron acceptor in their dehalorespiration and can dechlorinate VC to ethene efficiently (Fig. 1.8) (He *et al.,* 2003a; Cupples *et al.,* 2003; Müller *et al.,* 2004; Duhamel *et al.,* 2004; Sung *et al.,* 2006; Wei and Finneran, 2013). Among these *Dhc* strains GT and KB1/VC can dechlorinate TCE. In contrast, *Dhc* sp. CBDB1 has a different dechlorination spectrum. For instance, strain CBDB1 dechlorinates chlorobenzenes and dioxins (Adrain *et al.,* 2000). In the genus *Dhc*, dehalorespiration is solely an energy preservation system. These isolates exhibit a metabolic specialization, using only H_2 as an electron donor and chlorinated compounds as electron acceptors to support growth.

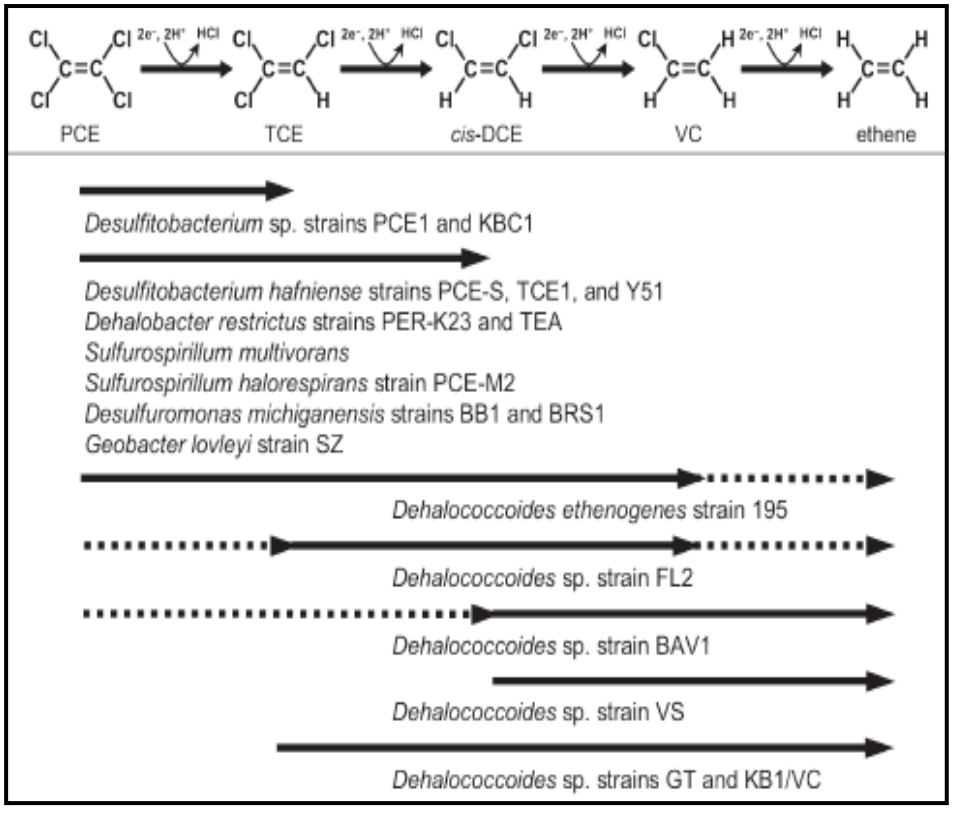


Figure 1.8: Reductive dechlorination pathways for chloroethenes by dehalorespiring bacteria (adapted from Futagami *et al.,* 2008)

1.3.2. Reductive Dehalogenases (RDases)

Reductive dechlorination reactions are catalyzed by the reductive dehalogenases (RDases). RDases are a class of enzymes found mostly in *Dhc* species and other dechlorinating organisms which catalyze the following reaction (Futagami *et al.,* 2008):

$$\mathbf{R—Cl + 2[H] \rightarrow R—H + H^+ + Cl^-}$$

Figure 1.9 illustrates some of the interactions associated with RDases on the cellular membrane in *Dhc* species. Hydrogenases are a crucial part of the reaction mechanism because they supply electrons to the reaction from H_2. In anoxic environments, the above reaction is thermodynamically favorable and chlorinated compounds can act as electron acceptors. However, it has been observed previously that hydrogenases are oxygen sensitive, whereas RDase may retain some activity following exposure to oxygen (Jaychandran *et al.*, 2004). 'Dehalorespiration' is defined as the process whereby energy from the above reaction is conserved and coupled to ATP synthesis in a chemisomotic mechanism (Holliger *et al.,* 1998). Dechlorinating organisms infer energy from the process and in many cases dechlorination activity can be linked to growth

(Duhamel *et al.,* 2004; He *et al.,* 2003b; Adrian *et al.,* 2007). Most RDases contain a twin arginine translocation (TAT) sequence that is involved with translocating the folded protein across the cytoplasmic membrane, two Fe-S clusters and a corronoid co-factor derived vitamin B_{12} (Futagami *et al.,* 2008). The latter appears to be crucial part of the reaction mechanism, as the addition of B_{12} has been shown to enhance growth and dechlorination rates (He *et al.,* 2007). There are a number of cases documenting the orientation of the enzyme to be facing the cell exterior (Nijenhuis and Zinder, 2005; Magnuson *et al.,* 2000; Hölscher *et al.,* 2003), but also evidence that this is not always the case (John *et al.,* 2006).

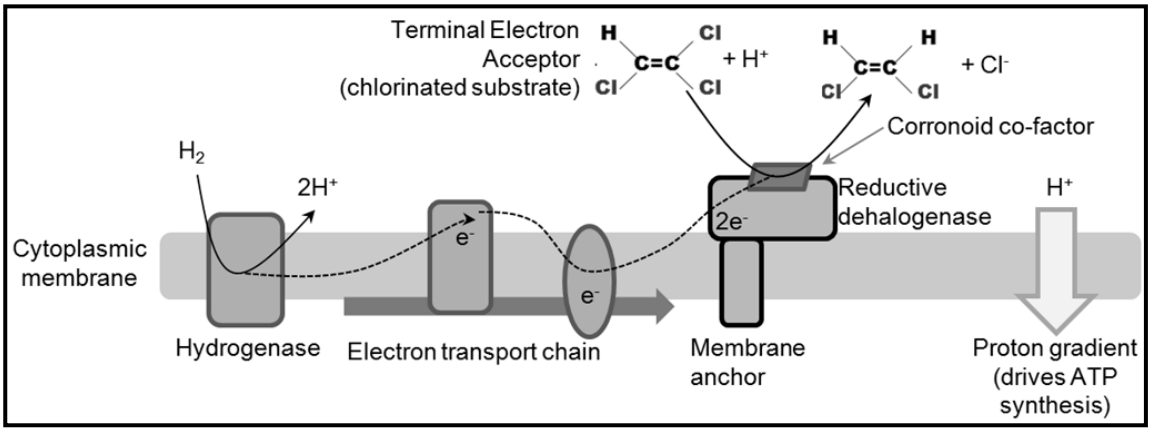


Figure 1.9: Schematic representation of dehalorespiration involving RDases on the cytoplasmic membrane in *Dhc* species (adapted from Chan, 2010)**.** The hydrogenses spilts hydrogen into protons, driving the proton gradient that is utilized for ATP synthesis; and electrons (e^-), which are carried through the electron transport chain to the dechlorination reaction, where the chlorinated substrate acts as a terminal electron acceptor. Reactions are proposed to take place with a corronoid co-factor and 2 Fe-S clusters.

To overcome the limitations of the 16S rRNA gene analysis, genes that correlate directly with dechlorination activity are being sought. Specific functions have been assigned to few *Dhc* RDases genes (Fig. 1.6) and a major task is to elucidate the substrate range of each functional RDases represented on the *Dhc* genomes (Behrens et al., 2008). This is pivotal for designing a comprehensive suite of molecular tools for monitoring abundance and expression of individual RDase genes and predicting dechlorination activity. The range of sequence variation between RDases is provided in Corrigenda. A TCE dehalogenase, encoded by the *tceA* gene was first discovered in *Dehalococcoides ethenogenes* strain 195, and is thought to be co- transcribed with the *tceB* gene encoding a small membrane anchor (Magnuson *et al.,* 2000). This gene has a

wide distribution among a range of environmental samples and those which contain *tceA* can degrade TCE, although not all TCE-degrading organisms contain *tceA* (Krajmalnik-Brown *et al.,* 2007). Two VC-RDases have been discovered, originating from two different isolates - *vcrA* from strain VS and *bvcA* from strain BAV1 (Müller *et al.,* 2004; Krajmalnik-Brown *et al.,* 2004). *Dhc* sp. strain VS carries a single copy of the *vcrA* gene and also contains a gene producing an RDase that is homologous to the *pceA* in strain 195 whose functions are different, as *vcr*A encodes to transform DCE, VC to ethene and *pce*A transforms PCE to TCE cometabolically. These are believed to be the distinguishing feature of *Dhc* from other dechlorinating organisms.

Table 1.6: *Dhc* RDase genes with assigned function

***Dhc* Strains**	**Known expression of RDase genes**	**Reaction Catalyzed**	**Molecular Mass (kDa)**
195	*pceA,* *tceA*	PCE → TCE TCE → VC	50,800 57,700
VS	*vcrA*	DCEs , VC → ethene	53,100
BAV1	*bvcA*	VC → ethene	52,800
FL2	*tceA*	TCE → VC	-
CBDB1	*pceA*	None, respires other chlorinated compounds	-
GT	**-**	TCE, cDCE, VC	-

1.4 Cleaning Up with Genomics: Applying Molecular Biological Tools to Bioremediation

Molecular biological tools (MBTs) are defined as tools that target biomarkers (e.g., specific nucleic acid sequences, peptides, proteins or lipids) to provide information about organisms and processes relevant to the assessment and/or remediation of contaminants (Stroo *et al.,* 2006). Our current knowledge of key biological processes in the subsurface remains insufficient, making it difficult to interpret MBT data or develop

the required biomarkers for bioremediation purpose (Stroo *et al*., 2006). More fundamental research is therefore recommended, specifically to identify and correlate biomarkers to evaluate community structure and assess the total degradative potential of a microbial population. However, the progress in molecular biology has been extraordinarily rapid from culture dependent pre-genomics practice to current 16S rRNA culture independent techniques such as dot blot, real time polymerase chain reaction (RT-PCR), phospholipid fatty acid (PLFA) analysis, enzyme probes, fluorescence *in situ* hybridization (FISH) and compound-specific isotope analyses (CSIA). Other MBTs such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and clone libraries are useful research tools that can provide important advances in the understanding of biodegradation processes.

Since the early 2000s, molecular techniques with higher throughput have been increasingly used to directly access the entire pool of environmental microbes without the limitations associated with lab-based cultivation of microbial strains. One of these techniques, 'metagenomics' is gaining popularity as it holds great promise for bioremediation (Gabor *et al*., 2007). Metagenomics (also known as ecological genomics, community genomics or environmental genomics) is a discipline that uses genomic methods to analyse natural ecological communities, namely the collective genomes in an environmental community (Riesenfeld *et al*., 2004). It allows the study of environmental communities in their whole complexity, which includes interactions between the community members. Above all, metagenomics has the potential to substantially enhance the discovery and characterization of bacterial and fungal metabolic pathways involved in the degradation of hazardous pollutants, many of which are still unknown. The wealth of data produced from metagenomic studies will help (i) identify functional traits in microbial communities that confer robustness to pollution and/or biodegradation capability, therefore allowing distinction between contaminated sites where natural attenuation is sufficient from sites where active bioremediation is necessary; (ii) design efficient monitoring tools for environmental damage and restoration; and (iii) expand gene catalogs for the design of novel biocatalysts using direct evolution approaches.

However, metagenomics is only about a decade old and its implementation in the context of bioremediation is even more recent. Hence, problems associated with this technique need to be solved before it can become commonplace in research

laboratories (George *et al*., 2010). The biggest challenges of metagenomics are storage, assembly and analysis of the 'data storm' generated through this approach (Uhlik *et al*., 2013). Moreover, it is envisioned that coupling genomic techniques to other 'meta-omics' such as bioinformatics, environmental genomics, transcriptomics, metabolomics and proteomics will be necessary to get a comprehensive understanding of which community features are associated with successful biodegradation.

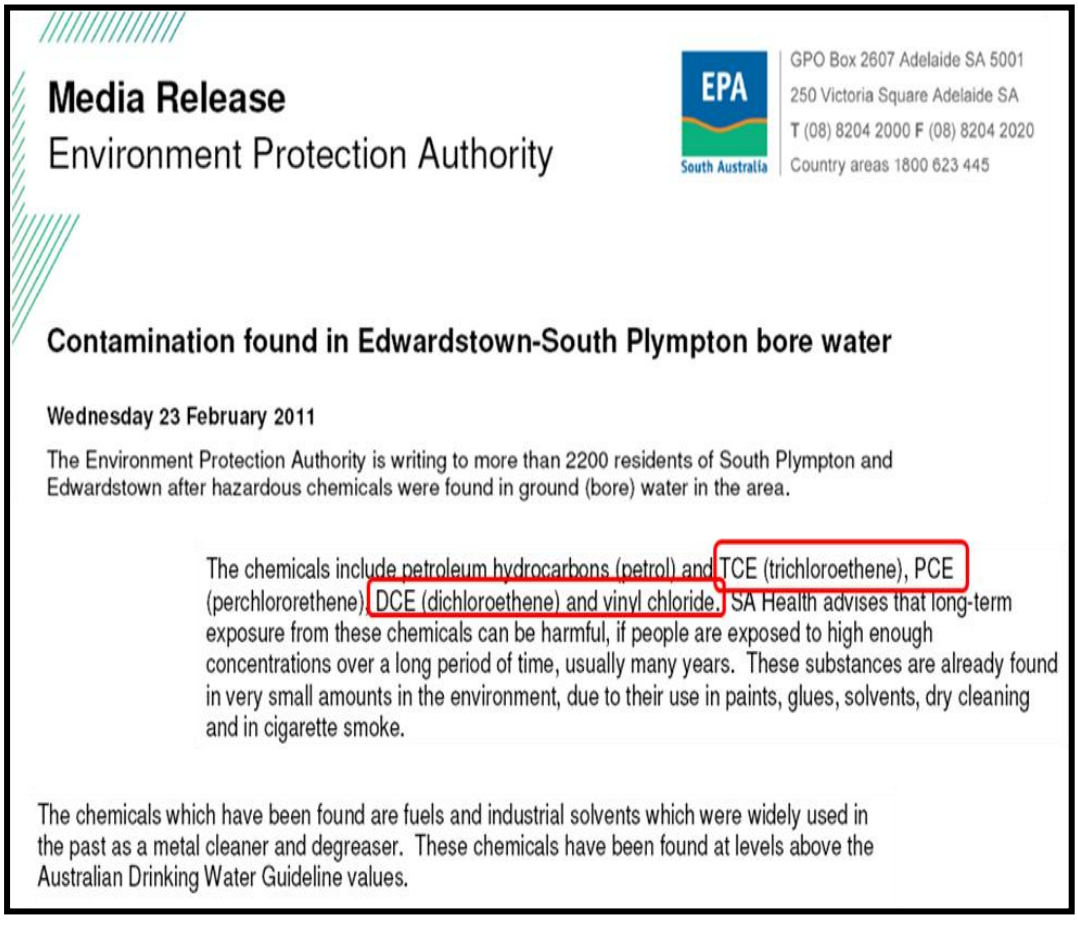
In this research project, various MBTs such as PCR based DGGE, quantitative PCR (qPCR), Sequencing are applied to understand microbial community structure, functional organization, dynamics and their performance during anaerobic degradation of PCE to unravel microbial interactions between community members.

1.5 Project Outline and Objectives

Increasing incidences of aquifer contamination by chloroethene solvents is a current concern in Australia. The news headline 'Contamination found in Edwardstown - South Plympton bore water' dated 23 February, 2011 in South Australia's leading daily newspaper 'The Advertiser' (http://www.adelaidenow.com.au/water-warning-delay-risks-public-safety/story-e6frea6u-1226011067372)drew the attention of environmentalists (Box 1.1).

As described earlier in the *Section 1.2*, due to the adverse effects of chloroethene contaminants to environmental and human well-being, it is of utmost importance to remediate contaminated sites effectively and efficiently. In this project, the issue of chloroethene contamination at Maidstone, Victoria is taken as a case study.

BOX 1.1: EPA media release



Media Release
Environment Protection Authority

EPA South Australia

GPO Box 2607 Adelaide SA 5001
250 Victoria Square Adelaide SA
T (08) 8204 2000 F (08) 8204 2020
Country areas 1800 623 445

Contamination found in Edwardstown-South Plympton bore water

Wednesday 23 February 2011

The Environment Protection Authority is writing to more than 2200 residents of South Plympton and Edwardstown after hazardous chemicals were found in ground (bore) water in the area.

The chemicals include petroleum hydrocarbons (petrol) and TCE (trichloroethene), PCE (perchlororethene), DCE (dichloroethene) and vinyl chloride. SA Health advises that long-term exposure from these chemicals can be harmful, if people are exposed to high enough concentrations over a long period of time, usually many years. These substances are already found in very small amounts in the environment, due to their use in paints, glues, solvents, dry cleaning and in cigarette smoke.

The chemicals which have been found are fuels and industrial solvents which were widely used in the past as a metal cleaner and degreaser. These chemicals have been found at levels above the Australian Drinking Water Guideline values.

1.5.1. The Site History

Since 1935, the study site located in Maidstone, Victoria in Australia (Fig. 1. 10) has a history of commercial industrial activities. The latest commercial activity at the site was foam manufacturing. Initial investigations identified chemicals of concern including PCE (up to 30 mg L^{-1}), TCE (> 10 mg L^{-1}), DCE in subsurface waters. Activities associated with storage and use of fuels, thinners for cleaning and adhesive chemicals on site, haphazard disposal of surfactants used within the foam hall to waste sump along with other waste chemicals has led to serious groundwater contamination over 3.4 ha. Both shallow (from 5 to 12 m bgs) and deep (from 15 to 40m bgs) aquifers underlying the industrial site were impacted by the contamination. Groundwater and site characterization data confirmed that spilled contaminants migrated through the aquifers where they formed DNAPL pools acting as long- term sources of contamination. Further site details are restricted due to confidential agreements.

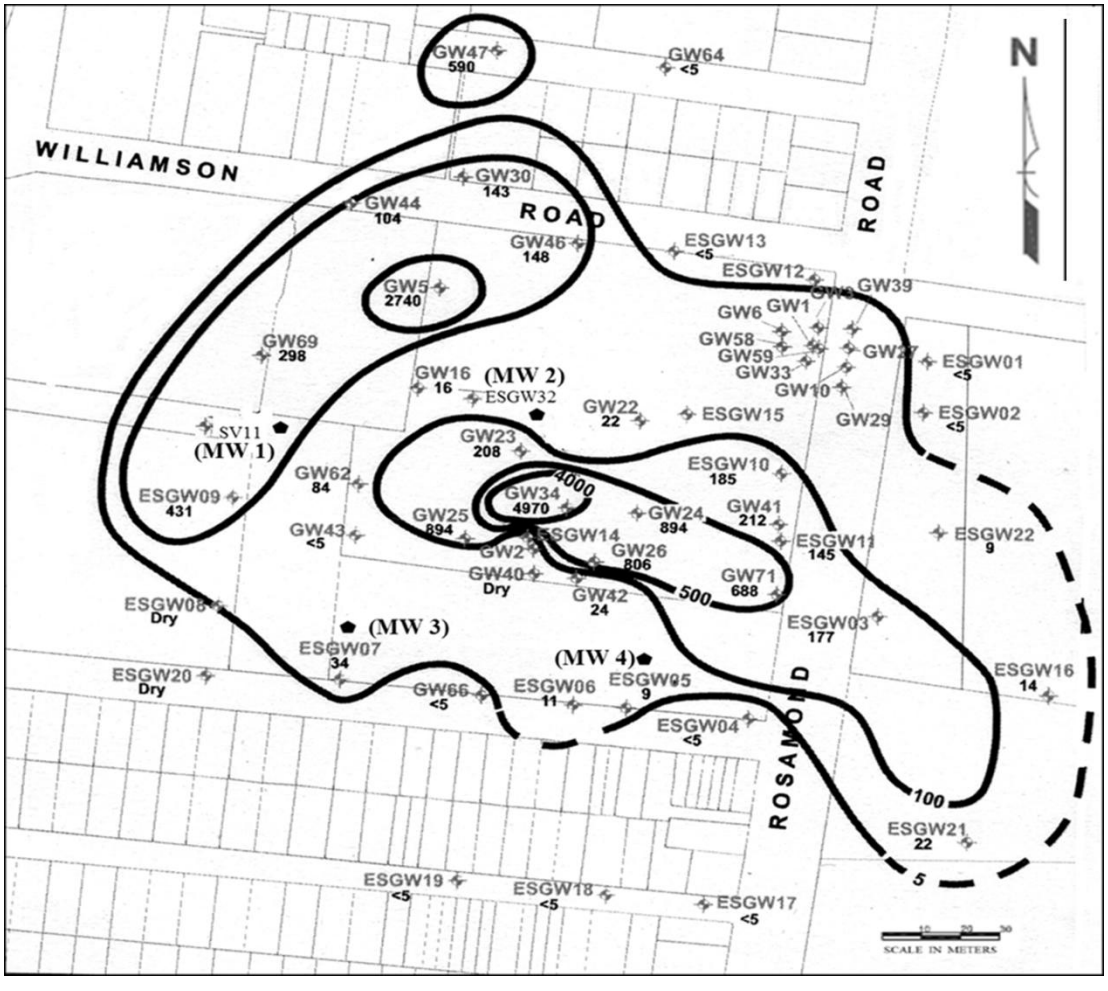


Fig. 1.10: Location of monitoring wells (MWs) at PCE-contaminated study site in Victoria, Australia (symbol⬟ indicates MWs selected for this study while contours designate PCE plume at 15 m bgs).

1.5.2. Aims of the Study

From the literature provided in this chapter, it is evident that significant *in situ* and *ex situ* research on the use of classic dehalorespiring bacteria especially *Dhc* and *Geobateriacea* for the bioremediation of chloroethene contaminated aquifers has been carried out. Comparatively, limited research has been done to shed the light on the other dechlorinating microorganisms which might possess the similar traits of complete dechlorination as shown by *Dhc* and *Geobacteriacea*. For successful commercial bioremediation, besides analyzing the contaminant degradation pattern, the better understanding of the total microbial community which carries degradation is

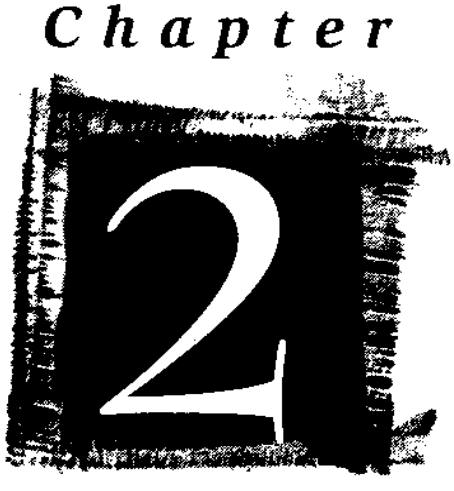
essential to assess and predict remediation progress. Especially, in the scenario as in this study, where strict EPA regulations preclude the injection of outsourced microbial consortia into the natural groundwater habitat, wise decision needs to be taken based on the site-specific characters. Hence, to fill the gap between current knowledge in the field of groundwater bioremediation the aims of this project were:

1] To study the functional organization and dynamics of indigenous non-*Dhc* dechlorinating community within chloroethene enrichment cultures to understand microbial behavior in correspondence with chloroethene removal;

2] To assess the applicability of three different bioremediation treatments such as biostimulation, biostimulation plus bioaugmentation and monitored natural attentuation by conducting laboratory scale comparative assays;

3] To assess *in situ* chloroethene degradation pattern and microbial community response by conducting commercial clean-up at a chloroethene contaminated site;

4] To explore microbial electric systems as an alternative technique to overcome the drawbacks associated with the current chloroethene remediation practices.



Materials and Methods

CHAPTER 2: Materials and Methods

2.1 Materials

All chlorinated ethenes, ethene and other chemicals for enrichment culture preparation and analytical measurements were purchased from Sigma-Aldrich (NSW, Australia) with minimum purity of 99.5%. All gases were ordered from BOC (SA, Australia).

2.2 Groundwater sample collection:

The protocol for groundwater (GW) sample collection from chloroethene contaminated site in Victoria, Australia is described below. The guidelines suggested by Ritalahti *et al*. (2010) were followed for sampling GW with few modifications depending upon site conditions. Aseptic techniques were employed to the extent possible while handling GW destined for laboratory analysis.

Before sampling:

The following blanks were prepared before sampling:

a) **Equipment blank:** One equipment blank (1L) was taken prior to the commencement of field work, from each set of sampling equipment to be used for that day.

b) **Trip blank:** A trip blank was required to accompany each volatile sample shipment. These blanks were prepared in the laboratory by filling a volatile organic analysis (VOA) bottle with distilled/deionized water (40 mL). To prepare a trip blank, the filtered water was taken to the well head at the site that was most likely to contain a dense microbial population and the previously prepared water was poured in to a plastic bottle (1L) just as would be done for any other sample. The bottle was sealed, labeled and stored on ice and shipped to the laboratory along with other samples.

1. GW samples were obtained using a dedicated pump or pump tubing, freshly installed pump tubing, a disposable pump that has not been used in another well or a new clean bailer and line.
2. Prior to sampling groundwater, MWs were purged using a low flow (100-500 $mLmin^{-1}$) technique (Puls *et al*., 1996).

3. The pump rate and the time at which the pump was started to determine the purge volume and the time required to reach stable parameters were recorded.
4. To ensure representative sampling, three water volumes of the groundwater MWs were pumped and discarded later. A minimum of one casing volume was purged before sample was taken.

During sample collection:

5. Groundwater samples from MWs below ground surface were collected using a submergible peristaltic pump (Waterra Pumps Ltd., Mississauga, ON) (Major *et al*., 2002).
6. A flow-through cell (YSI 556 Handheld Multi parameter Instrument, www.ysi.com) was connected to the tubing of the selected pump. The sample start time and geochemical parameters like pH, temperature, oxidation-reduction potential, specific conductance, dissolved oxygen and turbidity were recorded (Schaefer *et al*., 2010). The effluent from the pump was used to determine the concentration of ferrous iron, hydrogen sulphide and alkalinity and assessed using colorimetric field test kits model K-6010D, Hach kit model HS–C and Hach kit model AL-AP-MG-L respectively as per the manufacturer's instructions.
7. When geochemical parameters stabilized (i.e. constant readings were obtained), the flow–through cell was disconnected and samples were collected consecutively without flow interruption.
8. A polyethylene disposable bailer was lowered into the well to the midpoint of the screen and the bailer was moved up and down in the water column to surge the well.
9. While continuing to surge the well with the bailer, the flow-through cell was reconnected and the field parameters for pH, temperature, oxidation-reduction potential, specific conductance, dissolved oxygen and turbidity were recorded. The well was surged with steady motion avoiding rigorous mixing of sediment from the bottom of the well.
10. The GW was passed into N_2–flushed sterile high density polyethylene Nalgene bottles (Thermo Fisher Scientific Australia, NSW, Australia) using Teflon tubes attached to barbed bulkhead on screw caps with a positive meniscus to exclude air (minimal headspace).

11. After filling the bottles they were packed with bubble wrap and plastic bags to avoid leakage of samples during transport. Sample bottles were stored under N_2 atmosphere on ice for overnight transport to analytical laboratory.

The Maidstone study site characteristics data from where groundwater samples were collected for experimental chapters 3, 4 and 6 is presented in below Table 1.7.

Table 1.7 Field chemical characteristics of groundwater samples at the time of collection from Maidstone study site.

Selected Monitoring Wells	Temp (°C)	pH	Eh (mV)	Dissolved oxygen (ppm)	Alkalinity (ppm)	EC ($uScm^{-1}$)	Initial PCE concentration ($\mu g\ l^{-1}$)
SV 11 (MW 1)	19.8	6.90	184.9	10.12	960.0	12698	146.0
ESGW 32 (MW 2)	19.1	6.93	247.4	15.32	880.0	12665	3540.0
ESGW 07 (MW 3)	18.2	7.29	160.6	7.71	820.0	17214	130.0
ESGW 05 (MW 4)	18.0	7.68	110.3	1.16	260.0	19006	5.0

2.3 Anoxic mineral media preparation:

Anoxic mineral salt medium and microcosms were set up as per ATCC guidelines (American Type Culture Collection; www.atcc.org) and Löffler *et al.* (2005). The *Dhc* strains BAV1 (*Dehalococcoides* sp. ATCC® BAA-2100™), FL2 (*Dehalococcoides* sp. ATCC® BAA-2098™) and GT (*Dehalococcoides* sp. ATCC® BAA-2099™) were outsourced from the ATCC library for bioaugmentation experiment.

1] Preparation of MOPS buffered medium	
Salt Solution	20 mL
Trace element solution	1 mL
Se/Wo solution	1 mL
Resazurin solution	0.25 mL
Sodium acetate	0.41 g
Distilled water, bring volume up to 1000 mL.	
Medium was boiled at 100°C for 20 mins and cooled down to room temperature under the stream of N_2 and then following components were added:	
Na_2S	0.2 mmoL
L-cysteine	0.2 mmoL
2] The medium (75 mL) was dispensed into serum bottles (125 mL) under the flush with N_2. The bottles were closed without allowing air to enter and the stoppers secured with aluminum crimps. **3]** The bottles were autoclaved in an inverted position after the pink medium turned clear (i.e. the redox indicator resazurin was reduced). The bottles were removed from the autoclave, allowed to cool down to room temperature and the following stock solutions added.	
4] Addition of Stock Solutions	
$NaHCO_3$	0.1 mL/100 mL of medium
MOPS	2 mL/100 mL of medium
Ti(III)NTA	2 mL/100 mL of medium
3-vitamin solution	0.5 mL/100 mL of medium
6-vitamin solution	1 mL/100 mL of medium
Vitamin B12 solution	0.1 mL/100 mL of medium
5] Injection of substrates and *Dhc* strains: BAA-2098, strain FL2: 5 µL TCE/100 mL of medium. BAA-2099, strain GT: 5 µL TCE/100 mL of medium. BAA-2100, strain BAV1: 5 µL cis-DCE/100 mL of medium	

6. Groundwater was added as an inoculum (20 mL) using a gas tight syringe.
7. The headspace was filled with H_2 (2 to 20% in the headspace) with a gas tight syringe at a low partial pressure of 9 kPa to 30 kPa (5 to 10% of the headspace volume of the serum bottle).
8. All bottles were incubated statically upside down in an anaerobic glove box at room temperature (24°C–30°C) in the dark without shaking.

Notes:

- All vitamins and electron donors were added from a neutralized, anoxic, sterilized stock solution.
- All chlorinated compounds were added from saturated, anoxic, aqueous stock solution.
- To minimize the contact of the inoculums with the air present in the syringes during transfers, the syringes were reduced with a freshly prepared, filter sterilized 0.5 mM aqueous sulphide solution. This solution was kept in the syringe barrel for 5 to 10 min prior to use.

***Dhc* strains propagation procedure as per ATCC guidelines:**

1. 24 h prior to inoculation all components were placed in the anaerobic chamber.
2. The frozen vials of bacterial strains were placed in the anaerobic chamber and allowed to thaw. Using a gas tight syringe the entire contents of the vial were transferred into a single serum bottle.
3. The serum bottles were incubated at 24°C–30°C.
4. Headspace was maintained with H_2:N_2 (5:95%). The H_2 was kept at 2 to 20% in the headspace and added by syringe.

Stock solutions	
$NaHCO_3$	1M autoclaved
MOPS	1M pH adjusted to 7.45, filter sterilized
Ti(III)NTA	25mM, filter sterilized
Resazurin solution	
Resazurin	1 g
Distilled water	1.0 L
Salt solution	
NaCl	50.0 g
$MgCl_2.6H_2O$	20.5 g
NH_4Cl	13.5 g

KCl	26.0 g
$CaCl_2.2H_2O$	0.75 g
KH_2PO_4	10.0 g
Distilled water 1 L and stored at room temperature	
Trace element solution:	
HCl (25% w/w)	10 mL
$FeCl_2.4H_2O$	1.5 g
H_3BO_3	6.0 mg
$CoCl_2.6H_2O$	190.0 mg
$MnCl_2.4H_2O$	100.0 mg
$Na_2MoO_4.2H_2O$	36.0 mg
$ZnCl_2$	70.0 mg
$NiCl_2.6H_2O$	24.0 mg
$CuCl_2.2H_2O$	2.0 mg
Distilled water, 1 L and stored at room temperature in the dark.	
Se/Wo solution	
NaOH	0.5 g
$Na_2SeO_3.5H_2O$	6.0 mg
$Na_2WO_4.2H_2O$	8.0 mg
Distilled water, 1 L and stored at room temperature in the dark.	
3-vitamin solution	
Folic acid	1.0 mg
Riboflavin	2.5mg
DL-6,8-thioctic	2.5 mg
Distilled water, 250 mL and filter sterilized. Stored at 4˚C in the dark	
6-vitamin solution	
Biotin	1.0 mg

4-aminobenzoic acid	5.0 mg
Pantothenate	1.0 mg
Pyridoxamine	25.0 mg
Nicotinic acid	10.0 mg
Thiamine	2.0 mg
Distilled water, 100 mL and filter sterilized. Stored at 4°C in the dark.	
Vitamin B_{12} solution	
B_{12}	2.5 mg
Distilled water, 100 mL and filter sterilized. Stored at **4°C in the dark.**	

2.4 Setting an anaerobic chamber or glove box:

An anaerobic chamber (815–PGB 'La Petite', PLAS LABS, MI, USA) (Fig 2.1) was used throughout the project to set up anoxic enrichment cultures. The anaerobic chamber or glove box consisted of two main components: main working chamber and the transfer chamber.

i] The main working chamber

The following steps were followed for purging the main working chamber (31.5" W x 25.5" D x 21.5" H) (Fig 2.1) to bring in desirable anoxic conditions. The gloves were used as an indicator of pressure within the glove box. Positive pressure pushes the gloves out and negative pressure draws the gloves back into the chamber.

Step # 1: Incoming gas line (hose) containing N_2:CO_2(80:20%) was attached to the key cock valve on the lower right side of the glove box. A small vacuum pump was connected to the key cock valve on the lower left side of the glove box.

Step # 2: The incoming gas source or cylinder bottle regulator was set to 2" H_2O or 0.777 PSI (0.5 kPa maximum). The level of gas was raised until the gloves extended out of the glove box approximately 10" inches (25 cm).

Step # 3: The incoming gas was turned off and the vacuum pump was turned on. This exhausted the inner atmosphere until the gloves extended into the glove box. The

vacuum was left on until the gloves extended into the glove box approximately 10”inches (25 cm).

Step # 4: Steps # 2, #3 and # 4 were repeated at least eight or nine more times (purge cycles) then the gas and vacuum pump were turned off.

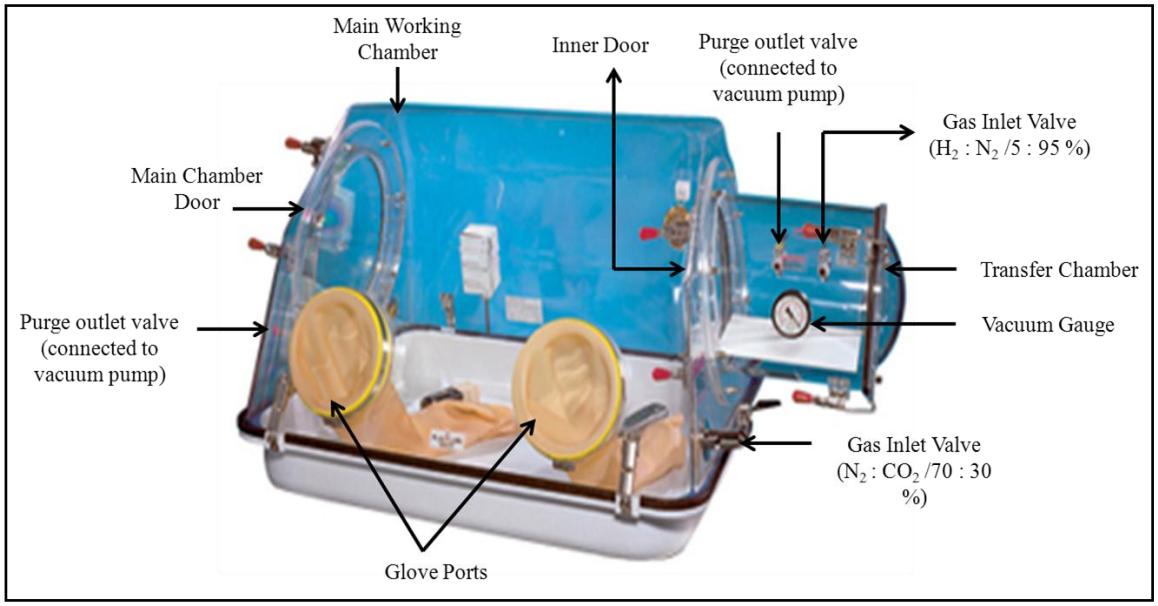


Figure 2.1: An anaerobic chamber or glove box used in this study

ii] The transfer chamber:

The transfer chamber (10” L x 9.75” I.D) (Fig 2.1) was used for inserting materials into and out of the main working chamber without disturbing the atmosphere in the main chamber.

Step # 1: A small vacuum pump was attached to the transfer chamber key cock valve labelled “VAC”. The incoming H_2:N_2 (5:95%) gas line hose was attached to the other key cock valve labeledN_2.

Step # 2: With the inner door closed and locked, the outer door was opened to place the desired materials inside the chamber. The outer door was then closed and locked.

Step # 3: The vacuum valve was turned on to vacuum the transfer chamber. The vacuum was drawn down to 20” of Hg. When the level was reached, the vacuum pump

was turned off and the vacuum valve closed. The key cock was opened to introduce the gas. This process was continued until the gauge reading displayed "0".

Step # 4: The whole process was repeated three times. Upon completion of this sequence, the inner door was opened and materials were transferred into the main chamber.

2.5 Microbial electric system (MES) setup:

For this study, we employed a typical two-chamber NCBE–type MES (National Centre for Biotechnology Education, Reading, U.K) with anode and a cathode compartments (60 x 70 x10 mm; 10 mL each) separated by a reinforced Nafion^{424} proton exchange membrane (PEM) 0.007" in thickness (Sigma-Aldrich, VIC, Australia) (Fig. 2.2; Bennetto, 1990). Chambers were kept watertight by placing rubber gaskets between chambers and also by bolting two perspex sheets together above and below the cells. The PEM was pre-treated by boiling in H_2O_2 (30%), then in 0.5M H_2SO_4 and finally in DI water, each for 1 h and then stored in deionized (DI) water prior to being used (Aulenta *et al*., 2007). The carbon fiber electrodes (3.2 x 4 cm) were soaked in DI water prior to use. Sampling ports were sealed with butyl rubber stoppers, while carbon electrodes were attached to copper wires from the top by feeding a wire through a butyl stopper in the sampling port. Electrochemical measurements and monitoring were performed using a Fluke 289 digital true RMS multimeter (RS Components, Australia).

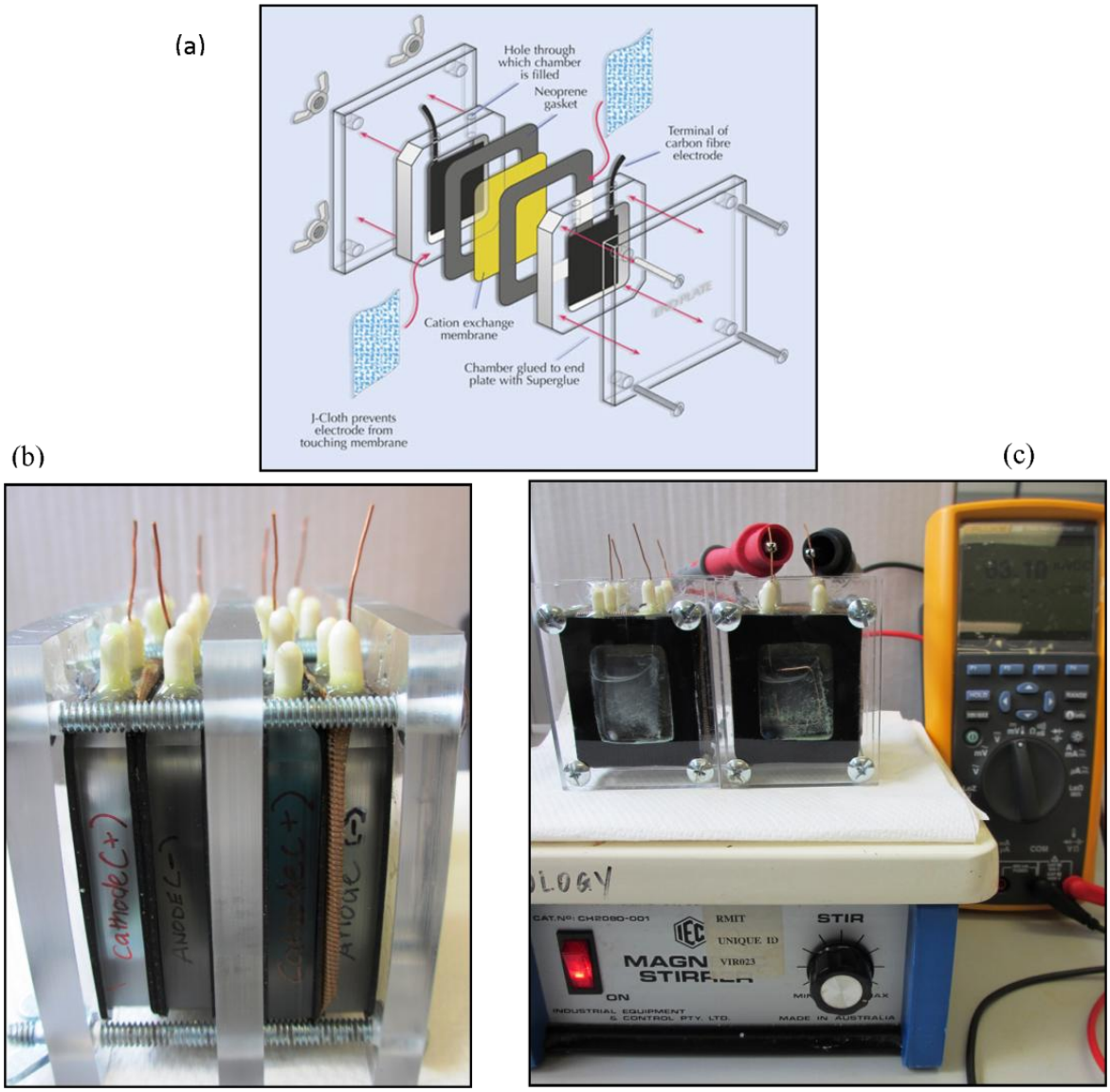


Figure 2.2: Design of two chamber NCBE–type MES used in this study showing (a) components of MES (Bennetto, 1990); (b) side view of MES and (c) MES in operation.

2.6 Microscopy

Cell viability and presence within cultures were visually observed under an Inverted Eclipse T*i* fluorescence microscope (Nikon, Australia) usinga Live/Dead® BacLight™ bacterial viability kit (Invitrogen, Australia) (Fig. 2.3). As Live/Dead cell viability kit stains live cells with green fluorescence and dead with red fluorescence, the greater presence of SYTO-9 stained green (live) cells indicates good viability. Equal volumes of

SYTO® 9 green–fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide (PI) were mixed thoroughly in a microcentrifuge tube. Dye mixtures of bacterial suspension (3µL/mL) were incubated in the dark for 15 min. The stained bacterial suspension (5 µL) was then trapped between a slide and an 18 mm square coverslip to observe the bacterial cells in a fluorescence microscope equipped with suitable filter sets. With an appropriate mixture of the SYTO® 9 and PI stain, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation/emission maxima for these dye is about 480/500 nm for SYTO 9 stain and 490/635 nm for PI.

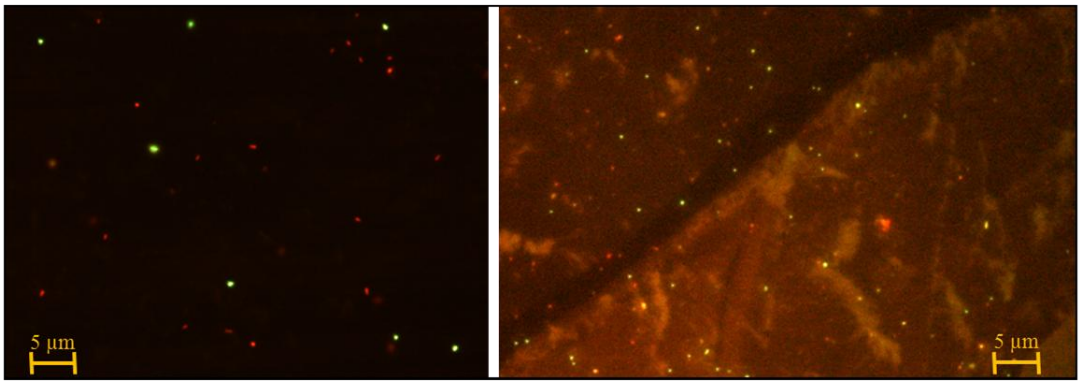


Figure 2.3. Fluorescent photomicrographs showing the presence and viability of cells grown in enrichment cultures from a PCE-contaminated groundwater assessed using Live/dead BacLight stain. Live bacterial cells stained green by SYTO-9 and dead bacteria stained red by PI. Morphotypes showed coccoid shaped bacterial cells with scale bar 5µm.

Chapter

Biostimulation of indigenous communities for the successful dechlorination of tetrachloroethene (perchloroethylene) - contaminated groundwater

Statement of Authorship

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1] Sayali S. Patil (Candidate)

Performed experiment design, analysis of samples, molecular and statistical data analysis, interpreted data, manuscript writing and evaluation, corresponding author

Signature  **Date** 07-10-2013

2] Eric M. Adetutu

Manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 07/10/2013

3] Arturo Aburto – Medina

Manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 13/10/2013

4] Ian R. Menz

Manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 12/10/2013

5] Andrew S. Ball

Assistance with experimental design, Interpreted data, manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 07 October 2013

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ORIGINAL RESEARCH PAPER

Biostimulation of indigenous communities for the successful dechlorination of tetrachloroethene (perchloroethylene)-contaminated groundwater

Sayali S. Patil · Eric M. Adetutu · Arturo Aburto-Medina · Ian R. Menz · Andrew S. Ball

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Abstract Chlorinated ethenes are of environmental concern with most reports of successful microbial-mediated remediation being associated with major dechlorinating groups such as *Dehalococcoides* (*Dhc*) species. However, limited information is available on the community dynamics and dechlorinating activities of indigenous non-*Dhc* groups. Here, we present evidence of dechlorination of tetrachloroethene (perchloroethylene, PCE) in groundwater samples by indigenous microbial communities. 100 % PCE conversion to ethene was observed in acetate-stimulated 24 week-microcosms (controls; 15 %). Microbial community profiles showed dominance by groups such as *Proteobacteria*, *Spirochaetes, Firmicutes, Methanomicrobiaceae* and *Methanosarcinaceae*. Pareto-Lorenz (PL) analyses suggested an adapted (45 % PL value) but variable bacterial community (55.5 % $\Delta_{t(week)}$) compared to Archaea (25 % PL value; 46.9 % $\Delta_{t(week)}$). Our findings provide evidence of dechlorinating potential of indigenous microorganisms and useful information on their dynamics which may be exploited for in situ groundwater bioremediation.

Keywords Dechlorinating microbial community · Groundwater · PCR-DGGE · Perchloroethylene · Reductive dechlorination · Tetrachloroethene

Introduction

Chlorinated compounds, such as tetrachloroethene (perchloroethylene, PCE), trichloroethene (TCE), dichloroethenes (DCE) and vinyl chloride (VC), are toxic but are widely used as industrial solvents, lubricants, degreasers and intermediates in chemical industries. Improper disposal of these compounds has led to the contamination of groundwater and soil (Löffler and Edwards 2006). VC in particular is dangerous to human health because of its carcinogenic properties.

Compared to conventional pump-and-treat and natural attenuation techniques, enhanced in situ anaerobic chloroethene bioremediation is a promising technology which involves the delivery of organic substrates into the subsurface. This delivery is designed to stimulate the existing microbial population (biostimulation) to carry

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S. S. Patil (✉) · E. M. Adetutu · I. R. Menz
School of Biological Sciences, Flinders University of South Australia, Adelaide, SA 5042, Australia
e-mail: pati0018@flinders.edu.au

S. S. Patil · A. S. Ball
Department of Applied Biosciences, Royal Melbourne Institute of Technology, Bundoora, VIC 3083, Australia

A. Aburto-Medina
Instituto Tecnológico y de Estudios Superiores de Monterrey (ITESM), 72800 Puebla, Mexico

out respiratory reduction of chlorinated solvents dissolved in groundwater. In some cases, microorganisms may be added (bioaugmentation) if the natural microbial population was incapable of performing the required transformations. Much of the current research activity is focused on organohalide respiration, where chlorinated compounds serve as terminal electron acceptors in support of microbial growth, using H_2, formate, acetate, pyruvate, lactate and butyrate as electron donors directly or indirectly. This process can be mediated by species such as *Dehalobacter, Dehalococcoides, Desulfitobacterium, Desulfuromonas, Dehalogenimonas, Geobacter and Sulfurospirillum* (Duhamel and Edwards 2006). One genus of particular scientific interest is '*Dehalococcoides' (Dhc)*. *Dhc* are obligate anaerobes that can completely reduce PCE to the environmentally-acceptable end-product, ethene which have been used individually or in mixed consortia for bioaugmentation treatments (Futagami et al. 2008).

Prior to deciding the appropriate bioremediation strategy on a full scale, it is necessary to assess the interactions between existing indigenous complex communities. Understanding the dynamics of these interactions is important for optimizing native dechlorination especially in microbial communities without major dechlorinating groups such as *Dhc* during in situ bioremediation. Hence, this study aims at documenting the composition, functional organization and dynamics of an indigenous complex microbial community (devoid of *Dhc*) within chloroethene-reducing ecosystems in order to predict in situ chlorinated ethene detoxification potential. A combination of chemical methods, molecular techniques based on polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) and community analysis tools were used for this purpose.

Materials and methods

Site description

The study site located in Victoria, Australia, has a history of commercial industrial activities dating back to 1935. Initial investigations identified chemicals of concern including PCE and TCE in subsurface waters. Activities associated with storage and use of fuels, thinners for cleaning and adhesive chemicals on site, haphazard disposal of surfactants used within the foam hall to a waste sump along with other waste chemicals have led to serious groundwater contamination. (Further site details are restricted due to confidential agreements.)

Groundwater sample collection

Groundwater samples with the highest PCE concentration (146 $\mu g\ l^{-1}$) were collected from a PCE-contaminated aquifer as per the protocol suggested by Ritalahti et al. (2010). Sample containers consisted of sterile and N_2-purged 4 l high density polyethylene Nalgene bottles with polypropylene screw caps (Thermo-Fisher Scientific Australia, NSW) and were filled to capacity. Bottles were stored on ice and then express shipped to the analytical laboratory. Upon arrival, samples were placed in the dark at 4 °C.

Enrichment culture preparation and growth conditions

Enrichment cultures (biostimulation) were prepared in Wheaton serum bottles (125 ml nominal volume) containing 75 ml growth medium and 20 ml PCE-contaminated groundwater as an inoculum which were sealed with Teflon-coated butyl rubber septa and aluminum crimp caps. Anoxic mineral salt medium was prepared as per Löffler et al. (2005) and ATCC guidelines and was amended with acetate (5 mM) as an electron donor and PCE (5 µl) as an electron acceptor. H_2 (5 % in 95 % N_2) was added in the headspace (5–10 % of the headspace volume of a bottle) of acetate fed cultures at a low partial pressure of 9 kPa. Cultures were prepared under strict anaerobic conditions in an anaerobic glove box (Thermo-Fisher Scientific Australia) using N_2:CO_2 (80 : 20 v/v) and maintained at a pH between 6.8 and 7.2. All chlorinated compounds, vitamins and electron donors were injected into cultures from anoxic, sterilized stock solutions using a gas-tight Hamilton syringe. To minimize the contact of the inoculum with the air present in the syringes during transfers, syringes were reduced with freshly prepared, filter sterilized 0.5 mM aqueous sulphide solution. All experiments were set up in duplicate with appropriate controls (no inoculum, electron donors). Culture bottles were incubated statically at room temperature (22–25 °C) in the dark. Immediately upon setup, all enrichment cultures turned clear from pink tint (given by resazurin redox

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indicator added to the groundwater) indicating establishment of reduced conditions. Samples were analyzed at predetermined time intervals (every 28 days) over 24 weeks (168 days) for dechlorination of PCE to TCE, *cis*-DCE (cDCE), VC and ethene using GC. Electron donors were replenished every time analyses indicated they were exhausted.

Analytical procedures

Chlorinated hydrocarbons were analyzed in 1 ml gas headspace using a GC system equipped with MS, flame ionizing detector detector and a Porabond Q column (0.32 mm by 25 m). The GC settings were: injector 200 °C; detector 300 °C; oven 3 min at 40 °C, followed by an increase of 10 °C min^{-1} to 70 °C, followed by an increase of 15 °C min^{-1} to 250 °C for 7 min; and carrier gas (He) at 2 ml min^{-1}. External standards from 0 to 30 µM were used for calibration. Methane gas analysis was carried out using an IBRID MX–6 Multi-gas analyzer (Air-Met Scientific, Australia).

DNA extraction and PCR amplification

Every 28 days, samples for DNA extraction were collected from enrichment cultures under aseptic and anaerobic conditions with a gas tight Hamilton syringe. These samples (1 ml) were centrifuged for 30 min at 16,000×*g*, 4 °C with pellets being re-suspended in phosphate buffer/saline and stored at −20 °C for 1 h to enhance cell lysis. Genomic DNA was then extracted with a Qiagen DNeasy Tissue Kit (Qiagen, NSW, Australia) according to the manufacturer's protocol. PCR amplification of 16S rRNA genes was carried out on T100 thermal cycler (Bio-Rad, NSW, Australia) to detect the presence of microorganisms associated with dechlorination. The bacterial (341f-GC/518r), archaeal (A109f/A934b) and *Dhc* population (1f-GC/259r) were targeted with the appropriate primer pair (Supplementary Table 1).

Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with a D-Code System (Bio-Rad, NSW, Australia) in accordance with the manufacturer's instructions. PCR amplification products (15 µl) were loaded onto 12 % (w/v) polyacrylamide gels (37.5:1, acrylamide:bisacrylamide) made with a denaturing gradient from 45 to 60 %. DGGE was run for 18 h at 60 °C and 60 V for microbial community analysis and was stained by silver staining as described by Patil et al. (2010).

Sequence analysis

Based upon strong band intensity, some dominant DGGE bands were excised using sterile razor blades and incubated overnight in nuclease free water at 60 °C for DNA elution. Re-amplification was performed using bacterial and archaeal primers. Re-amplified PCR products were purified using the Wizard SV gel and PCR clean up system (Promega, Madison, WI, USA) as per the manufacturer's instructions. The eluted DNA was quantified with a Nano-drop Lite spectrophotometer (Thermo Scientific Australia, NSW). Samples were then sent to the Australian Genome Research Facility (AGRF) for sequencing on the automated sequencer ABI 3730. Nucleotide sequences were analyzed using SEQUENCHER software (Sequencher Version 4.1.4) and homology searches were completed with the BLAST server of the National Centre for Biotechnology Information using a BLAST algorithm (http://www.ncbi.nlm.gov.library.vu.edu.au/BLAST/) for the comparison of a nucleotide query sequence against a nucleotide sequence database (blastn).

Statistical analyses

Relative band intensities or peaks on DGGE community profiles were analyzed using Phoretix 1D advanced analysis package (Phoretix Ltd, UK). Each band was considered to be a phylotype or species. The similarities between microbial communities within enrichment cultures were expressed as similarity clusters using unweighted pair group method using arithmetic averages (UPGMA). Moving window analysis (MWA) was carried out on the community fingerprints to study community dynamics (Dy) where Dy indicates the number of species that on average come to significant dominance above the detection limit at a given habitat, during a defined time interval (Marzorati et al. 2008). The more species observed per unit time and in proportion to the number already detected, the greater the changes in the community Dy. The rate of change (Δ_t) value in the respective native microbial communities was also calculated over the

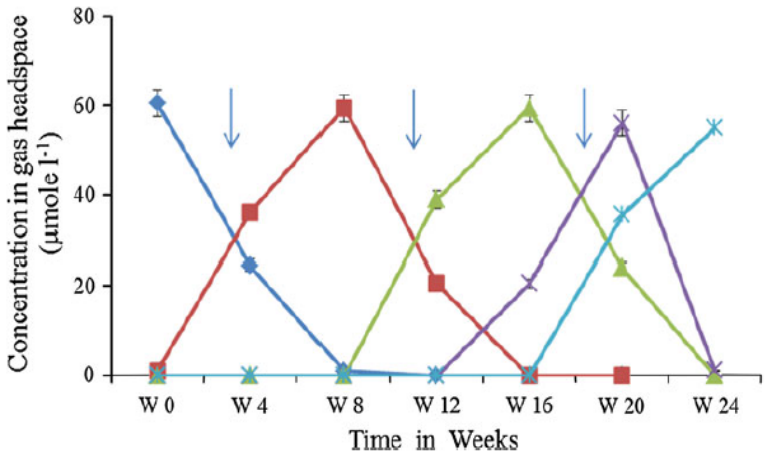

Fig. 1 Reductive dechlorination of tetrachloroethene (PCE) to ethene in enrichment cultures. The *arrow* indicates the addition of electron donors. Data are the average of the duplicates (duplicates differed by <10 %) and Y-axis *error bars* indicate standard error

incubation period as the average of the respective moving window curve data points. The higher the changes between the DGGE profiles of two consecutive sampling points, the higher the corresponding moving window curve data point will be and hence the higher the Δ_t values. Functional organization (Fo) indicates the result of the action of microorganisms that are most fitting to the ongoing environment-microbiological interactions (Marzorati et al. 2008). Functionality was assessed with Pareto-Lorenz distribution curves (PL curves) derived from the DGGE profiles by plotting normalized cumulative band intensities against their respective normalized cumulative band (numbers) (Marzorati et al. 2008).

Nucleotide sequence accession numbers

All sequences have been deposited in the NCBI database as follows; Bacterial nucleotide sequences: JX495100–JX495113; Archaeal nucleotide sequences: JX495114–JX495117.

Results

Reductive dechlorination of chlorinated ethenes

The performance of the acetate fed anaerobic enrichment cultures from a PCE-contaminated site was monitored over 24 weeks where PCE was dechlorinated to lesser chlorinated ethenes (Fig. 1). Intermediates TCE, cDCE and VC were produced prior to the formation of ethene. Dechlorination steadily continued over time. cDCE formation commenced in week 8 and reached its peak by week 16 when TCE fell below 20 µmol l^{-1}. Daughter products, cDCE and VC, co-existed until ethene was formed. Methane production was sustained throughout the period of dechlorination starting from week 8. However, during the experimental period negligible PCE dechlorination (15 %) and no methane production was observed in the controls without inoculum or electron donors (data not shown).

Identification of indigenous microbes from dechlorinating enrichment cultures

Throughout the dechlorination process, no *Dhc* amplicons were obtained from optimized touchdown PCR using specific primers against *Dhc* positive controls. This indicated either the absence of *Dhc* species or their presence below PCR detection limits in PCE contaminated groundwater samples. Bacterial and archaeal DGGE profiles for assessing community changes throughout the experiment are shown in. As dechlorination progressed, UPGMA dendrograms constructed from both bacterial and archaeal DGGE profiles (Fig. 2a, b) showed an increase in DGGE band numbers and intensities from weeks 0 to 24.

Analysis of the dominant DGGE bands led to the identification of species associated with the dechlorination process which belonged to four recognized phyla: *Firmicutes, Bacteroidetes, Spirochaetes* and *Proteobacteria*. As shown in Fig. 2a, different species identified as *Spirochaetes* (bands 1–4) and *Proteobacteria* (bands 5–8) which were observed from the

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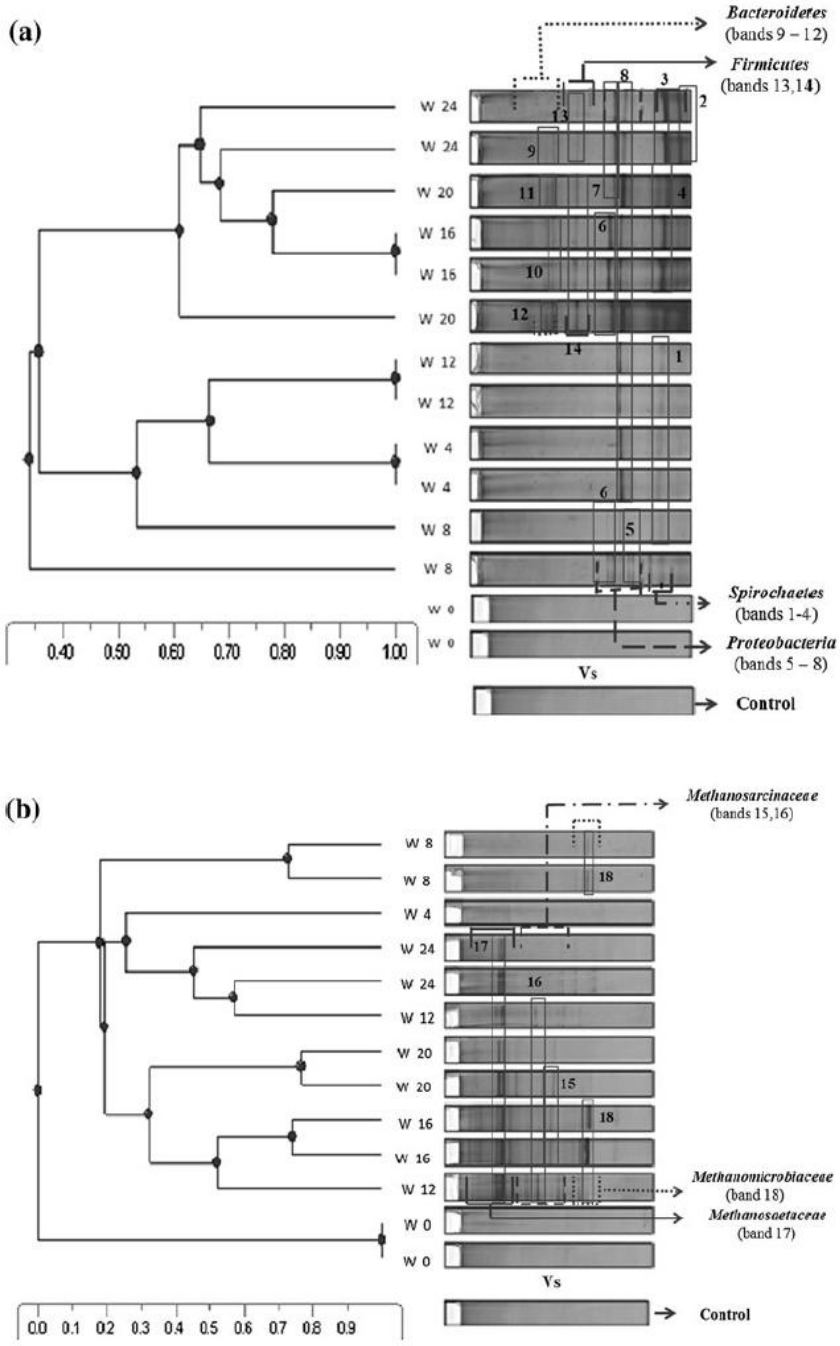

Fig. 2 Unweighted pair group method using arithmetic averages (UPGMA) dendrogram constructed from **a** bacteria and **b** archaea denaturing gradient gel electrophoresis (DGGE) community profiles. *Rectangular boxes* and numbers designate dominant bands excised and further sequenced which correspond to the band numbers in Supplementary Table 2 while, brackets indicate shift in microbial community associated with dechlorination. The *scale bar* represents percent similarity. Samples were analyzed in duplicates

beginning of first phase of the dechlorination (when PCE was thought to be converted to cDCE) became distinct from weeks 16 to 24 as DGGE band intensity increased in the later phase (cDCE to ethene transition). On the other hand, bacterial species grouped under the *Bacteroidetes* (bands 9–12) and *Firmicutes* (bands 13, 14) phylum were dominant from weeks 16 to 24 (cDCE to ethene transition phase). Detailed phylogenetic information on selected bands is presented in Supplementary Table 2.

Sequence analysis of dominant archaeal DGGE bands revealed the identity of methanogenic bacteria grouped under three major taxonomic classes: *Methanosarcinaceae*, *Methanosaetaceae* and *Methanomicrobiaceae*. Phylotypes affiliated with these three taxonomic classes are mentioned in Supplementary Table 2. Figure 2b showed the presence of methanogenic species under *Methanomicrobiaceae* group (band 18) from weeks 4 to 16 (initial phase of dechlorination) while species under *Methanosarcinaceae* (bands 15, 16) and *Methanosaetaceae* (band 17) phyla became dominant during weeks 12 to 24 at the cDCE to ethene transition phase.

Functional organization (Fo) and community dynamics (Dy) of the indigenous, dechlorinating-microbial community

Bacterial and archaeal community structure (species distribution) were investigated with PL distribution curves derived from their respective DGGE profiles (Fig. 3a–i). As a general rule, the more the PL curve deviates from the 45° diagonal (the theoretical perfect evenness line), the greater the shift in the evenness and potential functionality of the studied community. The 25, the 45 and the 80 % curves refer to communities with a low, medium, and high Fo but high, medium and low evenness respectively (Marzorati et al. 2008). The PL curve for the bacterial community showed an increase in the Fo over time (Fig. 3a–i) from 20 % (point-d, day 0) to 50 % (point-a, week 24). The average PL value over the experimental period was 45 % which was indicative of a community with medium evenness and functionality. However, the trend observed in the archaeal community was different (Fig. 3b-i). While, the PL values fluctuated (30 to 40 to 25 %, points f, e and g respectively) over 24 weeks, the archaeal community in general had low Fo but high evenness. No PL curve for week 0 was obtained because no amplicon or bands were detected in week 0 samples.

UPGMA dendrograms constructed from DGGE profiles in conjunction with MWA (Fig. 3a, b-ii), were used to interpret the Dy of indigenous microbial communities. Weekly deviations within bacterial communities were established using MWA (Fig. 3a-ii). For example, the correlation coefficient increased from ~20(W0–4) to 30 % (W8–12) and finally to 80 % (W20–24) indicating a highly dynamic bacterial community with new species becoming dominant over time. In the archaeal community, the correlation coefficient increased from ~5 % (W0–4) to the highest value of ~78 % (W12–16) before dropping to ~70 % (W20–24) (Fig. 3b-ii). Average values for these changes were normalized to weekly changes and expressed as the rate of change [$\Delta_{t(week)}$]. Consequently, it was observed that total bacterial community shifted more rapidly [$\Delta_{t(week)}$, 55.5 ± 22.7 %] than the archaeal community [$\Delta_{t(week)}$, 46.9 ± 19.1 %].

Discussion

Biostimulation with acetate addition supported complete dechlorination of PCE to ethene in the enrichment cultures. This study therefore highlights the importance of supplying appropriate electron donors in the bioremediation of groundwater contaminated with chlorinated compounds as limited dechlorination was observed in non-stimulated controls. The substantial dechlorination observed in this study also indicated that under the right nutrient conditions and microbial community (He et al. 2002), dechlorination can proceed efficiently in environments without classical dechlorinators such as *Dhc*. The fact that complete dechlorination occurred in the enrichment cultures shows that the potential for PCE degradation existed in these samples but was inherently limited by the absence of sufficient concentrations of nutrients (electron donors). This knowledge would be useful in the application of in situ bioremediation techniques to PCE contaminated sites.

Examination of the microbial community associated with dechlorination in the biostimulated samples showed a clear shift in their DGGE (microbial community) profiles. Some bacterial and archaeal species became dominant and active as the dechlorination progressed and their sequences were closely related to known microbial dechlorinators. However, bacterial community structure was more diverse than that of methanogenic archaea. *Spirochaetes* reported in this study have been found in other studies on PCE dechlorination in contaminated sites (Dong et al. 2011; Gu et al. 2004; Macbeth et al. 2004). *Spirochaetes* can either produce acetate from H_2 and CO_2 or ferment carbohydrates and other complex substrates to acetate and other substances which is utilized during organohalide respiration (Duhamel and Edwards 2006).

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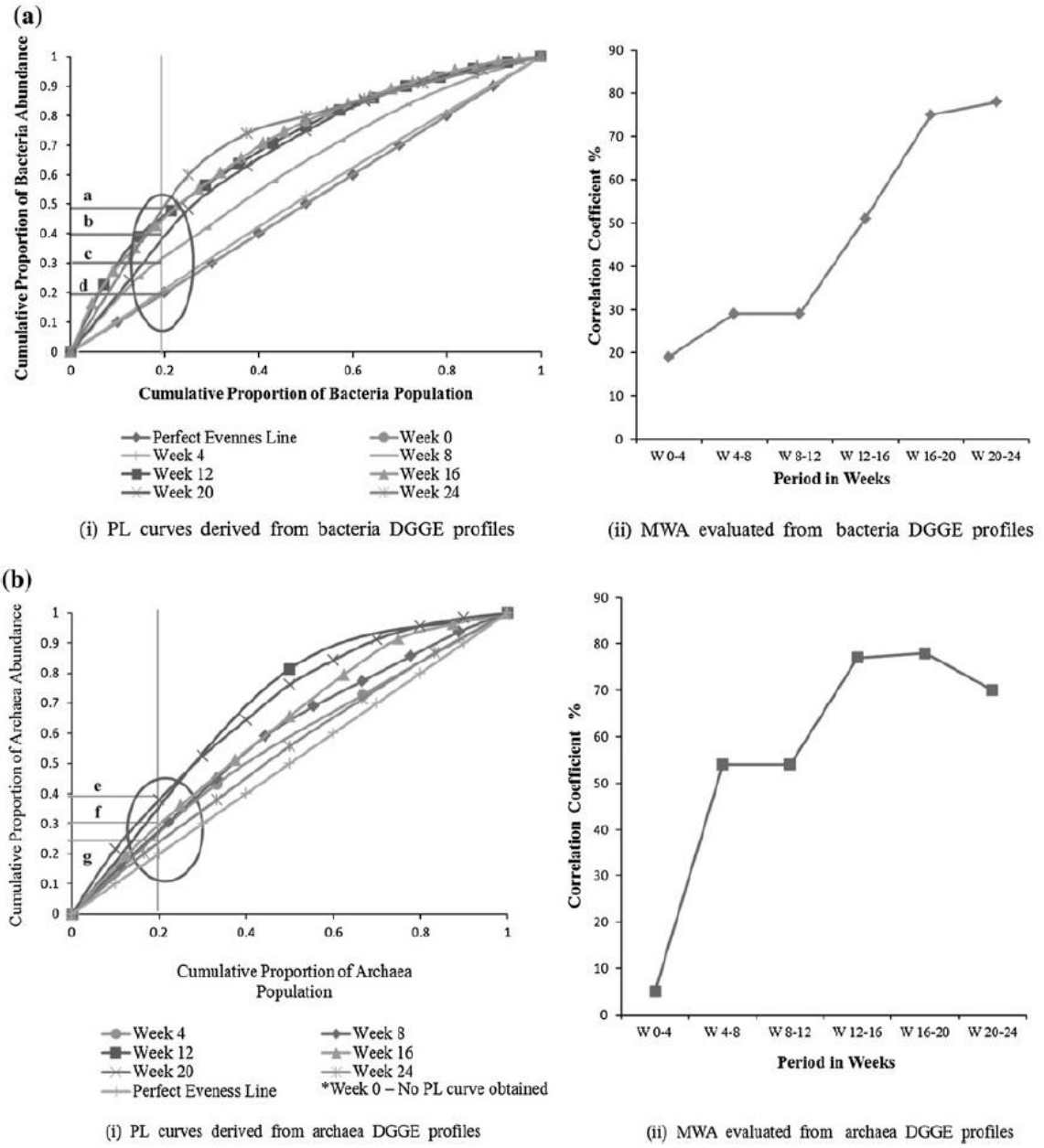

Fig. 3 Evaluation of (i) Pareto–Lorenz (PL) curves and; (ii) Moving window analyses (MWA) from DGGE profiles of **a** bacteria and; (**b**) archaea communities to assess functional organization (Fo) and dynamics (Dy) respectively. The *vertical line* is plotted at 0.2 *x*-axis in order to compare different Pareto values while the 45° *diagonal line* represents the perfect evenness of a community. *Letters a–g* indicate different range of Pareto values observed during weekly analysis. MWA and rate of change (Δ_t) values evaluates the level of community dynamics

Other detected groups such as, *Enterobacter* and *Desulfivibrio* spp., can also either reductively dechlorinate PCE to cDCE and other less toxic forms individually or syntrophically or support the formation of precursors which enhances the dechlorination process (Holliger et al. 1999; Sun et al. 2000). *Bacteriodetes* and *Firmicutes* species detected in these enrichment cultures may act as acetate fermenters

supplying small organic molecules or H_2 providing an energy and carbon source for dechlorinating microbes for their growth (Dong et al. 2011; Gu et al. 2004).

With regards to methanogenic archaea, Methanomicrobiales are the most frequently detected methanogen in H_2 or acetate amended cultures (Fathepure and Boyd 1988). Microorganisms with enzymes from the acetyl-coenzyme A pathway, including acetogens and acetoclastic methanogens may play a role in the first stage (PCE to cDCE) of dechlorination due to an abundance of transition metal coronoid cofactors. Also, coronoid-dependent (source of vitamin B_{12} or cofactor F_{430}) dechlorination has been demonstrated in methanogenic and acetogenic consortia for the second stage of reductive dechlorination of 1, 2 dichloroethane (1, 2-DCA) to ethene by *Methanosarcina bakeri* and *Methanosarcina thermophile* (Holliger et al. 1992). However, their role in reducing cDCE to ethene is unclear. Both methanogenic and non-methanogenic microbial groups can dechlorinate PCE to ethene co-metabolically. For example, a study conducted by Macbeth et al. (2004) showed the presence of methanogenic species *Methanomicrobium mobile* and *Methanosaeta thermophila* in a TCE contaminated deep fractured basalt aquifer further suggesting their syntrophic relationship with homoacetogenic bacterial dechlorinators.

Therefore the dominant microbial groups putatively identified in both the bacterial and archaeal communities would have played significant roles in the observed PCE dechlorination to ethene. Further analysis of the microbial community structure using PL curves showed that the bacterial community had a medium Fo and evenness compared to a low Fo archaeal community with high evenness. The bacterial community with medium Fo could be representative of an adapted community which can potentially deal with stress (contaminants) and changing environmental conditions, preserving its functionality (Marzorati et al. 2008). The bacterial communities associated with dechlorination would therefore appear to be functionally redundant (Marzorati et al. 2008), allowing reductive dechlorination of PCE to ethene to proceed irrespective of changing Dy. Communities with low Fo do not represent a well-defined internal structure in terms of species dominance and their role in reductive dechlorination are not as clearly defined as bacterial roles. The rapid shift observed in bacterial composition indicated a high Dy level as many species either came to dominance or were no longer detectable in the total microbial community resulting in broad dynamics. However, these changes in Dy seemed not to have interfered with the functionality of the system and were probably related to the emergence of new dominant species in response to the formation of different PCE secondary and tertiary products.

The phylogenetic signatures of certain known *Dhc* are frequently used as a proxy for the potential of indigenous bacteria to fully detoxify chlorinated solvents to ethene. However, a failure to detect *Dhc* in groundwater should not be taken to mean that dechlorination will cease at the level of DCE. Although *Dhc* was not detected in this study, complete PCE conversion to ethene was observed in acetate stimulation likely mediated by indigenous bacterial and archaeal dechlorinators, acetogens, methanogens and fermenters working synergistically. This point is important in planning in situ PCE bioremediation in environments without *Dhc* or other so called classical microbial dechlorinators.

Conclusions

The linking of PCR-DGGE community fingerprints and PL-curve distribution analyses to the microcosm degradation represents a useful technique for assessing initial characterization and community composition shifts during PCE dechlorination. This improves our understanding of microbial community dynamics associated with dechlorination, which may assist in predicting the fate of in situ PCE bioremediation. Given the shift away from bioaugmentation (of contaminated groundwater) in some countries such as Australia, biostimulation of indigenous communities for successful dechlorination of PCE-contaminated aquifer represents a legislative acceptable measure which may result in substantial savings in remediation costs. This study has shown that the application of an in situ biostimulation strategy may result in significant dechlorination at chloroethene contaminated sites.

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Chapter

Site-specific pre-evaluation of bioremediation technologies for chloroethene degradation

Statement of Authorship

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1] Sayali S. Patil (Candidate)

Performed experiment design, analysis of samples, molecular and statistical data analysis, interpreted data, manuscript writing and evaluation, corresponding author

Signature  **Date** 07-10-2013

2] Eric M. Adetutu

Manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 07/10/2013

3] Petra J Sheppard

Assistance with analytical measurements

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 15/10/2013

4] Paul Morrison

Assistance for analytical measurements

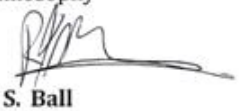
I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 22/10/2013

5] Ian R. Menz

Manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 15/10/2013

6] Andrew S. Ball

Assistance with experimental design, Interpreted data, manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 07 October 2013

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ORIGINAL PAPER

Site-specific pre-evaluation of bioremediation technologies for chloroethene degradation

S. S. Patil · E. M. Adetutu · P. J. Sheppard · P. Morrison · I. R. Menz · A. S. Ball

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Abstract Groundwater systems are important sources of water for drinking and irrigation purposes. Unfortunately, human activities have led to widespread groundwater contamination by chlorinated compounds such as tetrachloroethene (PCE). Chloroethenes are extremely harmful to humans and the environment due to their carcinogenic properties. Therefore, this study investigated the potential for bioremediating PCE-contaminated groundwater using laboratory-based biostimulation (BS) and biostimulation–bioaugmentation (BS-BA) assays. This was carried out on groundwater samples obtained from a PCE-contaminated site which had been unsuccessfully treated using chemical oxidation. BS resulted in complete dechlorination by week 21 compared to controls which had only 30 % PCE degradation. BS also led to a approximately threefold increase in 16S rRNA gene copies compared to the controls. However, the major bacterial dechlorinating group, *Dehalococcoides (Dhc)*, was undetectable in PCE-contaminated groundwater. This suggested that dechlorination in BS samples was due to indigenous non-*Dhc* dechlorinators. Application of the BS-BA strategy with *Dhc* as the augmenting organism resulted in complete dechlorination by week 17 with approximately twofold to threefold increase in 16S rRNA and *Dhc* gene abundance. Live/dead cell counts (LDCC) showed 70–80 % viability in both treatments indicating active growth of potential dechlorinators. The LDCC was strongly correlated with cell copy numbers ($r > 0.95$) suggesting its potential use for low-cost monitoring of bioremediation. This study also shows the dechlorinating potential of indigenous non-*Dhc* groups can be successfully exploited for PCE decontamination while demonstrating the applicability of microbiological and chemical methodologies for preliminary site assessments prior to field-based studies.

Keywords Chlorinated compounds · Biostimulation · Bioaugmentation · Quantitative PCR · Cell viability

Introduction

Improper disposal and storage of chlorinated compounds has led to widespread contamination of subsurface resources by chlorinated aliphatic contaminants such as tetrachloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE) and vinyl chloride (VC) (SCRD 2007). Due to their toxicity and suspected carcinogenic properties, monitoring the effective remediation of this group of contaminants has gained wide public and academic interest. Standard remedial approaches such as in situ chemical oxidation (ISCO) and pump-and-treat methods have proven to be ineffectual and costly in terms of removing these substances from the environment. To date, enhanced in situ bioremediation has proved to be a promising technique for chloroethene bioremediation (Aulenta et al. 2006; Ernst 2009). This strategy involves the delivery of organic substrates into the groundwater for the purpose of stimulating

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S. S. Patil (✉) · I. R. Menz
School of Biological Sciences, Flinders University of South Australia, Adelaide, SA 5042, Australia
e-mail: pati0018@flinders.edu.au

S. S. Patil · E. M. Adetutu · P. J. Sheppard · P. Morrison · A. S. Ball
Department of Applied Biosciences, Royal Melbourne Institute of Technology, Bundoora, VIC 3083, Australia

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growth and development of native microbial populations (biostimulation) by creating an anaerobic groundwater treatment zone generating hydrogen through fermentation reactions (ESTCP 2004, 2005). In some cases, specific microorganisms known for their dechlorinating capabilities may be added (bioaugmentation), but only if the natural microbial population is incapable of efficiently performing the required transformations (Löffler and Edwards 2006; Schaefer et al. 2010).

To stimulate the activity of desired indigenous dechlorinating microbes at contaminated sites, the redox conditions need to be created (especially in wells which are not completely anoxic) or maintained. This can be achieved by the addition of easily oxidizable organic carbon substrates such as acetate, lactate, butyrate, propionate, hydrogen releasing compounds (HRCs), vegetable oils and molasses (Ballapragada et al. 1997; Fennell et al. 1997; He et al. 2002; Ibbini et al. 2010; Lee et al. 2000). For bioaugmentation of chloroethene-contaminated sites, various members of the *Chloroflexi* phylum such as *Dehalococcoides ethenogenes strain* 195, GT, BAV—1, FL2, CBDB1, KB1/VC are commercially available, e.g. KB-1, Pinellas, Bio-Dechlor, SDC-9 cultures and are well known for completely reducing PCE to the environmentally safer ethene (Cichocka et al. 2010; Cheng et al. 2010; Cupples et al. 2003; Duhamel et al. 2004; Ellis et al. 2000; He et al. 2003, 2005; Hendrickson et al. 2002; Ibbini et al. 2010; Lendvay et al. 2003; Major et al. 2002; Müller et al. 2004; Schaefer et al. 2010; Sung et al. 2006). For site owners and bioremediation consulting companies, estimation of the degradation potential, the regulatory requirements and economics of the overall process at a contaminated site are important for selecting the appropriate remediation strategy. If biostimulation reduces the time and leads to meeting key bioremediation endpoints within the desired timeframe, it may well reduce the cost as less monitoring is required. On the other hand, some regulators like the Australian EPA discourage an introduction of foreign organisms to any environment that could stand a chance of causing mutation in indigenous organisms and adversely affecting the biome (Ball 2012). In such cases (as in this study), application of a bioaugmentation strategy can increase the time required for permits, inoculum and remediation costs.

This study was carried out to assess the remediation of PCE-polluted environments using biological strategies. Specifically, this study evaluated the impact of biostimulation alone (BS) and biostimulation combined with bioaugmentation (BS-BA) on the degradation of chloroethene in groundwater enrichment cultures. This involved assessment of the dechlorinating potential using laboratory-based experiments prior to future in situ anaerobic bioremediation. It is believed that the data obtained from this study would provide a better understanding of the feasibility of each treatment for site-specific applications. The bioremediation strategies were applied to PCE-contaminated groundwater obtained from a study site located in Victoria, Australia, which had been previously unsuccessfully treated using ISCO by injecting modified Fenton reagents such as hydrogen peroxide with iron chelate catalysts. To successfully apply the bioremediation approach over the failed ISCO attempt, we chose electron donors such as acetate for biostimulating dechlorinating organisms. A consortia of *Dehalococcoides* (*Dhc*) strains FL2, BAV-1 and GT was used for bioaugmentation. Time-intensive studies were performed intermittently to give a more detailed picture of comparative culture performance during which the biodegradation of electron donors and the formation of dechlorination products were documented. 16S rRNA dechlorinator-targeted quantitative real-time PCR (qPCR) was used to monitor the abundance of dechlorinating populations throughout the treatments to determine the extent of their growth in relation to the rate of chloroethenes removal. In addition, a live/dead cell count (LDCC)-based assay was conducted for quick monitoring of dechlorinating microbial cell viability during dechlorination.

Materials and methods

Chemicals

All chlorinated ethenes, ethene and other chemicals for microcosm preparation and analytical measurements were purchased from Sigma-Aldrich (NSW, Australia) with a minimum purity of 99.5 %. All gases were ordered from Coregas (VIC, Australia).

Groundwater sample collection

Since 1935, the study site located in Victoria, Australia, has a history of commercial industrial activities. The latest commercial activity at the site was foam manufacturing. Initial investigations identified chemicals of concern including PCE which has formed dense non-aqueous phase liquid (DNAPL) pools acting as a long-term source of contamination. Based upon varying PCE concentrations at different locations, groundwater samples from four different monitoring wells (MWs 1–4) were collected using the protocol suggested by Ritalahti et al. (2010). Temperature, pH, redox potential, specific conductance and dissolved oxygen were measured in groundwater that was pumped through a flow cell (YSI, VIC, Australia) onsite, with a pH/mV/EC/T/O_2 multi-parameter and corresponding probes (YSI) (Table 1).

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Table 1 Field characteristics of groundwater samples at the time of collection

Monitoring well	Initial PCE concentration (µg/l)	Temp (°C)	pH	Eh (mV)	Dissolved oxygen (ppm)	EC (uS/cm)	Alkalinity (ppm)	Soluble Iron (ppm)
MW 1	146.0	19.8	6.90	184.9	9.97	12,698	960.0	0.0
MW 2	3,540.0	19.1	6.93	247.4	10.02	12,665	880.0	0.0
MW 3	130.0	18.2	7.29	160.6	7.71	17,214	820.0	3.0
MW 4	5.0	18.0	7.68	110.3	1.16	19,006	260.0	2.0

Enrichment culture development

Two sets of enrichment cultures, A and B, were set up as per the guidelines presented by Löffler et al. (2005). Set 'A' designates biostimulation only (BS), while set 'B' designates biostimulation (BS) and bioaugmentation (BA) combined approaches for dechlorinating PCE. Both culture sets were prepared in Wheaton serum bottles (125 ml nominal volume) containing 75 ml of growth medium and 20 ml of groundwater as an inoculum which were sealed with Teflon-coated butyl rubber septa and aluminium crimp caps (Alltech, VIC, Australia). Anoxic mineral salt medium was prepared as per ATCC guidelines (American Type Culture Collection; www.atcc.org) and was amended with acetate (5 mM) as an electron donor and PCE (5 µl) as an electron acceptor. Hydrogen (5 in 95 % nitrogen) was added in the headspace (5–10 % of the headspace volume of a bottle) of acetate-fed cultures at a low partial pressure of 9 kPa (He et al. 2003). Cultures were prepared under strict anaerobic conditions and maintained in an anaerobic glove box (La-Petite, Thermo Fisher Scientific Australia, VIC) using N_2:CO_2 at the ratio of 80:20 %. The mixed consortia of well-known *Dhc* strains FL2 (ATCC® BAA-2098), GT (ATCC® BAA-2099) and BAV-1 (ATCC® BAA-2100) was injected only into set B (1.0×10^4 cells/ml); however, all chlorinated compounds, vitamins and electron donors were injected into both sets from anoxic, sterilized stock solutions using a Hamilton gas tight syringe (Alltech, VIC, Australia). To minimize the contact of the inoculum with the air present in the syringes during transfers, syringes were reduced with freshly prepared, filter-sterilized aqueous sulphide solution (0.5 mM). All experiments were repeated in duplicate and appropriate controls (without inoculum and/or electron donor, autoclaved) accompanied each experiment. Immediately upon setup, all enrichment cultures turned clear from pink tint (given by resazurin redox indicator added to the groundwater) indicating establishment of reduced conditions. Culture bottles were incubated statically at room temperature (22–25 °C) in the dark, and samples were analysed over 21 weeks (147 days). Since maintaining the optimum concentration of nutrients was essential for successful dechlorination, nutrient levels were monitored at predetermined time intervals by gas chromatography–mass spectrometry (GC/MS). Every 28 days, the samples from the cultures were analysed using GC/MS in order to measure the concentration of electron donors in addition to monitoring the conversion of PCE to secondary and tertiary products. Nutrient replenishments were carried out every time analyses indicated they were exhausted.

Analytical methods

Analytical procedures for both sets A and B were conducted as described by Zaan et al. (2010) with a few modifications. Chlorinated hydrocarbons were analysed in 1 ml gas headspace using a 5975C gas chromatographic (GC) system equipped with a mass spectrometry (MS), flame ionizing detector (FID) detector and a Porabond Q column (0.32 mm × 25 m) (Agilent Tech, Australia). The GC settings were: injector temperature 200 °C; detector temperature 300 °C; oven temperature 3 min at 40 °C, followed by an increase of 10 °C min^{-1} to 70 °C, followed by an increase of 15 °C min^{-1} to 250 °C for 7 min; and carrier gas (He) with a flow rate of 2 ml min^{-1}. External standards at six different concentrations from 0 to 30 µM were used for calibration. Chloride ion analyses on 1 ml diluted cultures were performed using a Chloride Analyser 926 (Ciba-Corning, Essex, England) as per the manufacturer's protocol.

Genomic DNA extraction

Every 28 days, cells for DNA extraction were collected from enrichment cultures under sterile and anaerobic conditions with a gas tight syringe. These samples (1 ml) were centrifuged for 30 min at 16,000*g*, 4 °C with pellets being re-suspended in 1x phosphate-buffered saline (PBS) buffer and stored at −20 °C for 1 h to enhance cell lysis. Genomic DNA was extracted with a Qiagen DNeasy Tissue Kit (Qiagen, NSW, Australia) according to the manufacturer's protocol with the following modifications: For improved cell lysis, 20 µl of lysozyme (100 mg/ml) and 180 µl enzymatic lysis buffer (20 mM Tris–Cl, pH 8.0, 2 mM sodium EDTA, 1.2 % Triton X-100) were added and the mixture was incubated for at least 30 min at 37 °C (Löffler

et al. 2005). The quality of the genomic DNA extracts was evaluated by electrophoresis at 110 V for 25 min on a 2 % w/v agarose gel stained with SYBR Safe (Invitrogen, Australia) and visualized by UV transillumination (Chemi Doc™ MP, BioRad, NSW).

Quantitative real-time PCR (qPCR)

Copy numbers of bacterial 16S rRNA genes from both sets A and B were quantified using universal bacterial 341F and 518 R primer set (Muyzer et al. 1993), while *Dhc*-specific genes were quantified using *Dhc*1F and *Dhc* 259R primers (Kim et al. 2010). Amplification was carried out using a MJ Mini Opticon™ real-time PCR detection system (BioRad, NSW) in reactions (25 µl) containing: Sybr Green Supermix (12.5 µl) (BioRad, NSW), distiled MilliQ water (8 µl), forward and reverse primers (1.25 µl; 6 pmol/µl) and DNA template (2 µl). The thermocycling program for 341F and 518 R was performed as described by Patil et al. (2010), while *Dhc* genes were quantified as per Smits et al. (2004). External standard curves showing the relationship between *Dhc* and 16S rRNA copy numbers, and *C(T)* values were generated with 3 × 10 fold serial dilutions. Amplification efficiencies were calculated from the slopes of the standard curves according to the formula: $E = 10^{-1/\text{slope}}$ (Rebrikov and Trofimov 2006). PCR efficiencies for both 16S rRNA and *Dhc* genes were between 1.8 and 1.95 with R^2 of 0.98 and 0.99, respectively (for standard curves see Fig. S1 in supplementary material). The *Dhc* target was normalized to the 16S rRNA target of the same sample using the following calculations: $\Delta C(T)_{\text{sample}} = \text{average } C(T)_{Dhc} - \text{average } C(T)_{16S\ rRNA}$. For the $2^{-\Delta\Delta C(T)}$ analysis, the normalized sample values were referenced to the values obtained for time point week 1 to study the x-fold increase in *Dhc* target, with the following equation: $\Delta\Delta C(T)_{\text{sample}} = \Delta C(T)_{\text{sample}} - \Delta C(T)_{\text{week 1}}$. The ratio of *Dhc* genes relative to the week 1 was estimated using $2^{-\Delta\Delta C(T)}$ (Livak and Schmittgen 2001; Treusch et al. 2005; Erkelens et al. 2012) (Table S1 in Supplementary material).

Cell viability test

Cell viability (live, dead and total cells) within sets A and B was measured periodically using Countess™ Automated Cell Counter (Invitrogen, Australia). Enrichment culture (10 µl) was mixed with 0.4 % trypan blue stain (10 µl), and then the sample mixture (10 µl) was loaded into the cell counting chamber slides to calculate cell count and viability as per the manufacturer's guidelines. Statistical significance was determined between different samples by *t* test analyses. Linear regression analysis was carried out to assess the relationship between cell concentration/ml obtained from LDCC analyses and cell copy numbers obtained from qPCR analyses. All statistical analyses were carried out in SPSS version 20.

Results and discussion

Reductive dechlorination of chlorinated compounds

Groundwater from MW 1–4 in set A (BS) and set B (BS-BA) was used to assess the biodegradative potential of natural microbiota upon stimulation by addition of nutrients or combined with the addition of microorganisms (Fig. 1). Monitoring of electron donor levels was carried out to ensure that optimum levels of these nutrients were maintained during the experimental period. Nutrient replenishments were carried out at week 9 (BS and BS-BA) and week 17 (BS only) as required (indicated with arrows in Fig. 1). Figure 1a shows the time course of 21 weeks (147 days) for the anaerobic PCE dechlorination by the enrichment cultures in MW 1–4. Although each of these enrichments displayed different dechlorination rates, PCE conversion began sequentially in all enrichments and the secondary products of dechlorination (TCE, *cis*-DCE and VC) coexisted until ethene was formed (Fig. 1a). The conversion of PCE to TCE commenced after about 25 days lag and complete conversion to *cis*-DCE occurred by week 13. The intermediate *cis*-DCE was completely dechlorinated to VC by week 17, and the complete dechlorination of VC to ethene was achieved by week 21. Dechlorination intermediates, *cis*-DCE and VC, were temporarily accumulated in all cultures. In contrast to the rapid conversion of PCE to *cis*-DCE, the dechlorination of *cis*-DCE to VC and ethene was relatively slow.

Figure 1b summarizes the results from the enrichment culture set B treated with nutrients and the *Dhc* consortia. In set B, PCE dechlorination commenced within 30 days and resulted in the rapid accumulation of TCE. Between weeks 5 and 9, accumulation of TCE and *cis*-DCE was observed. Its subsequent disappearance was coupled to VC formation. The hydrogenolysis of chloroethenes started at week 9 and resulted in a significant decrease in TCE and *cis*-DCE and a corresponding increase in VC and ethene concentrations by week 13 and 17, respectively. With set B cultures, the wells had different responses to dechlorination; however, in all cultures, PCE was dechlorinated sequentially to ethene by week 17 (119 days). In both sets of culture, the subsequent increase in chloride ion concentration observed during reductive dechlorination confirm biodegradation, since the chloride ion is a product of reductive dechlorination (Fig. 1a, b). PCE dechlorination did not progress beyond TCE and *cis*-DCE in the uninoculated and electron donor less autoclaved controls for both sets (data not shown).

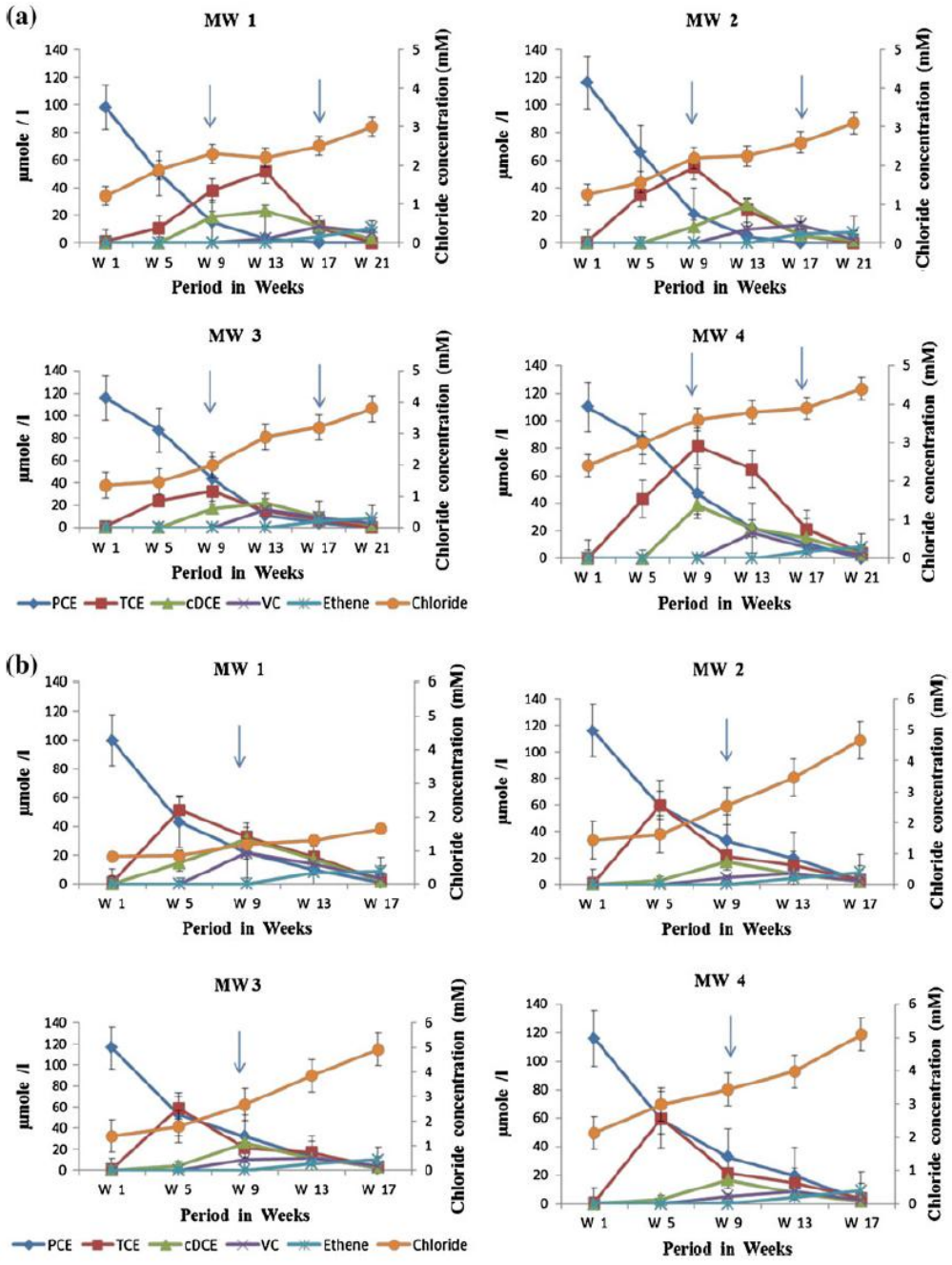

Fig. 1 PCE degradation and secondary product transformation in **a** BS only experiment (set A) and **b** BS-BA (set B) for MW 1–4 enrichment cultures. The *arrow* indicates the addition of electron donors. Data are the average of the duplicates (duplicates differed by <10 %). *Error bars* indicate standard error ($n = 2$)

The detection of some initial mass imbalance with PCE to TCE conversion in some wells is not unusual as this has been observed in other similar studies (Daprato et al. 2007; Yang et al. 2005). However, in this study, the system was stabilized after 20 days of incubation. In BS cultures, the electron donors enhanced complete dechlorination, indicating the presence of a native dechlorinating population whose dechlorinating activity was probably limited by the

lack of electron donors in the groundwater. Both BS and BS-BA treatments resulted in complete PCE conversion to ethene, but augmentation with *Dhc* consortia followed by stimulation was necessary to accelerate the rate and time of dechlorination in set B. Previously, a few studies carried out using only the biostimulation approach failed to attain complete dechlorination of PCE under laboratory and field conditions (Ibbini et al. 2010; Major et al. 2002; Lendvay et al. 2003). Incomplete dechlorination poses a major threat as accumulation of the intermediate dechlorination product VC is more toxic and carcinogenic than PCE and TCE (ASTDR 2007). Partial dechlorination is usually observed if the organisms that reductively dechlorinate ethene are not present or active at the contaminated site. However, this study reports the complete dechlorination of PCE via biostimulation, suggesting the presence of active indigenous dechlorinators in the groundwater collected from contaminated site. Fluxes of both H_2 and acetate might have controlled microbial redox processes in the biostimulation cultures that can synergistically sustain the complete reduction in PCE to benign ethene. Biostimulation increases the flux of H_2 and acetate (He et al. 2002), but competition for reducing equivalents, in particular for H_2 often limits its success (Sung et al. 2006). However, native dechlorinators stimulated in this study showed greater electron donor versatility towards acetate and H_2, indicating that an ecological niche in a well-controlled laboratory environment existed for organisms that could derive energy from the complete dechlorination of chlorinated solvents to ethene. To date, there is limited evidence using laboratory mixed cultures indicating that there might be organisms other than *Dhc* that can synergistically sustain complete reduction in PCE to ethene (Dong et al. 2011; Lee et al. 2011).

Growth-linked reductive dechlorination of chlorinated ethenes

16S rRNA gene-targeted qPCR was performed on sets A and B to study the correlation between rate of reductive dechlorination and growth of dechlorinating population in response to the BS and BS-BA treatments. Dechlorination of all four chlorinated ethenes was accompanied by an increase in 16S rRNA gene copies (Fig. 2). During BS, cell copies of indigenous microorganisms in enrichments MW 1–4 increased from week 1 to 21 from 2.01×10^4 to 1.43×10^5; 1.53×10^4 to 1.66×10^5; 2.63×10^4 to 1.79×10^5 and 2.27×10^4 to 1.77×10^5 cells/ml, respectively (Fig. 2a). Cell density in cultures MW 1 and 3 dropped in week 9 but increased by week 13. Most likely, the electron donors had become depleted as both dechlorination and growth accelerated by week 13, when nutrients were replenished. This suggests that the growth of dechlorinators is dependent on electron donors and dechlorination can slow down if these substrate are depleted (Cichocka et al. 2010). For the BS-BA experiment in set B, a mixed consortia of *Dhc* strains (1×10^4 cells/ml) was added into PCE-contaminated groundwater enrichment cultures followed by stimulation with electron donors. In enrichments MW 1–4, initial total cell concentration at week 1 was 3.65×10^4, 3.89×10^4, 3.08×10^4, 7.21×10^4 cells/ml which increased significantly to 7.98×10^5, 7.43×10^5, 6.98×10^5, 8.09×10^5 cells/ml by the end of dechlorination in week 17 (Fig. 2b). Similar to set A, a decrease in total cell copies in set B was observed for enrichments MW 3 and 4 during week 9 which increased in week 13 after nutrient replenishment. This emphasizes the fact that nutrients should be added to sustain native dechlorinators and *Dhc* activity throughout the process to achieve complete dechlorination. There was a distinct difference in the cell numbers between the two sets. The BS-BA culture contained at least an order of magnitude greater numbers of cells than in BS only cultures which is consistent with the higher ethene production rates in the augmented cultures (Fig. 2). However, over the experimental period, the number of cell copies did not increase significantly (week 1; 2.0×10^4 cell/ml and week 21 3.21×10^4 cells/ml) in the control sets where inoculum and electron donors were omitted from the system.

Analysis of groundwater from the BS enrichment culture with universal bacterial primers amplified 16S rRNA genes from indigenous dechlorinators; however, *Dhc*-specific 16S rRNA gene primers failed to detect *Dhc* population (Fig. 3a). This indicated the absence of *Dhc* species in the groundwater collected from PCE-contaminated site, although it was also possible that they could have been below the detection threshold of the primers used. Upon stimulation, enrichment cultures MW 1–4 showed ~3.86-, 2.58-, 2.96- and 3.73-fold increase in the abundance of 16S rRNA genes in set A at week 21 compared to week 1, respectively (Fig. 3a). As the absolute amounts of 16S rRNA copies measured in the same cultures stayed constant at week 1, the increase in the gene copies by the end of week 21 was found to be statistically significant (*t* test, 95 % confidence level). In order to analyse whether the *Dhc* species might be induced in set B, the *Dhc* target gene was normalized to the 16S rRNA target of the same culture (Treusch et al. 2005). Figure 3b displays the relative increase in *Dhc* genes normalized to 16S rRNA over the period of 17 weeks. While the abundance of 16S rRNA and *Dhc* genes was largely the same at week 1, bioaugmentation followed by stimulation resulted in approximately twofold and threefold increase in the abundance of 16S rRNA and *Dhc* genes by week 17, respectively (Fig. 3b). The comparison of the

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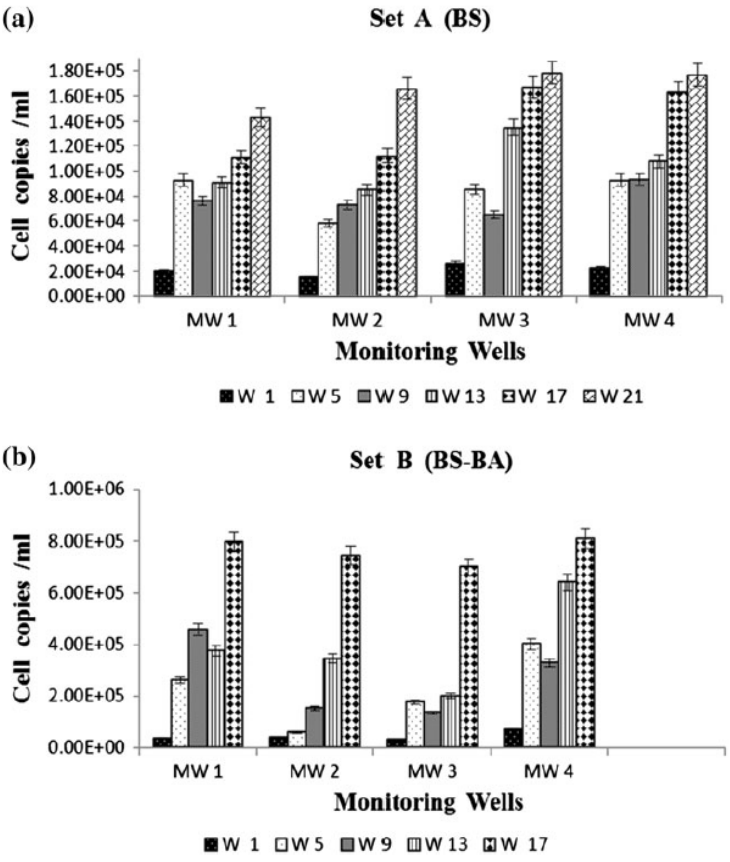

Fig. 2 Quantitative estimation of population abundance by qPCR in two PCE-dechlorinating enrichment sets of **a** BS only and **b** BS-BA over the period of 21 weeks. *Error bars* indicate standard error ($n = 2$)

bioremediation treatments and the quantitative analyses showed that rapid dechlorination in the BS-BA cultures could be enhanced due to simultaneous increase in *Dhc* and native dechlorinating microorganisms. The outcome of competition between *Dhc* and other chlorinated ethene respirers over electron donors, especially H_2 could possibly influence the rate and extent of PCE dechlorination (Becker 2006). The results obtained therefore suggested a syntrophic association between *Dhc* species and other native dechlorinators existed to carry out PCE reduction. Previously, an increase in *Dhc* gene copy numbers has been associated with enhanced reductive dechlorination of chlorinated compounds (Cichocka et al. 2010; Cupples 2008; Duhamel and Edwards 2006; Smits et al. 2004; Sung et al. 2006). Therefore, a approximately threefold increase in *Dhc* gene abundance over a approximately twofold increase in 16S rRNA genes might have synergistically contributed to the complete and rapid dechlorination of PCE in set B. BS and BS-BA treatments in enrichment cultures MW 1–4 enhanced dechlorination at different rates (Fig. 2) which could be due to varying abundance and response level of dechlorinators present at respective wells (Fig. 3).

Cell viability assay

The dechlorination process depends upon the presence and viability of an appropriate microbial population to facilitate the reaction (Major et al. 2002). The higher the viability of cells, the greater the ability of dechlorinating cells to reproduce in controlled anaerobic environment and therefore to reduce PCE by substrate utilization. Therefore, in addition to qPCR, we conducted cell viability assays to quantitatively distinguish between live and dead bacterial cells based on a total cell sample in a mixed population. For biostimulation, total cell concentration in enrichments MW 1–4 increased from weeks 1 to 21 from 2.05×10^4, 1.52×10^4, 2.69×10^4, 2.23×10^4 to 1.41×10^5, 1.67×10^5, 1.8×10^5, 1.76×10^5 cells/ml, respectively (Fig. 4a). The overall cell viability (live/dead cells based on total cell counts) throughout the dechlorination process was higher in MW 1 and 2 (80 %) than in MW 3 (75 %) and MW 4 (70 %).

For the BS-BA enrichments MW 1–4, initial total cell concentration at week 1 was 3.64×10^4, 3.87×10^4, 3.1×10^4, 7.24×10^4 cells/ml which increased to 7.96×10^5, 7.42×10^5, 6.98×10^5, 8.1×10^5 cells/ml

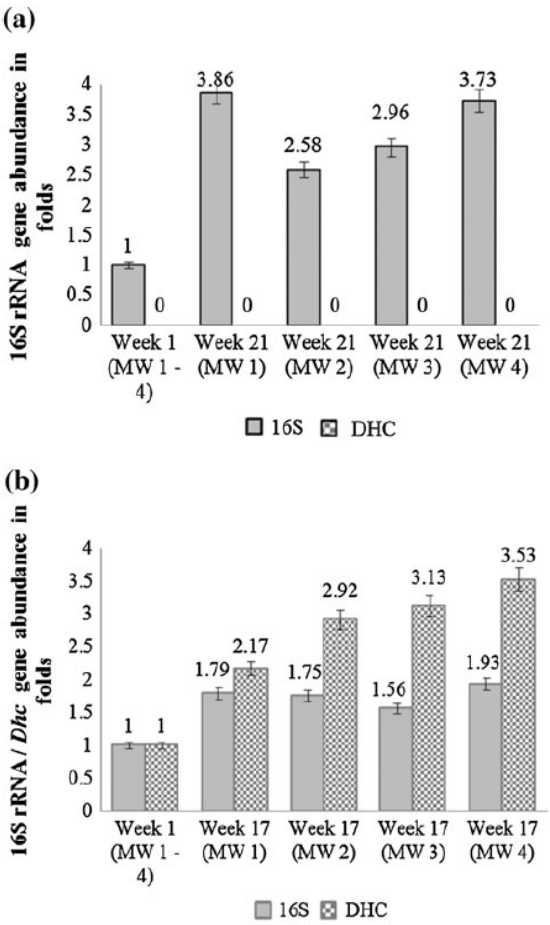

Fig. 3 Relative abundance of *Dhc* gene copies referenced to 16S rRNA of indigenous bacteria in the **a** set A with BS only and **b** set B with BS-BA microcosm experiments using qPCR analysis. *Dhc*-specific 16S rRNA genes failed to amplify in set A, indicating its absence in the groundwater. The fold increase over 21 weeks was calculated with normalized sample values and referenced to the week 1 values. *Error bars* indicate standard error ($n = 2$)

by the end of dechlorination in week 17 (Fig. 4b). Cell viability during the incubation period was approximately 75, 82, 78 and 79 % in cultures MW 1–4, respectively. Although total cell numbers of native cells increased in the BS, the numbers were at least an order of magnitude lower than BS-BA. In control samples (week 1; 2.0×10^4 cells/ml and week 21; 3.69×10^4 cells/ml), there was no significant increase in cell count and viability over the experimental period. In general, the total cell concentration within both sets increased with increasing cell viability as dechlorination progressed. Overall, the data indicated that higher cell viability can be correlated with active dechlorination and presumably growth within a microbial community.

We compared our total cell concentration results within both sets obtained using cell counter and qPCR (Figs. 2, 4). The comparative analyses demonstrated similar trend of increased total cell copies as dechlorination progressed, showing a good correlation between the LDCC and qPCR quantitative data ($r > 0.95$). In terms of methodology, this study therefore demonstrates the usefulness of using alternative low-cost quantification technique such as LDCC for monitoring total cell numbers and viability of organisms involved in reductive dechlorination. The LDCC assay provided insights into dechlorinating microbial viability which was highly similar to that obtained from qPCR analyses. LDCC could be a cheaper and faster alternative to the more rigorous qPCR method for quantitative analysis as it requires substantially less financial outlay compared to qPCR. It is also more portable and bioremediation practitioners can easily be trained in its use without the need for them to have a scientific background unlike in qPCR. Therefore, this could be of particular interest during field trials, where quick monitoring of microbial activities needs to be done to assess the progress of bioremediation.

Feasibility of bioremediation treatments

Bioremediation, both natural and enhanced, has proven to be a powerful approach for remediating chlorinated solvents (Cupples et al. 2004; Lee et al. 1997; Maymó-Gatell et al. 1997). But in recent years, there has been considerable debate over whether bioaugmentation is beneficial over biostimulation, particularly when it comes to commercial site application (ESTCP 2005). The decision to bioaugment or biostimulate is a function of several factors including economic, political and technical considerations. Within the last decade, basic research on natural microbial dechlorination mechanisms has shown that degradation of chlorinated compounds can be practically achieved by stimulating microbial reductive dechlorination (Ellis et al. 2000). However, conditions in some groundwater wells may not be completely anoxic, as observed in MW1-MW3 (Table 1) in this study. In order to ensure efficient microbial reductive dechlorination, low-H_2-generating organic substrates such as acetate, lactate or molasses could be added to create the required anaerobic conditions (Aulenta et al. 2006; ESTCP 2004; Fennell et al. 1997; He et al. 2002). Some researchers have stated that at the vast majority of sites, the desired activities will occur by stimulating existing environment and it is simply a matter of more time and more electron donors (Koenigsberg et al. 2003; Suthersan et al. 2002). Some environmental regulatory bodies are also wary of adding organisms, particularly mixed cultures in which not all of the organisms are fully characterized (Ball 2012). In such scenarios, bioremediation needs to be carried out by stimulating already present

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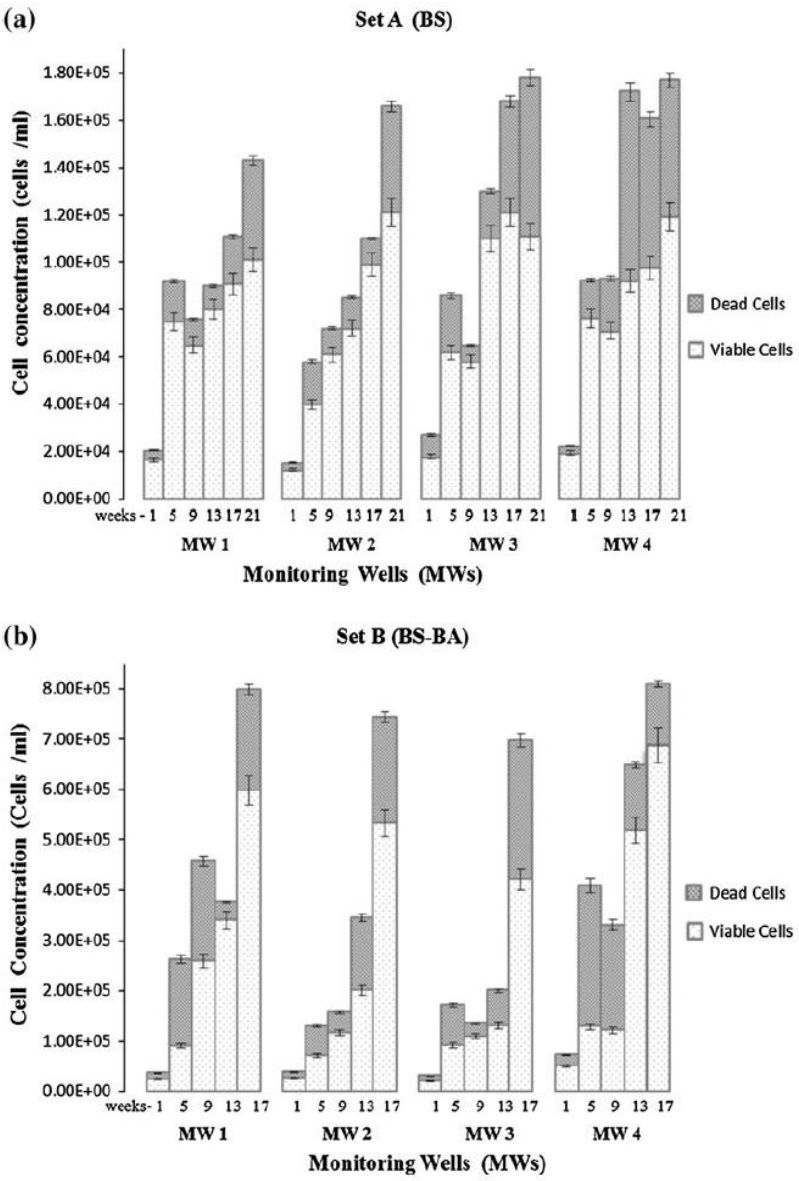

Fig. 4 Cell viability assay of **a** BS only (set A) and **b** BS-BA experiment (set B) for MW 1–4 enrichment cultures over the period of 21 weeks. *Error bars* indicate standard error ($n = 2$)

native microorganisms that are well suited for subsurface environments and well distributed spatially within the subsurface.

Studies demonstrating biostimulation followed by bioaugmentation with an enrichment culture capable of complete dechlorination of PCE to ethene indicated some benefits of this strategy over a biostimulation only approach (Ellis et al. 2000; He et al. 2003; Sung et al. 2006; Cichocka et al. 2010; Ibbini et al. 2010; Major et al. 2002). Bioaugmentation may offer a solution for contaminated sites where dechlorination is not occurring naturally (where the appropriate organisms are lacking) or where it is too slow to be practical (very low numbers of dechlorinating organisms) (Major et al. 2002). Even at sites where competent *Dhc* are present, bioaugmentation may decrease the lag time prior to the onset of dechlorination. This is particularly true for sites desiring rapid remediation due to an impending property transaction and stringent regulatory or commercial deadlines. Although costs for the culture solutions needed for bioaugmentation are decreasing, the inoculum itself can still be a significant expense at relatively large sites. The choice of electron donor can also affect the decision whether to or not to bioaugment. Bioaugmentation with *Dhc*-containing cultures may not

always ensure that complete dechlorination of PCE can be sustained in the presence of PCE to *cis*-DCE dechlorinating specialist unless adequate electron donor can be specifically delivered to *Dhc* populations (Becker 2006). In order for bioaugmentation to succeed, a niche must be created that is specifically available for augmented microorganisms (Ellis et al. 2000). The fate of electron donors and their fermentation products including not only H_2 but also other intermediates is of critical importance for understanding the response of dechlorinating communities. Using high-strength soluble donors (such as lactic acid, molasses) added at frequent intervals may make bioaugmentation more attractive, because it is relatively expensive to operate the system for even a few months without achieving complete dechlorination (ESTCP 2005). On the other hand, bioaugmentation may be less attractive when using long-lasting, less soluble donors (such as chitin, HRC™ or vegetable oil), because the time and additional operational and maintenance needed to achieve complete dechlorination may represent a relatively small incremental cost (Ibbini et al. 2010). Overall, this fundamental information on the ecology and biophysical interaction of community members involved in the partial and complete dechlorination process should help to better understand and design appropriate remediate strategies for chloroethene-contaminated sites.

Conclusion

This study reported a comparative pre-evaluation of BS and BS-BA approaches for the PCE remediation on groundwater samples obtained from a PCE-contaminated site. Both approaches resulted in complete dechlorination in samples, with the BS-BA approach resulting in a shorter dechlorination time frame. However, even when bioaugmentation cannot be applied either due to costs or legislative difficulties, biostimulation strategy can still be effectively applied leading to remediation of site. 16S rRNA-based qPCR and LDCC analyses were used to assess the dechlorinating community potential during PCE dechlorination. Regression analysis showed that LDCC represents a low-cost and 'low-tech' approach to monitoring the dechlorinating potential of community resulting in substantial time and financial savings. Given the variety of environmental factors expected in different contaminated sites, this study has demonstrated the value of site-specific pre-evaluation of PCE-contaminated sites using a combination of microbiological and chemical approaches; a crucial step to design a successful in situ field-based bioremediation strategy.

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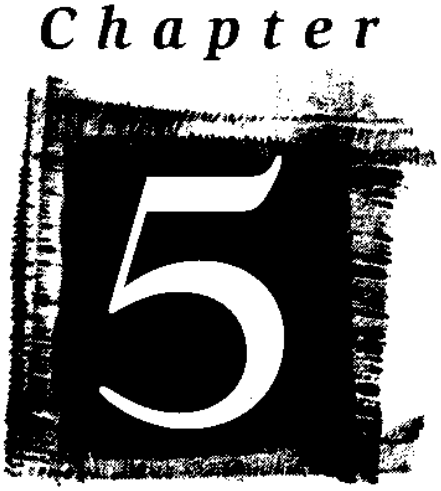

Application of molecular biological tools for the assessment of the *in situ* bioremediation potential of TCE

Statement of Authorship

"Application of molecular biological tools for the assessment of the *in situ* bioremediation potential of TCE", Submitted to *Groundwater Remediation and Monitoring* (Under Review)

1] Taylor D. Gundry

Performed experimental design, sampling, molecular and statistical data analysis, Interpreted data, partial manuscript writing, first author (50% contribution to experimental data generation)

I give consent for S.S. Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature Taylor Gundry **Date** 12/02/2014

2] Sayali S.Patil

Performed experimental design, sampling, chemical analysis of samples, quantitative data analysis, Interpreted data, partial manuscript writing, corresponding author (50% contribution to experimental data generation)

Signature **Date** 11/02/2014

3] Andrew S. Ball

Assistance with experimental design and data interpretation, manuscript evaluation

I give consent for S.S. Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature Andrew Ball **Date** 11/02/2014

Application of molecular biological tools for the assessment of the *in situ* bioremediation potential of TCE

Taylor D. Gundry [1], Sayali S. Patil* [1, 2] and Andrew S. Ball [1]

[1] Department of Applied Biosciences, Royal Melbourne Institute of Technology, Bundoora, VIC - 3083

[2] School of Biological Sciences, Flinders University of South Australia, Adelaide, SA - 5042

* **For correspondence:** Phone: (+61 03 99256678), Fax: (+61 03 99257110); E-mail: pati0018@flinders.edu.au

Abstract

Trichloroethene (TCE) is a common groundwater pollutant that can be difficult to remediate by conventional methods. Three major enhanced bioremediation strategies involving biostimulation only (BS), biostimulation-bioaugmentation (BS-BA) and monitored natural attenuation (MNA) were designed for the removal of TCE (40-150 µg/L) at a commercial site in Victoria, Australia. Although, molecular biological tools (MBTs) have been widely used for laboratory based microcosm studies, their real world applications are limited to basic molecular analyses. A detail insight into the dechlorinating microbial community structure, dynamics and their response to remediation can greatly assist in designing appropriate remediation plans.Hence, the aim of this study was to investigate *in situ* microbial community dynamics in TCE contaminated groundwater during three remediation treatments using a culture independent fingerprinting method involving PCR based denaturant gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR). All three remediation strategies were successful in depleting the TCE however only the BS-BA and BS method were able to completely remediate the TCE with the depletion of the intermediate products below maximum concentration level. DGGE analysis and sequencing revealed the presence of *Dehalococcoides, Geobacteriaceae, Sulfurospirillum* and *Pseudomonas* species across the site.There was no statistically significant difference between the diversity and equitability index between treatment groups ($p = 0.084$, $p= 0.083$ respectively) however over the course of the trial the diversity amongst all groups increased slightly.

The application of MBTs allowed the monitoring of the microbial community fitness throughout the trials, giving the option to change nutrient regime if it had been necessary.

Introduction

Among halogenated hydrocarbons, chlorinated hydrocarbons are the most potent and environmentally persistent pollutants due to their strong C-Cl bonds. They have been extensively used in agriculture and industrial applications such as pesticides, solvents and in the plastics industry (SCRD 2007). In particular, the dry cleaning agent tetrachloroethene also known as perchloroethene (PCE) and the industrial solvent trichloroethene (TCE) are usually recalcitrant under oxic conditions; however, they can be effectively biodegraded under anaerobic conditions such as those prevailing in aquifers by microbes performing reductive dechlorination i.e. the substitution of chlorine by a hydrogen atom. Bacteria performing reductive dechlorination fall into three phylogenetic clusters, among which the *Chloroflexi* group is of particular interest because several of its member strains *Dehalococcoides ethenogenes* (*Dhc*) 195, FL2, GT and BAV1 can completely dechlorinate chloroethenes to environmentally benign ethene (Cichocka et al. 2010; Cheng et al. 2010). However, in nature and in the most robust laboratory cultures, reductive dechlorination is performed by microbial consortia rather than pure cultures (Duhamel et al. 2004; Ellis et al. 2000; He et al. 2003). Hydrogen (H_2), primarily supplied by syntrophic organic fermenters is known to be a key electron donor for reductive dechlorination by *Dhc* (Hendrickson et al. 2002; Lendvay et al. 2003; Major et al. 2002; Müller et al. 2004).

For commercial chloroethene remediation, the decision to follow biostimulation (addition of nutrients or substrates) or bioaugmentation (injection of known microbial consortia) depends upon economical, local legislative and site-specific technical factors. Site-specific pre-evaluation of contaminated sites using combined microbial and analytical approaches provides an insight for designing effective remediation strategies (Patil et al. 2013). So far, several laboratory studies have extensively assessed the role of *Dhc* mixed cultures for chloroethene removal by using molecular biological tools (MBTs) like PCR based denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (qPCR) (Cichocka et al. 2010; Cheng et al. 2010; Duhamel et al. 2004; He et al. 2003). However, the real world

applications of these techniques to monitor commercial bioremediation potential are still limited. For example, field trials conducted by Lookman et al. (2007), Major et al. (2002), Schaefer et al. (2010) successfully demonstrated chloroethene degradation by biostimulating and augmenting *Dhc* cultures in PCE contaminated aquifers; however, the results were primarily based on basic PCR detection indicating only the presence or absence of the classic dechlorinator, *Dhc*. Microbial profiling tools such as DGGE and qPCR hold great promise to unravel microbial community structure, dynamics, functionality and abundance throughout the remediation process (Cupples 2008; Marzorati et al. 2008). Fundamental knowledge of these factors is quintessential to understand the site characteristics and to further predict the contaminant degradation pattern (Patil et al. 2013). If adequate potential dechlorinators already exist within the contaminated zone it may only need the injection of appropriate substrates to enhance the degradation rates otherwise, known microbial consortia may be added to facilitate dechlorination. In some cases, where stringent environmental regulations preclude the addition of microbial consortia in contaminated aquifers, appropriate decisions could be made based on microbiological data obtained from preliminary site screening by applying MBTs. DGGE and qPCR can be effectively used to assess the overall remediation progress by studying microbial shifts by correlating dechlorination rates with specific or total community response during *in situ* trials. Moreover, preliminary and during treatment assessment by molecular and analytical tools can provide indications of substrate depletion or microbial cell abundance. For commercial bioremediation practitioners, where cost is always crucial, these parameters can set guidelines to follow subsequent economically viable and effective steps to monitor the remediation process.

In situ chloroethene bioremediation remains to be optimized based on an extensive knowledge of the microbes and pathways involved in the process. Considering the advantages MBTs has to offer for commercial bioremediation, here, we describe the applications of MBTs for monitoring *in situ* bioremediation involving three treatments, biostimulation only (BS), biostimulation plus bioaugmentation (BS-BA) and monitored natural attenuation (MNA) for treatment of a TCE contaminated plume. Dechlorinating microbial community structure and dynamics before and after the three bioremediation treatment were also studied.

Methods

Site Characterization

The study site (Figure 1) located in Mordialloc, a southern suburb of Victoria in Australia has historically been used for light industrial activities including manufacture, machining and warehousing of metal products, which have resulted in impacts to fill material and groundwater, predominantly metals and halogenated organic compounds, respectively. Previous environmental assessment identified a TCE contamination source zone. Soils from the targeted zone consisted of orange brown, soft to firm, clay, minor gravel, sand. Soil porosity ranged between 40-45%. Hydraulic conductivity estimated using slug test data was 0.02 m/day. Groundwater velocity was approximately 0.005 m/day. Further site details are restricted due to confidential agreements. For this study, twelve wells with various TCE levels ranging from 40 to 150 µg/L were selected. Out of twelve MWs, MW 22, MW 11A, MW 15A, MW 17 were dedicated for BS only treatments while, GW 8A, GW 1B, MW 16B, GW 4B were allocated for BS-BA treatment and the rest of the wells including GW 6A, MW 20A, GW 5 and MW 21 were allocated for MNA treatment (Fig. 1). A control well, MW 10A with no TCE contamination was used for comparative treatment analysis. This study was conducted over a period of nine months from March to November 2013. Construction details of selected wells are listed in Supplementary Information (SI) Table 1.

Groundwater Collection

Groundwater samples were collected prior to bioremediation treatments (PT) in March 2013; during treatment (DT) from April-October 2013 and post-treatment (PST) in November 2013. A total volume of 4 L of groundwater was collected from the screen interval between 5 to 8 mbTOC from all thirteen wells using polypropylene bailer (Bunnings Warehouse, VIC, Australia). A flow-through cell (YSI, VIC, Australia) recorded pH, oxidation-reduction potential (ORP), specific conductance, temperature,

dissolved oxygen (DO) and turbidity of groundwater. When geochemical parameters were stabilized the flow-through cell was disconnected, and replicate samples were collected consecutively without flow interruption. Pre-treatment groundwater characteristics are described in Table 1. Sample containers consisted of sterile and N_2 -purged 4 L high density polyethylene Nalgene bottles with polypropylene screw caps (Thermo Scientific Australia, NSW) were filled to the capacity. Upon collection, bottles were transported to the analytical laboratory. All samples were stored in the dark at 4°C until further use.

Analytical Procedures

For analytical measurements, groundwater samples were collected at an interval of 30 d starting from the month of April to October. Samples were analysed for chlorinated ethenes using a 5975C gas chromatographic (GC) system equipped with a mass spectrometry (MS), flame ionizing detector (FID) and a Porabond Q column (0.32 mm by 25 m) (Agilent Tech, Australia). Chlorinated hydrocarbons were analysed in a 1 mL gas headspace. The GC settings were: injector temperature 200°C; detector temperature 300°C; oven temperature 3 min at 40°C, followed by an increase of 10°C min^{-1} to 70°C, followed by an increase of 15°C min^{-1} to 250°C for 7 min; and carrier gas (He) with a flow rate of 2 mL min^{-1}. External standards at six different concentrations from 0 to 30 µM were used for calibration. Acetate and bromide (tracer) were measured using an ion chromatograph (ICS-1100, Dionex; Thermo Scientific Australia, NSW).

Biostimulation and Bioaugmentation Treatments

Injections of the electron donor sodium acetate were performed on BS and BS/BA wells (1 kg/well). Sodium acetate was mixed with groundwater extracted from each of the BS and BS-BA wells. The mixture was heated to dissolve acetate quickly and allowed to cool down before re-injecting in to the wells. A tracer test was conducted by adding sodium bromide (500g/well). *Dhc* strains FL2 (*Dehalococcoides* sp. ATCC® BAA-2098™), BAV1 (*Dehalococcoides* sp. ATCC® BAA-2100™) and GT (*Dehalococcoides* sp. ATCC® BAA-2099™) outsourced from ATCC (American Type Culture Collection) were used as the source material to scale-up the inoculum to be injected at the contaminated site. This culture was grown using anoxic minimal salt medium as described by Löffler

et al. (2005) and ATCC guidelines (www.atcc.org). Scale up (5 L) was conducted through serial 10% (v/v) transfers as described by Vainberg et al. (2009). Cultures were incubated in the dark at room temperature for up to 14 d. All cultures were spiked with an aqueous TCE to a final concentration of 5 µL/L. After 2 weeks of sodium acetate injection, bioaugmentation began with the addition of 1 L of *Dhc* mixed consortia ($1.5x10^3$ cells/L) into each BS-BA well.

Microbial Profiling

For microbial analysis, a total volume of 2 L was filtered using sterile 0.22 µm cellulose acetate filters (Satorius Stedim Biotech, Germany). Microbial community DNA was extracted using a PowerWater DNA Isolation Kit (MoBio laboratories) as per the manufacturer's instructions. Extracted genomic DNA was amplified on a T100 thermal cycler (BioRad, NSW, Australia) using the universal bacterial primer pair 314F-GC clamp and 907R (Sapp et al. 2007) as per the program described in Table 2. Amplified 500 bp PCR fragments were further analysed using a DGGE D-Code System (BioRad, NSW, Australia) in accordance with the manufacturer's instructions. The 45 – 60% denaturing gradient gel was run for 18 h at 60°C and 60 V and was silver stained as described by Patil et al. (2010). Upon staining, dominant DGGE bands were excised using sterile razor blades and incubated in 100 µL of nuclease free water for overnight at 37°C and stored at -20°C until re-amplification. Re-amplification was performed using 341F without GC clamp/907R primers (Table 2) and then re-amplified products were purified using the Wizard® SV gel and PCR clean up system (Promega, Madison, WI, USA) as per the manufacturer's instructions. The eluted DNA was checked for concentration and purity using a Nanodrop Lite spectrophotometer (Thermo Scientific Australia, NSW). Samples were then processed as per the guidelines set by the Australian Genome Research Facility (AGRF) and sequenced using an automated sequencer ABI 3730. Nucleotide sequences were analysed using SEQUENCHER software (Sequencher Version 4.1.4, GeneCodes Copr., Ann Arbor, MI, USA) and homology searches were completed with the BLAST server of the National Centre for Biotechnology Information (NCBI) using a BLAST algorithm (http://www.ncbi.nlm.gov.library.vu.edu.au/BLAST/) for the comparison of a nucleotide query sequence against a nucleotide sequence database (blastn).

Real-time Quantification

From the beginning to the end of the treatments total microbial abundance was measured using qPCR. This was performed with a Rotor-Gene PCR machine (Qiagen, Australia) in the 72-well rotor using *Dhc*, *Geobacteriaceae* (*Geo*) specific and bacterial universal primer sets (Table 2). Amplification was carried out using a reactions mixture (25 µL) containing: Sybr Green Supermix (12.5 µL) (BioRad, NSW), distilled MilliQ water (8 µL), forward and reverse primers (1.25 µL; 6 pmol/µL), and DNA template (2 µL) (Table 2). A standard curve was included routinely using a triplicate dilution series.

Statistical Analyses

Phoretix 1D advanced analysis package (Phoretix Ltd, UK) was used to measure relative band intensities or peaks on DGGE community profiles. The noise levels and minimum peak thresholds of the software were set to optimum values in order to reduce background noise peaks. Each band was considered to be a phylotype or species and the band densities were then used to calculate the Shannon Weaver diversity (H') and equitability index (J'). The Shannon Weaver diversity index is a general diversity value which increases as the number of species (bands) increases while the equitability index is a measure of the relative abundance of the different species (bands) in the sample (Dilly et al. 2004). The raw data generated from DGGE gels used for calculating H' and J indices is supplied in SI Table 3. A matrix for similarities for densitometric curves of the band patterns was calculated based on the Dice – Sorenson coefficient and dendrograms were created using unweighted pair group method using arithmetic averages (UPGMA) linkage. All statistical analyses were carried out in Minitab (version 14 Student).

Results and Discussion

Reductive dechlorination of TCE to ethene

Figure 2 compares the three bioremediation treatments, BS-BA (Fig. 2a), BS only (Fig. 2b) and MNA (Fig. 2c) in terms of the reductive dechlorination occurring in the groundwater converting TCE to ethene. All three treatments were successful in the depletion of TCE to below the maximum contaminate level (MCL) of drinking water set by the US EPA of 5.0 µg/L. The US EPA also sets the MCL for *cis*-DCE and VC in drinking water at 70 µg/L and 2.0 µg/L respectively (US EPA 2012a, 2012b). However only the

BS-BA and BS treated wells showed the complete degradation through to the depletion of the daughter product VC to below the MCL of 2.0 µg/L (BS-BA 1.87 µg/L; BS 1.84 µg/L and MNA 4.3 µg/L). For the two enhanced bioremediation techniques, VC was present above the MCL for about 100 days, while it was still present above the MCL in the MNA wells by the end of the trial period.

These results highlighted the benefits of enhanced bioremediation compared to the alternate passive technique for the complete remediation of TCE. As an indigenous dechlorinating community was already present pre-trial, biostimulation could be hypothesised to be an effective treatment as demonstrated by Patil et al. (2014). If no indigenous dechlorinating community was present the wells would need to be augmented with an inoculum of dechlorinatiors as demonstrated by Ernst (2009), Major et al. (2002) and Okutsu et al. (2012) which showed that complete remediation in the presence of active *Dhc* mixed consortia was most effective.

Physical and Chemical Groundwater Characteristics

Table 1 describes the groundwater characteristics such as pH, DO, ORP, temperature and colour for PT, DT and PST. The pH values within 6.5- 7.5 are considered to be optimal for bacteria growth (Burea Verita, 2011). At the study site, the reported pH values fell within 6.2 – 7.8 (acceptable range) indicating that conditions did not preclude microbiological activity and may help to explain the success of all three treatments. Also, anaerobic bacteria function best at DO concentrations less than 0. 5 mg/L. The pre-treatment DO readings (1.98-2.96 mg/L) suggested hypoxic conditions at DO >2.0 mg/L (Australian Government, 2013) under which reductive dechlorination is unlikely to occur. However, with the addition of substrates in the plumes anaerobic conditions were maintained with subsequent decreased DO levels (0.3-0.4 mg/L). The observed ORP readings of -70 mV (MW 15A/BS) and -38 mV (GW1B/BS-BA) indicated the possibility of reductive dechlorination occurring within the plume. Overall, throughout the remediation process conditions were suitable to facilitate the microbial reduction of TCE.

Tracer Test

By the end of the trial period (November) the bromide ion tracer had been detected in four downstream wells (MW17, GW4B, MW16B and MW21), indicating that the treatments had travelled between wells. This was unexpected as the hydraulic conductivity data suggested a flow rate of only 0.02 m/day indicating that the bromide ions should not have migrated so far. Another anomaly with the data is that acetate ions appeared in the control wells (MW10A) in the months of September and October, even though it was upstream from all the other wells on site. This highlighted the inherent uncertainties of undertaking field demonstrations, where the results are still valid, by virtue of replicates, but with greater uncertainty than would be expected from bench top studies due to factors such as the heterogeneity of the aquifer.

Addition of excessive electron donors may not necessarily benefit or fasten the dechlorination, while it may increase treatment cost (Wei and Finneran, 2013). Hence, during this trial a low amount of acetate (1 kg/BS and BS-BA well) was injected initially and then the re-injections (1 kg) were carried out upon donor depletion. This represented a logical and economically viable approach towards successful bioremediation avoiding the risk of generating excessive methane and clogging of soil pores. There is also the potential to use qPCR in conjunction with the IC results to monitor if the electron donor injections were being successful, by comparing the rate of decrease of the electron donor with the increase in the microbial cell abundance. This method would allow remediators to adjust their supplement application regime accordingly.

Microbial community profiling and dynamics during TCE remediation

Major bacterial sequences that were found in the ground water across the site are listed in Table 3. The rest of the sequences and their accompanying DGGE gels are shown in the SI Table 2 and Figure 1. Known dechlorinators found across the site were; *Sulfurospirillum sp.* (PT in well GW1B (BS-BA)), *Geobacter lovleyi* strain (DT in well GW4B (BS-BA)), *Pseudomonas sp.* (PST in well MW16B (BS-BA)) and *Dehalococcoides sp. DG* (PST in well MW16B (BS-BA). When compared with the DGGE gels, the bands these sequences were extracted from were present across the site in all three treatments, indicating the existence of a strong indigenous dechlorinating population.

Several earlier studies have reported bacterial diversity decrease with the presence of pollutants (De Lipthay et al., 2008; Lowe et al., 2002). The average Shannon Weaver diversity (H') was therefore expected to be lower in the contaminated wells than in the control wells. However the opposite was observed in this study. Figure 3a showed a less diversified community in the control well than that of the contaminated wells initially. This could indicate that the indigenous microbial community was not very diverse before the contamination of the chloroethenes. Two-way ANOVA of the H' index showed no statistically significant difference ($p = 0.083$), between either the treatments or time. However, a discriminant analysis test was able to match successfully 11 out of 12 wells to their correct treatment group. The contradictions of these two tests therefore suggest a slight difference between the treatments in relation to the H' index. The Equitability index (J') (Figure 3b) showed no observable trend in the data in relation to the time of the treatment. This indicated that over time bacterial abundance increased that were above the threshold of detection through PCR and DGGE. However, the J' indicated no trend of an increase or decrease suggesting none or few bacterial species were able to grow dominantly in the population. These results of the H' and J' indices demonstrate that augmentation of a *Dhc* consortia did not apply enough competitive pressure on the other microbes for them to become depleted or dominated, as a high J' and low H' would have indicated. This could aid in the argument for the reassessment of bioaugmentation guidelines for commercial bioremediation.

Microbial abundance during bioremediation of TCE

Injection of high dosages up to 160 – 200 L does not guarantee complete and rapid dechlorination which can lead to substantial financial losses (Schaefer et al. 2010). As a result, we precisely determined the amount of culture needed for a study site through preliminary site evaluation using qPCR and the cell density was measured periodically until ethene formation. Figure 4a, 4b and 4c details the bacterial abundance of; *Geo, Dhc* species and total microbial community respectively throughout the remediation process. The initial bacterial abundances of; *Geobacter sp.* and *Dhc sp.* were 200 ± 15 cells/L and 150 ±17 cells/L respectively across the site. Based on these values, 1.5×10^3 cells/L of *Dhc* mixed consortia was initially injected in BS-BA wells. By the end of treatment period, the final abundance of *Dhc sp*. in the BS-BA, BS, MNA and Control was 2.53×10^7 cells/L, 2.68×10^5 cells/L, 2.24×10^3 cells/L and 2.98×10^2 cells/L respectively.

On the other hand, the final abundances of *Geo sp.* in the wells per treatment were BS-BA (2.26 x10^6 cells/L), BS (2.13 x10^5 cells/L), and MNA (1.01 x10^4 cells/L) compared to the Control (2.99 x10^2 cells/L). These results clearly demonstrated that the enhanced treatments had a benefit of increasing the dechlorinating community. Confirmatory results obtained using microcosm studies by Patil et al. (2013) have shown that a BS-BA combination is able to increase the abundance of the dechlorinating community greater than the BS only treatments.

Commercial competitiveness of MBTs based bioremediation

The cost of a remediation method represents an important factor in selecting a commercial remediation strategy. In this study, the site was intended for further residential development so a delay in approval by the appropriate authorities was also a cost to the owners. Bioremediation was successfully achieved by using MBTs within 221 days for the BS-BA and BS treatments, while the VC still remained in the MNA wells (4.2 µg/L). Major et al. (2005) demonstrated that bioaugmentation represented a cost effective measure when compared to biostimulation. Such a claim could not be drawn from the results of these trials as all wells had *Dhc* present.

Conclusions

In summary, this study reported three different bioremediation techniques (BS, BS-BA and MNA) to successfully decontaminate a TCE plume on a commercial scale. Considering the high cost and long timeframes required for few other pilot studies, this case study represents a short and inexpensive way of chloroethene bioremediation. So far, the application of MBTs for commercial site clean-up is restricted to basic PCR detection and qPCR. Hence, the knowledge governed from PCR based DGGE molecular fingerprinting assays throughout this field trial provided additional information about microbial community shift and diversity in response to TCE degradation. Overall, this case study highlighted that the bioaugmentation process does not necessarily deplete microbial biodiversity. Also the study confirmed that careful preliminary laboratory testing is crucial to design a successful and cost-effective bioremediation plan and MBTs can be potentially applied to assess the success rate of commercial remediation.

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List of Figures

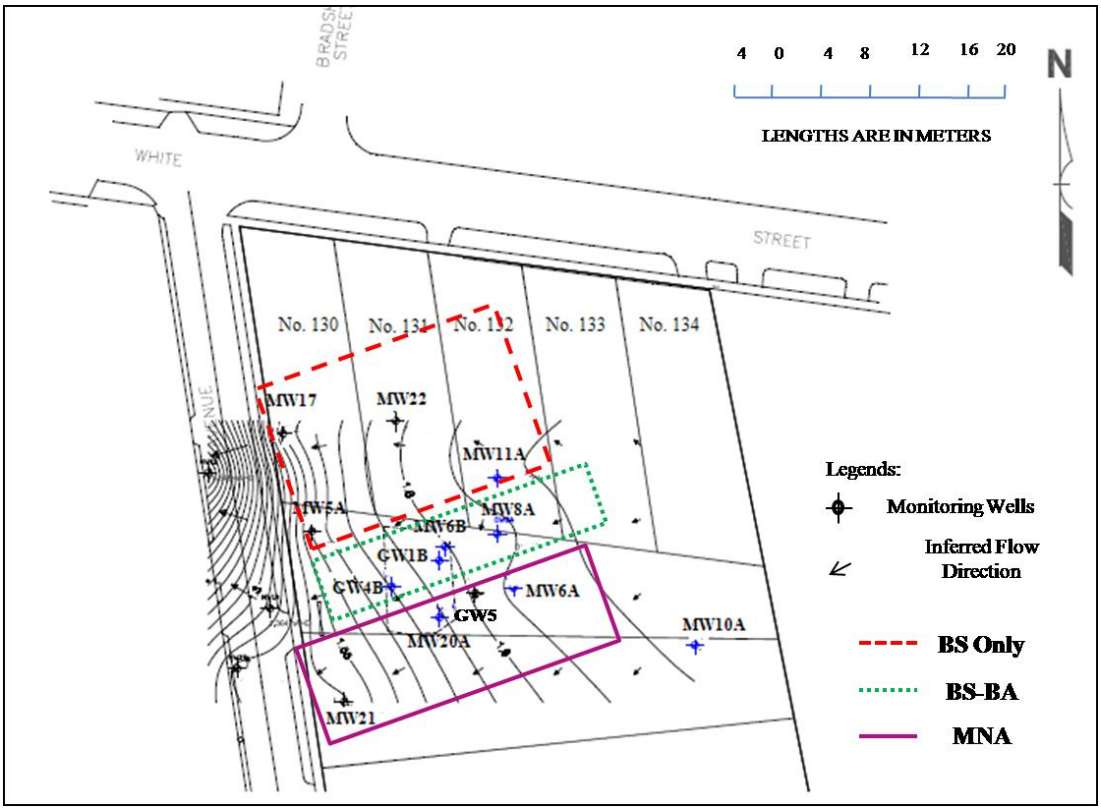


Figure 1. TCE contaminated study site with groundwater contour plan. Boxes with red dashed, green dotted and purple clear lines indicate the wells selected for BS only, BS-BA and MNA treatment zones, respectively. The contours designate TCE plume at 10 m bgs.

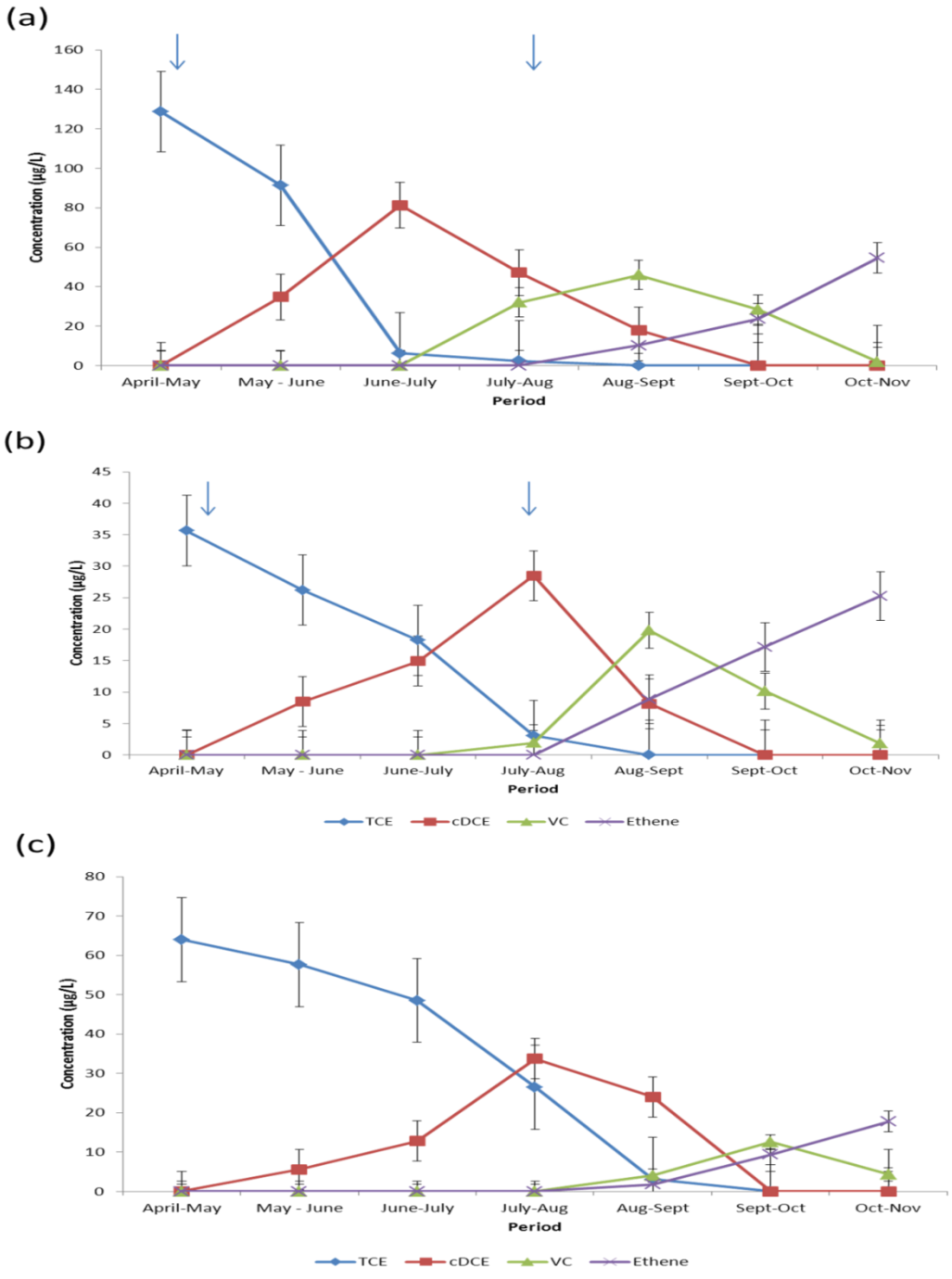


Figure 2. Sequential dechlorination of TCE to ethene within groundwater plume over the period of seven months from Apr – Nov' 2013 by **(a)** BS - BA, **(b)** BS only and **(c)** MNA treatments. Results are the means of four replicate wells for each treatment. The error bars represent standard error. The arrows indicate injection of electron donors into the wells.

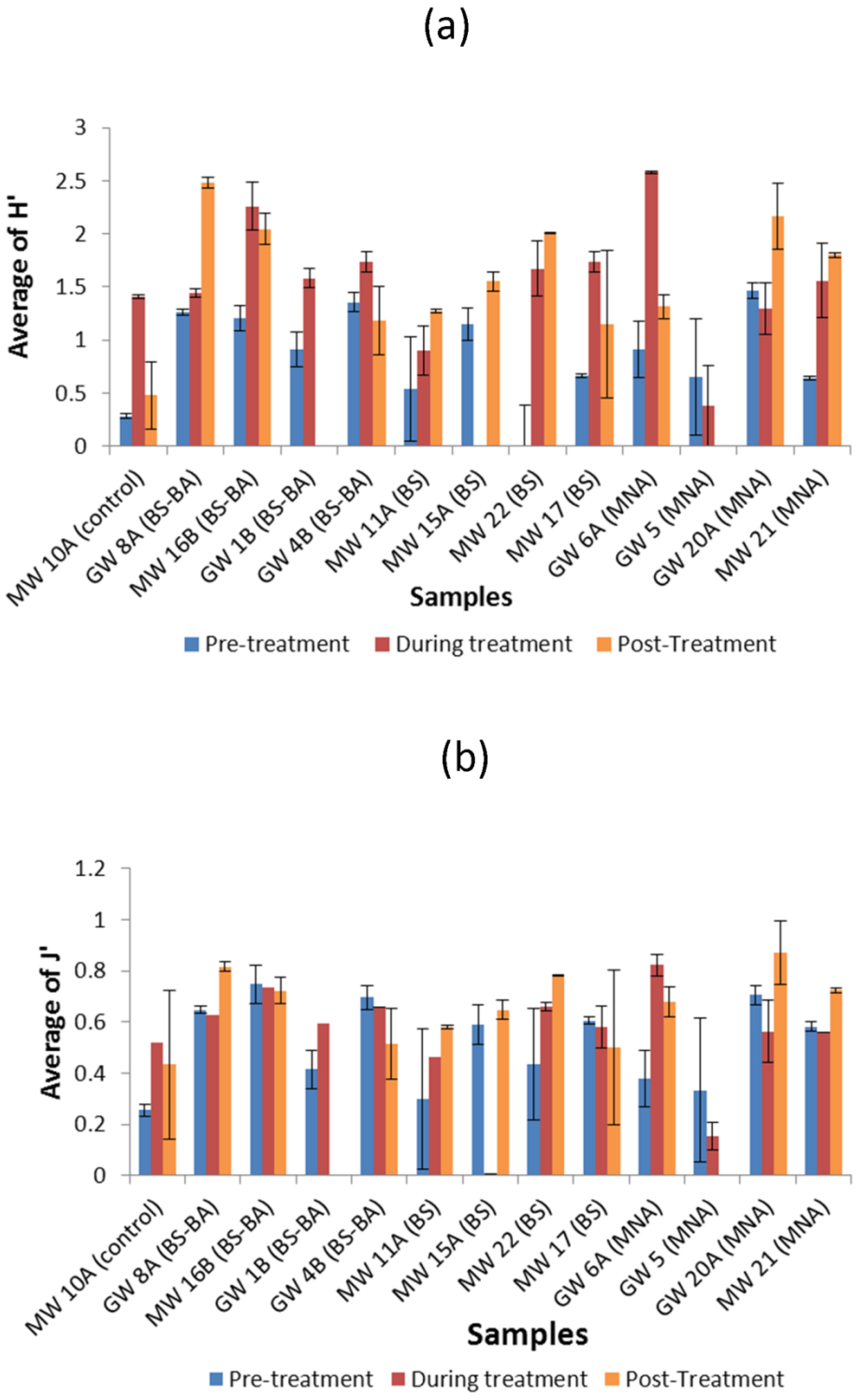


Figure 3 (a) Shannon Weaver diversity index (H') and **(b)** Equitability index (J') for the groundwater microbial community, derived from DGGE gels of each well for PT, DT and PST phases.

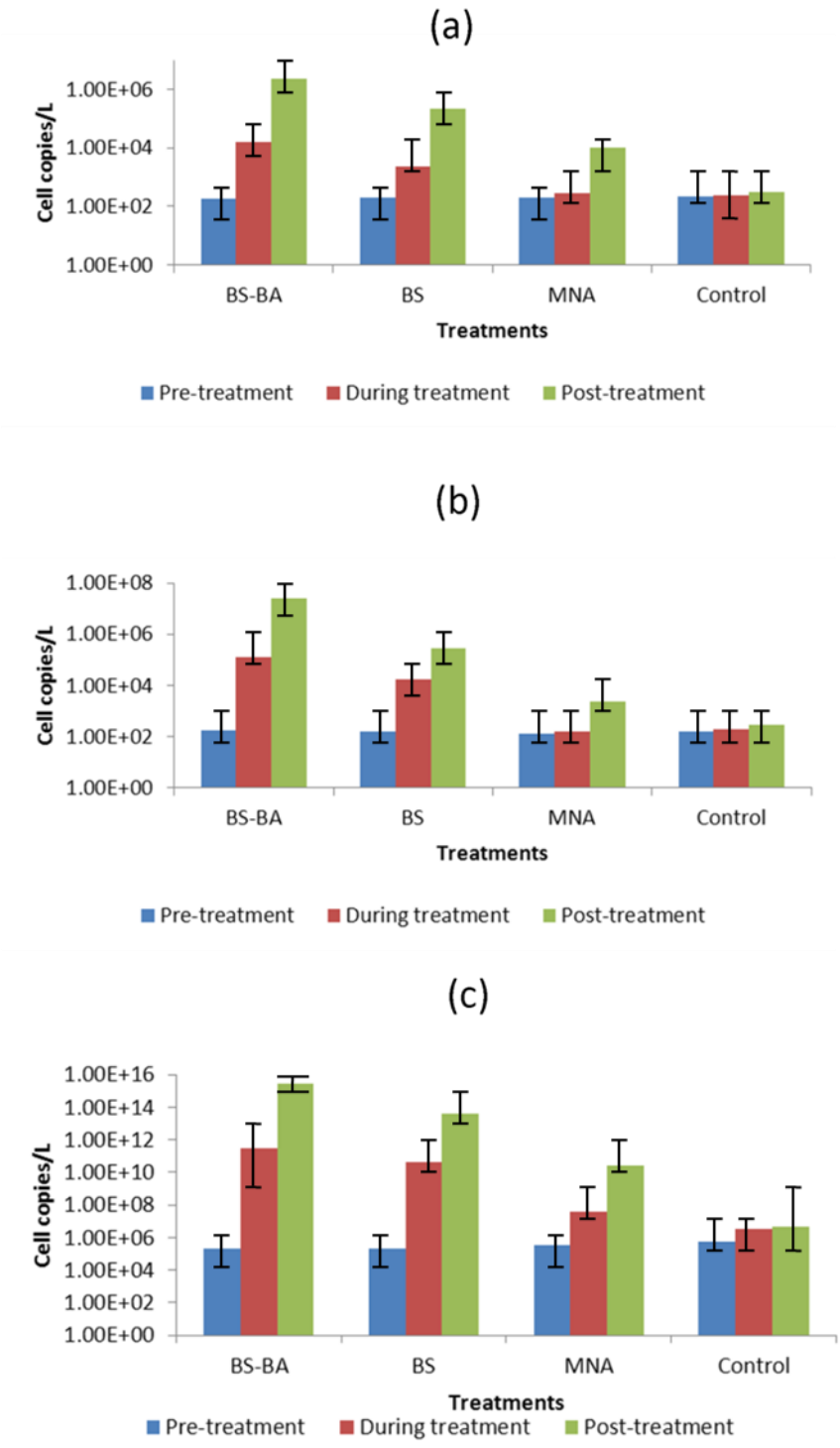


Figure 4. Microbial community abundance in the groundwater wells obtained using qPCR with specific and universal bacterial primers for **(a)** *Geo* sp, **(b)** *Dhc* sp, and **(c)** total community for pre-treatment (March 2013); during treatment (April-October 2013) and post-treatment (November 2013).

List of Tables

Table 1. Physical and chemical groundwater characteristics for pre-treatment, during treatment and post-treatment analysis

Treatment	Well ID	DO (ppm)			pH			ORP (mV)			Temp (°C)	PT TCE Conc (µg/L)
		PT	DT	PST	PT	DT	PST	PT	DT	PST		
	MW 22	1.98	1.84	0.65	6.84	7.5	7.06	2	3	-12	14.9	30.03
BS	MW 11 A	0.77	0.73	0.54	6.20	7.2	7.18	101	98	31	15.2	39.07
	MW 15 A	0.08	0.14	0.32	6.95	7.5	7.57	-87	-83	-70	15.6	41.43
	MW 17	0.72	0.91	0.39	7.06	7.3	6.88	-51	-48	-52	12.5	32.09
BS-BA	GW 8A	0.92	0.76	0.42	6.88	7.5	7.37	40	37	-16	13.8	123.98
	GW 1B	0.66	0.98	0.59	7.53	7.9	7.88	-50	-48	-38	16.7	148.09
	MW 16B	2.96	1.98	0.76	6.95	7.6	7.21	45	47	33	15.1	112.83
	GW 4B	0.36	0.32	0.41	6.29	7.4	6.93	48	43	49	15.4	129.54
MNA	GW 6A	0.32	0.98	0.53	6.75	7.8	7.48	-135	-128	-65	17.9	57.87
	MW 20A	0.24	0.76	0.52	6.98	7.4	7.19	-38	-40	-56	17.0	79.09
	GW 5	2.15	1.54	0.73	7.44	7.8	7.18	-10	-12	-34	15.0	47.21
	MW 21	0.39	0.76	0.41	6.43	7.5	6.72	36	34	45	16.4	71.78
Control	MW 10A	0.36	0.54	0.39	6.00	6.2	6.98	49	52	48	15.5	1.29

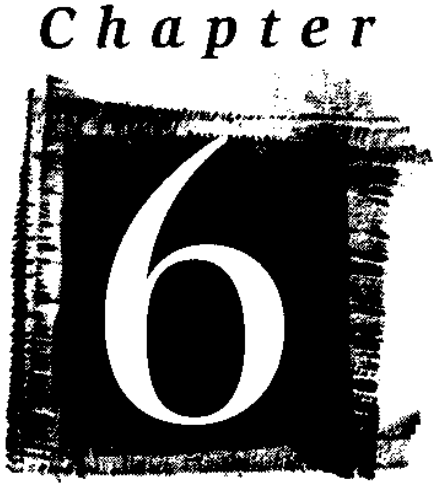
DO = Dissolved Oxygen; ORP = Oxidation reduction potential; PT = pre-treatment characteristics; DT = during treatment characteristics; PST = post-treatment characteristics

Table 2. List of primers and PCR protocols used in this study

Primer type	Primers	Primer Sequences	PCR Programs
Universal bacterial	341F with GC clamp	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG – 3'	Touchdown PCR started with a denaturing step at 94°C for 5 mins. Every cycle consisted of 3 steps each for 1 min: 94°C, annealing temperature and 72°C. The initial annealing temperature of 65°C was decreased by 0.5°C per cycle until a touchdown of 55°C at which temperature 12 additional cycles were carried out. Final primer extension was performed at 72°C for 10 mins followed by 22 cycles starting at 71°C and decreasing by 1°C per cycle.
	341F without GC clamp	5'-CCTACGGGAGGCAGCAG-3'	
	907R	5'- ATTACCGCGGCTGCTGG- 3'	
***Dhc* specific**	DHC-1F	5'-GATGAACGCTAGCGGCG-3'	Initial denaturation for 15 min at 94°C; followed by 40 cycles of 94°C for 30 s, 20 s 58°C and and 72°C for 30 s (after which acquisition took place using Sybr channel). Finally, a melting curve from 55°C - 95°C with increment of 0.5°C for 10 s.
	DHC-259R	5'-CAGACCAGCTACCGATCGAA-3'	
***Geo* specific**	Geo 494F	5'- AGGAAGCACCGGCTAACTCC-3'	Initial denaturation for 20 s at 95°C; followed by 40 cycles of 95°C for 3 s (denaturing), 30 s 61.5°C (annealing) (after which acquisition took place using Sybr channel). Finally, a melting curve from 60°C - 95°C with increment of 1°C for every 30 s (extension).
	Geo 825R	5'- TACCCGCRACACCTAGT-3'	

Table 3. Major of phylogenetic affiliations of bacterial 16S rDNA sequences obtained from excised DGGE bands compared with the NCBI database

Treatments	Wells	Excised DGGE bands	Accession Number	Closest relative (NCBI) database	Max Identity (%)	Phylum
PT	GW1B	1C	AF407413.1	Uncultured bacterium clone RB13C10 16S ribosomal RNA gene, partial sequence	97	/
DT	GW4B	21A	AB713999.1	Uncultured *Sulfurospirillum* sp. gene for 16S ribosomal RNA, partial sequence.	99	Proteobacteria
PST	MW16B	10A	JN982204	*Geobacter lovleyi* strain Geo7.1A 16S ribosomal RNA gene, partial sequence.	80	Proteobacteria
PST	MW16B	10D	JQ627628	*Dehalococcoides* sp. DG 16S ribosomal RNA gene, partial sequence.	94	Chloroflevi
PST	MW21	19B	AM935015	Uncultured *Pseudomonas* sp. partial 16S rRNA gene, clone AMKB12.	79	Proteobacteria



Sustainable remediation: electrochemically assisted microbial dechlorination of tetrachloroethene contaminated groundwater

Statement of Authorship

Sustainable remediation: electrochemically assisted microbial dechlorination of tetrachloroethene contaminated groundwater, *Microbial Biotechnology* doi: 10.1111/1751-7915.12089

1] Sayali S. Patil (Candidate)

Performed experiment design, analysis of samples, molecular and analytical data analysis, interpreted data, manuscript writing and evaluation, corresponding author

Signature  **Date** 07-10-2013

2] Eric M. Adetutu

Manuscript evaluation

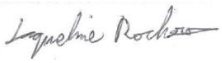
I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 07/10/2013

3] Jacqueline Rochow

Supplied essential materials for experiments and manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 10/10/2013

4] James G. Mitchell

Manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 8/10/13

5] Andrew S. Ball

Assistance with experimental design, Interpreted data, manuscript evaluation

I give consent for S.S.Patil to present this scientific manuscript for examination towards the Doctor of Philosophy

Signature  **Date** 07 October 2013

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Sustainable remediation: electrochemically assisted microbial dechlorination of tetrachloroethene-contaminated groundwater

Sayali S. Patil,[1,2*] Eric M. Adetutu,[2] Jacqueline Rochow,[1] James G. Mitchell[1] and Andrew S. Ball[2]
[1]*School of Biological Sciences, Flinders University of South Australia, Adelaide, SA 5042, Australia.*
[2]*Department of Applied Biosciences, Royal Melbourne Institute of Technology, Bundoora, Vic. 3083, Australia.*

Summary

Microbial electric systems (MESs) hold significant promise for the sustainable remediation of chlorinated solvents such as tetrachlorethene (perchloroethylene, PCE). Although the bio-electrochemical potential of some specific bacterial species such as Dehalococcoides and Geobacteraceae have been exploited, this ability in other undefined microorganisms has not been extensively assessed. Hence, the focus of this study was to investigate indigenous and potentially bio-electrochemically active microorganisms in PCE-contaminated groundwater. Lab-scale MESs were fed with acetate and carbon electrode/PCE as electron donors and acceptors, respectively, under biostimulation (BS) and BS-bioaugmentation (BS-BA) regimes. Molecular analysis of the indigenous groundwater community identified mainly *Spirochaetes*, *Firmicutes*, *Bacteroidetes*, and γ and δ-*Proteobacteria*. Environmental scanning electron photomicrographs of the anode surfaces showed extensive indigenous microbial colonization under both regimes. This colonization and BS resulted in 100% dechlorination in both treatments with complete dechlorination occurring 4 weeks earlier in BS-BA samples and up to 11.5 μA of current being generated. The indigenous non-*Dehalococcoides* community was found to contribute significantly to electron transfer with ~61% of the current generated due to their activities. This study therefore shows the potential of the indigenous non-*Dehalococcoides* bacterial community in bio-electrochemically reducing PCE that could prove to be a cost-effective and sustainable bioremediation practice.

Received 4 June, 2013; revised 29 July, 2013; accepted 18 August, 2013. *For correspondence. E-mail pati0018@flinders.edu.au; Tel. (+61) 03 9925 6678; Fax (+61) 03 9925 7110.
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Introduction

Chlorinated ethenes such as tetrachloroethene (perchloroethylene, PCE), trichloroethene (TCE) and dichloroethene (DCE) are among the most frequently detected groundwater pollutants (Moran *et al.*, 2007), posing a serious threat to the environment and human well-being because of their carcinogenic properties (ATSDR, 2007). The current *in situ* and onsite bioreactor-engineered approaches for the bioremediation of chlorinated contaminants typically involve the addition of molecular hydrogen (H_2) or H_2-releasing organic substrates to stimulate the metabolism of reductive dechlorinating microorganisms. This stimulation facilitates the reduction of PCE to environmentally benign ethene. The problems often associated with this approach include the extensive competition for carbon and H_2 between dechlorinators and non-dechlorinating sulphate reducers, methanogens and homoacetogens, and accumulation of large amounts of fermentation products in the subsurface. These problems can result in deterioration of groundwater quality, possible aquifer clogging because of excessive biomass growth and even explosion hazards through excessive methane production (Aulenta *et al.*, 2009a).

A ground-breaking alternative to this approach is the use of insoluble electrodes to directly and selectively deliver electrons instead of chemicals via microbial electric system (MES) to dechlorinating communities growing as biofilms at the electrode surfaces (Lohner and Tiehm, 2009; Lovley, 2011). A wide diversity of microorganisms is able to convert the chemical energy stored in the chemical bonds of organic compounds to electrical energy through the catalytic reactions under anaerobic conditions (Lovley, 2012). The most important step in MES is the transfer of electrons from bacteria to the electrode (Rabaey *et al.*, 2004). During this process, some microorganisms require soluble redox mediators such as methylene blue, viologens, thionines, ferricyanides and quinoid compounds that serve as an electron shuttle between the cells and the electrodes to stimulate the bio-electrochemical conversion process. As an example, Aulenta and

colleagues (2007) reported the cessation of TCE dechlorination in the absence of the low-potential redox mediator, methyl viologen. However, TCE degradation resumed when methyl viologen was added. The proposed MES process carries several advantages resulting from the use of electrodes to stimulate biological reduction in the subsurface. Among them are continuous monitoring and direct delivery of electrons to dechlorinating microorganisms in terms of current and potential. In addition, no chemicals are required to be injected, which eliminates the need for transport, storage, dosing and post-treatment (Aulenta *et al*., 2009b).

Reductive dechlorination or dehalogenation (i.e. the substitution of chlorine by a hydrogen atom) is the main pathway used by dechlorinating microorganisms for the stepwise reduction of PCE to TCE, *cis*-DCE (cDCE) and vinyl chloride (VC) before forming the environmentally safe end-product ethene (Futagami *et al*., 2008). A clear understanding of how microbial ecology within MES brings about reductive dechlorination is important for its wider application (Rabaey *et al*., 2004). Pronounced enrichment of microorganisms from *Geobacteraceae*, *Desulfuromonas*, *Desulfitobacteriacea* and *Dehalococcoides* (*Dhc*) groups in mixed consortia have been extensively observed to electrochemically interact with electrodes. This interaction that involves directly donating or accepting electrons from electrode surfaces is exploited in MES to assist in the reductive dechlorination of chlorinated compounds with energy production (Bond *et al*., 2002; Bond and Lovley, 2003; Aulenta *et al*., 2007; 2008; 2009a,b; Strycharz *et al*., 2008). However, a wide diversity of other, as-yet-undefined microorganisms may function in a similar manner. The ability of other dechlorinating populations compared with the mentioned 'classical' bacterial groups associated with bio-electrochemical reductive dechlorination has to date been poorly investigated (Lovley, 2012). Hence, we developed a system where MES were fed with PCE-contaminated groundwater consisting of a biostimulated natural microbial population [biostimulation (BS) treatment] and a stimulated population augmented with a dechlorinating consortia *Dhc* strains BAV1, GT and FL2 [BS-bioaugmentation (BS-BA) treatment]. These treatments were compared with control MES with no inoculum or nutrient stimulation. We postulated that it is important to understand the multispecies interactions among the dechlorinating community in order to successfully assess the potential for stimulating the process of decontamination of groundwater. If stimulation of indigenous microbial community can lead to bio-electrochemical PCE transformation, then it could serve as a cost-effective *in situ* remediation practice as it would restrict the need for BA of contaminated subsurfaces. Furthermore, given the recent move in some countries to discourage the use of bio-augmenting agents (Ball, 2013), this approach may reduce the risk of damaging or causing mutation in the natural biome.

The purpose of this study was therefore to identify and evaluate the ability of an indigenous non-*Dhc* dechlorinating community present in PCE-contaminated groundwater that could evolve in MES to accomplish reductive dechlorination along with bioenergy production. In addition, an assessment of the contribution of this indigenous non-*Dhc* dechlorinating population in comparison with classical dechlorinating microorganisms such as *Dhc* was performed. These investigations were carried out using electrochemical analysis and culture-independent polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE)-based molecular techniques.

Results and discussion

MES-assisted reductive dechlorination of PCE

In this study, we employed a bio-electrochemical system (Fig. 1) to study the microbial reductive dechlorination of PCE under BS and BA regimes. Figure 2 illustrates the cumulative formation of PCE-dechlorinating intermediate products and simultaneous current flow during both BS and BS-BA treatments, when MES were fed with acetate as an electron donor and PCE/electrodes as acceptors. During BS treatment, PCE was completely reduced to ethene over a period of 16 weeks (Fig. 2A). PCE was consumed by week 4, with the subsequent production of TCE. As the TCE concentration was reduced to 15 μmol l^{-1}, cDCE was detected by week 6. Daughter products, cDCE and VC co-existed until ethene was formed. In week 16, only after VC was respired did ethene concentration reach its peak. Current production was negligible for first 3 weeks, but as dechlorination progressed, current production increased from week 4 and stabilized between 6.27 and 6.98 μA over the period of 16 weeks of complete dechlorination (Fig. 2A). In contrast, PCE dechlorination did not progress beyond TCE, and current generation was also negligible in the control 1 MES without acetate stimulation (Table 1). These findings showed that BS was beneficial to dechlorination and that the indigenous microbial community (BS) were most likely involved in complete reductive dechlorination given that no *Dhc* were detected in the groundwater samples used for this study. Reductive dechlorination was also accompanied by simultaneous bioenergy production (Fig. 2A).

In BS-BA-treated MES, dechlorination was faster, and the current production was ~1.6-fold higher than MES run on BS-only treatment (Fig. 2B). PCE dechlorination started immediately, as indicated by the rapid accumulation of TCE by week 2. During BS-BA treatment, PCE was transformed into ethene over 12 weeks (Fig. 2B). Current generation started from week 2 and was stabilized

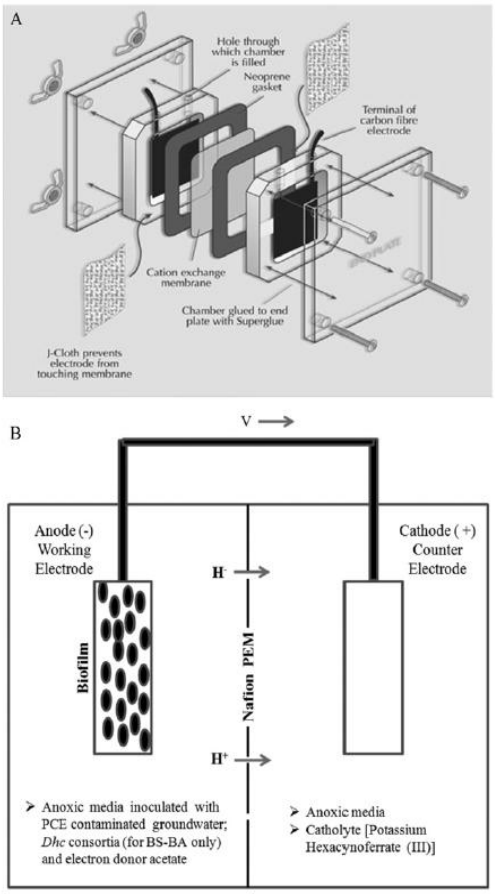

Fig. 1. (A) Components of two-chamber NCBE-type MES used in this study (Bennetto, 1990); (B) schematics explaining mechanism of MES.

between 11.08 and 11.45 µA by the end of dechlorination in week 12 (Fig. 2B). The *Dhc* and non-*Dhc* communities altogether led to complete PCE dechlorination, where ~61% of energy production (from Eqn 1 in experimental procedures) was observed to be contributed by non-*Dhc* activities. This indicated the significant role played by non-*Dhc* community in association with the *Dhc* consortia to optimize dechlorination and current output. In contrast, over the experimental period, PCE dechlorination stopped at TCE, and negligible current was observed in control 2 MES, when the *Dhc* microbial culture and acetate were omitted from the poised electrode system (Table 1). This indicates both nutrient stimulation and the electrochemically active *Dhc* bacterial community are important for enhancing the complete reductive dechlorination of PCE to ethene. Interestingly, no methane was produced in the cultures probably because of the presence of mixed culture of a lower number of methanogens compared with dechlorinators. This could prove advantageous for MES as it eliminates the competition for H_2 between dechlorinating and non-dechlorinating communities.

In both the BS and BS-BA-treated MES, the periodic conversion of the dark yellow catholyte potassium hexacyanoferrate [$K_3Fe(CN)_6$] to pale yellow or colourless resulted in the cessation of current flow and dechlorination. However, replenishment of $K_3Fe(CN)_6$ caused a resumption of current flow and dechlorination, indicating its definitive effect on the electron transfer mechanism of MES. When the catholyte was available as a dissolved compound, the measured rate of PCE dechlorination and current flow was unexpectedly higher from BS-only- and BS-BA-treated MES than that measured in the control 3 MES (Table 1). Wei and colleagues (2012) have also reported $K_3Fe(CN)_6$ as an excellent cathodic electron acceptor for two-chambered MES to obtain high power output. While there are some reports on the benefits of BS or BA, or the combined treatment on PCE dechlorination (Aulenta *et al.*, 2007; 2008; 2009a,b), most of these studies have been on samples with *Dhc*, with scant attention being paid to the role of indigenous non- *Dhc* species. The potential roles of these non-*Dhc* species have therefore been comparatively poorly investigated to date. In addition, most studies have been focused on either BS or

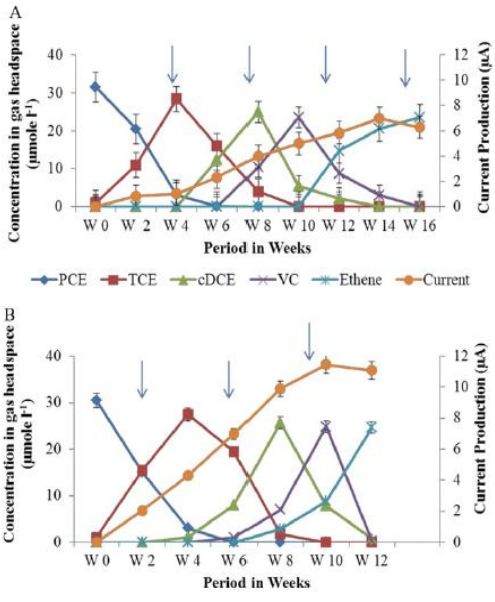

Fig. 2. MES-assisted reductive dechlorination of PCE and simultaneous current production in (A) MES 1 and 2 with BS and (B) MES 3 and 4 with BS-BA treatments. Values are average of duplicate cultures. The arrows show when fresh catholyte and electron donors were added.

Table 1. Comparative response of BS only and BS-BA treatments against controls during MES assisted PCE dechlorination.

Chloroethene	BS only	BS-BA	Control 1 (without acetate)	Control 2 (without acetate and *Dhc* inoculum)	Control 3 (without catholyte)	Control 4 (without electrodes)
PCE	+	+	+	+	+	+
TCE	+	+	+	+	+	+
cDCE	+	+	–	–	–	+
VC	+	+	–	–	–	–
Ethene	+	+	–	–	–	–

+ Presence; – Absence; Control MES (1) medium, electrodes, PCE-contaminated groundwater, catholyte but no acetate; (2) medium, electrodes, PCE-contaminated groundwater, catholyte but no acetate and *Dhc* inoculum; (3) medium, electrodes, PCE-contaminated groundwater, acetate but no catholyte; (4) medium, PCE-contaminated groundwater, acetate, catholyte, but no electrodes.

BA, or BS-BA alone in different systems. Hence, one highlight of this research is that both BS and BS-BA treatments were set up with the same sample under similar experimental conditions allowing for objective comparison of these strategies (unlike in most studies).

Community analysis

Microbial community fingerprints were obtained from DGGE analysis during the MES-assisted reductive dechlorination of PCE from BS-only, BS-BA and control treatments (Fig. 3). No bands were obtained from the control MES (Fig. 3) presumably because of either PCR detection constraints relating to the small volume used for DNA extraction or the absence of nutrient stimulation. Similarly, no amplicons were obtained from archaea-specific PCR indicating the absence or below-detection level of a methanogenic archaea community in the groundwater. No *Dhc* species were detected in PCR-based assays carried out on groundwater samples. To identify the likely electrochemically active bacterial species carrying out reductive dechlorination in BS and BS-BA MES, the dominant bands (operational taxonomic units) based on strong band intensity were excised from the DGGE gel and sequenced. Sequencing results indicated significant phylogenetic diversity in the species identified. Microbial community belonging to facultative anaerobic bacteria including the taxa *Spirochaetes*, *Firmicutes*, γ-*Proteobacteria*, δ-*Proteobacteria* and *Bacteroidetes* were detected in both BS-only- and BS-BA-treated MES, while *Chloroflexi* was detected only in BS-BA MES (Table 2).

Community detected within BS-only-treated MES. In addition to the non-detection of *Dhc* in the original groundwater samples, no *Dhc* amplicons were obtained from optimized PCR assays during the dechlorination process, within BS-only treated MES. This indicated either the absence of *Dhc* species or their presence below PCR detection limits and possible ecological insignificance in groundwater sample. However, the *Spirochaetes* showed 96–98% similarity to uncultured bacterial clones DCE33, TANB18, DPF05 and *Spirochaeta* sp. (Table 2). DGGE bands that showed 96% similarity with an unidentified bacterial clone DCE25 was grouped under *Firmicutes*, while bands putatively assigned to the phyla γ-*Proteobacteria* showed 98% and 96% sequence similarity to *Enterobacter* species and the bacterial clone ALO1_GLFRUDD03F0MQ1, respectively. Other bands

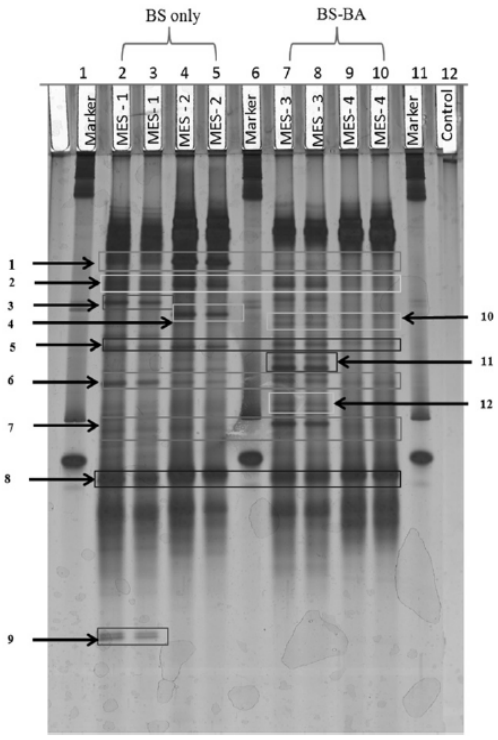

Fig. 3. Microbial community fingerprint from MES analysed using DGGE. MES 1 and 2 represent BS only (lanes 2–5); MES 3 and 4 with BS-BA treatment (lanes 7–10), while lane 12 indicates control MES. Lanes 1, 6 and 11 represent marker. Band numbers designate dominant bands excised from DGGE gel for sequence analysis that correspond to the band numbers in Table 2.

Table 2. Overview of the bacterial species identified based on the occurrence of a dominant DGGE pattern obtained from MES-assisted PCE dechlorination.

Excised DGGE Bands	Accession No.	Closest matches overall (NCBI database)	Maximum % similarity	Phylum	Detected within (treatment)
1	AF349763.2	Uncultured bacterium DCE33 16S ribosomal RNA gene, partial sequence	97	Spirochaetes	BS and BS-BA
2	AY667253.1	Uncultured bacterium clone TANB18 16S ribosomal RNA gene, partial sequence	96	Spirochaetes	BS and BS-BA
3	GQ377125.1	Bacterium enrichment culture clone DPF05 16S ribosomal RNA gene, partial sequence	96	Spirochaetes	BS and BS-BA
4	AF357916.2	*Spirochaeta* sp. 16S ribosomal RNA gene, partial sequence	98	Spirochaetes	BS and BS-BA
5	AJ249227.1	Bacterium DCE25 16S rRNA gene	96	Firmicutes	BS and BS-BA
6	JF920024.1	*Enterobacter* sp. 16S ribosomal RNA gene, partial sequence	98	γ-Proteobacteria	BS and BS-BA
7	JF689075.1	Bacterium enrichment culture clone ALO1_GLFRUDD03F0MQ1 16S ribosomal RNA gene, partial sequence	96	γ-Proteobacteria	BS and BS-BA
8	DQ903931	*Desulfovibrio* Sp. GmS2 (SRB enrichment clone) 16S ribosomal RNA gene, partial sequence	97	δ-Proteobacteria	BS and BS-BA
9	HM488066.1	Uncultured bacterium clone ZM4-54 16S ribosomal RNA gene, partial sequence	97	Bacteroidetes	BS and BS-BA
10	AY165308.1	*Dehalococcoides* sp BAV1 16S rRNA gene, partial sequence	100	Chloroflexi	BS-BA only
11	AY914178.1	*Dehalococcoides* sp GT, 16S rRNA, partial sequence	100	Chloroflexi	BS-BA only
12	AF357918.2	*Dehalococcoides* sp FL2, 16S rRNA gene, partial sequence	100	Chloroflexi	BS-BA only

showed 97% similarity to the well-known dechlorinator *Desulfovibrio* species under the taxonomic group of δ-*Proteobacteria* and uncultured bacterial clones ZM4-54 within the phylum *Bacteroidetes*.

The detection of *Spirochaetes* group in this study is not unusual as they have also been reported in other PCE-reducing cultures inoculated with a sample from a chloroethene-contaminated site (Gu *et al*., 2004; Macbeth *et al*., 2004; Dong *et al*., 2011). *Spirochaetes* either utilize H_2 or ferment carbohydrates and other complex substrates to acetate and other substances that are utilized during organohalide respiration. Under the γ-*Proteobacteria*, clone ALO1_GLFRUDD03F0MQ1 is a known 1,2dichloroethane dechlorinator (Low *et al*., 2011), while *Enterobacter* species are the only facultative anaerobes reported so far to reductively dechlorinate PCE to cDCE (Holliger *et al*., 1999). Studies by Löffler and colleagues (2003), and Sun and colleagues (2000) have shown the contribution of a marine dechlorinating *Desulfivibrio* species in the reductive dechlorination that forms syntrophic associations with other dechlorinating bacteria to produce hydrogen by the transformation of organic compounds added to the medium. The hydrogen produced can then be transferred to dehalogenating bacteria and thus support microbially mediated reductive dechlorination (Drzyzga *et al*., 2001; Eydal *et al*., 2009). Bacterial clones ZM4-54 under the *Bacteriodetes* may, through fermentation, supply small organic molecules or H_2 necessary for the growth of dechlorinating bacteria (Tancsics *et al*., 2010); however, the metabolic function of this organism is still unclear. The bacterium DCE25 in TCE and cDCE cultures acts as an acetate-fermenting organisms providing energy and a carbon source for the dechlorinating microbes (Flynn *et al*., 2000).

Community detected within BS-BA-treated MES. In addition to the earlier H_2-utilizing bacterial communities, only in MES run with BS-BA treatment augmented with *Dhc* strains BAV1, GT and FL2 were the *Chloroflexi* phyla detected (Table 2). The *Dhc* strains GT, FL2 and BAV1 have previously been well documented to cometabolically transform PCE to ethene (Futagami *et al*., 2008).

The faster dechlorination and almost twofold increase in current flow in BS-BA-treated MES when compared with the BS-only MES could be due to synergistic activity between the dechlorinating *Dhc* and non-*Dhc* species. Members of the indigenous non-*Dhc* community were believed to have contributed significantly to electron transfer as calculations using Eqn 1 showed that they could have been responsible for ~61% of the total current generated within BS-BA-treated MES. In spite of the absence of *Dhc* strains, BS-MES cultures containing an indigenous non-*Dhc* community were equally capable of completely reducing PCE. Overall, these comparative

treatments highlighted the potential of mixed non-*Dhc* bacterial communities evolved in MES, which most likely contributed to the electron transfer mechanism supporting complete reductive dechlorination of PCE with and without *Dhc*. A study by Aulenta *et al.* (2009b) also reported the key role played by β-, δ- and γ-*Proteobacteria*, and *Firmicutes* besides *Dhc* in a mixed culture carrying out the dechlorination of TCE to non-chlorinated end-products within MES. Although the dechlorinating capabilities of these detected indigenous non-*Dhc* population have been well-studied (Gu *et al.*, 2004; Macbeth *et al.*, 2004; Dong *et al.*, 2011), knowledge about their electrochemical properties is currently limited.

Anode biofilms

The analysis of the anode electrode surfaces via environmental scanning electron microscope (ESEM) conducted at the end of the experiment revealed that the surfaces of carbon fibre electrodes in both BS-only- and BS-BA-treated MES had been colonized (Fig. 4A and B). In contrast, no bacterial cells were observed at the anode surface of the controls 1–3 MES (Fig. 4C) that could be due to the lack of nutrient stimulation and/or biologically active microorganisms. This could have led to incomplete PCE degradation observed in these control samples unlike in BS-only and BS-BA samples. Mixed bacterial culture in BS-BA-treated MES formed complex cellular aggregates compared with sparsely distributed cells in BS-only MES (Fig. 4A and B). Microbial cells did not support complete dechlorination and energy production when the supply of electrons to the electrode was discontinued in control 4 MES (Table 1). Overall, this result demonstrated that the bacterial community present in BS-only and BS-BA sets of MES could bio-electrochemically interact with electrodes as electron acceptors by forming a stable, attached population that can produce electrical current via reductive dechlorination coupled to electron transfer to the electrodes.

Reductive dechlorination and bacterial electrochemical activity

In this study, DGGE band sequencing yielded sequences similar to those of several previously described H_2-oxidizing *Dhc* and non-*Dhc* bacteria. These findings suggest that facultative anaerobic bacterial species are capable of growing in MES using the electrode as an acceptor, further indicating their electrochemical potential or at least redox controlling properties. MES enhances the growth of bacteria that can use the electrode as an electrode acceptor as bacteria have been observed to gain more energy when using an electrode as an electron acceptor than when they use protons (Rabaey *et al.*,

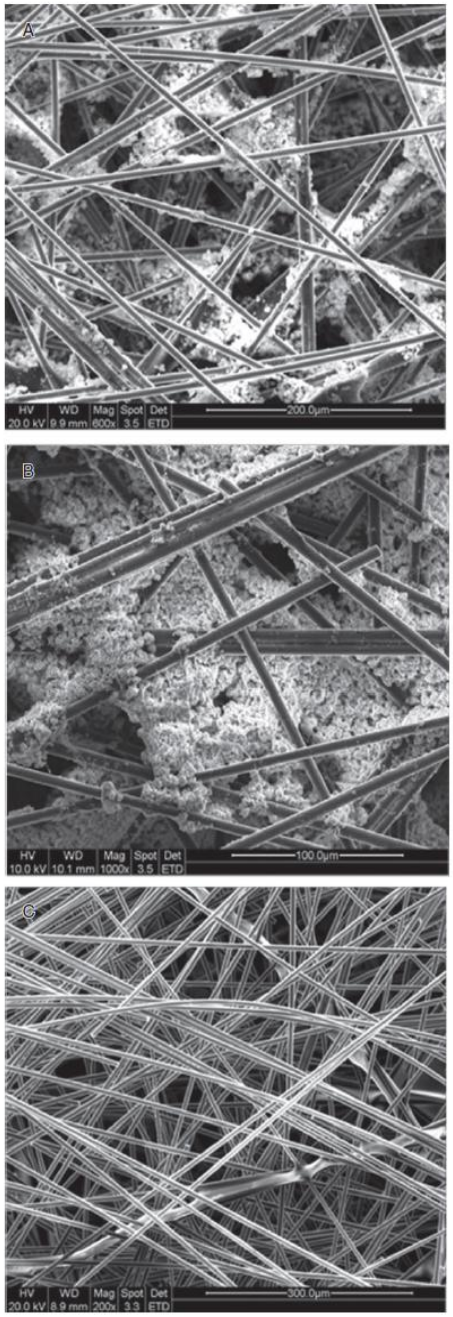

Fig. 4. Environmental scanning electron photomicrographs of bacterial biofilms grown on carbon fibre anode surfaces during PCE dechlorination within (A) MES with BS only (B) MES with BS-BA and (C) control 1–3 MES at the end of experiment. No biofilm was observed in controls 1–3 MES; however, thick biofilm was noticeable in MES run with BS and BS-BA treatments.

2004). In recent years, it has been demonstrated that a direct electron transfer between bacteria and electrodes is possible within MES (Strycharz *et al*., 2008). This study seems to indicate that a similar approach might have pursued by non-*Dhc* bacteria especially with regards to ethene formation that strive to access electrons throughout PCE dechlorination that was utilized for energy production.

As previous reports on this topic have been limited to a few microbes (Lovley, 2012), this study focused on other organisms to elucidate their electrochemical mechanism during PCE remediation. We report here that non-*Dhc* microorganisms within MES supplied with acetate as an electron donor and PCE/electrode as acceptors seemed to play a role in the complete PCE dechlorination with current production. However, to implement this strategy competitively with BS-BA, a clear understanding about electron transfer mechanisms between non-*Dhc* species and electrodes with nutrient stimulation is necessary to optimize the dechlorination rates and current output. In order to scale-up this strategy for successful *in situ* application, extensive testings based on subsurface characteristics and site-specific design needs to be studied. Williams and colleagues (2010) for the first time demonstrated the *in situ* applicability of graphite electrodes in the subsurface serving as electron acceptors for microbial stimulation during uranium bioremediation at Rifle site in Colorado. A similar approach may be employed for the remediation of chlorinated solvents. Preliminary investigations of environmental factors and complex microbial interactions at contaminated sites will decide the potential of MES for PCE bioremediation. If a native dechlorinating community was found to be capable of self-mediated electrochemical conversion of PCE, it would further eliminate the need of bio-augmenting the subsurface that will eventually reduce remediation cost. Future research necessitates investigating the possibility of electrode-dependent, microbially catalysed PCE degradation where non-*Dhc* bacteria can utilize the electrode as the sole electron donor. Altogether, this strategy could prove advantageous, especially where electron donor delivery to subsurface has always been a challenge. Nevertheless, it will be of fundamental importance to focus on mechanisms involved in the extracellular electron transfer process between microorganism and the electrodes to develop strategies to maximize dechlorination rates.

To conclude, this study highlighted the electrochemical potential of indigenous non-*Dhc* dechlorinators compared with *Dhc* species during the complete dechlorination of PCE to ethene via MES. Although the direct involvement of mixed *Dhc* culture in the electron transfer process was expected in BS-BA run MES, the potential of non-*Dhc* dechlorinators exhibiting similar mechanisms within BS-MES was observed. Microbial communities in the MES evolved specifically as an optimized biocatalyser generating a stable power output, opening a perspective for the development of a new sustainable bioremediation strategy. Clearly, research is needed to further elucidate the electrochemical mechanism of these as-yet-undefined non-*Dhc* dechlorinators in order to advance this field in a rational manner.

Experimental procedures

Materials

All chlorinated ethenes, ethene and other chemicals including potassium hexacyanoferrate (III), sulphuric acid and hydrogen peroxide required for the experimental setup and analytical measurements were purchased from Sigma-Aldrich (Sydney, NSW, Australia) with a minimum purity of 99.5%. All gases were ordered from Coregas (Melbourne, Vic., Australia).

Groundwater sample collection

For this study, we selected a chloroethene contaminated site located in Victoria, Australia. A sample of contaminated groundwater (4 l) with a PCE concentration of 130 $\mu g\ l^{-1}$ was collected from the monitoring well as per the protocol suggested by Ritalahti *et al.* (2010). A flow-through cell (YSI, Melbourne, Vic., Australia) recorded pH, oxidation-reduction potential, specific conductance, temperature, dissolved oxygen and turbidity of groundwater. When geochemical parameters were stabilized, the flow-through cell was disconnected and replicate samples collected consecutively without flow interruption. Sample containers consisted of sterile and N_2^- purged high-density polyethylene Nalgene 4 l bottles with polypropylene screw caps (Thermo Fisher Scientific Australia, Sydney, NSW, Australia). During sampling, bottles were filled to capacity and stored on ice that was then express-shipped to the analytical laboratory. Upon arrival, samples were placed in the dark at 4°C.

Media preparation

An anoxic PCE-dechlorinating mineral media was enriched and maintained as per the guidelines presented by Löffler and colleagues (2005) and the American Type Culture Collection (ATCC; http://www.atcc.org). Media was prepared in Wheaton serum bottles of 125 ml nominal volume containing 75 ml of growth medium and 20 ml of PCE-contaminated groundwater as an inoculum amended with 5 mM acetate as an electron donor and 5 µl of PCE as an electron acceptor. The bottles were sealed with Teflon-coated butyl rubber septa and aluminium crimp caps (Alltech, Melbourne, Vic., Australia). Hydrogen (5% in 95% nitrogen) was added in the headspace (5–10% of the headspace volume of a bottle) at a low partial pressure of 9 kPa. Cultures were prepared under strict anaerobic conditions maintained in an anaerobic glove box (La-Petite, Thermo Fisher Scientific Australia) using $N_2 : CO_2$ at the ratio of 80%:20%. Resazurin redox indicator was added to the groundwater to denote reduced conditions. Immediately upon setup, media turned clear from pink tint

(given by the resazurin redox indicator added to the groundwater) indicating establishment of reduced conditions.

MES construction and operation

For this study, we employed typical two-chamber NCBE-type MES (National Centre for Biotechnology Education, Reading, UK). The MES chamber ($7.5 \times 9.0 \times 5.5$ cm) consisted two electrode compartments ($60 \times 70 \times 10$ mm; 10 ml each) separated by a reinforced Nafion^{424} proton exchange membrane (PEM) 0.007″ thickness (Sigma-Aldrich) (Fig. 1A; Bennetto, 1990). Compartments were kept watertight by placing rubber gaskets between chambers and by bolting two Perspex sheets together. The PEM was pretreated by boiling in H_2O_2 (30%), then in 0.5 M H_2SO_4 and finally in de-ionized (DI) water, each for 1 h, and then stored in DI water prior to being used. The carbon fibre electrodes (3.2×4 cm) were soaked in DI water prior to use (Aulenta *et al.*, 2007). Sampling ports were sealed with rubber stoppers, while carbon electrodes were attached to copper wires fed through rubber stoppers in the sampling port.

Two out of the four MES were run on a BS-only approach where MES were fed with groundwater comprising an indigenous microbial population. The remaining two MES were dedicated to the BS-BA treatment where the same groundwater was amended with a dechlorinating mixed consortia of *Dhc* species FL2 (*Dehalococcoides* sp. ATCC® BAA-2098™), BAV1 (*Dehalococcoides* sp. ATCC BAA-2100™) and GT (*Dehalococcoides* sp. ATCC BAA-2099™) outsourced from ATCC (http://www.atcc.org). MES were established by supplying acetate as electron donor and PCE/electrodes as acceptors. Ten millilitres of anoxic media inoculated with PCE-contaminated groundwater and acetate was transferred anaerobically from 125 ml Wheaton bottles into the working electrode chambers of all four MES using a gas-tight syringe. In parallel, the counter electrode was filled with an equal volume of a reduced mineral media and was spiked with 20 mM potassium hexacyanoferrate (III) [$K_3Fe(CN)_6$, catholyte] in phosphate buffer (Fig. 1B). The *Dhc* mixed consortia (1×10^3 cells ml^{-1} each) was injected only into the working electrode compartment of MES 3 and 4. Chambers were then flushed with 80:20 $N_2 : CO_2$ mixed gases. The whole process was carried out in an anaerobic chamber under strict anoxic conditions. Once sealed, MES were maintained at 22–25°C in the dark under gentle magnetic stirring in an attempt to promote the growth and adhesion of dechlorinating bacteria on the surface of carbon fibre electrodes. The working electrode was poised at −450 mV (versus standard hydrogen electrode). Electrochemical measurements were taken using a Fluke 289 digital true RMS multimeter (RS Components, Melbourne, Vic., Australia). Cells were monitored over 16 weeks (112 days) with current and voltage being recorded throughout the dechlorination process. The energy production (%) from BS and BS-BA treatments was calculated as follows:

$$a = (a_1 \div c) \times 100; b = 100 - a \quad (1)$$

where a_1 = current from BS, a = % current from BS, b = % current from BA, and c = total current from BS-BA. The experiment was followed by multiple control MES with: (i) medium, electrodes, PCE-contaminated groundwater, catholyte but no acetate; (ii) medium, electrodes, PCE-contaminated groundwater, catholyte but no acetate and *Dhc* inoculum; (iii) medium, electrodes, PCE-contaminated groundwater, acetate but no catholyte; (iv) medium, PCE-contaminated groundwater, acetate, catholyte, but no electrodes.

Microbial community analysis

Genomic DNA extraction from groundwater samples treated with MES was carried out as described by Löffler and colleagues (2005) and 16S rRNA gene fragments were amplified with bacterial universal primer set 341 F-GC/518R (Muyzer *et al.*, 1993) to detect the presence of indigenous and likely dechlorinating bacterial communities. The *Archaea*-specific primers A109f and A934b were also used for detection of methanogens under domain *Archaea* as described by Høj *et al.* (2008). *Dhc*-specific primers 1F-GC and 259R with *Dhc* strain GT, FL2 and BAV1 (positive controls) were used for the detection of *Dhc* in groundwater samples using touchdown PCR that was optimized as described by Kim and colleagues (2010). Amplified 200 bp PCR fragments were further analysed by DGGE as described by Patil and colleagues (2010). Dominant DGGE bands were excised using sterile razor blades and incubated in two volumes of DNA elution buffer (0.5 mmol l^{-1} ammonium acetate, 10 mmol l^{-1} magnesium acetate, 1 mmol l^{-1} ethylenediamine tetraacetic acid pH 8 and 0.1% sodium dodecyl sulfate) overnight at 37°C. DNA was then precipitated with two volumes of absolute ethanol, air-dried, resuspended in 20 µl nuclease-free water and stored at −20°C until re-amplification (McKew *et al.*, 2007). Re-amplification was performed using 341F/518R primers. Re-amplified PCR products were purified using the Wizard® SV gel and PCR clean up system (Promega, Madison, WI, USA) as per the manufacturer's instructions. The eluted DNA was checked for concentration and purity using a Nanodrop Lite spectrophotometer (Thermo Scientific Australia). Samples were then sent to the Australian Genome Research Facility for sequencing using an automated sequencer, ABI 3730. Nucleotide sequences were analysed using SEQUENCHER software (Sequencher Version 4.1.4, GeneCodes Corp., Ann Arbor, MI, USA), and homology searches were completed with the BLAST server of the National Centre for Biotechnology Information (NCBI) using a BLAST algorithm (http://www.ncbi.nlm.gov.library.vu.edu.au/BLAST/) for the comparison of a nucleotide query sequence against a nucleotide sequence database (blastn).

Analytical procedures

Every 2 weeks, 50 µl chlorinated ethenes were removed from the gas headspace of both working and counter electrode compartments using a gas tight, sample-lock Hamilton syringe (Alltech) and analysed by an HP 6890 gas chromatographic (GC) system equipped with a 5973 mass spectrometry and flame ionizing detector and a Porabond-Q column (0.32 mm by 25 m) (Agilent Tech, Melbourne, Vic., Australia). The GC settings were: injector temperature 200°C; detector temperature 300°C; oven temperature 3 min at 40°C, followed by an increase of 10°C min^{-1} to 70°C, followed by an

increase of 15°C min^{-1} to 250°C for 7 min; and carrier gas (He) with a flow rate of 2 ml min^{-1}. External standards at six different concentrations from 0 to 30 μM were used for calibration. Electron donors were replenished every time analyses indicated they were exhausted.

Microscopy

At the end of the experiment, anodes from all MES were removed, cut into small pieces using a sterile razor blade and washed with phosphate buffer (pH 7.0) to remove loosely attached cells. Subsequently, samples were observed using a Quanta 200 ESEM (FEI Company, Melbourne, Vic., Australia). The ESEM was operated at 10–20 kV, and images were captured digitally.

Nucleotide sequence accession numbers

All bacterial sequences have been deposited in the NCBI database under accession numbers JX495100–JX495111.

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Conflict of interest

None declared.

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General Discussion

And

Conclusions

Chapter 7. General Discussion and Conclusions

Subsurface and soil contamination by toxic chlorinated compounds such as PCE, TCE, DCE and VC is still a serious cause of environmental concern (SCRD, 2007). Due to their detrimental impact on human health and the environment, it is of public and scientific community interest to find efficient and effective solutions for chloroethene removal. This study focused on enhanced reductive dechlorination as an effective bioremediation strategy and provides new information on the interactions within dechlorinating microbial communities and their response during the sequential PCE degradationl from contaminated sources like groundwater.

7.1 Exploring the potential of non-*Dehalococcoides* dechlorinating communities for complete PCE degradation

The use of laboratory microcosms containing contaminated groundwater collected from a PCE contaminated site in Victoria, Australia provided an insight into indigenous dechlorinating community structure and their degradation potential which successfully led to the sequential breakdown of PCE to the environmentally safer end product, ethene. In the absence of members of the *Chloroflexi* group (which includes *Dhc* species), microbial communities associated with *Proteobacteria, Spirochaetes, Firmicutes, Bacteroidetes, Methanomicrobiaceae, Methanosaetaceae* and *Methanosarcinaeceae* were the major detectable indigenous groups that led to complete PCE breakdown under acetate stimulation. Furthermore, laboratory based MES fed with acetate and carbon electrode/PCE as electron donors and acceptors respectively under BS and BS-BA regimes indicated the bio-electrochemical PCE degradation potential of the indigenous groundwater community including *Proteobacteria, Spirochaetes, Firmicutes* and *Bacteroidetes*. Earlier research was largely focused on the activities of strict anaerobic reductive dechlorinating microorganisms such as *Dehalococcoides, Dehalobacter, Desulfitobacterium, Desulfuromonas, Geobacteriaceae* and *Sulfurospilillum* (Duhamel and Edwards, 2006). Therefore, the information generated in this study on the dechlorinating activities of microoganisms other than those previously well studied microbial groups, is a significant outcome. These organisms play different roles in the microbial community, as some of the organisms carry dechlorination by directly supplying the electrons or substrates which are utilized during organohalide respiration (Dong *et al*., 2011); other organisms act

indirectly by forming precursors to enhance the dechlorination process (Gu *et al*., 2004). However, the electrochemical mechanisms involved in bioenergy production during PCE transformation need further research.

7.2 Dechlorinating community flux during PCE degradation

Cluster analysis of the PCR-DGGE profile using an UPGMA-dendrogram indicated the association of distinct microbial groups with the particular chloroethene transition phase. Bacterial species associated with *Proteobacteria* and *Spirochaetes* were found throughout the PCE to ethene transition, however *Firmicutes* and *Bacteroidetes* were observed only during cDCE to ethene transition. On the other hand, methanogenic species under *Methanomicrobiaceae* were persistent during the PCE to cDCE transition compared to *Methanosaetaceae* and *Methanosarcinaeceae* groups been abundant only during the later phase of cDCE to ethene transformation. Furthermore, PL curve and MWA analyses showed that apart from microbial community shifts there was a distinct trend between community dynamics and functional organization in response to PCE dechlorination. The 45% and 25% PL curve indicated medium and low functionally organized bacterial and archaeal communities, respectively. In addition, the rapid bacterial community shift highlighted a more dynamic and diverse bacterial community compared to the less dynamic and diverse archaeal community. These findings suggest that bacteria may contain more functionally redundant species than archaea, allowing reductive dechlorination to occur smoothly irrespective of changing dynamics. Most studies on dechlorinating microbial community dynamics have been carried out using conventional methods of analyzing community fingerprints such as those involving the use of Shannon diversity and equitability indices (Lee *et al*., 2011; Macbeth *et al*., 2004). The advent of newer methods such as PL distribution curves and MWA for analyzing community structure means that novel and more detailed information can be obtained from microbial community fingerprints generated using PCR-DGGE assays (Marzorati *et al*., 2008). Therefore, one major highlight of this study was the use of PL curves and MWA analysis to understand the association between functional organization and dynamics within a potential native dechlorinating population.

Moreover, the application of quantitative real time PCR established the relationship between the rate of dechlorination and relative microbial cell abundance. Increased chloride concentration as a result of PCE degradation coincided with an

increase in indigenous non-*Dhc* and augmented *Dhc* cell abundance, which suggested the presence and higher viability of these dechlorinating populations to facilitate the reaction. Previously, increases in *Dhc* gene copies have been associated with reductive dechlorination (Cichocka *et al*., 2010; Cupples, 2008). From the outcome of the qPCR analyses conducted in this project it could be hypothesized that syntrophic associations between *Dhc* and other non-*Dhc* dechlorinators existed to enhance the PCE reduction.

7.3 Advances in the current bioremediation practices

The ability of the indigenous dechlorinating communities to bio-electrochemically transform PCE completely to ethene with bioenergy production suggests that MES may be appropriate as a future sustainable technique. The significant contribution of indigenous non-*Dhc* species to bioenergy production (61%) compared to the contribution from the *Dhc* species indicated that if an indigenous non-*Dhc* dechlorinating community was found to be capable of the self-mediated electrochemical conversion of PCE, it would further eliminate the need of bioaugmenting the subsurface which will eventually reduce the bioremediation cost. Overall, this system can prove advantageous especially where electrodes could be used as electron donors to stimulate microbial activity. In addition, no chemicals or substrates need to be injected externally which eliminates the need of continuous monitoring and dosing (Aulenta *et al*., 2009b). Although, MES showed significant potential as a sustainable way of remediating PCE, the real world applications are still limited due to low energy output and rigorous structural designs (Du *et al*., 2007). Clearly, further research is needed in order to make MES readily applicable for *in situ* bioremediation by improving their performance and reducing construction and operating costs.

Cost is usually an issue for the commercial bioremediation of PCE contaminated sites (ESTCP, 2004). Monitoring the dechlorinating potential of a site is therefore crucial in order to ensure economical and successful remediation. This is generally done with quantitative qPCR which requires a substantial financial outlay. This study for the first time has revealed an application of a novel LDCC approach that can effectively be used as a cheaper, rapid and simple alternative to expensive and rigorous qPCR. This technique could prove more useful and widely adapted, where simple and

rapid monitoring of microbial activities needs to be assessed to track the bioremediation progress.

7.4 Preliminary site evaluation: an important aspect of commercial bioremediation

No contaminated site is the same and differences in microbial population and sub-surface environmental conditions can vary substantially (ESTCP, 2005).Hence, the site-specific pre-evaluation of contaminated sites using a combination of microbiological and chemical approaches is a crucial step to design a successful *in situ* field based bioremediation strategy involving either BS, BA or both. In our case, it was important to understand the site-specific requirements needed for effective and complete dechlorination of the historically PCE-contaminated study site. This was especially important given the failure of earlier non-biological *in situ* chemical oxidation treatment which had resulted in substantial financial loss. Therefore, it was quintessential to develop a strategy that is not just efficient but also cost effective. Our comparative BS only and BS-BA based laboratory studies have shown that biostimulation can work effectively provided the groundwater was adjusted with electron donors to make it completely anoxic to microbially assist PCE removal. In addition, given the shift away from bioaugmentation of contaminated groundwater in some countries like Australia (Ball, 2014), biostimulation of indigenous communities for successful dechlorination of PCE contaminated aquifer represents a legislatively acceptable approach. Therefore, it was also important in light of this change that the success of PCE decontamination in groundwater without detectable population of major dechlorinators such as *Dhc* be investigated and demonstrated. Most reports (Cichocka *et al*., 2010; Imfeld *et al*., 2008; Lendvay *et al*., 2003; Dugat-Bony *et al*., 2012) on biostimulation have been focused on groundwater in the presence of *Dhc.* This study therefore demonstrates that indigenous non-*Dhc* microbial groups can also be successfully stimulated for decontamination of PCE contaminated environments. This will be encouraging news to bioremediation practitioners in Australia. Although, BS-BA approach has some benefits over the BS only strategy (Major *et al*., 2002) decision to implement either treatment relies on site specific characteristics and legislations employed by local environmental protection agencies.

7.5 From micro to macro study

Microcosm studies conducted on groundwater samples collected from PCE contaminated site provided a detail insight into the dechlorinating microbial community structure, dynamics and their response to sequential PCE degradation. In Chapter 4, the detection of some initial mass imbalance with PCE to TCE conversion in some wells is observed, which is not unusual as this has been noticed in other similar studies (Daprato et al. 2007; Yang et al. 2005). I believe that, this could probably be due to the inability of indigenous microorganisms augmented with *Dhc* mixed consortia to establish an ecological niche in early days of incubation under well-controlled laboratory environment that are also competing to derive energy through reductive dechlorination. This seems not to be a case in Chapter 3, where indigenous communities were not augmented with *Dhc* strains. However in this study, the system was stabilized by approximately 20 days of incubation showing the production of the intermediates TCE, DCE and VC prior to the formation of ethene. The similar cause of inability of indigenous microorganisms augmented with *Dhc* consortia to settle well under laboratory conditions could be responsible for the initial imbalance in energy conversion process further causing variability in chloride ions released during reductive dechlorination pathway (Fig 1, page 57). Though, initial chloride concentrations in MWs 1 and 4 varied between BS and BS-BA sets, the difference is minimal (40 µmole/l to 35 µmole/l; 60 µmole/l to 54.2 µmole/l respectively). On the other hand, initial chloride concentrations of MWs 2 and 3 were similar (40µmole/l) between BS and BS-BA treatments. This variation could be due to the mechanical or technical error in the measurements. However, the error bars plotted in the Figure 1, Chapter 4 reduces the anomaly in the results presented.

Based on the microcosm studies and preliminary site assessment by using laboratory molecular techniques, three major bioremediation strategies involving BS, BS-BA and MNA were designed for *in situ* removal of TCE from a contaminated site. Considering the high cost and long timeframes required for other pilot studies (Ellis *et al*., 2000; Ibbini *et al*., 2010) this case study represented a short and inexpensive way of decontaminating chloroethene plume. The addition of excessive electron donors may not necessarily benefit or increase the rate of dechlorination, which in turn will increase treatment cost (Wei and Finneran, 2013). Hence, during this trial low

concentrations of acetate were injected initially and then the re-injections were done upon donor depletion. This indicated a logical and economically viable approach towards successful bioremediation avoiding the risk of generating excessive methane and aquifer clogging. A similar approach was implemented for *Dhc* injections for BA treatments. Injection of high dosages up to 160 – 200L does not guarantee complete and rapid dechlorination which can lead to substantial financial loss (Schaefer *et al*., 2010). As a result, we precisely determined the amount of culture needed for a study site through preliminary site evaluation using qPCR and the cell density was measured periodically until ethene formation. Overall, the knowledge governed from the application of MBTs throughout this commercial site-clean-up trial provided additional information about microbial community shift and diversity in response to TCE degradation. Moreover, this case study highlighted that careful considerations for preliminary laboratory testing along with site-specific hydro-geo characteristics and seasonal variations are crucial parameters to design successful bioremediation plan and MBTs can be potentially applied to assess the success rate of commercial remediation.

7.6 Conclusions

Bioremediation poses a great potential for subsurface chloroethene decontamination. The phylogenetic signatures of certain known *Dhc* species are frequently used as a proxy for the potential of indigenous bacteria to fully detoxify chlorinated solvents to ethene. However, a failure to detect *Dhc* in groundwater should not be taken to mean that dechlorination will occur intermittently. The present work demonstrated the potential of indigenous non-*Dhc* dechlorinating microorganisms in successfully degrading PCE to ethene by investigating their structure, dynamics and functional organization. The data obtained throughout this study using a combination of molecular techniques like PCR-DGGE, quantitative qPCR and rapid, simple LDCC together with analytical and statistical tools gave a concise portrait of microbial community dynamics and their response to PCE degradation on a laboratory scale which can be used as a management tool for commercial bioremediation. Moreover, field trials were conducted to test the response of indigenous and augmented dechlorinating microbial community to chloroethene degradation under natural environmental conditions. This study further ventured into the application of microbial

electric systems as a future sustainable alternative to overcome the drawbacks associated with existing bioremediation practices. However, detailed insight into bio-electrochemical potential of detected indigenous non-*Dhc* community is required to advance this field in a rational manner. The work done throughout this project will assist in designing the appropriate bioremediation regimes, a crucial step for commercial bioremediation industries globally. Further next generation metagenomics studies are needed to evaluate the functional and metabolic response of dechlorinating communities during *in situ* remediation of chlorinated compounds to understand the potential benefits for commercial bioremediation. These experiments lie beyond the scope of the PhD project, but have commenced.

References, Appendices and Corrigenda

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Appendices

Appendix 1: ISME' 14 Poster Presentation

RMIT UNIVERSITY
Melbourne, Australia

Flinders UNIVERSITY
Adelaide, Australia

Indigenous Microbial Community Dynamics During Reductive Dechlorination of Groundwater At a Chloroethene Contaminated Site In Victoria, Australia

Sayali S. Patil [1, 2*], Eric M. Adetutu[1], Arturo Aburto – Medina [1,2], Ian R. Menz[1] and Andrew S. Ball [2]
[1] School of Biological Sciences, Flinders University of South Australia, Adelaide, SA - 5042
[2] Department of Applied Biosciences, Royal Melbourne Institute of Technology, Bundoora, VIC – 3083

Introduction

Halogenated organic compounds (tetrachloroethene, PCE, trichloroethene, TCE, dichloroethene, DCE) represent a large group of hazardous chemicals of environmental concern in many contaminated sites. Previously, many studies have focused on *Dehalococcoides* group that perform complete dechlorination of chloroethenes. In contrast, the functional organization (Fo) and dynamics (Dy) of indigenous dechlorinators from PCE - contaminated sites are poorly investigated. Therefore the aims of this study are;

(a) To monitor indigenous microbial community composition by PCR - DGGE while documenting dechlorination of chloroethene compounds;

(b) To evaluate indigenous microbial community dynamics [$\Delta t_{(week)}$] and distribution by Moving Window Analysis (MWA) and Pareto – Lorenz (PL) curves characterization, respectively.

Materials & Methods

Chloroethene contaminated groundwater from four monitoring wells (MWs) was collected from a study site located in Victoria, Australia. Anaerobic enrichment cultures were set up in a glove box (Fig 1) as per the guidelines of Löffler *et al* (2005) and ATCC (American Type Culture Collection, www.atcc.org). Analytical procedures were conducted using 5975C gas chromatographic (GC) system equipped with a mass spectrometry (MS) detector (Agilent Tech, Australia). PCR based DGGE was carried out as described by Patil *et al* (2010). Based on PL curves and rate of change ($\Delta t_{(week)}$) values community functionality and dynamics was evaluated (Marzorati *et al*., 2008).

Fig1. Mesocosm preparation in 125 ml Wheaton bottles within anaerobic glove box

Results & Discussion

(i) Reductive dechlorination of chlorinated ethenes:

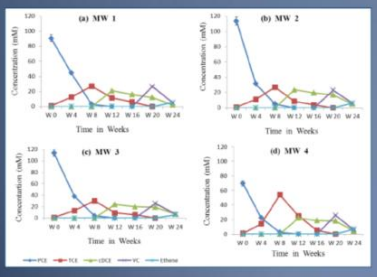

Fig. 2 Reductive dechlorination of PCE to ethene in (a) MW 1; (b) MW 2; (c) MW 3 and; (d) MW 4 mesocosms over the period of 24 weeks. Data are the average of the duplicates (duplicates differed by < 10%) and error bars indicate SDs.

(ii) Identification of indigenous dechlorinators from mesocosms: Sequence analysis indicated the increasing abundance of *Proteobacteria* and *Spirochaetes* phylum during weeks 0 to 24, while *Firmicutes* and *Bacteroidetes* were dominant from weeks 16 to 24 [Fig. 3 (a)]. Under the archaea domain, *Methanomicrobiaceae* was observed from weeks 4 to 16, while *Methanosarcinaceae* and *Methanosaetaceae* were dominant from weeks 12 to 24 [Fig. 3 (b)].

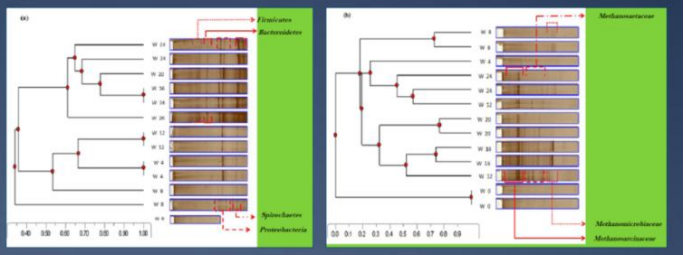

Fig. 3 UPGMA dendrogram constructed from (a) bacteria and (b) archaea DGGE community profiles from week 0 to 24 for represented MW 4. Designated lines indicate dominant DGGE bands which were excised and further sequenced. The scale bar represent percent similarity. Samples were analyzed in duplicates.

(iii) Functional organization (Fo) and dynamics (Dy) of indigenous dechlorinators: As a general rule, the 25%, 45% and 80% curves refer to a low, medium, and high Fo respectively. For the bacterial community, PL curve deviated from 20% to 45% from week 0 to 24 indicating low to medium Fo [Fig. 4 (a) i]. However, the archaeal community showed 25% to 40% evenness depicting low Fo [Fig. 4 (b) i]. MWA calculated average values of weekly deviations ($\Delta t_{(week)}$) as described by Marzorati *et al* (2008). The total bacterial community shifted more rapidly ($\Delta t_{(week)}$, 55.5% ± 22.7%) than the archaeal community ($\Delta t_{(week)}$, 46.9% ± 19.1%) indicating high 'Dy' level [Fig. 4 (a) & (b) ii].

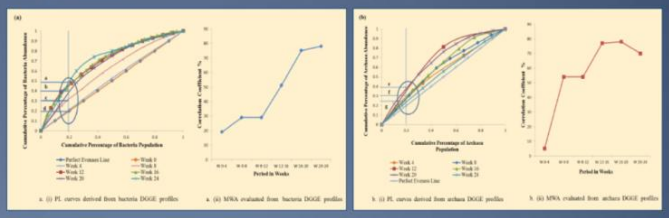

Fig. 4 Evaluation of (i) Pareto – Lorenz curves and; (ii) MWA from DGGE profiles of MW 4 (a) bacteria and; (b) archaea communities to assess Fo and Dy respectively. The vertical line is plotted at 0.8 x - axis in order to compare different Pareto values while the 45° diagonal line represents the perfect evenness of a community. Letters 'a' - 'g' indicate different range of Pareto values observed during weekly analysis. MWA and rate of change (Δt) values evaluates the level of community dynamics.

Conclusions

1] This study showed that acetate or H_2 alone can be sufficient to promote the complete reductive dechlorination of PCE to ethene, provided a native microbial community consisting of dechlorinating populations, syntrophic acetate - oxidizers and H_2/CO_2 acetogens exists;

2] Shifts in community structure occurred during initial (PCE – cDCE) and later (cDCE – ethene) phase of dechlorination;

3] High Dy level, as many species came to dominance leaving the total community in broad dynamics without loss of functionality;

3] Detection of these signature members and their role can help to explain whether complete or partial dechlorination may or may not occur as well as various degradation patterns observed at contaminated sites.

Acknowledgements

The authors thank the team of Environmental Earth Sciences Pty Ltd, Victoria for providing groundwater samples and Flinders Advanced Analytical Laboratory, Adelaide for assisting in gas chromatography analyses. Our sincere thanks also goes to the Flinders University of South Australia for offering Scholarship and Conference Travel Award.

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Appendix 2. Patil, S.S., Adetutu E.M., Aburto–Medina, A., Menz I.R., Ball A.S. 2014.Biostimulation of indigenous communities for the successful dechlorination of tetrachloroethene (perchloroethylene) - contaminated groundwater. *Biotechnology Letters*36, 75 – 83 doi: 10.1007/s10529-013-1369-1

Supplementary Table 1 List of primers and PCR protocols used in this study

Primer type	Primer	Primer sequence	PCR Protocol
Universal bacterial	341f-GC	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG – 3'	5 min initial denaturation at 95°C, 4 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 30 s of denaturation, annealing and extension followed by 10 min of final extension at 72°C.
	341f	5'CCTACGGGAGGCAGCAG3'	
	518 r	5'- ATTACCGCGGCTGCTGG - 3'	
Universal archaeal	A109f	5'-ACKGCTCAGTAACACGT-3'	Initial denaturation at 94°C for 3 min, followed by 32 cycles of 94°C for 45 s, 52°C for 1 min,

	A934b	5’-GTGCTCCCCCGCCAATTCCT-3’	72°C for 1 min, followed by a final extension at 72°C for 7 min
Dhc- specific	1f-GC	5’-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGATGAACGCTAGCGGCG-3’ (Target - 49 – 65 bp nucleotide positions of the *D. ethenogenes* 16S rDNA,)	Initial denaturation for 5 min at 94°C; 20 cycles of 94°C for 45 s, annealing for 45 s, and 72°C for 45 s (with the annealing temperature decreasing from 65 to 50°C at 0.5°C/cycle); an additional 15 cycles with annealing at 50°C; and a 5 min final extension at 72°C
	259r	5’-CAGACCAGCTACCGATCGAA-3’ (Target - 402 – 422 bp nucleotide positions of the *D. ethenogenes* 16S rDNA,)	

Supplementary Table 2 Phylogenetic affiliations of indigenous bacterial and archaeal 16S rRNA sequences obtained from PCE dechlorinating enrichment cultures compared with National Centre of Biotechnology Information (NCBI) database. DGGE bands 1 to 14 refer to bacterial species while bands 15 to 18 refer to archaeal species

Excised DGGE Bands	Accession No.	Closest matches overall (NCBI database)	Phylum	% Similarity
1	AF349763.2	Uncultured bacterium DCE33 16S ribosomal RNA gene, partial sequence	Spirochaetes	96%
2	AY667253.1	Uncultured bacterium clone TANB18 16S ribosomal RNA gene, partial sequence	Spirochaetes	96%
3	GQ377125.1	Bacterium enrichment culture clone DPF05 16S ribosomal RNA gene, partial sequence	Spirochaetes	98%

4	AF357916.2	*Spirochaeta* sp. Buddy 16S ribosomal RNA gene, partial sequence	Spirochaetes	98%
5	JF689075.1	Bacterium culture clone ALO1_GLFRUDD03F0MQ1 16S ribosomal RNA gene, partial sequence	γ -Proteobacteria	97%
6	JF920024.1	*Enterobacter* sp. 16S ribosomal RNA gene, partial sequence	γ - Proteobacteria	98%
7	FJ627782	*Desulfovibrio* sp (SRB 35) 16S ribosomal RNA gene, partial sequence	δ-Proteobacteria	96%

8	DQ903931	*Desulfovibrio* sp. GmS2 (SRB enrichment clone) 16S ribosomal RNA gene, partial sequence	δ-Proteobacteria	97%
9	JF502582.1	Uncultured bacterium clone G10 16S ribosomal RNA gene, partial sequence.	Bacteroidetes	97%
10	HM481392.1 .	Uncultured bacterium clone FL283 16S ribosomal RNA gene, partial sequence	Bacteroidetes	98%
11	FJ458042	Uncultured bacterium clone B19_E08 16S ribosomal RNA gene, partial sequence.	Bacteroidetes	96%
12	HM488066	Uncultured bacterium clone ZM4-54 16S ribosomal RNA gene, partial sequence.	Bacteriodetes	98%

13	AB610563	Clostridium sp. K12 gene for 16S rRNA, partial sequence	Firmicutes	98%
14	AJ249227.1	Uncultured bacterium DCE25 16S rRNA gene, partial sequence	Firmicutes	99%
15	M59140	*Methanosarcina thermophila* sp. 16S rRNA gene, partial sequence	Methanosarcinaceae	98%
16	AJ012094	*Methanosarcina bakeri* sp. 16S rRNA gene, partial sequence	Methanosarcinaceae	98%
17	M59141	*Methanosaeta thermophila* sp. 16S rRNA gene, partial sequence	Methanosaetaceae	99%
18	M59142	*Methanomicrobium mobile* sp. 16S rRNA gene, partial sequence	Methanomicrobiaceae	99%

Appendix 3. Patil, S.S., Adetutu E.M., Sheppard P.J., Morrison P., Menz I.R., Ball, A.S. 2014. Site - specific pre-evaluation of bioremediation technologies for chloroethene degradation. *International Journal of Environmental Science and Technology* 11 (7), 1869 – 1880.

Fig. S1 Standard curve showing the relationship between (a) *Dhc* and (b) 16S rRNA gene copy numbers and *C(T)* values in the serial dilutions of a known copy number. Both standard curves revealed amplification efficiencies over 1.8 with R^2 of 0.98 and 0.99, respectively

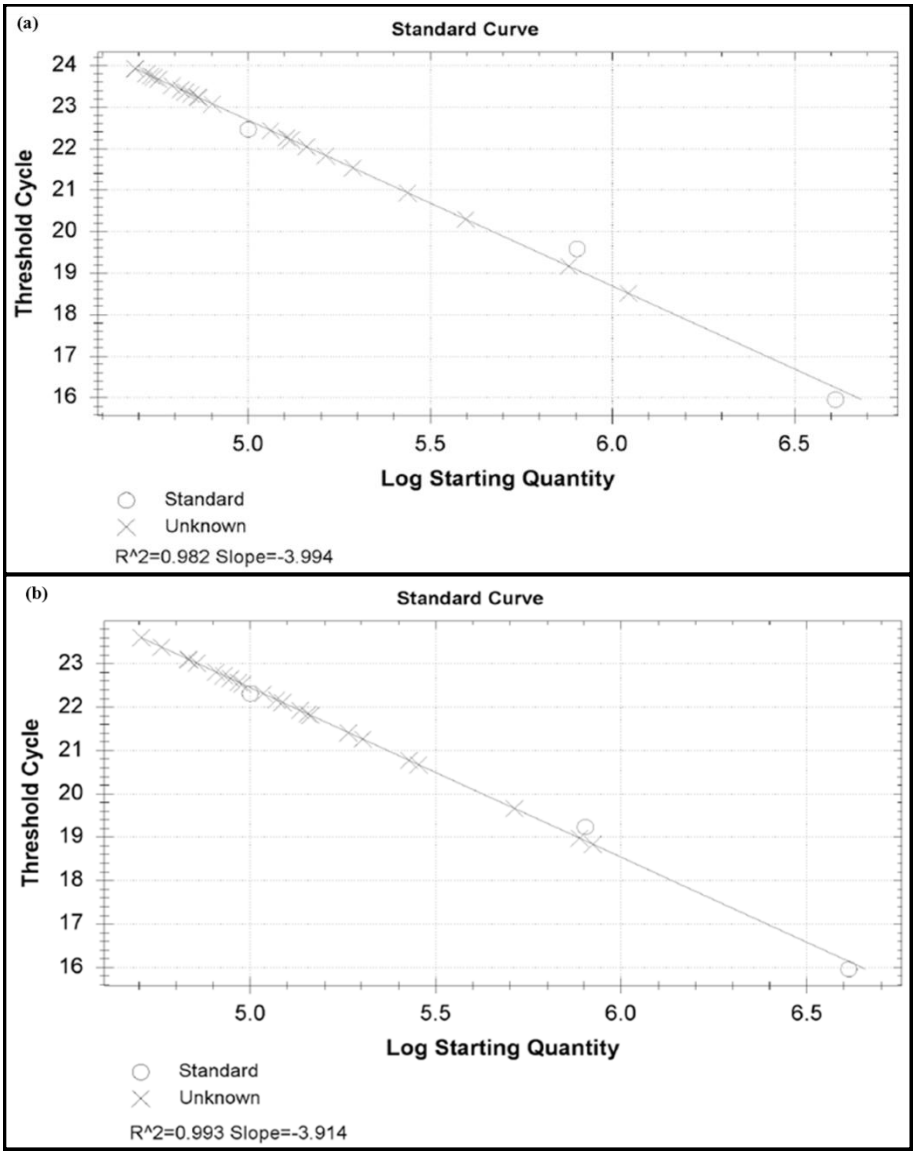


Table S1 Relative amounts of *Dhc* genes from *Dehalococcoides* species are measured by quantitative real time PCR, referenced to the amount of 16S rRNA for each sample [=$\Delta C(T)$] and to time week 1[=$\Delta\Delta C(T)$], then displayed as $2^{-\Delta\Delta(C(T)}$values. Avg, average; *C(T)*, cycle threshold; S.E, standard error

Set A	MW 1	Avg $C(T)_{Dhc}$ ± S.E	Avg $C(T)_{16S}$ ± S.E	$\Delta C(T)$ avg$C(T)_{Dhc}$ - avg Δ $C(T)_{16S}$	ΔΔ avg $C(T)$ avg $\Delta C(T)$ - avg $\Delta C(T)_{sample\ W1}$	$2^{-\Delta\Delta C(T)}$
	Week 1 (16S)	0	25.13 ± 0.77	25.13	0	1
	Week 21 (16S)	0	23.18 ± 0.01	23.18	-1.95	3.86
	MW 2					
	Week 1 (16S)	0	23.36 ± 0.19	23.36	0	1
	Week 21 (16S)	0	21.99 ± 0.72	21.99	-1.37	2.58
	MW 3					
	Week 1 (16S)	0	24.17 ± 0.87	24.17	0	1
	Week 21 (16S)	0	22.6 ± 0.11	22.6	-1.57	2.96
	MW 4					
	Week 1 (16S)	0	24.98 ± 0.03	24.98	0	1
	Week 21 (16S)	0	23.08 ± 0.87	23.08	-1.9	3.73
Set B	**MW 1**	**Avg $C(T)_{Dhc}$ ± S.E**	**Avg $C(T)_{16S}$ ± S.E**	**$\Delta C(T)$ avg$C(T)_{Dhc}$ - avg Δ $C(T)_{16S}$**	**ΔΔ avg $C(T)$ avg $\Delta C(T)$ - avg $\Delta C(T)_{sample\ W1}$**	**$2^{-\Delta\Delta C(T)}$**
	Week 1 (16S)	32.46 ± 0.3	23.03 ± 0.77	9.43	0	1
	Week 1 (Dhc)	32.60 ± 0.01	22.18 ± 0.01	10.42	0	1
	Week 17 (16S)	31.97 ± 0.62	23.83 ± 0.08	8.59	-0.84	1.79
	Week 17 (Dhc)	32.88 ± 0.99	23. 38 ± 0.14	9.5	-0.92	1.89
	MW 2					
	Week 1 (16S)	43.04 ± 0.42	29.55 ± 1.29	13.49	0	1
	Week 1 (Dhc)	42.00 ± 1.18	25.66 ± 1.0	16.34	0	1
	Week 17 (16S)	36.29 ± 0.92	23.61 ± 0.5	12.68	-0.81	1.75
	Week 17 (Dhc)	38.44 ± 0.28	23.65 ± 1.1	14.79	-1.55	2.92
	MW 3					
	Week 1 (16S)	37.21 ± 1.75	22.74 ± 0.57	14.47	0	1
	Week 1 (Dhc)	37.96 ± 2.89	21.87 ± 0.78	16.09	0	1
	Week 17 (16S)	35.62 ± 1.82	21.80 ± 0.70	13.82	-0.65	1.56
	Week 17 (Dhc)	36.81 ± 1.59	22.37 ± 0.34	14.44	-1.65	3.13
	MW 4					
	Week 1 (16S)	38.09 ± 0.08	35.28 ± 3.00	2.81	0	1
	Week 1 (Dhc)	36.13 ± 0.54	33.98 ± 0.61	2.15	0	1
	Week 17 (16S)	37.94 ± 0.45	36.08 ± 2.1	1.86	-0.95	1.93
	Week 17 (Dhc)	37.13 ± 1.7	36.8 ± 3.7	0.33	-1.82	3.53

Appendix 4: Gundry, T. D., **Patil, S. S**., Ball, A. S. 2014. Application of molecular biological tools for the assessment of the *in situ* bioremediation potential of a trichloroethene contaminated plume, Submitted to *Groundwater Monitoring and Remediation* (Under review)

Supplementary Information Table 1. Groundwater monitoring well construction details

Well ID	Treatment	Screen Interval (mbTOC)	Measured Depth (mbTOC)	DTW (mbTOC)	Relative Water level (mAHD)	Surveyed TOC Elevation
MW 22	BS 1	8.0 – 5.0	9.06	2.70	1.786	3.700
MW 11 A	BS 2	8.0 – 5.5	8.80	2.92	1.896	3.980
MW 17	BS 3	8.0 – 6.0	9.28	2.97	1.564	3.500
MW 15 A	BS 4	8.0 – 6.0	8.46	2.34	1.614	3.240
GW 8A	BS-BA 1	8.0 – 5.	8.95	3.00	1.820	4.040
GW 1B	BS-BA 2	07.0 – 4.0	7.86	2.77		3.780
MW 16B	BS-BS 3	8.0 – 5.	9.04	2.86	1.769	3.870
GW 4B	BS-BA 4	08.0 – 5.0	9.06	2.77	1.698	3.680
GW 6A	MNA 1	8.0 – 5.0	8.58	2.72	1.865	3.790
MW 20A	MNA 2	19.0 – 25.0	24.34			4.060
GW 5	MNA 3	7.0 – 1.5	6.30	2.98	1.811	3.990
MW 21	MNA 4	8.0 – 5.	9.03	2.30	1.489	3.790
MW 10A (Control)	No treatment	08.0 – 5.0	8.69	2.95	1.915	4.010

mbTOC = meters below top of casing; DTW = Depth to water; mAHD = Elevation in meters with respect to Australian Height Datum; Well diameter = 50 mm for all wells; BS = Biostimulation only; BS-BA = Biostimulation plus bioaugmentation; MNA = Monitored natural attenuation

Supplementary Information Table 2. Phylogenetic affiliations of all of the bacterial 16S rDNA sequences obtained from excised DGGE bands compared with the NCBI database

Excised DGGE bands	Wells	Closest relative (NCBI) database	Max Identity (%)	Accession No.
Pre-Treatment				
1C	GW1B	Uncultured bacterium clone RB13C10 16S ribosomal RNA gene, partial sequence	97	AF407413.1
2E	MW10A	Uncultured bacterium clone EDW07B005_144 16S ribosomal RNA gene, partial sequence	99	HM066626.1
5D	MW20A	Uncultured organism clone SBZI_4852 16S ribosomal RNA gene, partial sequence	97	JN527344.1
6B	MW22	Uncultured bacterium clone ncd2607a02c1 16S ribosomal RNA gene, partial sequence.	97	JF228136
7E	GW6A	Uncultured bacterium clone DR132 16S ribosomal RNA gene, partial sequence.	85	JF429176
During Treatment				
6A	GW5	*Bacillus licheniformis* strain A12 16S	97	KC434968

		ribosomal RNA gene, partial sequence		
6B	GW5	*Bacillus licheniformis* partial 16S rRNA gene, partial sequence	100	FR687205.1
13A	MW6A	*Bacillus licheniformis* strain CPH6 16S ribosomal RNA gene, partial sequence	99	JX912559.1
20B	GW1B	*Bacillus licheniformis* strain CPH6 16S ribosomal RNA gene, partial sequence	99	JX912559.1
21A	GW4B	Uncultured Sulfurospirillum sp. gene for 16S ribosomal RNA, partial sequence	99	AB713999.1
Post-Treatment				
A2A	MW17	Uncultured delta proteobacterium gene for 16S rRNA, partial sequence	89	AB074948.1
A14A	GW4B	Uncultured bacterium clone FLSED19 16S ribosomal RNA gene, partial sequence	86	EU552851.1
A23B	GW5	Uncultured Gamma proteobacterium partial 16S rRNA gene, clone AMJF11	91	AM934908.1
A24A	MW10A	Uncultured bacterium clone 2C229167 16S ribosomal RNA gene, partial sequence	96	EU800921.1
2A	MW17	Uncultured bacterium clone MI1EUBR_d04 16S ribosomal RNA	97	JX472542.1

		gene, partial sequence		
10A	MW16B	*Geobacter lovleyi* strain Geo7.1A 16S ribosomal RNA gene, partial sequence.	80	JN982204
10D	MW16B	*Dehalococcoides* sp. DG 16S ribosomal RNA gene, partial sequence	94	JQ627628
11A	GW8A	*Geobacter lovleyi* strain Geo7.3B 16S ribosomal RNA gene, partial sequence	89	JN982208
12A	GW8A	*Geobacter lovleyi* strain Geo7.1A 16S ribosomal RNA gene, partial sequence	91	JN982204
15C	GW4B	*Geobacter lovleyi* strain Geo7.1A 16S ribosomal RNA gene, partial sequence	91	JN982204
19A	MW21	Uncultured bacterium clone FLSED29 16S ribosomal RNA gene, partial sequence	87	EU552860
19B	MW21	Uncultured Pseudomonas sp. partial 16S rRNA gene, clone AMKB12	79	AM935015

Supplementary Information Table 3. DGGE band scoring data from **(a)** pre – treatment **(b)** mid-treatment and **(c)** post-treatment DGGE gels for analysing Shannon Weaver diversity index (H') and Equitability index (J).

A.

Pre-Treatment Data

				Equitability Index (J)							Shannon Diversity Data (H')		
Samples	Replicate A	Number of bands/ Species (n*i*) Replicate B	Av. Bands (S)	LN n*i* Rep A	Rep B	Equitability Index (J) Rep A	Rep B	Average of J	STD	SE	SDI (H) Rep A	Rep B	SDI Average
MW10A	3	3	3	1.098612	1.098612289	0.27787961	0.232257	0.255068534	0.032259733	0.022811	0.305282	0.255160897	0.280221426
GW8A	7	7	7	1.94591	1.945910149	0.661737281	0.634129	0.647933357	0.019521696	0.013804	1.287681	1.233958899	1.260820095
MW16B	5	5	5	1.609438	1.609437912	0.673033782	0.822235	0.747634492	0.105501335	0.074601	1.083206	1.323336506	1.203271296
GW1B	9	9	9	2.197225	2.197224577	0.339063645	0.489479	0.414271388	0.10635981	0.075208	0.744999	1.075495576	0.910247275
GW4B	7	7	7	1.94591	1.945910149	0.649859226	0.744088	0.696973772	0.066630031	0.047115	1.264568	1.447929012	1.356248337
MW11A	6	6	6	1.791759	1.791759469	0.024206607	0.575458	0.299832317	0.389793617	0.275626	0.043372	1.031082377	0.537227394
MW15A	7	7	7	1.94591	1.945910149	0.666130039	0.510051	0.588090315	0.110364836	0.07804	1.296229	0.992512624	1.144370912
MW22	6	6	6	1.791759	1.791759469	0.219028472	0.653391	0.436209903	0.307140926	0.217181	0.392446	1.170720111	0.781583225
MW17	3	3	3	1.098612	1.098612289	0.618310197	0.590619	0.604464779	0.019580378	0.013845	0.679283	0.648861687	0.664072434
GW6A	11	11	11	2.397895	2.397895273	0.267691511	0.488946	0.378318904	0.156450759	0.110627	0.641896	1.172442013	0.907169111
GW5	7	7	7	1.94591	1.945910149	0.614151486	0.050612	0.332381676	0.398482687	0.28177	1.195084	0.098486143	0.646784877
MW20A	8	8	8	2.079442	2.079441542	0.741182349	0.669338	0.70525995	0.050801943	0.035922	1.541245	1.391848311	1.466546839
MW21	3	3	3	1.098612	1.098612289	0.601416716	0.563399	0.582407873	0.026882563	0.019009	0.660724	0.618957098	0.639840446

B.

Mid – Treatment Data

Samples	Replicate A	Number of bands/Species (n*i*) Replicate B	Av. Bands(S)	LN n*i* Rep A	LN n*i* Rep B	Equitability Index (J) Rep A	Equitability Index (J) Rep B	Average of J	STD	SE	Shannon Diversity Data (H') SDI (H) Rep A	SDI (H) Rep B	SDI Average
MW10A	15	15	15	2.70805	2.708050201	0.525337476	0.514374	0.519855545	0.007752621	0.005482	1.42264	1.392949569	1.407794913
GW8A	10	10	10	2.302585	2.302585093	0.64174118	0.609346	0.625543728	0.022906657	0.016197	1.477664	1.403071649	1.440367662
MW16B	21	23	22	3.044522	3.135494216	0.816657162	0.649717	0.733187312	0.118044195	0.08347	2.486331	2.03718534	2.261758197
GW1B	15	14	14.5	2.70805	2.63905733	0.549560262	0.635033	0.592296427	0.060438064	0.042736	1.488237	1.675887414	1.582062096
GW4B	15	13	14	2.70805	2.564949357	0.605233042	0.713151	0.659192215	0.076309794	0.053959	1.639001	1.829197195	1.734099328
MW15A/ MW11A	7	7	7	1.94591	1.945910149	0.342003747	0.584329	0.463166492	0.171349997	0.121163	0.665509	1.137052191	0.901280377
	0	0	0	0	0	0	0	0	0	0	0	0	0
MW22	11	14	12.5	2.397895	2.63905733	0.589373447	0.732359	0.66086604	0.101105795	0.071493	1.413256	1.932736419	1.672996111
MW17	20	20	20	2.995732	2.995732274	0.612834583	0.548677	0.580756014	0.045365947	0.032079	1.835888	1.64369073	1.739789535
GW6A	23	23	23	3.135494	3.135494216	0.820141128	0.825094	0.822617373	0.003501939	0.002476	2.571548	2.587076266	2.579312014
GW5	12	0	6	2.484907	0	0.305761587	0	0.152880793	0.216206091	0.152881	0.759789	0	0.3798945
MW20A	10	10	10	2.302585	2.302585093	0.668624543	0.458835	0.56372955	0.148343921	0.104895	1.539565	1.056505611	1.298035258
MW21	15	17	16	2.70805	2.833213344	0.445157663	0.674209	0.559683195	0.161963561	0.114526	1.205509	1.910177164	1.557843231

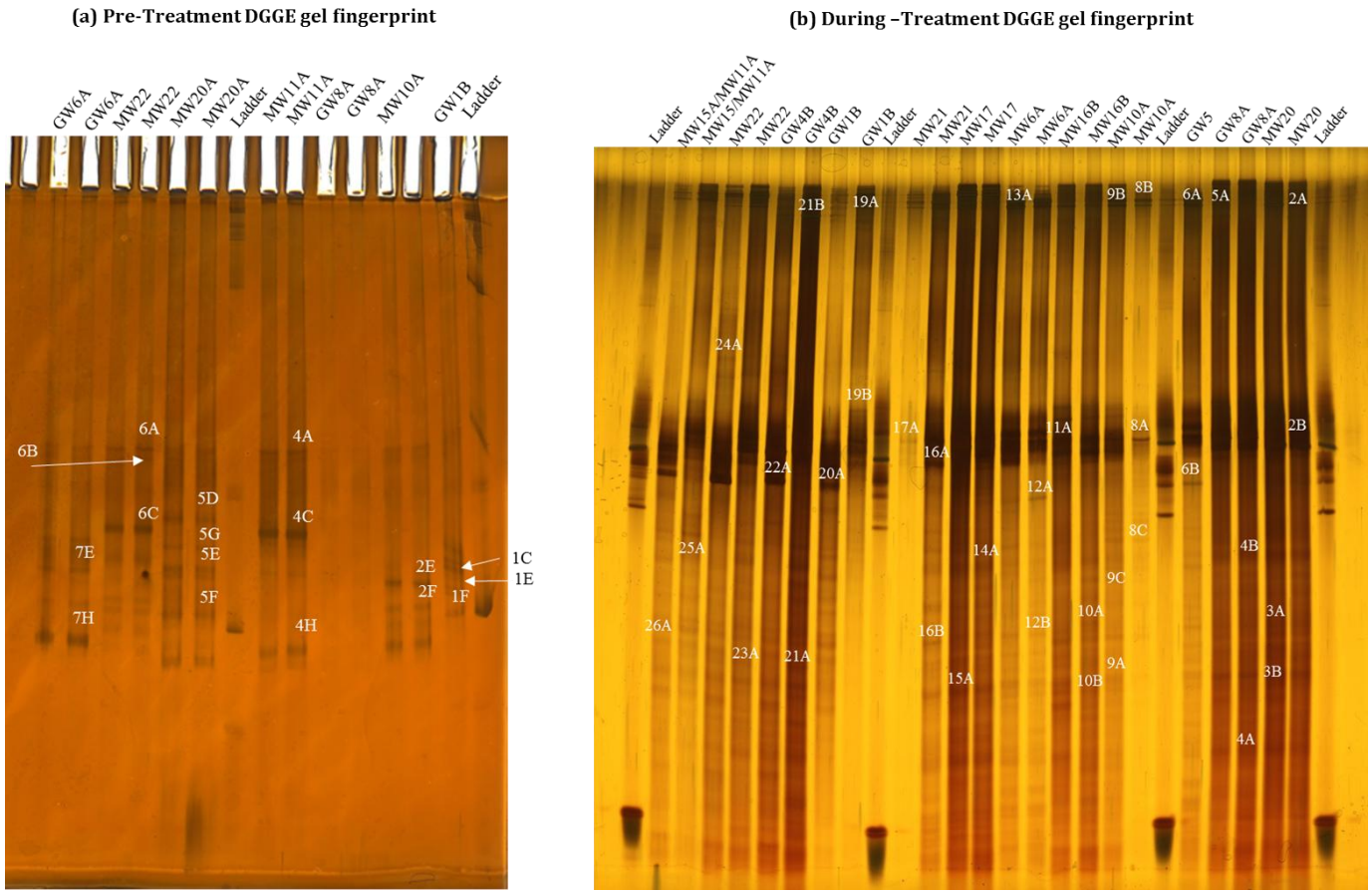
C.

Post - Treatment Data

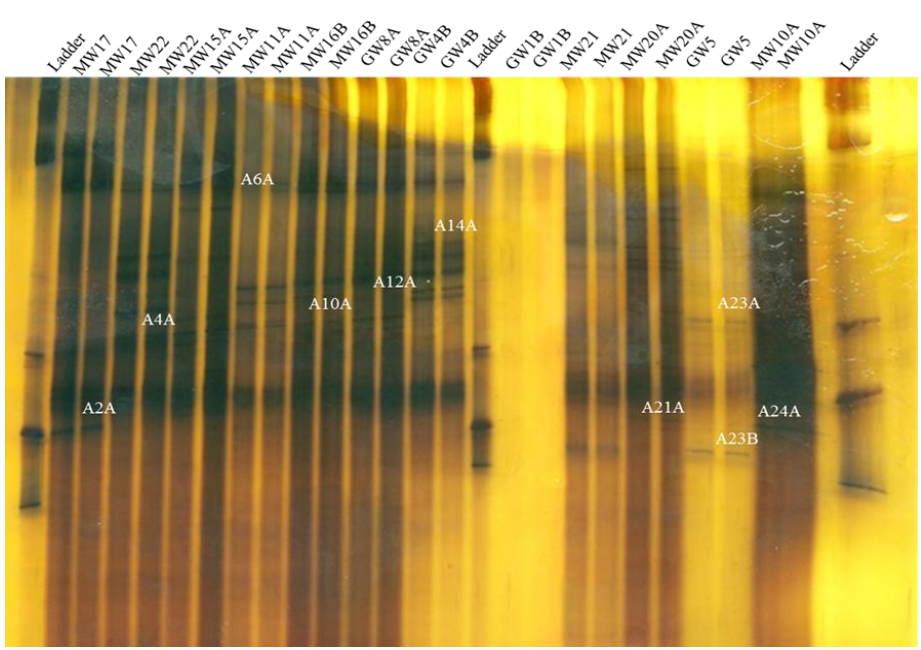
Equitability Index (J) **Shannon Diversity Data (H')**

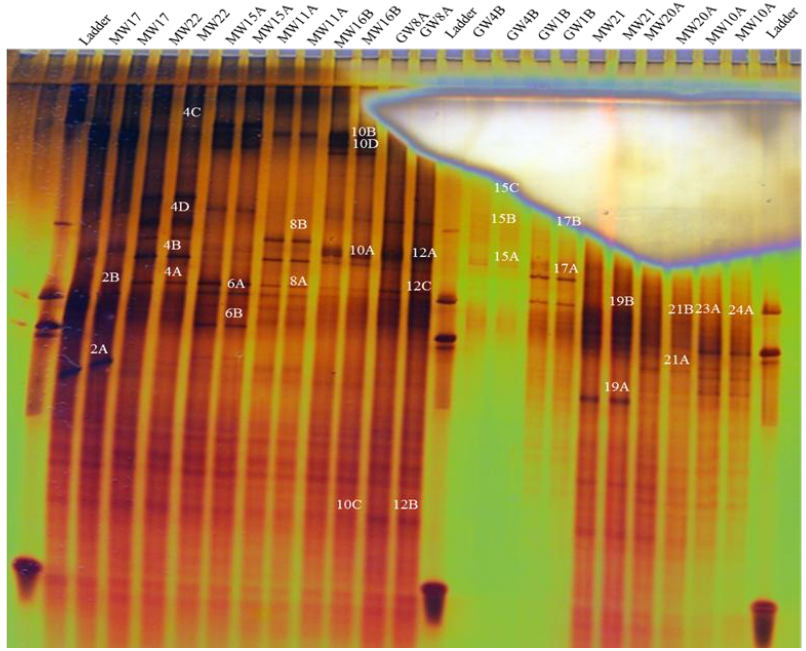
Samples	Replicate A	Number of bands/Species (n*i*) Replicate B	Av. Bands(S)	LN n*i*		Equitability Index (J)		Average of J	STD	SE	SDI (H)		
				Rep A	Rep B	Rep A	Rep B				Rep A	Rep B	SDI Average
MW10A	9	9	9	2.197225	2.197224577	0.140668321	0.617207	0.378937851	0.238269531	0.168482	0.30908	1.356143229	0.83261156
GW8A	4	4	4	1.386294	1.386294361	0.561190245	0.764512	0.662851358	0.101661113	0.071885	0.777975	1.059839327	0.918907099
MW16B	8	8	8	2.079442	2.079441542	0.177203269	0.336917	0.257060009	0.079856739	0.056467	0.368484	0.700598682	0.534541261
GW1B	9	9	9	2.197225	2.197224577	0.454743275	0.264327	0.359534932	0.095208343	0.067322	0.999173	0.580784877	0.789978989
GW4B	5	5	5	1.609438	1.609437912	0.354392833	0.410828	0.382610337	0.028217504	0.019953	0.570373	0.661201902	0.615787582
MW15A/ MW11A	4	4	4	1.386294	1.386294361	0.591144766	0.314365	0.45275483	0.138389936	0.097856	0.819501	0.43580228	0.627651468
	7	7	7	1.94591	1.945910149	0.736209289	0.111322	0.423765686	0.312443603	0.220931	1.432597	0.216622772	0.82460995
MW22	5	5	5	1.609438	1.609437912	0.466388729	0.015678	0.241033269	0.225355459	0.15935	0.750624	0.025232462	0.387928082
MW17	1	1	1	0	0	0	0	0	0	0	0	0	0
GW6A	11	11	11	2.397895	2.397895273	0.738729792	0.258575	0.822617373	0.003501939	0.002476	2.571548	2.587076266	2.579312014
GW5	17	17	17	2.833213	2.833213344	0.590806707	0.557707	0.152880793	0.216206091	0.152881	0.759789	0	0.3798945
MW20A	5	5	5	1.609438	1.609437912	0.079018802	0.597076	0.56372955	0.148343921	0.104895	1.539565	1.056505611	1.298035258
MW21	4	4	4	1.386294	1.386294361	0.371650678	0.889291	0.559683195	0.161963561	0.114526	1.205509	1.910177164	1.557843231

Supplementary Information Figure 1. DGGE gels of 16 rDNA gene sequences from amplified DNA extracted from groundwater samples with excised band locations for **(a)** PT **(b)** DT and **(c)** PST. Gels with letters indicate where the bands were excised from for further sequencing.



(c) Post-Treatment DGGE gel fingerprint



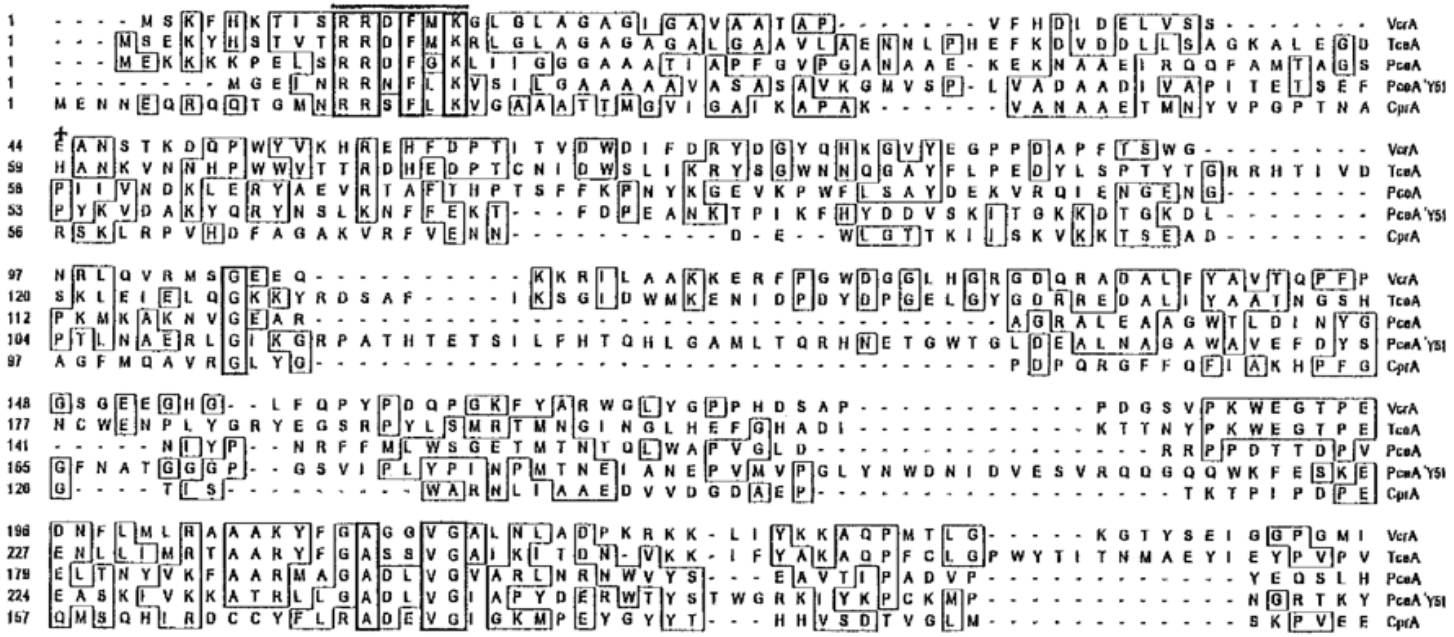


CORRIGENDA

This section aims to provide corrections and further clarifications from thesis chapters 1, 3 and 4.

Chapter 1

- **Range of sequence variation between RDases *pce*A, *tce*A, *vcr*A is as below:**



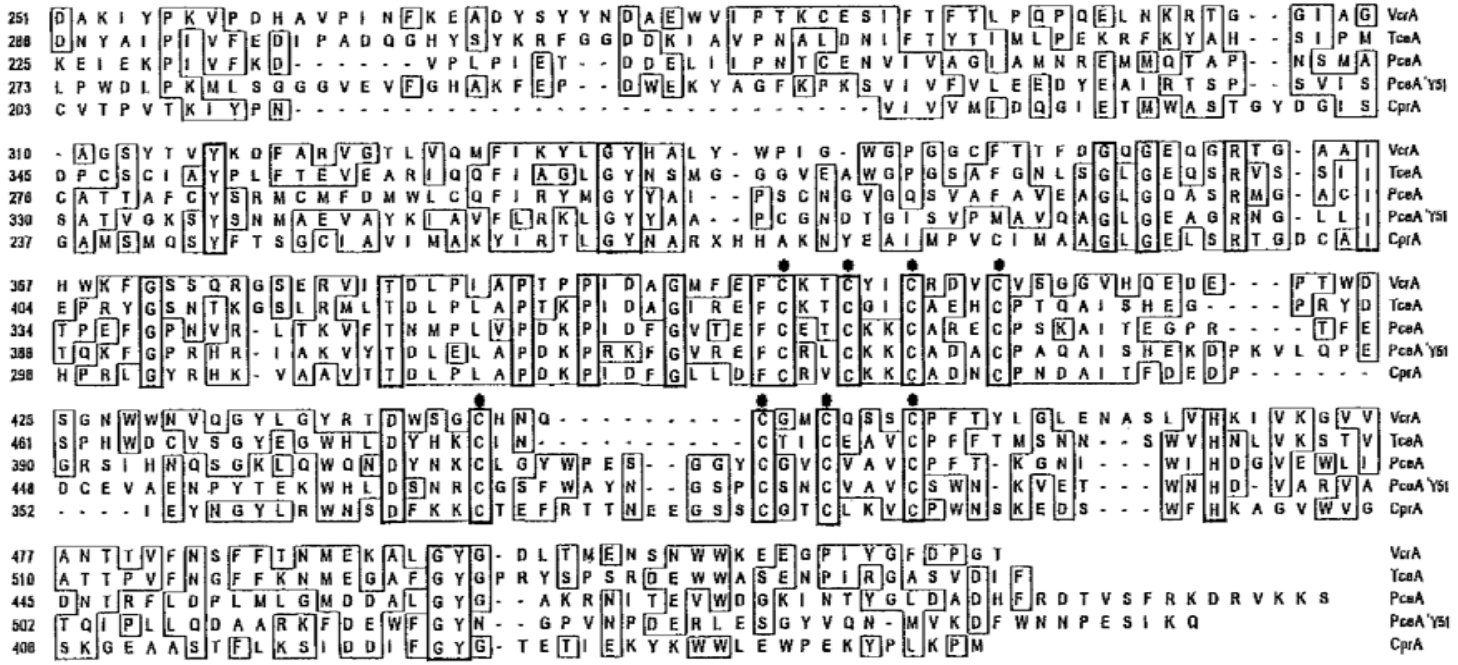


Figure. Amino acid sequence alignment of *Vcr*A from *Dehalococcoides* sp. strain VS (SEQ ID NO: 2) with *Tce*A from *D. ethenogenes* (accession number AF228507, SEQ ID NO: 41), PceA from *S. multivorans* (accession number AF022812, SEQ ID NO:42), *Pce*A from *Desulfitobacterium* sp. strain Y51 (accession number AB070709, SEQ ID NO:43), and *Cpr*A from *D. dehalogenans* (accession number AF204275, SEQ ID NO:44). Amino acid residues identical in all 5 sequences are highlighted in black. Functionally similar amino acid residues (2 distance units) and amino acid residues that are conserved in only some of the sequences are boxed. Horizontal bar, twin-arginine motif; plus sign, first amino acid residue, E 44, of the mature VcrA; asterisks, conserved cysteines (adapted from Spormann et al., 2013).

Published Chapter 3

- **Missing legend for Figure 1 is now provided :**

- **Missing word "Figure 2" form sentence 2, paragraph 2 of results section on page 44 is now provided.** The sentence now reads as **–** "Bacterial and archaeal DGGE profiles for assessing community changes throughout the experiment are shown in Figure 2".

- Raw DGGE band scoring data for both bacterial and archaeal DGGE gels is presented in below table. This data obtained using Phoretix 1D advanced analysis package was analyzed to construct PL curves and MWA based on the DGGE band intensities.

Raw Bacterial DGGE Band Scoring Data Used for constructing PL Curves and MWA Analyses

Week 4 Band No	Gaussian Vol	Band No	Gaussian Vol	Ave	Ave Sort	X Proportion	Y Proportion	X cum proportion of bands 0	Week 4 0
1	112465.22	1	130070.04	121267.6	121267.6	0.25	0.494496967	0.25	0.49449697
2	40206.71	2	49788.89	44997.8	44997.8	0.25	0.183488996	0.5	0.67798596
3	23168.37	3	50199.35	36683.86	42285.04	0.25	0.172427065	0.75	0.85041303
4	40063.26	4	44506.81	42285.04	36683.86	0.25	0.149586972	1	1
					245234.3	1	1		

Week 8 Band No	Gaussian Vol	Band	Gaussian	Ave	Ave Sort	X Proporation	Y Proportion	X cum proportion of bands 0	Week 8 0

		No	Vol						
1	43984.56	1	46113.35	45048.96	50843.44	0.055555556	0.082578562	0.055555556	0.08257856
2	47387.28	2	54299.6	50843.44	45048.96	0.055555556	0.073167313	0.111111111	0.15574588
3	49761.23	3	38176.64	43968.94	43968.94	0.055555556	0.071413174	0.166666667	0.22715905
4	48981.11	4	32765.67	40873.39	42680.74	0.055555556	0.069320922	0.222222222	0.29647997
5	55818.62	5	29542.86	42680.74	40873.39	0.055555556	0.066385472	0.277777778	0.36286544
6	41228.17	6	26464.52	33846.35	37999.19	0.055555556	0.061717273	0.333333333	0.42458272
7	36100.27	7	35105.53	35602.9	35602.9	0.055555556	0.057825282	0.388888889	0.482408
8	25707.69	8	34296.86	30002.28	33846.35	0.055555556	0.054972332	0.444444444	0.53738033
9	30958.48	9	32324.63	31641.56	32855.6	0.055555556	0.053363183	0.5	0.59074351
10	28671.17	10	29750.52	29210.85	32250.36	0.055555556	0.052380176	0.555555556	0.64312369
11	39080.58	11	26630.61	32855.6	31641.56	0.055555556	0.051391371	0.611111111	0.69451506
12	33413.22	12	19679.82	26546.52	31368.43	0.055555556	0.050947769	0.666666667	0.74546283
13	37999.19			37999.19	30002.28	0.055555556	0.048728896	0.722222222	0.79419173
14	29899.59			29899.59	29899.59	0.055555556	0.048562118	0.777777778	0.84275384
15	31368.43			31368.43	29210.85	0.055555556	0.047443477	0.833333333	0.89019732
16	32250.36			32250.36	26546.52	0.055555556	0.043116151	0.888888889	0.93331347
17	21025.94			21025.94	21025.94	0.055555556	0.034149772	0.944444444	0.96746324
18	20032.81			20032.81	20032.81	0.055555556	0.032536757	1	1
					615697.8	1	1		

Week 12								X cum proportion of bands	Week 12
Band No	**Gaussian Vol**	**Band No**	**Gaussian Vol**	**Ave**	**Ave Sort**	**X Proportion**	**Y Proportion**	**0**	**0**
1	55905.14	1	62895.77	59400.46	66851.54	0.111111111	0.174568908	0.111111111	0.17456891

2	77686.62	2	56016.45	66851.54	59400.46	0.111111111	0.155111959	0.222222222	0.32968087
3	55535	3	51663.71	53599.36	53599.36	0.111111111	0.13996359	0.333333333	0.46964446
4	50290.88	4	39233.14	44762.01	44762.01	0.111111111	0.116886698	0.444444444	0.58653115
5	37182.56	5	25868.53	31525.55	39667.42	0.111111111	0.103583234	0.555555556	0.69011439
6	25444.95	6	24700.15	25072.55	31762.25	0.111111111	0.08294051	0.666666667	0.7730549
7	30027.71	7	30594.32	30311.02	31525.55	0.111111111	0.082322417	0.777777778	0.85537732
8	22648.59	8	40875.9	31762.25	30311.02	0.111111111	0.079150924	0.888888889	0.93452824
9	39667.42			39667.42	25072.55	0.111111111	0.06547176	1	1
					382952.1	1	1		

Week 16								**X cum proportion of bands**	**Week 16**
Band No	**Gaussian Vol**	**Band No**	**Gaussian Vol**	**Ave**	**Ave Sort**	**X Proportion**	**Y Proportion**	**0**	**0**
1	59273.9	1	46960.51	53117.21	53117.21	0.125	0.23451139	0.125	0.23451139
2	50739.8	2	39093.34	44916.57	44916.57	0.125	0.198305751	0.25	0.43281714
3	26696.21	3	22308.34	24502.28	27161.95	0.125	0.11991944	0.375	0.55273658
4	25055.97	4	20590.76	22823.37	24502.28	0.125	0.10817705	0.5	0.66091363
5	18147.37	5	13148.18	15647.78	22823.37	0.125	0.100764697	0.625	0.76167833
6	19030.12	6	16856.76	17943.44	20389.03	0.125	0.090017135	0.75	0.85169546
7	21874.84	7	18903.21	20389.03	17943.44	0.125	0.079219926	0.875	0.93091539
8	28700.92	8	25622.97	27161.95	15647.78	0.125	0.069084611	1	1
					226501.6	1	1		

Week 20								**X cum proportion of bands**	**Week 20**
Band No	**Gaussian Vol**	**Band**	**Gaussian**	**Ave**	**Ave Sort**	**X Proportion**	**Y Proportion**	**0**	**0**

		No	Vol						
1	40624.87	1	37593.15	39109.01	39279.81	0.111111111	0.144624952	0.111111111	0.14462495
2	35226.5	2	35590.28	35408.39	39109.01	0.111111111	0.14399608	0.222222222	0.28862103
3	37476.57	3	34500.43	35988.5	37609.62	0.111111111	0.138475453	0.333333333	0.42709648
4	32780.03	4	27525.1	30152.57	35988.5	0.111111111	0.132506625	0.444444444	0.55960311
5	17648.9	5	18332.02	17990.46	35408.39	0.111111111	0.130370709	0.555555556	0.68997382
6	16916.2	6	10075.43	13495.82	30152.57	0.111111111	0.111019204	0.666666667	0.80099302
7	8983.9	7	36143.24	22563.57	22563.57	0.111111111	0.083077164	0.777777778	0.88407019
8	36797.25	8	38421.99	37609.62	17990.46	0.111111111	0.066239358	0.888888889	0.95030955
9	39279.81			39279.81	13495.82	0.111111111	0.049690454	1	1
					271597.7	1	1		

Week 24								**X cum proportion of bands**	**Week 24**
Band No	**Gaussian Vol**	**Band No**	**Gaussian Vol**	**Ave**	**Ave Sort**	**X Proportion**	**Y Proportion**	**0**	**0**
1	25587.14	1	77350.3	51468.72	70173.74	0.111111111	0.188227669	0.111111111	0.18822767
2	83865.41	2	56482.06	70173.74	51468.72	0.111111111	0.138055031	0.222222222	0.3262827
3	47878.98	3	42449.75	45164.37	45164.37	0.111111111	0.121144801	0.333333333	0.4474275
4	38229.63	4	41620.88	39925.26	39925.26	0.111111111	0.107091887	0.444444444	0.55451939
5	36863.09	5	34013.96	35438.53	39801.96	0.111111111	0.106761158	0.555555556	0.66128054
6	28435.84	6	21197.38	24816.61	38442.13	0.111111111	0.103113686	0.666666667	0.76439423
7	31481.23	7	48122.68	39801.96	35438.53	0.111111111	0.095057089	0.777777778	0.85945132
8	35810.81	8	19352.74	27581.78	27581.78	0.111111111	0.073982854	0.888888889	0.93343417
		9	38442.13	38442.13	24816.61	0.111111111	0.066565826	1	1
					372813.1	1	1		

Raw Archaeal DGGE Band Scoring Data Used for PL Curves and MWA Analyses

Week 4								X cum proportion of bands	Week 4
Band No	**Gaussian Vol**	**Band No**	**Gaussian Vol**	**Ave**	**Ave Sort**	**X Proporation**	**Y Proportion**	**0**	**0**
1	56657.86	1	59996.75	58327.305	58327.305	0.333333333	0.433587002	0.333333333	0.433587002
2	39429.77	2	39586.15	39507.96	39507.96	0.333333333	0.293689858	0.666666667	0.72727686
3	37372.06	3	36002.86	36687.46	36687.46	0.333333333	0.27272314	1	1
					134522.725	1	1		

Week 8								X cum proportion of bands	Week 8
Band No	**Gaussian Vol**	**Band No**	**Gaussian Vol**	**Ave**	**Ave Sort**	**X Proporation**	**Y Proportion**	**0**	**0**
1	10586.96	1	28084.87	19335.915	20659.23	0.111111111	0.158379674	0.111111111	0.158379674
2	17737.44	2	23581.02	20659.23	19335.915	0.111111111	0.148234756	0.222222222	0.30661443
3	4901.38	3	16592.45	10746.915	18949.8	0.111111111	0.145274686	0.333333333	0.451889116
4	7436.88	4	8918.24	8177.56	18133.78	0.111111111	0.139018839	0.444444444	0.590907955
		5	10932.44	10932.44	12954.49	0.111111111	0.099312893	0.555555556	0.690220848
		6	18133.78	18133.78	10932.44	0.111111111	0.083811269	0.666666667	0.774032117
		7	18949.8	18949.8	10746.915	0.111111111	0.08238898	0.777777778	0.856421098
		8	12954.49	12954.49	10551.04	0.111111111	0.080887346	0.888888889	0.937308443
		9	10551.04	10551.04	8177.56	0.111111111	0.062691557	1	1
					130441.17	1	1		

Week 12								X cum proportion of bands	Week 12
Band No	Gaussian Vol	Band No	Gaussian Vol	Ave	Ave Sort	X Proporation	Y Proportion	0	0
1	33811.04	1	40257.64	37034.34	37034.34	0.5	0.814336268	0.5	0.814336268
2	7725.79	2	9161.42	8443.605	8443.605	0.5	0.185663732	1	1
					45477.945	1	1		

Week 16								X cum proportion of bands	Week 16
Band No	Gaussian Vol	Band No	Gaussian Vol	Ave	Ave Sort	X Proporation	Y Proportion	0	0
1	28877.8	1	29322.02	29099.91	37607.42	0.125	0.195935893	0.125	0.195935893
2	28399.76	2	27422.19	27910.975	31546.07	0.125	0.164356061	0.25	0.360291954
3	24103.55	3	21515.86	22809.705	29099.91	0.125	0.151611487	0.375	0.51190344
4	9335.38	4	9121.24	9228.31	27910.975	0.125	0.145417096	0.5	0.657320536
5	41148.41	5	34066.43	37607.42	26600.265	0.125	0.138588254	0.625	0.79590879
6	33374.68	6	29717.46	31546.07	22809.705	0.125	0.118839312	0.75	0.914748102
7	26315.95	7	26884.58	26600.265	9228.31	0.125	0.048079798	0.875	0.962827901
8	7354.02	8	6915.41	7134.715	7134.715	0.125	0.037172099	1	1
					191937.37	1	1		

Week 20								X cum proporation of bands	Week 20
Band No	Gaussian Vol	Band No	Gaussian Vol	Ave	Ave Sort	X Proporation	Y Proportion	0	0
1	36991.04	1	32118.11	34554.575	50989.06	0.1	0.215440644	0.1	0.215440644

2	67710.12	2	34268	50989.06	38618.715	0.1	0.163173058	0.2	0.378613702
3	32108.28	3	24804.61	28456.445	34554.575	0.1	0.14600112	0.3	0.524614822
4	30246.72	4	8065.91	19156.315	28456.445	0.1	0.120235102	0.4	0.644849924
5	31237.96	5	24328.57	27783.265	27783.265	0.1	0.11739076	0.5	0.762240684
6	38447.49	6	38789.94	38618.715	19156.315	0.1	0.080939889	0.6	0.843180573
7	17869.78	7	15022.3	16446.04	16446.04	0.1	0.069488346	0.7	0.912668919
8	4949.88	8	2642.64	3796.26	10264.525	0.1	0.043370007	0.8	0.956038925
9	7519.42	9	5696.89	6608.155	6608.155	0.1	0.027920993	0.9	0.983959918
10	10842.19	10	9686.86	10264.525	3796.26	0.1	0.016040082	1	1
					236673.355	1	1		

Week 24								**X cum proportion of bands**	**Week 24**
Band No	**Gaussian Vol**	**Band No**	**Gaussian Vol**	**Ave**	**Ave Sort**	**X Proporation**	**Y Proportion**	**0**	**0**
1	32752.68	1	21612.03	27182.355	35886.77	0.166666667	0.202260272	0.166666667	0.202260272
2	25937.53	2	21035.2	23486.365	31595.055	0.166666667	0.178071875	0.333333333	0.380332146
3	23999.06	3	47774.48	35886.77	31510.225	0.166666667	0.177593767	0.5	0.557925913
4	43492.78	4	19527.67	31510.225	27767.895	0.166666667	0.156501741	0.666666667	0.714427655
5	25083.24	5	38106.87	31595.055	27182.355	0.166666667	0.153201598	0.833333333	0.867629253
6	39425.89	6	16109.9	27767.895	23486.365	0.166666667	0.132370747	1	1
					177428.665	1	1		

Published Chapter 4

- **Raw data used for the construction of standard curve in quantitative PCR (qPCR) analysis is presented below.**

Standard Curve Spreadsheet Raw Data

Fluor	Well	Type	Ident. SQ	Rep Mean	SD	Ct	Log SQ Mean	SQ	SD	SQ Point	SQ	Ct	Ct	Set
FAM	A01	Std	-	1		16.25	-3.000	1.00E-03		1.00E-03	0.00E+00	16.25	0.000	N/A
FAM	A02	Unkn	-	3		24.65	-5.500	3.16E-06		3.16E-06	0.00E+00	24.65	0.000	N/A
FAM	A03	Unkn	-	11		24.95	-5.587	2.59E-06		2.59E-06	0.00E+00	24.95	0.000	N/A
FAM	B01	Std	-	2		19.67	-4.000	1.00E-04		1.00E-04	0.00E+00	19.67	0.000	N/A
FAM	B02	Unkn	-	4		20.30	-4.201	6.30E-05		6.30E-05	0.00E+00	20.30	0.000	N/A
FAM	B03	Unkn	-	12		26.08	-5.925	1.19E-06		1.19E-06	0.00E+00	26.08	0.000	N/A
FAM	C01	Std	-	3		22.95	-5.000	1.00E-05		1.00E-05	0.00E+00	22.95	0.000	N/A
FAM	C02	Unkn	-	5		24.70	-5.515	3.06E-06		3.06E-06	0.00E+00	24.70	0.000	N/A
FAM	D02	Unkn	-	6		24.86	-5.562	2.74E-06		2.74E-06	0.00E+00	24.86	0.000	N/A
FAM	E02	Unkn	-	7		20.97	-4.402	3.96E-05		3.96E-05	0.00E+00	20.97	0.000	N/A
FAM	F02	Unkn	-	8		24.74	-5.526	2.98E-06		2.98E-06	0.00E+00	24.74	0.000	N/A
FAM	G01	Unkn	-	1		17.90	-3.486	3.26E-04		3.26E-04	0.00E+00	17.90	0.000	N/A
FAM	G02	Unkn	-	9		25.11	-5.635	2.32E-06		2.32E-06	0.00E+00	25.11	0.000	N/A
FAM	H01	Unkn	-	2		21.85	-4.663	2.17E-05		2.17E-05	0.00E+00	21.85	0.000	N/A
FAM	H02	Unkn	-	10		19.10	-3.845	1.43E-04		1.43E-04	0.00E+00	19.10	0.000	N/A

Where, **Ct** is threshold cycle and **SQ** is a starting quantity, copy number

- **The graphical presentation of control experiment run against BS and BS-BA treatment sets is presented below.**

Figure. The control set showing that PCE dechlorination did not progress beyond TCE in the un-inoculated and electron donor less, autoclaved control sets under both BS and BS-BA treatments.