FACULTY OF ENGINEERING UNIVERSITY OF PORTO

Microbial bioelectrochemical remediation of chlorinated organics

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Abbreviation

1,1,1-TCA	1,1,1-Trichloroethane
1,1,2,2-TeCA	1,1,2,2-Tetrachloroethane
1,1,2-TCA	1,1,2-Trichloroethane
1,1-DCE	1,1-Dichloroethene
1,2-DCA	1,2-Dichloroethane
AQ	Anthraquinone
AQDS	Anthraquinone-2,6-disulfonate
BES	Bioelectrochemical system
CAH	Chlorinated aliphatic hydrocarbons
CARD	Catalyzed reported deposition
CE	Coulombic efficiency
CF	Chloroform
CH_4	Methane
cis-DCE	cis-Dichloroethene
CM	Chloromethane
CT	Carbon tetrachloride
CVs	Cyclic voltammograms
DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane
DI	Deionized
DNAPL	Dense non aqueous phase liquid
EDS	Energy-dispersive X-ray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
EET	Extracellular electron transfer
ETH	Ethene

FID	Flame ionization detector
FISH	Fluorescence *in situ* hybridization
GC	Gas chromatograph
HMDS	Hexamethyldisilazane
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IC	Inorganic carbon
IET	Interspecies electron transfer
MCL	Maximum contamination level
MES	Microbial electrochemical system
MQ	Ultrapure
MV	Methyl viologen
NPs	Nanoparticles
NPOC	Non purgeable organic carbon
OCP	Open circuit potential
ON	Over night
PBS	Phosphate buffer solution
PCE	Tetrachloroethene
PEM	Proton exchange membrane
PNA	Peptide nucleic acid
POC	Purgeable organic carbon
PVC	Polyvinyl chloride
RD	Reductive dechlorination
RDP	Ribosomal data project
RT	Room temperature
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy

SHE	Standard hydrogen electrode
TC	Total carbon
TCD	Thermal conductivity detector
TCE	Trichloroethene
TE	TrisHCl + EDTA
TOC	Total organic carbon
trans-DCE	trans-Dichloroethene
USEPA	U.S. environmental protection agency
VC	Vinyl chloride
VFA	Volatile fatty acids

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Firstly I would like to thank my supervisors, which helped me to broaden my horizons, overcome adversities, think outside the box and always look ahead.

I would also like to thank CERENA (Porto), the Department of Mining Engineering and the Faculty of Engineering of the University of Porto. A special reference to LEPABE, for the help in several subjects.

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Most importantly I must thank my family and friends, for the unconditional support throughout the different phases of my life, which made yet another challenge possible. You were always there for me in the good and bad moments, no matter what.

Finally I must apologize to everyone for, "sometimes", being unbearable.

Abstract (en)

Due to the extensive use as well as improper storage, handling and disposal practices, toxic chlorinated ethanes are frequently found as subsurface and groundwater contaminants all over the World, including Portugal. Because of their high toxicity, physicochemical properties and impact on living organisms, research of effective remediation techniques of these compounds are required.

In this thesis, bioelectrochemical systems (BES) for the anaerobic reductive dechlorination of chlorinated ethanes are examined as a novel, more sustainable and effective remediation technique.

The main studied parameters include the influence of the set cathode potential and the presence of a redox mediator, either in solution or grafted on the cathode surface, on the chloroethanes' bioelectrochemical dechlorination rate and efficiency.

The dynamics of microbial communities directly and indirectly involved in the dechlorination process were also tracked during each experiment. The newly developed PNA-FISH biomolecular technique was adapted for the detection and monitoring of two strains of a key dehalorespiring microorganism. Additionally, conductive minerals were tested for their role as conduits for the syntrophic dechlorination of 1,2-dichloroethane (1,2-DCA).

Initial tests provided a set of recommended parameters for process optimization in a simplified BES, while other tests with anthraquinone-2,6-disulfonate (AQDS) addition demonstrated an increase in dechlorination rate, for both AQDS in solution and grafted on the cathode, although at varying degrees. Protocols for AQDS grafting are also reported.

In addition, the use of conductive minerals led to a 3-fold increase in dechlorination rate. It is assumed that the tested mineral is used in interspecies extracellular electron transfer, facilitating catalytic electron transport.

This work demonstrates the potential of bioelectrochemical systems for anaerobic biodegradation of chlorinated ethanes and provides a detailed description of relevant optimized parameters as well as several novel protocols for redox mediator immobilization, biomolecular monitoring tools, etc. These protocols can be applicable to a wide variety of different situations and contaminants.

Resumo (pt)

A utilização intensiva de compostos etano-clorados bem como práticas inadequadas de armazenamento, manuseamento e eliminação faz com que estes compostos sejam frequentemente detetados como contaminantes do subsolo por todo o mundo, incluindo em Portugal. Desta forma investigação em técnicas de remediação é necessária devido à sua elevada toxicidade, propriedades físico-químicas e impacto em organismos vivos.

Nesta tese, foi investigada a possibilidade da aplicação de sistemas bioelectroquímicos, como técnica de remediação alternativa para o tratamento anaeróbico de etanos clorados, mais económica do que os sistemas de bioremediação tradicionais.

Os principais parâmetros estudados incluem a influência do potencial do cátodo e a adição de mediadores redox, fornecidos em solução ou imobilizados no cátodo, sobre a taxa e eficiência de descontaminação bioelectroquímica de cloroetanos.

As comunidades microbianas, responsáveis por este processo foram monitorizadas durante cada teste. A técnica biomolecular PNA-FISH foi adaptada para a deteção e monitorização de duas estirpes de um microrganismo crucial no tratamento destes compostos. O papel de minerais condutores foram testados na descloração sintrófica de 1,2-dicloroetano (1,2-DCA).

Os testes iniciais forneceram um conjunto de parâmetros para a otimização de um BES simplificado, enquanto outros testes, com a adição de um mediador de redox Antraquinona-2,6-Disulfonato (AQDS) demonstraram um aumento na taxa de remediação, tanto para a adição de AQDS em solução como imobilizado no elétrodo, embora em grau variável. Também é apresentado o protocolo para imobilizar o AQDS no elétrodo.

O uso de minerais condutores resultou num aumento de três vezes na taxa de remediação. Este resultado leva a assumir que o mineral testado possa ser utilizado na transferência extracelular de eletrões entre espécies, facilitando o transporte catalítico de eletrões.

Este trabalho demonstra o potencial dos sistemas bioelectroquímicos na biodegradação anaeróbia de etanos clorados e fornece uma descrição da optimização de parâmetros relevantes, bem como vários protocolos inovadores para a imobilização do mediador redox, ferramentas de monitorização biomolecular, etc. Estes protocolos podem ser aplicáveis a uma ampla variedade de diferentes situações e contaminantes.

Abstract (it)

A causa del vasto uso industriale, nonché di pratiche di stoccaggio, movimentazione e smaltimento improprie, gli etani clorurati sono tra i più frequenti inquinanti di suoli ed acque sotterranee in tutto il mondo, incluso il Portogallo. A causa della loro elevata tossicità, delle loro peculiari proprietà fisico-chimiche che ne comportano una elevata presistenza, grande attenzione è rivolta allo sviluppo di opportune tecniche di bonifica per questi composti.

In questa tesi, sono stati studiati e sviluppati nuovi processi bioelettrochimici (BES) in grado di accelerare la declorazione riduttiva anaerobica di etani clorurati. I principali parametri di processo studiati includono l'influenza del potenziale di lavoro del catodo e la presenza di un mediatore redox, sia in soluzione o adeso alla superficie dell'elettrodo stesso. L'effetto di tali parametri è stato valutato rispetto alla velocità e resa del processo di declorazione riduttiva. La dinamica delle comunità microbiche direttamente e indirettamente coinvolte nel processo di declorazione sono stati monitorati durante ogni esperimento. La tecnica biomolecolare PNA-FISH di nuova concezione è stato applicata per il rilevamento e il monitoraggio dei due ceppi di un microrganismo declorante chiave. Inoltre, minerali conduttivi sono stati testati per il loro ruolo come condotti elettronici in processi sintrofici coinvolti nella declorazione riduttiva dell'1,2-dicloroetano (1,2-DCA).

La prima fase della sperimentazione ha consentito di identificare il valore ottimale del potenziale catodico nel processo di declorazione riduttiva in assenza di mediatori redox aggiunti. I test condotti in presenza del mediatore antrachinone-2,6-disolfonato (AQDS), solubile o immobilizzato, hanno messo in evidenza un aumento del tasso di declorazione riduttiva e delle corripsondenti rese. Infine, l'uso di minerali conduttivi ha portato ad un incremento di 3 volte il tasso di declorurazione rispetto a controlli non ammendati. Si presume che il minerale testato sia utilizzato in processi di trasferimento interspecie di elettroni.

In conclusione, questo lavoro dimostra le potenzialità dei sistemi bioelettrochimici per stimolare la declorazione riduttiva anaerobica dei etani clorurati e fornisce una descrizione dettagliata dei relativi parametri di processi, dei diversi protocolli per l'impiego ed immobilizzazione di mediatori redox nel processo, nonché di strumenti di monitoraggio biomolecolari, ecc. Questi protocolli possono essere applicabile ad una vasta gamma di diverse situazioni e contaminanti.

Layout

This thesis is divided in 8 chapters and 12 appendices:

Chapter 1 – Introduction. In this chapter an overview of the relevance and motivation for this thesis is presented and its objectives are outlined. Also general properties and interactions with the environment of chlorinated organics are discussed. A more detailed description is made regarding the chlorinated ethanes 1,2-Dichloroethane (1,2-DCA) and 1,1,2-Trichloroethane (1,1,2-TCA), compounds that were the main focus of this thesis. The principles and applications of bioelectrochemical systems (BES) are described, as well as the use of redox mediators, conductive minerals and biomolecular tools in bioremediation technologies.

Chapter 2 to 6 – These chapters are based on scientific articles, which were either published in (chapters 2, 3 and 6) or submitted to (chapters 4 and 5), international peer-reviewed journals.

Chapter 7 – Concluding remarks. This chapter describes the main findings and considerations of this work. Additionally suggestions for future research in this topic are detailed.

Chapter 8 – The engineering implications of the research developed in this thesis is presented in this chapter.

The appendices present more detailed information regarding methods, equipment and experimental procedures used. Because this PhD was performed in two different research centers, Rome (Italy) and Porto (Portugal), equipment varied depending on the location. Therefore, when applicable, this distinction will be duly noted in the appendices.

Scientific literature output

Articles in international peer-reviewed journals:

Leitão, P., Bellagamba, M., Rossetti, S., Nouws, H., Danko, A., Aulenta, F. (2017). Grafting AQDS onto graphite electrodes using a commercial anion exchange membrane to promote the bioelectrochemical reductive dechlorination of 1,2-dichloroethane. Submitted.

Leitão, P., Aulenta, F., Rossetti, S., Nouws, H., Danko, A. (2016). Impact of magnetite nanoparticles on the syntrophic dechlorination of 1,2-dichloroethane. Submitted.

Leitão, P., Rossetti, S., Danko, A., Nouws, H., Aulenta, F. (2016). Enrichment of *Dehalococcoides mccartyi* spp. from a municipal activated sludge during AQDS-mediated bioelectrochemical dechlorination of 1,2-dichloroethane to ethene. Bioresource Technology, 214, 426-431.

Leitão, P., Rossetti, S., Nouws, H., Danko, A., Majone, M., Aulenta, F. (2015). Bioelectrochemically-assisted reductive dechlorination of 1,2-dichloroethane by a *Dehalococcoides*-enriched microbial culture. Bioresource Technology, 195, 78-82.

Danko, A., Fontenete, S., Leite, D., Leitão, P., Almeida, C., Schaefer, C., Vainberg, S., Steffan, R., Azevedo, N. (2014). Detection of *Dehalococcoides* spp. by peptide nucleic acid fluorescent in situ hybridization. Journal of Molecular Microbiology and Biotechnology, 24, 142-149.

Oral communications in scientific meetings

Leitão, P., Rossetti, S., Nouws, H., Danko, A., Aulenta, F. (2015). Bioelectrochemical dechlorination of 1,2-DCA mediated by the humic acid analogue antraquinone-2,6-disulfonate. 3rd Symposium on Subsoil Characterization and Remediation.

Leitão, P., Santos, R., Fontenete, S., Leite, D., Danko, A., Azevedo, N. (2013). *Dehalococcoides* spp. detection in rock samples using PNA-FISH. 2nd Symposium on Subsoil Characterization and Remediation.

Posters in scientific meetings

Leitão, P., Bellagamba, M., Rossetti, S., Nouws, H., Danko, A., Aulenta, F. (2016). Reductive dechorination of 1,2-DCA with Antraquinone-2,6-Disulfonate modified electrode.

3rd European Meeting of the International Society for Microbial Electrochemistry and Technology.

Leitão, P., Rossetti, S., Nouws, H., Danko, A., Aulenta, F. (2015). Bioelectrochemical dechorination of 1,2-DCA mediated by the humic acid analogue Anthraquinone-2,6-Disulfonate. 6th European Bioremediation Conference.

Leitão, P., Rossetti, S., Nouws, H., Danko, A., Majone, M., Aulenta, F. (2014). Microbial reductive dechlorination of 1,2-dichloroethane (1,2-DCA) with graphite electrodes serving as electron donors. 2nd European meeting of the International Society for Microbial Electrochemistry and Technologies.

Leitão, P., Santos, R., Fontenete, S., Leite, D., Danko, A., Azevedo, N. (2013). Peptide nucleic acid fluorescent in situ hybridization (PNA-FISH) detection of *Dehalococcoides* spp. in mixed cultures and environmental samples. 12th International UFZ-Deltares Conference on Groundwater-Soil-Systems and Water Resource Management, AquaConSoil.

1 Introduction

1.1 Chlorinated organics and interactions with the environment

Chlorinated organics are used in several industrial applications such as chemical and dry cleaning, textile dyeing, solvent formulations, among others. Due to improper use, handling, storage and disposal over the last decades they are among the most common organic groundwater contaminants throughout Portugal [including downgradient from the chemical complex of Estarreja (Branco, C., 2007)], Europe and worldwide (Huang, B, 2014).

These contaminants are highly toxic, some are mutagenic and/or suspected to be carcinogens or proven carcinogens. Therefore, the presence of chlorinated organics in groundwater, even in small amounts, is undesirable (Huang, B, 2014). Because they are usually denser than water and have low aqueous solubilities, a Dense Non Aqueous Phase Liquid (DNAPL) tends to form in groundwater (Pankow, J., 1996). This phenomenon occurs when a portion of contaminant migrates through the aquifer and forms large clusters. Contamination by DNAPLs poses as a major challenge for remediation actions since these compounds are constantly being dissolved in the water flow, hence being a continuous source of contamination (Schaefer, C., 2010).

Degradation of organic solvents in groundwater can be governed by several reactions, either abiotic or biotic, the latter usually being the predominant (Tobiszewski, M., 2012).

This distinction can be somewhat difficult since many times one process occurs as a consequence of the other and/or both processes occur simultaneously.

To better understand the reactions involving chlorinated organics it is necessary to take into account the properties of these compounds. Chlorinated aliphatic hydrocarbons (CAHs), a class belonging to chlorinated organics, are composed of a simple hydrocarbon chain to which at least one chlorine atom is covalently bonded. CAHs can be further divided into three categories based on common structural characteristics: chlorinated methanes, chlorinated ethanes and chlorinated ethenes.

The behavior of CAHs in the subsurface is controlled, to a large extent, by their physical and chemical properties. The most relevant properties, which govern the fate and transport in the subsurface of chlorinated methanes, ethanes and ethenes are summarized in Table 1.1.

Table 1.1 - Physical and chemical properties of some chlorinated organic solvents at 25 °C.

Compound	Formula	Molecular weight (g/mol)	Carbon oxidation state[a]	Density (ρ) (g/mL)	Solubility (S) (mg/L)	Vapor pressure (p⁰) (torr)	Henry's law constant (K_H) (10^{-3} atm.m³/mol)	Log (K_{ow})	Log (K_{OC})[b]	PV (mg/L)[c]	MCL (mg/L)[d]
Chlorinated methanes											
Tetrachloromethane	CCl_4	153.8	+IV	1.59	800	153.8	28.9	2.64	1.9	NR[e]	0.005
Trichloromethane	$CHCl_3$	119.4	+II	1.49	8200	196.8	1.97	1.97	1.52	0.10[f]	0.10[g]
Dichloromethane	CH_2Cl_2	84.9	0	1.33	13200	415	1.25	1.25	---	NR	0.005
Chloromethane	CH_3Cl	50.5	-II	0.92	5235	4275	0.91	0.91	---	NR	NR
Chlorinated ethanes											
1,1,2,2-tetrachloroethane	$C_2H_2Cl_4$	167.9	+I	1.60	2962	5.9	0.44	2.39	1.9	NR	NR
1,1,2-trichloroethane	$C_2H_3Cl_3$	133.4	0	1.44	4394	24.2	0.96	2.38	---	NR	0.005
1,2-dichloroethane	$C_2H_4Cl_2$	99.0	-I	1.25	8606	79.0	1.20	1.48	1.52	0.003	0.005
Chloroethane	C_2H_5Cl	64.5	-II	0.90	5700	120	1.8	1.43	---	NR	NR
Chlorinated ethenes											
Tetrachloroethene	C_2Cl_4	165.8	+II	1.63	150	18.1	26.3	2.88	2.29	0.10	0.005
Trichloroethene	C_2HCl_3	131.4	+I	1.46	1100	74.2	11.7	2.53	1.53	0.10	0.005
cis-1,2-dichloroethene	$C_2H_2Cl_2$	96.9	0	1.28	3500	203	7.4	1.86	---	NR	0.07
trans-1,2-dichloroethene	$C_2H_2Cl_2$	96.9	0	1.26	6260	333	6.8	1.93	---	NR	0.1
1,1-dichloroethene	$C_2H_2Cl_2$	96.9	0	1.22	3344	604	23.0	2.13	---	NR	0.007
Chloroethene	C_2H_3Cl	62.5	-I	0.91	2763	2660	79.2	1.38	---	0.005	0.002

Unless otherwise noted, all values were taken from Mackay, D. (1993).

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[a] Average value calculated using oxidation states for H = +I and Cl = -I.
[b] When available, log(K_{oc}) values were obtained from Nguyen, T., 2005.
[c] PV - Parametric Value. Directive 98/83/EC of the European Council
[d] MCL - Maximum Contamination Level. Source: USEPA (2016).
[e] NR= Not regulated
[f] Sum of concentrations of specified compounds. The specified compounds are: chloroform, bromoform, dibromochloromethane, bromodichloromethane.
[g] For total trihalomethanes, which is defined as the summed concentration of chloroform, bromoform ($CHBr_3$), bromodichloromethane ($CHBrCl_2$), and dibromochloromethane ($CHBr_2Cl$).

An overview that links the physical and chemical properties of chlorinated solvents to their presence in the subsurface phases is presented next. The processes by which pure phase chlorinated solvents dissolve into groundwater and their partitioning between the three phases present in the subsurface (aquifer solids, water and air) can be schematically represented as presented in Figure 1.1.
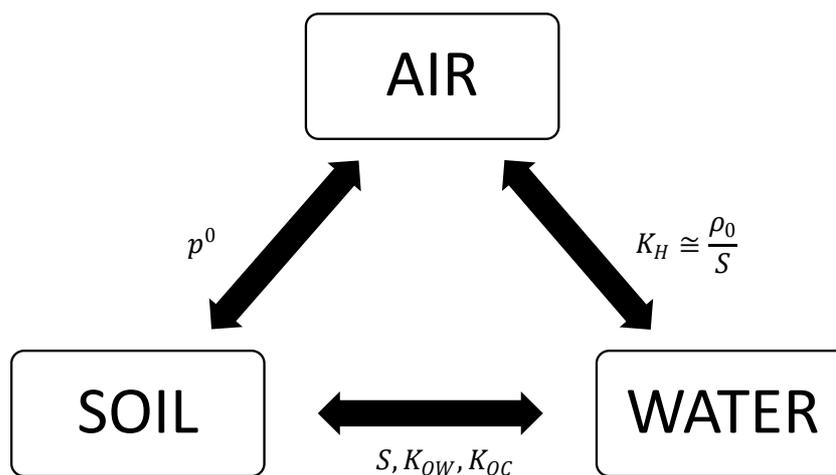
Figure 1.1 - The three major phases present in the subsurface and properties of chlorinated solvents that rule the partitioning between phases (Cwiertny, D., 2010).

At room temperature, most CAHs are colorless liquids with densities (ρ) greater than water. The dissolution extent of these solvents in groundwater is controlled by the solvent's aqueous solubility (S), defined as the maximum amount of a chlorinated solvent that will partition into water at a given temperature (Lyman, W., 1982). CAHs can be classified as sparingly soluble in water (Table 1.1), however, their aqueous solubilities are relatively high when compared to the U.S. Environmental Protection Agency's (USEPA) MCLs (Pankow, J., 1996) which, combined with its recalcitrant nature, contributes to their persistence as groundwater pollutants. Another very important consequence of their low solubility, as stated previously, is their tendency to form DNAPLs in the subsurface, separate liquid phases at the base of an aquifer.

The transport rate in the subsurface is affected by the partitioning of CAHs between aquifer solids and water. CAHs can be considered moderately hydrophobic but when compared to other organic pollutants their affinity to sorb onto aquifer solids is much lower. A measure of a compound's hydrophobicity is the octanol-water partitioning coefficient (K_{ow}), which is defined as the equilibrium concentration of the CAH in octanol relative to its equilibrium concentration in water, in a two-phase system containing octanol and water:

$$K_{OW} = \frac{C_{octanol}}{C_{water}}$$

For laboratory investigations of hydrophobicity, octanol is chosen as a convenient reference solvent (immiscible with water). K_{ow} is also important to evaluate the tendency of a given compound to accumulate into organisms.

To describe the processes in the subsurface in a more pertinent way, values of K_{oc} should be considered, which represent the chemical equilibrium partitioning between water and the organic carbon fraction of aquifer solids:

$$K_{OC} = \frac{C_{organic\ carbon}}{C_{water}}$$

Values of both K_{ow} and K_{oc} generally increase as the number of chlorine substituents of a compound increases. These larger values of solid-water partitioning coefficients will result in slower rates of subsurface transport. An inverse relationship between aqueous solubility and K_{ow} (or K_{oc}) values is also observed in Table 1.1.

CAHs are relatively volatile compounds. Therefore, air-water partitioning is expected to take place when contaminated groundwater comes into contact with air, as is the case in unsaturated subsurface zones (i.e., the vadose zone). In such instances, the equilibrium partitioning between gas phase and liquid phase (water) is typically described by Henry's Law, which is applicable to dilute solutions of a chlorinated solvent in water. The Henry's Law constant, K_H, relates the equilibrium concentration of the chlorinated solvent in air to its equilibrium concentration in water:

$$K_H = \frac{C_{gas}}{C_{liquid}}$$

Unlike reported values of S, K_{ow} and K_{oc}, the K_H values presented in Table 1.1 do not reveal any significant trend within or across the different classes of chlorinated solvents.

The last partitioning process to consider is the one that occurs between aquifer solids and air. As with volatilization between air and water, several chemical and environmental factors are at play in solid-air partitioning processes (Thomas, R., 1982), a process still poorly understood. One noteworthy variable is the vapor pressure (p^0) of a CAH, which represents the maximum attainable concentration of CAH in air (Schwarzenbach, R., 2003). Compounds with high values of p^0 tend to partition more readily between air and

sediments (and similarly, between air and water). Values of p^0 tend to decrease with increasing chlorination, although some exceptions can be frequently observed.

Although not included in Figure 1.1, transformation reactions (degradation) are another critical pathway that impacts the fate of CAHs in groundwater. Rates and products of transformation reactions will depend on many of the chemical and physical properties discussed above, but also with the average oxidation state of carbon in the CAH (Table 1.1). For the CAHs listed in Table 1.1 this value ranges from -II to +IV. A positive oxidation state corresponds to a species in an oxidized form and prone to reduction. On the other hand, chlorinated solvents with more reduced carbon centers are more susceptible to oxidation.

Depending on the contaminant and on the environmental redox conditions, degradation can proceed via aerobic or anaerobic conditions. A trend can be established between sorption onto surface material, aerobic and anaerobic degradation, and the degree of chlorination, this later defined as the number of chloride atoms divided by the number of carbon atoms (Figure 1.2). CAHs with a lower degree of chlorination are more prone to aerobic degradation and show lower sorption, whereas CAHs with a higher degree of chlorination are more susceptible to anaerobic degradation and sorption.
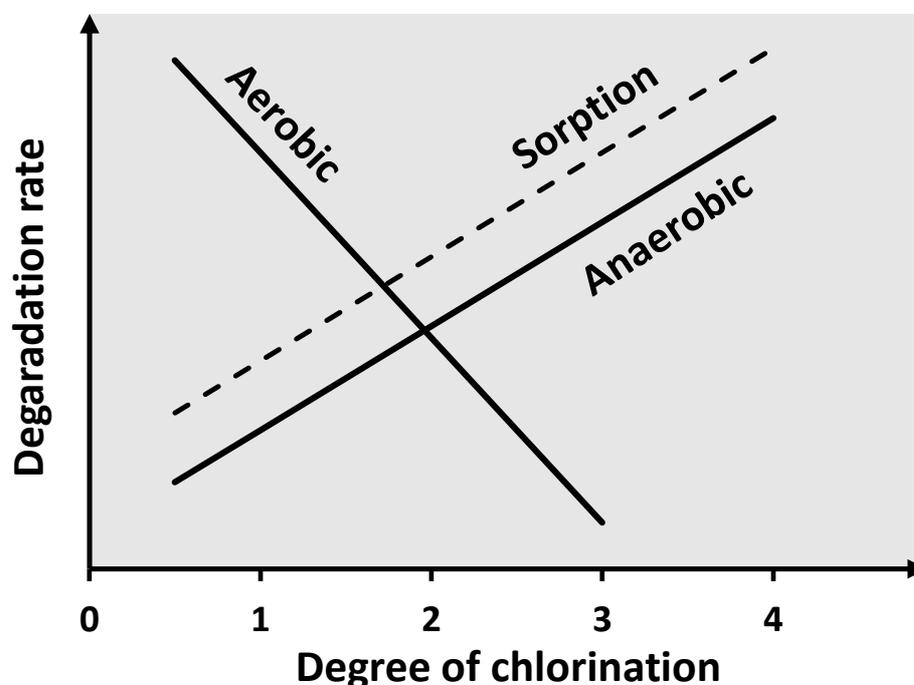
Figure 1.2 - Relation between degree of dechlorination and aerobic and anaerobic degradation and sorption (Adapted from Norris, R., 1993).

Aerobic degradation processes are associated with CAHs that have a reducing character, i.e. CAHs that can be used as electron donors, oxygen as electron acceptor and

present in oxidizing environments. Vinyl Chloride (VC) can be cited as one example that is susceptible to this degradation process and has been reported to be used as the sole source of carbon and energy under aerobic conditions (Hartmans, S., 1992).

Anaerobic dechlorination can be governed either by oxidative or reductive reactions, where in the first reaction the chlorinated compound is used as an electron donor (the electron acceptor is different from oxygen), while in the second reaction the chlorinated compound is used as an electron acceptor. Some CAHs have an oxidizing character due to the strong electro-negativity of chlorine atoms, making them more readily used as electron acceptors and therefore more prone to anaerobic reductive dechlorinating processes. A well-known CAH example which is more likely to undergo such process is tetrachloroethene (PCE) (Maymó-Gatell, X., 1999).

Most anaerobic dechlorinating reactions fit into four categories (Figure 1.3): the first two are substitution reactions (not involving redox processes); the latter two are redox reactions, each involving two electrons. In the first reaction, water or another nucleophilic agent substitutes a chlorine atom, releasing chloride. In the dehydrohalogenation reaction hydrogen and a chlorine atom are removed simultaneously, resulting in a less chlorinated compound and in a double bond. In the third reaction, dihaloelimination, two chlorine atoms are removed and reduced, and a double bond is formed. In reductive dechlorination, a chlorine atom is reductively replaced by hydrogen.
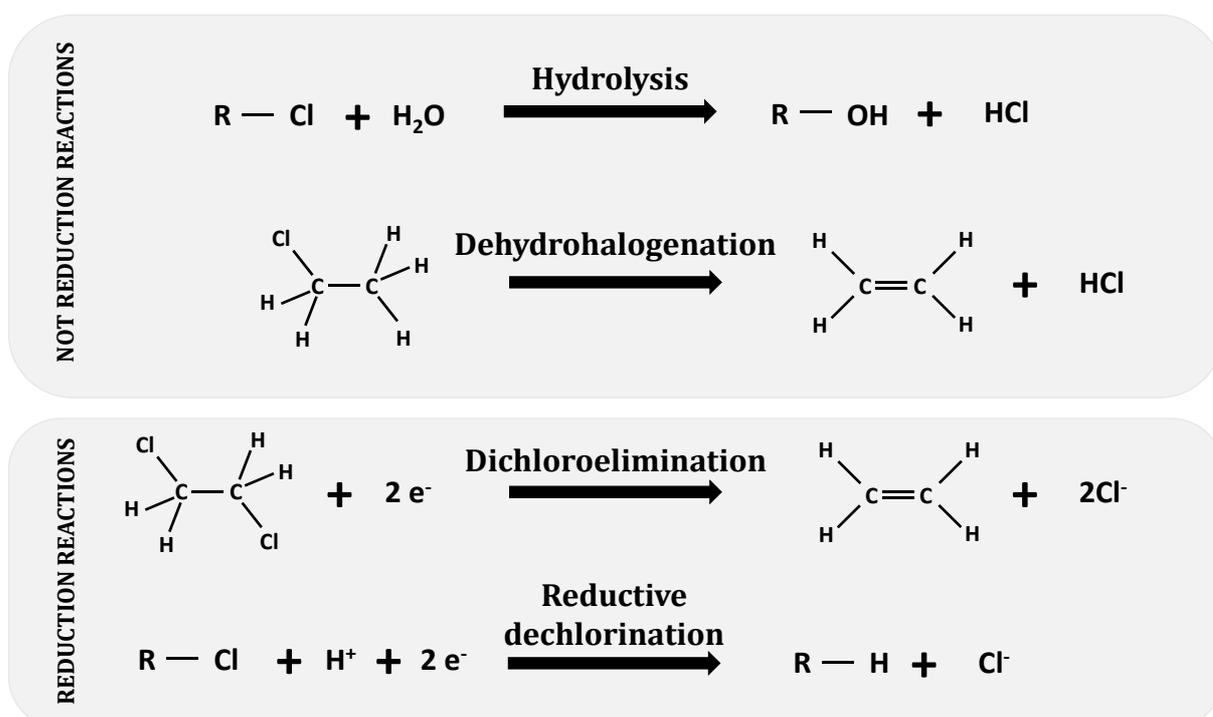
Figure 1.3 – Anaerobic transformation reactions of chlorinated aliphatic hydrocarbons (Adapted from De Wildeman, S., 2003).

Yet another form by which CAH can be degraded is a fascinating feature displayed by microorganisms a process called co-metabolism (Wackett, L., 1995). Co-metabolism is the process by which microorganisms, while degrading a primary substrate, can also transform other compounds. This reaction happens because enzymes expressed to degrade the primary substrate can fortuitously degrade other coumponds, including CAH. Examples of cometabolism process includes the oxidation of chlorinated solvents by oxygenases, known as cooxidation (Alvarez-Cohen, L., 2001) and the reductive dehalogenation of chlorinated solvents by reduced enzyme cofactors (Gantzer, C., 1991; Krone, U., 1989) under anaerobic conditions.

1.2 Relevance and Motivation

Available remediation techniques for the treatment of CAHs have evolved over the last twenty years from traditional pump-and-treat, air sparging, and soil vapor extraction technologies, to the widespread use of bioremediation technologies.

Bioremediation in particular takes advantage of biotic reactions and can be facilitated by naturally occurring or added microorganisms (bioaugmentation). In anaerobic environments microorganisms use the contaminants as electron acceptors and existing or added substrates as electron donors. These electron donors can be hydrogen or organic electron donors that are slowly fermented into hydrogen and organic acids.

Over the last years several dechlorinating microorganisms have been isolated that can couple CAH reductive dechlorination (RD) to energy conservation and growth, with a wide variety of metabolic requirements, pathways and rates. Among the metabolic requirements are the usage of only one electron donor by some bacterial communities, such as *Dehalobacter* and *Dehalococcoide*s, where others can use several electron donors, such as *Dehalospirillum* and *Desulfitobacterium*.

This thesis focuses on anaerobic microbial chloroethane degradation techniques, more specifically of 1,2-DCA and 1,1,2-TCA. Several reasons have led to this choice. Firstly, they have a high water solubility, 8606 mg/L and 4394 mg/L, respectively, and combined with high half-life in reducing conditions. Secondly, from an engineering point of view, anaerobic aquifers are important reservoirs for these pollutants, due to the low oxygen level of many aquifers and therefore reductive environments (De Wildeman, S., 2003), combined with their high resistance towards reduction reactions, anaerobic processes are imperative to understand and master. Furthermore, several studies have already focused on chlorinated ethenes, e.g. TCE (Aulenta, F., 2009), but no research was

performed regarding chloroethanes using the techniques applied in the present work. Finally, their toxicity and frequent occurrence (e.g. downgradient from the chemical complex in Estarreja, Portugal) further justify the choice of these compounds.

1,2-DCA is a clear, manufactured liquid, and does not occur naturally in the environment. Its annual production is estimated to be 17.5 million tonnes, mostly in the United States, Western Europe and Japan (Field, J., 2004). The most common use of 1,2-DCA is to produce other chemical products such as VC, a precursor to make a variety of plastic and vinyl products. It is also used as a solvent and added to leaded gasoline to remove lead. In the past it was also found in products such as degreasing agents, cleaning solutions and pesticides. When released into water or soil it generally evaporates, but some remains in the soil and can migrate through the ground into water. The environmental half-life of 1,2-DCA is 50 years at temperatures between 10 and 20 °C for abiotic hydrolysis or dehydrohalogenation (Vogel, T., 1987). In Europe, the Water Framework Directive (Directive 2000/60/EC) classified 1,2-DCA as one of the 33 priority pollutants (EU, 2000) and it has been identified similarly by the USEPA, (2015).

Regarding anaerobic dechlorination reactions, the degradation process in focus in this thesis, 1,2-DCA can undergo two different anaerobic dechlorination pathways: via reductive dechlorination or via dichloroelimination (Hunkeler, D., 2002).

Several microorganisms have been recognized to carry out 1,2-DCA catalyzed dehalorespiration: *Dehalococcoides* spp. (Maymo-Gatell, X., 1999; Grostern, A., 2006), *Dehalobacter* spp. (Grostern, A., 2006), *Desulfitobacterium* spp. (De Wildeman, S., 2003) and *Dehalogenimonas spp.* (Dillehay, J., 2014).

1,1,2-TCA, the other CAH focused in this thesis, is a colorless, sweet-smelling liquid. This compound dissolves in water, evaporates easily and boils at a higher temperature than water. It is mainly used as a solvent for chlorinated rubbers, fats, oils, waxes, and resins and as an intermediate in the production of 1,1-DCA. 1,1,2-TCA can also be present as an impurity in other chemicals and may be formed in landfills when 1,1,2,2-Tetrachloroethane (1,1,2,2-TeCA) is broken down. When it is released into the environment, most 1,1,2-TCA ends up in the air, but some may infiltrate in the groundwater. Breakdown in air and groundwater is slow. This chemical has an environmental half-life of 170 years at temperatures between 10 and 20 °C for abiotic hydrolysis or dehydrohalogenation (Vogel, T., 1987). The USEPA has classified 1,1,2-TCA as a Group C, possible human carcinogen (ATSDR, 1989).

Anaerobic dechlorination of 1,1,2-TCA can procede by two pathways: via reductive dechlorination to 1,2-DCA or via dichloroelimination to VC (Hunkeler, D., 2002).

Some examples of known microorganisms that have been shown to use 1,1,2-TCA as a respiratory electron acceptor are *Dehalobacter* spp. (Grostern, A., 2006), *Desulfitobacterium* (Zhao, S. 2015) and *Dehalogenimonas* spp. (Dillehay, J., 2014).

Anaerobic degradation of 1,2 DCA and 1,1,2-TCA can be naturally occurring in groundwater but are severely limited. Remediation techniques are therefore required to enhance this degradation, in particular the microbial processes in anaerobic conditions.

Microbial dechlorination is one of the most promising approaches to *in situ* remediation of groundwater contaminated with chlorinated solvents. This remediation technique requires the supply of electron donors. However there are several limitations to the use of this approach. The needed electron donors, molecular hydrogen (H_2) or H_2-releasing organic substrates, are often used inefficiently since they are added many times over the required stoichiometric amount, in order to ensure complete reduction. As a result, there is the stimulation of unwanted reactions, including the production of methane and the accumulation of fermentation products and biomass. This is caused by the extensive competition for carbon and H_2 by microbial populations (dechlorinators, methanogens, acetogens, nitrate reducers, sulfate reducers, etc). Therefore, manipulating and controlling the microbial communities that carry out these dehalogenating reactions can be difficult. These problems can result in groundwater quality deterioration, possible aquifer clogging because of excessive biomass growth and even explosion hazards through excessive methane production (Aulenta, F., 2006).

A promising alternative to minimize some of these limitations is the combined use of biological and electrical processes to enhance biodegradative processes, especially when they minimize the need for substrate or cosubstrate supply and/or make the remediation process more specific and reliable. Certain microorganisms (often referred to as "electroactive" or "electrochemically-active") are capable of respiring insoluble electron donors or acceptors, including solid-state electrodes in bioelectrochemical systems (BES) (Figure 1.4). This process does not require the addition of electron acceptors or donors which are substituted with the appropriate electrochemical potential. The latter can be tuned at the desired value and it is a more flexible and robust way to drive and control relevant microbial reactions. Since no chemicals need to be added (just electrons) this eliminates the need for transport, storage, dosing, and post-treatment.
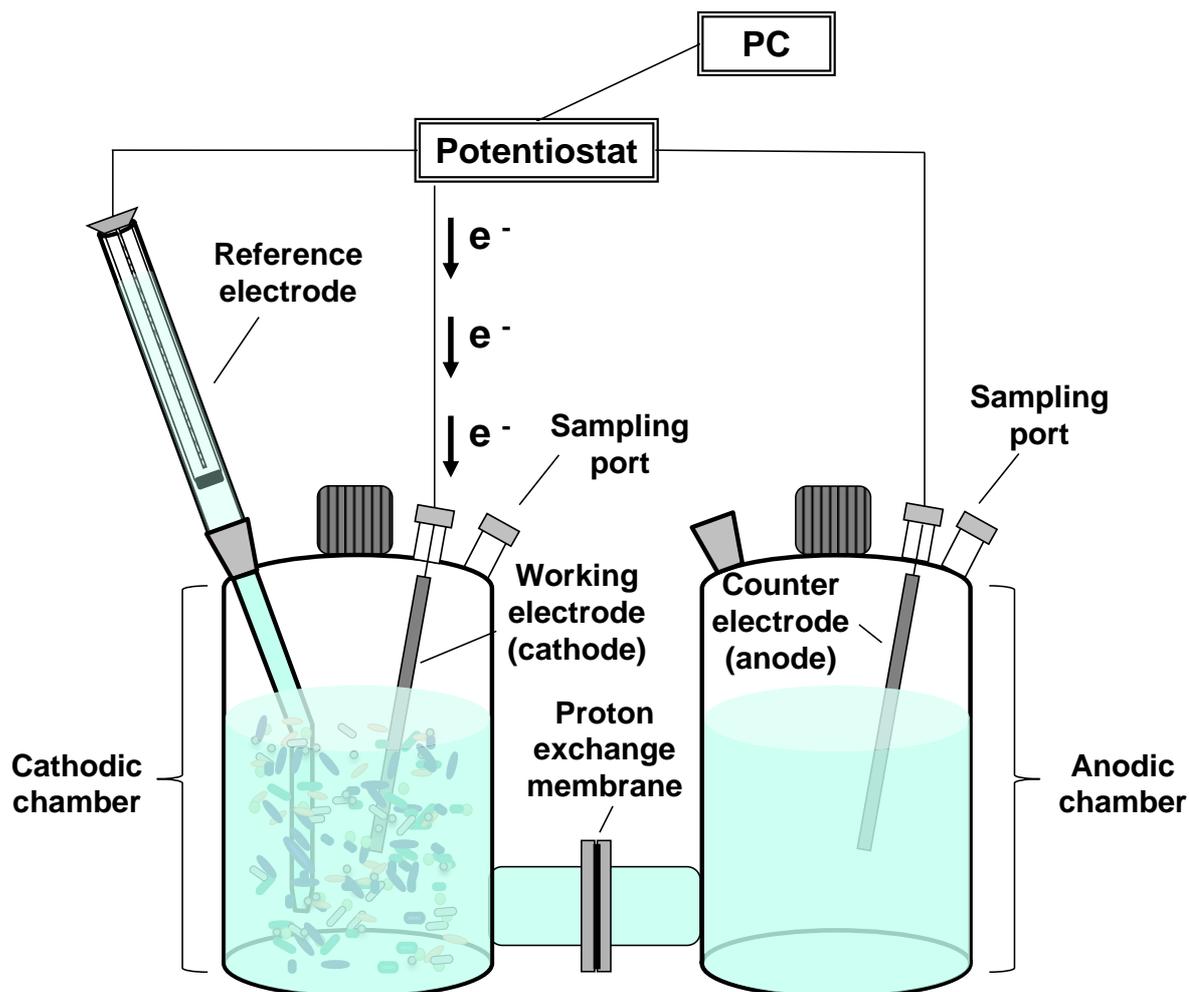
Figure 1.4 – Schematic representation of a bioelectrochemical system.

Several studies have provided evidences that in a BES the cathode can serve as a constant supply of electrons needed for the reductive dechlorination process of chlorinated organics, coupled or complemented with anodic oxidization of lower chlorinated organic compounds for further degradation.

A study performed with the pollutant tetrachloroethene (PCE) suggested the ability of *G. lovleyi* to microbially catalyze its reductive dechlorination to trichloroethene (TCE) in the cathodic chamber of a BES (Strycharz, M, 2008). Research on TCE bioelectrochemical treatment with electrodes serving as electron donors (Aulenta, F. 2009).

To further treat the end products from the reductive dechlorination of chloroethenes, research has been performed into the use of sequential reductive - oxidative bioelectrochemical techniques (Lohner, S., 2009, 2011). Bioanodes can also be employed for the oxidation of chlorinated solvents in BES, where microorganisms catalyze the oxidation of substrates, converting part of the chemical energy available in the substrates into electrical energy. As an example, an anodophilic consortium was capable of

oxidizing 1,2-DCA to CO_2, ethene glycol and acetate, under airtight bioelectrochemical conditions (Pham, H., 2009).

Interactions between microorganisms and electron conductors are crucial processes to take into account, when bioremediation methods in general and microbial electrochemical systems in particular, are used. These interactions can be either capacitive or faradaic processes. The first arises as a consequence of the decrease in electrochemical capacity at the double layer of the electrode. This occurs when a biofilm is attached (or detached) on the electrode, resulting in the movement of water molecules and ions which create a balancing electric current. Faradaic processes, on the other hand, are based on redox reactions between microorganisms and the molecular counterpart with which it can perform extracellular electron transfer (EET) and includes pseudo-capacitive and electrocatalytic processes (Schroder, U., 2015).

EET mechanisms refer to the electron exchange reactions performed by microorganisms, which can be divided as direct or indirect (Rosenbaum, M., 2011). Direct extracellular electron transfer describes the electron mechanisms between the electrode and a microorganism at close proximity. This electron transfer mechanism is by direct contact using transmembrane redox-proteins such as cytochromes (Bond, D., 2012), or pili (Reguera, G., 2005). Indirect electron transfer mechanisms are the processes by which microorganisms perform the electron chain facilitated by molecular compounds, which serve as conduits. These conduits can be self-produced substances [e.g. flavins (Marsili, E., 2008)], extracellular polymeric substances (EPS) of biofilms (Cao, B., 2011), substances present in the environment [e.g. conductive minerals (Gacitúa, M., 2014)] or added substances [e.g. humic acid analogs (Aulenta, F., 2010), methyl viologen (Steinbusch, K., 2010), neutral red (Park, D., 1999)]. The distance of microbial EET can vary between nanometers to more than a centimeter (Shi, L., 2016). EET can also occur between microorganisms, via direct or indirect mechanisms (Thrash, J., 2008).

Nevertheless, the interaction between dechlorinating communities, soil and aquifer geochemical components remains largely unknown. Although electroactive microorganisms have found applications as electrocatalysts in a number of different bioelectrochemical processes, from bio-energy generation to groundwater bioremediation, the ecological and evolutionary bases of EET remain poorly elucidated, and practical strategies for boosting EET are only marginally explored. Recent studies have suggested that, in sedimentary environments, microorganisms with EET capabilities may take advantage of the electric currents running through conductive minerals, which connect spatially segregated bio-geochemical redox processes (Nielsen, L., 2010). Redox-active minerals, such as those that

contain iron and manganese, are abundant in soils and in aquatic and subsurface sediments, in which they can electrically support microbial growth.

Enhancement of electron transfer by redox mediators, with different structures and properties, on the reductive dechlorination of different chlorinated solvents has been tested during the last two decades. Redox mediators are organic molecules that can reversibly be oxidized and reduced, conferring the capacity to serve as electron carriers in multiple redox reactions. These compounds accelerate reactions by lowering the activation energy of the total reaction. For instance, the humic acid analogue Anthraquinone-2,6-disulfonate (AQDS), has been previously reported to enhance TCE bioelectrochemical remediation (Aulenta, F., 2010), as well as the reductive decolorization of azo dyes (Cervantes, F., 2010).

The key players in anaerobic bioremediation process are the microorganisms capable of performing dehalorespiration. Several molecular methods are employed for the identification and quantification of active dechlorinating bacteria or target specific genes (Illman, W., 2009). The most common for field conditions are Polymerase Chain Reaction, quantitative Polymerase Chain Reaction and Phospholipids fatty acids analysis (Beller, H., 2002; Van Raemdonck, A., 2006; Cupples, A., 2008). Fluorescence *In Situ* Hybridization (FISH) and Catalyzed Reported Deposition Fluorescence *In Situ* Hybridization (CARD-FISH) techniques are also increasingly been used at lab scale conditions due to low cost and ease to use (Matturro, B., 2012, 2013a, 2013b).

The FISH technique relies on a labeled DNA or RNA sequence which is used as a probe to identify or quantify the naturally occurring counterpart of the sequence in a biological sample. However a disadvantage of applying *in situ* hybridization techniques is the difficulty in identifying targets that have low DNA and RNA copies.

To overcome this limitation researchers have improved FISH to allow for signal amplification, using catalyzed reporter deposition (CARD) (Kerstens, H., 1995). CARD is easy, quick, extremely sensitive, and efficient, making it a good option for detecting low copies of nucleic acids and antigens *in situ*. CARD-FISH is a powerful methodology with a growing number of applications for the quantitative evaluation of microbial populations of complex ecosystems.

Another FISH technique that has been increasingly applied in the food and health areas is the Peptide Nucleic Acid (PNA) probes. In PNA-FISH the backbone of the synthetic probe is neutrally charged, leading to more affinity for duplexes with DNA and RNA than

traditional DNA probes (Armitage, B., 2003). As a result, shorter probes can be synthetized, which can more easily diffuse through the microbial cell wall and hence increase the robustness of the FISH method.

1.3 Objectives

The main goal of this thesis is to remediate chlorinated ethanes using anaerobic biodegradation, either in bioelectrochemical systems or with conductive mineral enhancement, in order to evaluate the feasibility and efficiency of these techniques. This class of chemicals was selected due to their toxicity as well as their widespread use and contamination (including in Portugal). Additionally, and despite these facts, research on the remediation of these chemicals is scarce.

The specific research objectives are:

1) Enrichment and identification of a stable electro-active microbial cultures able to completely dechlorinate the target contaminants, using polarized electrodes as insoluble electron donors.

2) Evaluation of the impact of added redox mediators on the reductive dechlorination kinetics in bioelectrochemical systems.

3) Development of an easily reproducible and straightforward procedure to immobilize redox mediators on the electrode in order to decrease time and costs of chlorinated ethanes remediation.

4) Assessment of the potential use of conductive minerals to enhance reductive dechlorination kinetics in microcosms amended with organic electron donors.

5) Application of a novel biomolecular detection technology (PNA-FISH) to distinguish between two strains of a key chlorinated ethane degrading microorganism (*Dehalococcoides* spp.).

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2 Bioelectrochemically - assisted reductive dechlorination of 1,2-dichloroethane by a *Dehalococcoides* - enriched microbial culture

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2.1 Highlights

- 1,2-Dichloroethane is dechlorinated to ethene with an electrode as electron donor.

- 1,2-Dichloroethane dechlorination rate and yield depend on the set cathode potential.

- *Dehalococcoides* is responsible for H_2-mediated bioelectrochemical dechlorination.

2.1.1 Keywords

Bioelectrochemical dechlorination; 1,2-Dichloroethane; Reductive Dechlorination; Microbial biocathode.

2.2 Graphical abstract
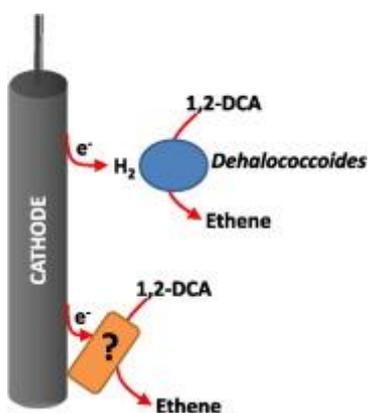
2.3 Abstract

The aim of this study was to verify the possibility to use a polarized graphite electrode as an electron donor for the reductive dechlorination of 1,2-dichloroethane, an ubiquitous groundwater contaminant. The rate of 1,2-DCA dechlorination almost linearly increased by decreasing the set cathode potential over a broad range of set cathode potentials (i.e., from -300 mV to -900 mV vs. the SHE). This process was primarily dependent on electrolytic H_2 generation. On the other hand, reductive dechlorination proceeded (although quite slowly) with a very high Coulombic efficiency (near 70 %) at a set cathode potential of -300 mV, where no H_2 production occurred. Under this condition, reductive dechlorination was likely driven by direct electron uptake from the surface of the polarized electrode. Taken as a whole, this study further extends the range of chlorinated contaminants which can be treated with bioelectrochemical systems.

2.4 Introduction

The chlorinated aliphatic hydrocarbon 1,2-dichloroethane (1,2-DCA) is extensively used in the production of vinyl chloride, a precursor in the chemical synthesis of polyvinylchloride, and has also been used as a solvent and degreasing agent in a variety of industries (van der Zaan, B., 2009). Due to improper storage, handling, and disposal practices, 1,2-DCA has become one of the most common soil and groundwater pollutants and has been identified as one of the 33 priority pollutants by the European Water

Framework Directive and by the USEPA (Majone, M., 2015). Concentrations of 1,2-DCA in groundwater typically vary in the range of 0.1–100 mg/L, with a maximum solubility in water of approximately 8000 mg/L (Klecka, M., 1998). The presence of 1,2-DCA in the environment poses serious environmental concerns, particularly due to the fact that it is a suspected carcinogen and is highly persistent under anaerobic conditions, with an estimated half-life of more than 50 years (Premaratne, S., 1995; Vogel, T., 1987).

Bioremediation, via anaerobic reductive dechlorination, is one of the most promising approaches for the treatment of groundwater contaminated with 1,2-DCA (Arjoon, A., 2013; Baric, M., 2014; De Wildeman, S., 2003, 2002; Grostern, A., 2006; Hirschorn, S., 2007; Klecka, M., 1998; Marzorati, M., 2010). This approach typically requires the subsurface injection of fermentable substrates that serve as electron donors to stimulate autochthonous microbial populations using 1,2-DCA as a respiratory electron acceptor (Duhamel, M., 2007). Typically, the used electron donors are commercial products such as soluble molasses or other fermentable (i.e., H_2-releasing) organic compounds. However, this approach suffers of a number of drawbacks, including the inefficient use of the added electron donors, stimulation of unwanted side reactions, and in general, poor control over reaction conditions (Aulenta, F., 2007). Furthermore, in order to achieve remediation goals, a long period of time and multiple electron donor additions are frequently needed.

In order to overcome some of these limitations, the use of polarized graphite electrodes as the sole electron donors for the microbial reductive dechlorination (RD) processes has been recently proposed (Aulenta, F., 2009; Aulenta, F., 2007; Aulenta, F., 2010; Aulenta, F., 2011; Strycharz, S., 2010, 2008). However, no attempts have been made so far to apply a bioelectrochemical approach to promote the reductive transformation of 1,2-DCA. Hence, in order to further extend the field of applicability of this novel remediation approach, this study explored the possibility to stimulate the microbial RD of 1,2-DCA with a graphite electrode (cathode) serving as the sole electron donor. Attention was given to examining the influence of the set cathode potential on the rate and efficiency of 1,2-DCA dechlorination, as well as on the electron transfer mechanisms possibly involved in the biological process. Molecular analyses of the involved microbial communities (both planktonic and attached onto the electrode surface) were also carried out in order to shed light on the identity of the microorganisms responsible for the electricity-driven dechlorination of 1,2-DCA.

2.5 Methods

2.5.1 Bioelectrochemical setup

The bioelectrochemical cell setup used in this study consisted of two gastight borosilicate glass bottles (each having a total volume of 270 mL and working volume of 150 mL) separated by a 3-cm^2 cross-sectional area Nafion^{TM} 117 proton exchange membrane (PEM). Prior to its use, the PEM was boiled successively in four separate solutions for 2 h each: H_2O_2 (3 % v/v), deionized (DI) water, 0.5 M H_2SO_4 and DI water, and then finally stored in DI water until use. The cathode and anode were graphite rods (6 mm diameter, Sigma Aldrich, Milano, Italy). The nominal surface area of the cathode (calculated by taking into account only the part of the electrode that was immersed in the liquid phase) was 9.7 cm^2. The distance between the anode and the cathode was approximately 10 cm. Prior to their use, the graphite electrodes were pretreated as described elsewhere (Gregory, K., 2004). A reference electrode (KCl-saturated Ag/AgCl, +199 mV vs. the standard hydrogen electrode (SHE); Amel S.r.l., Milano, Italy) was placed in the cathodic chamber. The catholyte and anolyte consisted of anaerobic medium as described below. Throughout the manuscript, all potentials are reported with respect to SHE.

At the beginning of the study, the cathode compartment of the cell was inoculated with approximately 100 mL of a dechlorinating culture, previously enriched in the laboratory with 1,2-DCA as electron acceptor and H_2 as electron donor. Briefly, the culture was maintained in an anaerobic serum bottle (total volume 120 mL) that was sealed with a Teflon-faced butyl rubber stopper and aluminium crimp seal, and covered with aluminium foil to prevent growth of phototrophic organisms. The serum bottle was operated in a fill-and-draw mode, receiving a weekly dose of 1,2-DCA (0.5 mmol) and H_2 (3.75 mmol). Before each refeeding, the culture was purged with a N_2/CO_2 gas mixture to remove all volatile compounds. On average, 22.5 mL of suspended culture was removed weekly and replaced with fresh medium. The average cell retention time was 25–30 days. The temperature was maintained at 25 °C and the pH at 7.2–7.5. This 1,2-DCA dechlorinating culture was maintained for over 80 days before being inoculated in the bioelectrochemical cell.

2.5.2 Bioelectrochemical experiments

The bioelectrochemical system (BES) experiments were carried out over a period of 280 days, during which six different cathode potentials were evaluated, namely OCP, -300 mV, -500 mV, -600 mV, -700 mV and -900 mV. Throughout the experimental period, the

BES was operated in a fill-and-draw mode. Briefly, every week the cathode and anode compartments of the BES were purged (30 min) with a N_2/CO_2 (70:30 % v/v) gas mixture to remove volatile compounds, then a fixed liquid volume was removed from each compartment and replaced with fresh anaerobic medium, in order to maintain an average cell retention time of 68 days. Thereafter, the cathode compartment was spiked with 1,2-DCA at a nominal concentration of 0.5 mmol/L. The pH of the solution was maintained at 7.2–7.5. The BES was incubated at 25 °C (via a water bath), in the dark and received constant magnetic stirring. The bioelectrochemical cell was connected to an IVIUM-n-STAT multichannel electrochemical analyzer (Ivium Technologies, The Netherlands) which allowed setting the cathode potential at the desired value and recording the resulting electric current. Electrochemical data were processed using the Ivium Soft® software package.

The cumulative electric charge (expressed as electron equivalents, $(e - eq_i)$) that was transferred at the electrodes was calculated by integrating the current (i) over the period of electrode polarization. Cumulative reducing equivalents ($e - eq_{RD}$) that were used for the reductive dechlorination of 1,2-DCA were calculated from the measured amounts of ethene, considering the corresponding molar conversion factor of $2e - eq$/mol The Coulombic Efficiency (CE) for the reductive dechlorination process was calculated as follows:

$$CE\ (\%) = \frac{(e - eq_{RD})}{(e - eq_i)} \times 100$$

To quantify the extent of purely electrolytic hydrogen evolution at the cathode potentials investigated in this work, abiotic BES experiments were also performed. These experiments were carried out under conditions identical to those reported above, with the only exception being the lack of a microbial culture in the cathode compartment.

2.5.3 Medium

The medium contained the following components: NH_4Cl (0.5 g/L), $MgCl_2.6H_2O$ (0.1 g/L), K_2HPO_4 (0.4 g/L), $CaCl_2.2H_2O$ (0.05 g/L), trace metal solution (10 mL/L) (Zeikus, J., 1977), vitamin solution (10 mL/L) (Balch, W., 1979), and $NaHCO_3$ (15 mL/L, 10 % w/v). All solutions were purged for at least 0.5 h with a N_2/CO_2 (70:30 % v/v) gas mixture before use. The pH value of the medium was 7.5.

2.5.4 Microbiological analysis

Liquid samples from the source culture (i.e., the inoculum) and from the cathode compartment of the bioelectrochemical cell, as well as biofilm samples from the graphite cathode, were taken for microbiological analysis. Samples from the bioelectrochemical cell were taken at the end of the study, after the cell had been operated for 3 successive cycles at -300 mV (vs. SHE).

Liquid samples (0.45 mL) were immediately fixed with formaldehyde (2 % v/v final concentration), filtered through 0.2 mm polycarbonate filters (Ø 47 mm, Millipore) by gentle vacuum (<0.2 bar) and stored at -20 ºC until use.

To fix the biomass from the biofilm formed at the electrode, the surface of the electrode was carefully scraped with a sterile spatula. The detached biomass was initially collected in a solution composed of 45 mL of PBS buffer and 5 mL of 37 % (w/v) formaldehyde solution, and then filtered as described above.

Catalyzed Reporter Deposition-Fluorescence *In Situ* Hybridization (CARD-FISH), carried out following previously published protocols (Di Battista, A., 2012; Fazi, S., 2008), was applied on the filtered samples to characterize their microbial composition. Specifically, CARD-FISH oligonucleotide probes targeting *Bacteria* (EUB338mix probes) and *Archaea* (ARC195 probe), were employed, as well as genus-specific probes targeting known dechlorinating bacteria such as *Dehalococcoides mccartyi* (Dhe1259c and Dhe1259t probes), *Dehalobacter* spp. (DHB643 probe), and *Sulfurospirillum* spp. (SULF220ab probe). The probes, labeled at the 50-end with HRP, were purchased from BIOMERS (http://www.biomers.net). Details of the oligonucleotide probes used are available at http://www.microbial-ecology.net/probebase. Oligonucleotide probes were always added together with 4',6-diamidino-2-phenylindole (DAPI) for the quantification of total cells. Slides were examined by epifluorescence microscopy (Olympus, BX51) and the images were captured with an Olympus F-View CCD camera and processed and analyzed with AnalySIS software (SIS, Germany).

2.5.5 Analytical methods

Volatile components, namely 1,2-DCA, vinyl chloride (VC), ethene (ETH), ethane (ETA) and methane (CH_4) were quantified by injecting 50 µL of headspace (taken with a gas-tight syringe) into a Perkin–Elmer GC 8500 gas chromatograph (2 m x 2 mm glass column packed with 60/80 mesh Carbopak B/1 % SP-1000 Supelco; N_2 carrier gas 18

mL/min; oven temperature 190 ^{0}C; flame ionization detector temperature 250 ^{0}C). H$_2$ was analyzed by injecting 50 μL of headspace with a gas-tight syringe in a PerkinElmer Auto System GC (4.6 m x 2.1 mm stainless steel column packed with 60/80 Carboxen-1000 support Supelco; N$_2$ carrier gas 40 mL/min; oven temperature 225 ^{0}C; thermal conductivity detector temperature 250 ^{0}C). Headspace concentrations were converted to aqueous-phase concentrations using tabulated Henry's law constants (Chen et al., 2012).

2.5.6 Chemicals

1,2-DCA (99.8 + %), hydrogen (99.5 + %) and all the other chemicals were purchased from Sigma–Aldrich (Milano, Italy, except where indicated differently). The chemicals used to prepare the mineral medium were of analytical grade and used as received.

2.6 Results and discussion

2.6.1 Bioelectrochemical reductive dechlorination of 1,2-DCA

During each feeding cycle, the time course of the 1,2-DCA dechlorination and competing reactions (i.e., methanogenesis) were monitored through daily analyses of headspace samples of the cathode compartment of the bioelectrochemical cell. Regardless the set cathode potential, the removed 1,2-DCA was almost stoichiometrically dechlorinated to ETH via dichloroelimination, with only low amounts of harmful VC (deriving from dehydrochlorination of 1,2-DCA) accumulating over the course of the cycle. Low levels of CH$_4$ were also occasionally detected.

As an example, Fig. 2.1 presents the results of a typical feeding cycle, with the cathode potential potentiostatically fixed at -700 mV. The added 1,2-DCA was reductively dechlorinated at a rate of 40 μeq/L d. At the end of the cycle, the removed 1,2-DCA (181 μM, corresponding to 40 % of the initial dose) was almost completely converted into ETH (140 μM, corresponding to 77 % of the removed 1,2-DCA) and a very low amount of VC (0.11 μM, corresponding to about 0.05 % of the removed 1,2-DCA) was detected. Notably, the observed pathway of 1,2-DCA dechlorination and relative distribution of dechlorination products was very similar to the one observed in the source culture which was used as inoculum of the bioelectrochemical cell, where H$_2$ was supplied as electron donor (data not shown).
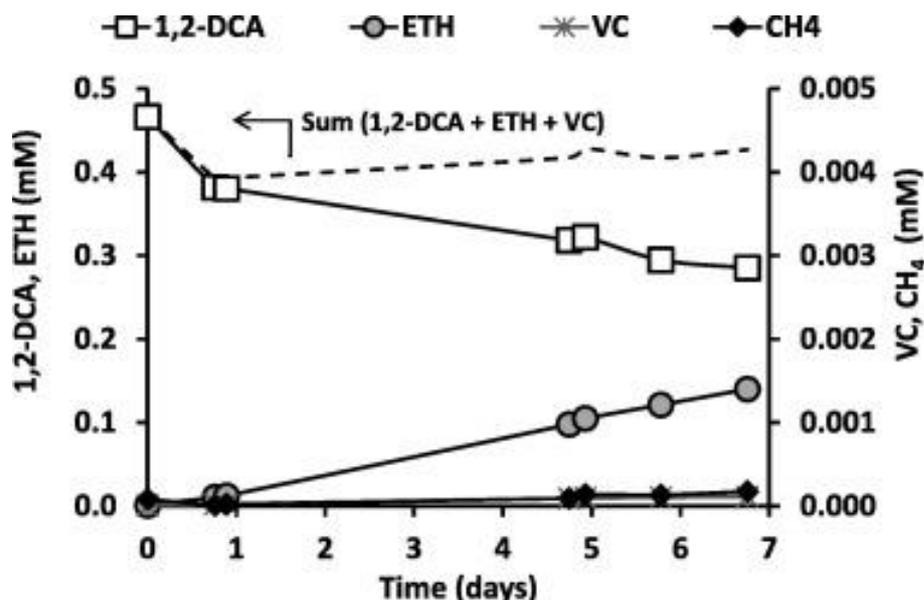
Figure 2.1 - Time course of bioelectrochemical 1,2-DCA dechlorination and methane formation during a typical 7-day feeding cycle, with the cathode potentiostatically set at -700 mV.

2.6.2 Effect of cathode potential on the rate and yield of 1,2-DCA dechlorination

Throughout an experimental period of 23 weeks, the bioelectrochemical cell was operated at different cathode potentials, randomly changed (and replicated) in the range from -300 mV to -900 mV. Open circuit control experiments were also carried out to quantify the background reductive dechlorination activity of the mixed microbial culture in the bioelectrochemical cell.

The rate of 1,2-DCA dechlorination was found to generally increase (from 10 ± 4 µeq/L d, up to 37 ± 10 µeq/L d) with the decrease of the set cathode potential from -300 mV to -900 mV (Fig. 2.2). In open circuit tests, the reductive dechlorination proceeded slowly (4 ± 3 µeq/L d) and was most likely driven by electrons deriving from endogenous cell metabolism or from reduced metabolites released into the medium during feeding cycles with the cathode set at more reducing values.
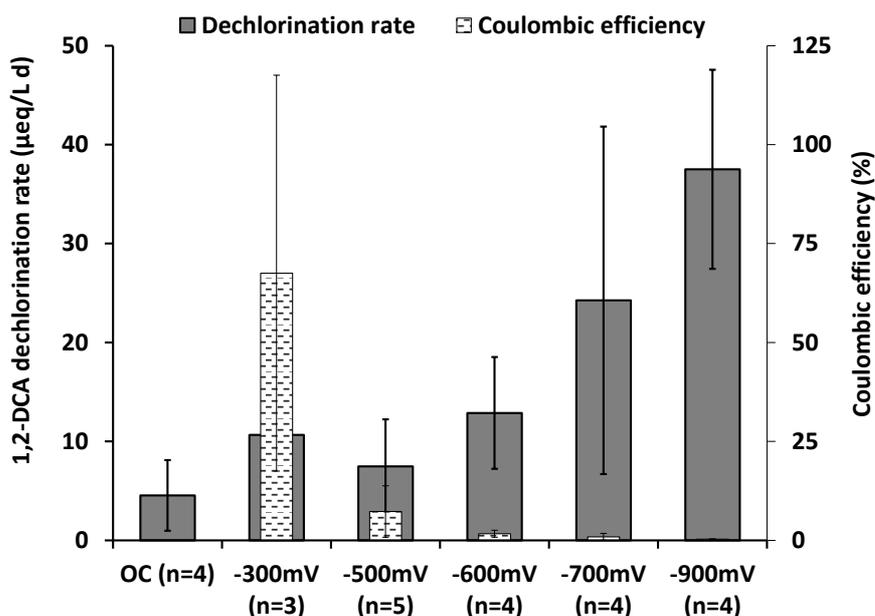
Figure 2.2 - Effect of the cathode potential on the rate of 1,2-DCA dechlorination and the Coulombic efficiency (data were corrected to account for the reductive dechlorination measured in an Open Circuit Control). For each experimental condition, the number of replicated batch experiments (each consisting of a 7-day feeding cycle) is indicated.

Coulombic Efficiency (CE), i.e. the yield of current utilization for the reductive dechlorination process, was calculated at the end of each feeding cycle (Fig. 2.2). The highest value (68 ± 50 %, corrected to account for the reductive dechlorination observed in open circuit controls) was observed when the cathode was fixed at the less reducing value (-300 mV). Substantially lower and decreasing values were observed as the cathode was set at lower potentials. For example, the CE was 7 ± 6 % at -500 mV and lower than 2 % for cathode potentials lower than -600 mV.

Since molecular H_2 is known to be a key electron donor in the microbial reductive dechlorination of 1,2-DCA, batch tests were carried out with abiotic (i.e., non-inoculated) cells to quantify the effect of the cathode potential on the purely electrolytic H_2 production.

Consistent with the results of previous studies (Villano, M., 2011), production of H_2 with the graphite electrode was observed only at cathode potentials lower than -500 mV, whereas H_2 remained below instrumental detection limits during the course of the tests carried out at -300 mV. As expected, the rate of abiotic H_2 production increased almost exponentially (from 0.19 ± 0.02 meq/L d to 13.5 ± 3.0 meq/L d) when the cathode potential was decreased from -500 mV to -900 mV. Notably, an almost linear correlation was found between the rate of dechlorination and the rate of abiotic H_2 production, pointing to a direct role of electrolytically-generated H_2 in the electricity-driven and microbially catalyzed reductive dechlorination process (Fig. 2.3). On the other hand, the

fact that the rate of 1,2-DCA dechlorination was substantially higher at -300 mV than that observed in OCP experiments and proceeded with an extremely high CE strongly suggests that it was driven by direct electron transfer, without the intermediate production of H_2.
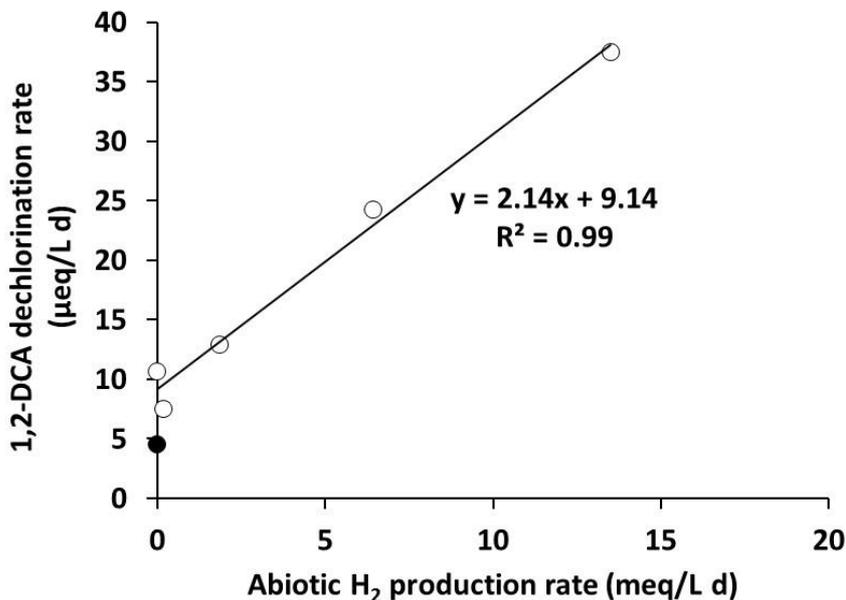
Figure 2.3 - Correlation between the rate of 1,2-DCA dechlorination and abiotic H_2 production.

Overall, the herein reported results compare favorably with those obtained in previous study (Aulenta, F., 2011) whereby the effect of the cathode potential (in the range from -250 mV to -750 mV) was analyzed with reference to TCE dechlorination, using a continuous-flow bioelectrochemical reactor. Also in that case, the rate of dechlorination increased by decreasing the cathode potential (and in turn increasing the H_2 availability), while the highest Coulombic efficiency (almost 100 %) was obtained when the cathode was operated at the highest potential (i.e., -250 mV) when the dechlorination was most likely driven by direct electron transfer rather than by H_2. Collectively the results of this and the previous study lead to a trade-off between rate and efficiency, suggesting the existence of an optimal cathode potential range which satisfies both.

2.6.3 Microbiological characterization

In order to gain insights on the identity of the microorganisms responsible for the bioelectrochemical reductive dechlorination process, at the end of the study the microbial communities in the cathode compartment of the BES (planktonic state and attached to the cathode surface) were analyzed by CARD-FISH. For comparative purposes, the H_2-fed suspended culture that was used as the inoculum for the bioelectrochemical cell was also analyzed.

In the inoculum, *Bacteria* and *Archaea* accounted for 63 ± 14 % and 5 ± 3 % of DAPI-stained cells, respectively (Fig. 2.4a). Notably, almost 90 % of *Bacteria* could be identified as *Dehalococcoides*, using genus-specific CARD-FISH probes (Fig. 2.4b). The predominance of *Dehalococcoides* is consistent with its known ability to metabolically dechlorinate 1,2-DCA to ethene (and traces of VC) using H_2 as the primary electron donor (Maymo-Gatell et al., 1999; Schmidt et al., 2014; Tandoi et al., 1994). Other known dechlorinating microorganisms such as *Dehalobacter* and *Sulfurospirillum* together accounted for less than 2 % of total Bacteria, suggesting a marginal contribution to the observed dechlorinating activity.
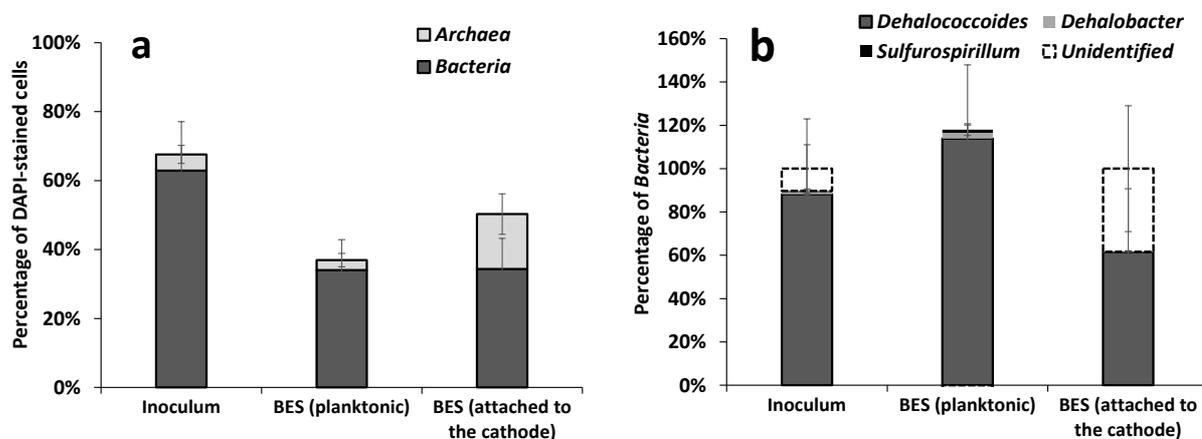
Figure 2.4 - CARD-FISH analysis of the microbial communities in the inoculum, in the bulk liquid of the BES cathode chamber, and on the surface of the graphite cathode. (a) Percentage of *Bacteria* and *Archaea*, relative to the total DAPI-stained cells. (b) Percentage of *Dehalococcoides*, *Sulfurospirillum*, and *Dehalobacter* relative to the total Bacteria.

In the BES, both planktonic and attached cells also primarily consisted of *Bacteria*. A slightly higher percentage of *Archaea* (relative to total DAPI-stained cells) was observed in the microbial community attached onto the cathode compared to planktonic cells (16 ± 6 % vs. 3 ± 2 %). This is possibly due to the fact that H_2 was produced at the electrode or that attached *Archaea* were protected more against the inhibitory effects of chlorinated compounds compared to cells suspended in the bulk liquid.

Remarkably, *Dehalococcoides* accounted for virtually all planktonic bacterial cells in the BES cathode chamber, whereas a substantial portion (40 %) of bacterial cells collected from the electrode remained unidentified, since they did not bind with any of the tested genus-specific, CARD-FISH probes. A previous study (Di Battista, A., 2012) showed that the presence of Dehalococcoides in bioelectrochemical dechlorinating systems is linked to the capacity of this microorganism to thrive on electrolytically produced H_2. This agrees with the observed predominance of this microorganism in the bulk liquid of the BES in the system in this study. On the other hand, the occurrence of other as-yet-unidentified

Bacteria at the surface of the cathode could be linked to their capacity of direct electron uptake from the surface of the polarized electrode.

2.7 Conclusions

This study demonstrates that graphite electrodes could be used to stimulate the microbially catalyzed reductive dechlorination of 1,2-DCA to ethene, thereby further broadening the range of toxic chlorinated subsurface contaminants which can be treated with BES technology. The set cathode potential turned out to be a key parameter affecting the rate and yield of the reductive dechlorination process.

In conclusion, this study provides further evidence that bioelectrochemical systems represent a versatile and tunable technology to drive the biodegradation of a range of subsurface contaminants.

2.8 Acknowledgements

2.9 References

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3 Enrichment of *Dehalococcoides mccartyi* spp. from a municipal activated sludge during AQDS-mediated bioelectrochemical dechlorination of 1,2-dichloroethane to ethene

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3.1 Highlights

- 1,2-DCA can be treated in potentiostatically controlled bioelectrochemical systems.

- AQDS drives stoichiometric dechlorination of 1,2-DCA to ethene.

- *Dehalococcoides mccartyi* enriched during the bioelectrochemical dechlorination.

- Results provide new insights into the biogeochemical fate of 1,2-DCA in humics-rich aquifers.

3.1.1 Keywords

Activated sludge; 1,2-Dichloroethane; Bioelectrochemical systems; Redox mediator Anthraquinone-2,6-disulfonate (AQDS); *Dehalococcoides mccartyi*.

3.2 Graphical abstract
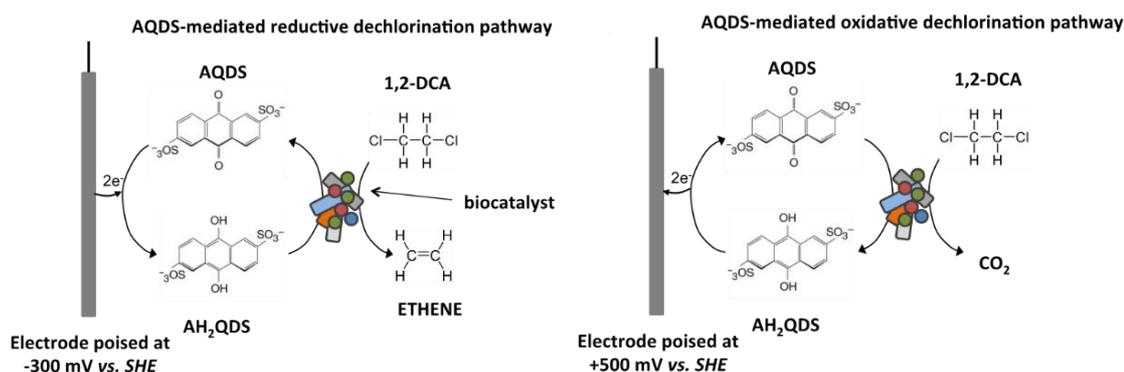
3.3 Abstract

The application of bioelectrochemical systems (BES) for the treatment of chloroethanes has been so far limited, in spite of the high frequency that these contaminants are detected at contaminated sites. This work studied the biodegradation of 1,2-dichloroethane (1,2-DCA) in a lab-scale BES, inoculated with a municipal activated sludge and operated under a range of conditions, spanning from oxidative to reductive, both in the presence and in the absence of the humic acid analogue anthraquinone-2,6-disulfonate (AQDS) as a redox mediator. The results showed stable dechlorination of 1,2-DCA to ethene (up to 65 ± 5 µmol/L d), when the BES was operated at a set potential of -300 mV vs. SHE, in the presence of AQDS. Sustained filled-and-draw operation resulted in the enrichment of *Dehalococcoides mccartyi*. The results of this work provide new insights into the applicability of BES for groundwater remediation and the potential interaction between biogeochemistry and 1,2-DCA in humics-rich contaminated aquifers.

3.4 Introduction

Clorinated aliphatic hydrocarbons (CAH), such as trichloroethene (TCE), 1,2-dichloroethane (1,2-DCA), and 1,1,2-trichloroethane (1,1,2-TCA) are widely used as degreasing agents, industrial solvents and as precursors in the manufacture of many valuable chemicals (Doherty, R., 2000; Moran, M., 2007). Improper use, handling, and disposal practices have lead to numerous accidental releases of these compounds into the environment. As such, CAH are nowadays among the most common groundwater

contaminants, particularly in industrialized countries. The majority of CAH are extremely toxic (some are known or suspected carcinogens) and therefore their presence in the environment poses significant health risks (McCarty, P., 2010).

Enhanced in situ bioremediation is regarded as a sustainable and cost-effective alternative to the pump & treat method for the treatment of CAH-contaminated aquifers (Aulenta, F., 2006a; Majone, M., 2015). This remediation option typically involves the manipulation of environmental conditions in order to stimulate autochthonous microorganisms to degrade the chlorinated contaminants. This can be achieved through the addition of nutrients, electron donors or acceptors, and/or through pH, redox and temperature control (Boopathy, R., 2000; Juwarkar, A., 2010). Although the viability of this approach has been extensively demonstrated in the literature, it typically suffers from a number of limitations such as the poor control over biodegradation kinetics and efficiency, the high costs associated with the supply of substrates (e.g., electron donors or acceptors), competition between different microbial groups for substrates, and finally the uncontrolled accumulation of undesired (often harmful) end-products (Aulenta, F., 2007).

In recent years, BES have emerged as a novel technology for the oxidative or reductive degradation of a variety of environmental contaminants, including CAH (Aulenta, F., 2013, 2011, 2008; Leitão, P., 2015; Pous, N., 2015; Sleutels, T., 2012; Venkidusamy, K., 2016; Viggi, C., 2015; Wang, H., 2013). The underlying principle behind the application of BES is that carbon electrodes suitably positioned within the contaminated aquifer can stimulate biodegradation processes by serving as direct (or indirect) electron acceptors or donors in microbial metabolism. The main advantage of BES over conventional (*in situ*) bioremediation processes is that the electron flow associated with contaminant degradation (oxidation or reduction), or with the supply of electron donors or acceptors, can be easily monitored and fine-tuned, thereby providing a unique tool for controlling and manipulating the rate and yield of the biodegradation processes. Furthermore, compared to traditional bioremediation processes, BES allows co-localizing the electron donor, the degrading bacteria and the contaminants, which typically tend to adsorb on the carbon-based electrodes used as electron donors or acceptors.

So far, numerous lab-scale studies have provided convincing evidence of the efficacy of bioelectrochemical remediation technologies, although field-scale applications remain extremely limited.

As far as CAH are concerned, a number of studies have clearly demonstrated that chloroethenes, such as TCE, can be dechlorinated at the microbial biocathode of a BES,

which resulted in the formation of lower chlorinated (or eventually non-chlorinated) end products, such as cis-dichloroethene (cis-DCE), vinyl chloride (VC), or ethene (ETH) (Aulenta, F., 2011; Di Battista, A., 2012). Typically, when the biocathode is polarized at potentials lower (more reducing) than -550 mV vs. the SHE, the reductive dechlorination (RD) process proceeds at high rates and is primarily driven by abiotically generated H_2 (Aulenta, F., 2011, 2008). Due to the simultaneous occurrence of hydrogenophilic methanogenesis, the efficiency of H_2 utilization in the RD process is, however, extremely low (<1 %). Conversely, when the microbial biocathode is potentiostatically controlled at potential values higher than -350 mV (vs. SHE), the RD of TCE proceeds primarily via direct electron transfer (i.e., without intermediate H_2 generation) with current utilization efficiencies approaching 100 %, although at extremely low dechlorination rates (Aulenta et al., 2011).

One possible strategy to combine high degradation rates with high current efficiencies is the use of selective redox mediators (Aulenta, F., 2009). As an example, in a previous study, the reduced form of the humic acid analogue anthraquinone-2,6-disulfonate (AQDS) served as a model thermodynamically 'targeting' electron donor capable of selectively stimulating respiratory processes relevant to the bioremediation of perchlorate over competing processes such as methanogenesis and sulfate reduction (Van Trump, J., 2009). The use of AQDS as a redox mediator in BES to catalyze both oxidative and reductive reactions has been widely documented in the literature (Bond, D., 2003; Dumas, C., 2008; Zhang, T., 2012), although its application in the bioelectrochemical treatment of CAH is limited. In a recent study, AQDS was shown to accelerate the bioelectrochemical reductive dechlorination of TCE to cis-DCE (Aulenta, F., 2010). Although, no further dechlorination of cis- DCE was observed, even in the presence of a specialized cis-DCE dechlorinating culture highly enriched in *Dehalococcoides mccartyi* species.

In contrast to chloroethenes, the application of BES for the treatment of chloroethanes has received very little attention, in spite of the frequency that these contaminants are detected at contaminated sites. A recent study investigated the influence of the set cathode potential on the reductive dechlorination of 1,2-DCA to ethene. The results obtained were similar to those with TCE with relatively high dechlorination rates (up to 18 µmol/L d, at -900 mV vs. SHE) and a low current efficiency (<1 %) when the process was driven by electrolytic H_2 generation. In contrast, very low dechlorination rates (5 µmol/L d) with a high current efficiency approaching 70 % were

obtained at a set cathode potential of -300 mV (vs. SHE), where no H_2 production occurred (Leitão, P., 2015).

Notably, in a pioneering study, Pham and colleagues provided the first experimental evidence that microbial bioanodes could anaerobically oxidize 1,2-DCA with an anode serving as electron acceptor (Pham, H., 2009). However, the involved degradation pathway and microbial community composition were not completely identified.

In the present study, the application of BES for the treatment of 1,2-DCA was further explored. To this aim, a lab-scale BES was setup and operated under a range of operating conditions, spanning from oxidative to reductive, both in the presence and in the absence of the humic acid analogue AQDS as a redox mediator.

3.5 Materials and methods

3.5.1 Bioelectrochemical system setup

The BES used in this study consisted of two gastight borosilicate glass bottles (total volume 270 mL per bottle) separated by a 3 cm^2 cross-sectional area Nafion^{TM} 117 proton exchange membrane (PEM) (Fig. 3.1). Prior to use, the PEM was pretreated as described elsewhere (Leitão, P., 2015). The working electrode and the counter electrode were graphite rods (6 mm diameter, Sigma Aldrich, Milan, Italy) with a nominal surface area (calculated by taking into account only the part of the electrode that was immersed in the liquid phase) of 9.7 cm^2. Prior to use, the electrodes were treated as previously described (Gregory, K., 2004). The distance between the working and the counter electrode was approximately 10 cm. A KCl-saturated Ag/AgCl reference electrode (+199 mV vs. standard hydrogen electrode, SHE; Amel S.r.l., Milano, Italy) was also placed in the proximity of the working electrode, via a Luggin's capillary.
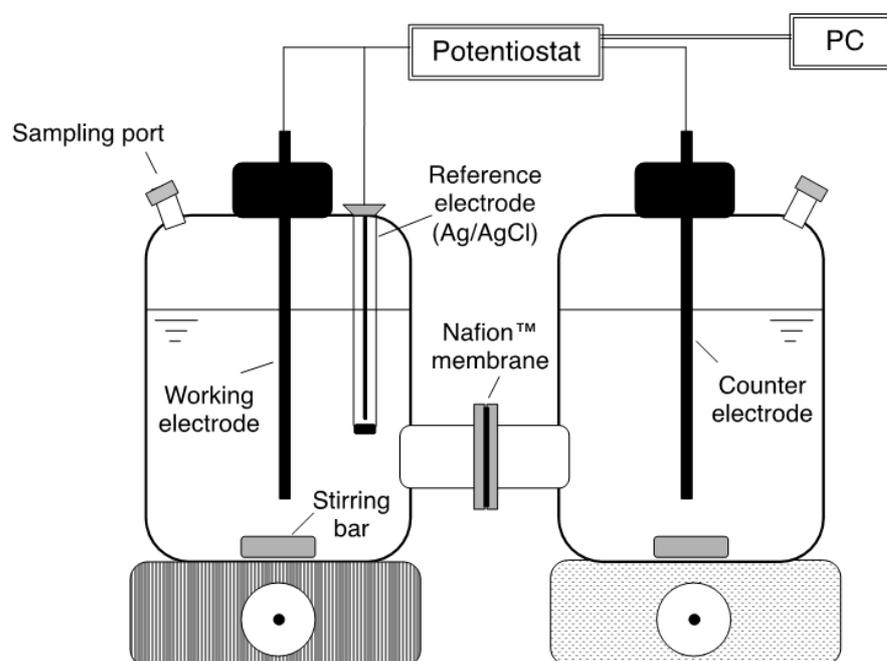
Figure 3.1 - Schematic drawing of the 2-chamber bioelectrochemical system.

3.5.2 Bioelectrochemical system operation

At the start of the study, the working electrode compartment of the BES was filled with 130 mL of anaerobic mineral medium and a 20 mL inoculum consisting of activated sludge from the ''Roma Nord'' municipal wastewater treatment plant. The medium contained the following components: NH_4Cl (0.5 g/L), $MgCl_2.6H_2O$ (0.1 g/L), K_2HPO_4 (0.4 g/L), $CaCl_2.2H_2O$ (0.05 g/L), trace metal solution (10 mL/L) (Zeikus, J., 1977), vitamin solution (10 mL/L) (Balch, W., 1979), and $NaHCO_3$ (15 mL/L, 10 % w/v). All solutions were purged for at least 0.5 h with a N_2/CO_2 (70:30 % v/v) gas mixture before use. The pH value of the medium was approximately 7.5.

The counter electrode compartment was filled with 150 mL of anaerobic mineral medium with the same composition.

Throughout the study the BES was operated in a fill and draw mode. Briefly, every week, which corresponded to a feeding cycle, the working and counter electrode compartments of the BES were purged (30 min) with a N_2/CO_2 (70:30 % v/v) gas mixture to remove volatile compounds and to maintain anaerobic conditions. Afterwards, a fixed volume (15 mL) of liquid phase was removed from each compartment and replaced with fresh anaerobic medium in order to maintain an average hydraulic and biomass retention time of approximately 70 days. From the fourth feeding cycle onward, AQDS was supplied weekly to the working electrode's compartment at a final concentration of 0.5 mmol/L.

At the start of each feeding cycle, the working electrode's compartment was spiked with 1,2-DCA or 1,1,2-TCA at a nominal concentration (i.e., neglecting partitioning into the gas phase) of approximately 0.6 mmol/L. Afterwards, the working electrode was polarized at the desired value by means of an IVIUMnSTAT multichannel potentiostat (Ivium Technologies, The Netherlands). Under each operating condition, the BES was operated for at least 3 feeding cycles in order to verify reproducibility.

3.5.3 Bioelectrochemical system monitoring

Over the course of the study, the BES was monitored for volatile components (i.e., the spiked chlorinated contaminant and its reductive dechlorination products) and for the consumed electric charge. Volatile components [i.e, 1,1,2-TCA, 1,2-DCA, VC, ETH, and methane (CH_4)] were measured by injecting 50 µL of headspace (taken with a gas-tight syringe) into a Perkin–Elmer GC 8500 gas cromatograph (2 m x 2 mm glass column packed with 60/80 mesh Carbopak B/1 % SP-1000 Supelco; N_2 carrier gas 18 mL/min; oven temperature 190 °C; flame ionization detector temperature 250 °C). The consumed electric charge (Coulombs) was calculated by integrating the measured electric current (A) over the period of electrode polarization (s) using Ivium Soft (Ivium Technologies, The Netherlands).

To characterize the electrochemical activity of AQDS, cyclic voltammetric tests at different scan rates (from 1 mV/s to 20 mV/ s) were also carried out on an identical BES but did not contain the inoculum.

3.5.4 CARD-FISH analysis

At the end of selected feeding cycles, mixed liquor samples (0.45 mL) were taken from the working electrode's compartment, immediately fixed with formaldehyde (2 % vol/vol final concentration), filtered through 0.2 mm polycarbonate filters (Ø 47 mm, Millipore) by gentle vacuum (<0.2 bar) and stored at -20 °C until use.

CARD-FISH assays were performed on filter-harvested cells to quantify members of the *Archaea* (Arc915 probe), *Bacteria* (Eub338 I, II, III probes), *D. mccartyi* spp. (Dhe1259 c and Dhe1259 t probes), and *Desulfitobacterium* spp. (Dsf440 probe plus Dsf475 helper probe), according to previously published procedures (Di Battista, A., 2012).

The probes, labeled at the 50-end with HRP, were purchased from BIOMERS (http://www.biomers.net). Details of the used oligonucleotide probes used are available at http://www.microbial-ecology.net/probebase. Oligonucleotide probes were always

added together with DAPI for the quantification of total cells. Slides were examined by epifluorescence microscopy (Olympus, BX51) and the images were captured with an Olympus F-View CCD camera and processed and analyzed with AnalySIS software (SIS, Germany). The cell counts and error bars (calculated as standard deviations) were determined by using at least 10 microscopic grids for each filter. All samples were analyzed in duplicate.

3.5.5 Chemicals

All chemicals were purchased from Sigma–Aldrich (Milano, Italy). The chemicals used to prepare the mineral medium were of analytical grade and used as received.

3.6 Results and discussion

3.6.1 Cyclic voltammetry

A preliminary cyclic voltammetric study was carried out to assess the electrochemical behavior of AQDS. For this purpose, Cyclic Voltammograms (CVs) were recorded in anaerobic mineral medium supplemented with the redox mediator at a concentration of 0.5 mmol/L, both in the presence and in the absence of chlorinated contaminants (i.e., 1,2-DCA and 1,1,2-TCA). For comparative purposes, CVs in mineral medium without AQDS were also recorded (Fig. 3.2).
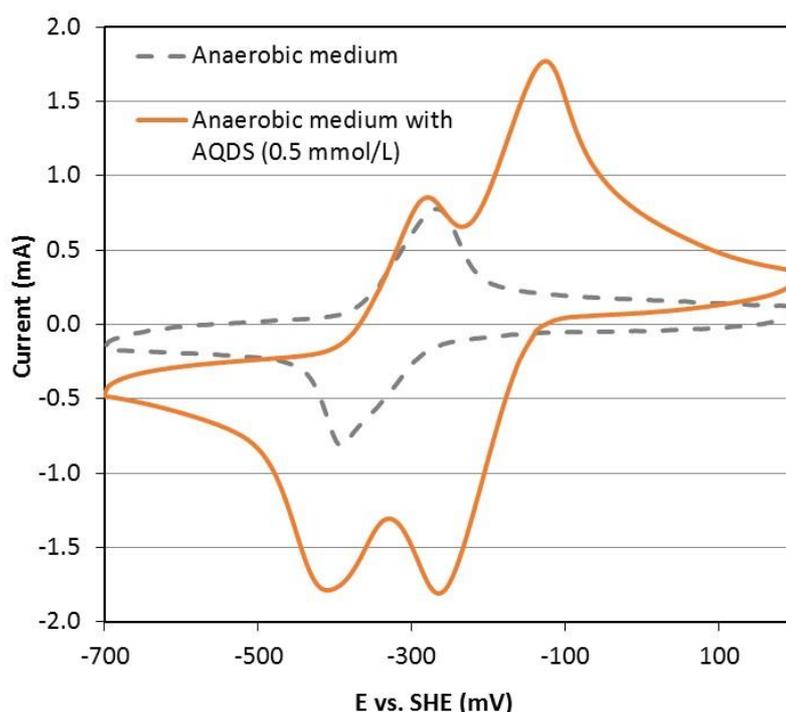
Figure 3.2 - Cyclic voltammogram (10 mV/s) of the anaerobic medium in the presence and in the absence of AQDS (0.5 mmol/L).

As shown in Fig. 3.3a, the CVs of AQDS in mineral medium revealed the presence of two distinct redox couples. One was characterized by a formal redox potential of -197 ± 2 mV which can be attributed to AQDS while the other one was characterized by a formal redox potential of -347 ± 3 mV, which could be attributed to other redox active species contained in the mineral medium (e.g., Fe^{2+}/Fe^{3+}). Regarding the AQDS redox couple, the peak separation (ΔEpotential = $E_{p,Anode}$ - $E_{p,Cathode}$) substantially increased with the scan rate clearly indicating that the redox system was not reversible. Furthermore, the anodic and cathodic peak heights linearly correlated with the square root of the scan rate pointing to a diffusion-controlled regime and suggesting that the redox mediator did not largely adsorb onto the electrode surface (Harnisch and Freguia, 2012).
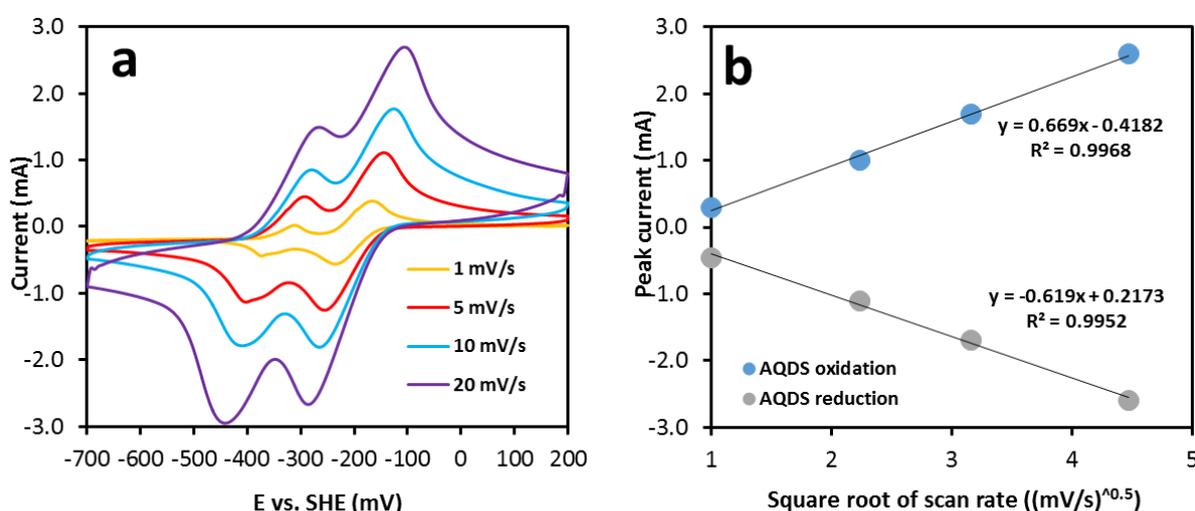
Figure 3.3 - (a) Effect of the scan rate (1-20 mV/s) on cyclic voltammograms of AQDS (0.5 mmol/L) in anaerobic mineral medium. (b) Linear dependency of cathodic and anodic AQDS peak current on the square root of the scan rate.

The CVs recorded in the presence of AQDS and 1,2-DCA or 1,1,2- TCA, (with the chlorinated compounds added to a final nominal concentration of approximately 0.5 mmol/L) were almost indistinguishable from those recorded in the absence of chlorinated compounds (data not shown), indicating that these compounds were not abiotically reduced or oxidized, at least within a potential window ranging from -700 mV to +200 mV (vs. SHE).

Finally, CVs also showed that within the examined potential window (-700 mV to +500 mV vs. SHE) evolution of oxygen (from the electrochemical oxidation of water) or hydrogen (from electrochemical reduction of water) did not occur.

3.6.2 Bioeletrochemical dechlorination

In the present study, the possibility to stimulate the anaerobic degradation of chlorinated ethanes (i.e., 1,2-DCA and 1,1,2-TCA) in a bioelectrochemical system under a range of operating conditions was investigated.

At the beginning of the study, the bioelectrochemical reactor was inoculated with an activated sludge treating municipal wastewater and spiked with 1,2-DCA (0.6 mmol/L). During the first 3 feeding cycles (i.e., Phase I), the reactor was operated in a fill and draw mode with the working electrode potentiostatically kept at +500 mV vs. SHE in order to verify the capability of the inoculated microorganisms to anaerobically oxidize the chlorinated compound with the polarized electrode serving as direct electron acceptor. However, only a very slow 1,2-DCA removal (not correlated with electric current generation, as discussed later) was observed during these initial cycles (i.e., approximately 0.004– 0.013 mmol/cycle) (Fig. 3.2).
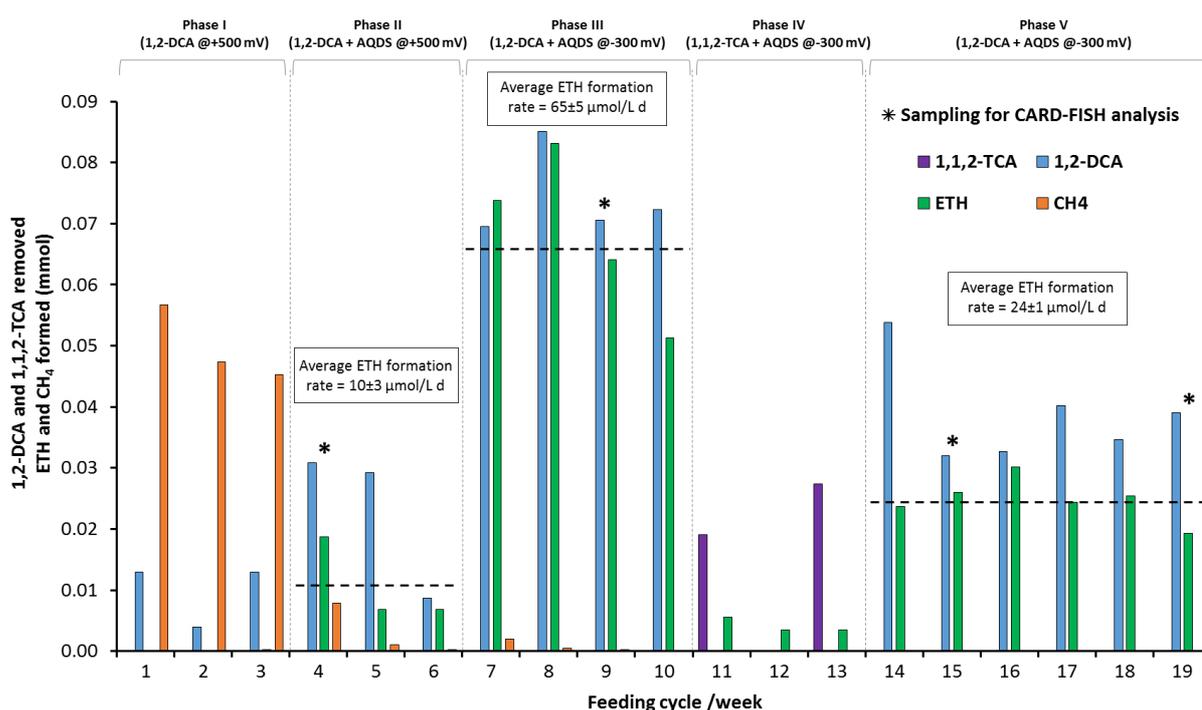
Figure 3.4 - Performance of the bioelectrochemical system as a function of the applied operating conditions, in terms of weekly removal of chlorinated compounds (1,2-DCA or 1,1,2-TCA) and formation of ETH and CH$_4$.

In spite of the highly oxidizing potential applied to the electrode, a substantial amount of methane was produced and accounted for approximately 0.05–0.06 mmol/cycle. This most probably was derived from the degradation of the organic matter associated with the inoculum. The bioavailability of oxidizable organic matter was further confirmed by the generation of an (anodic) electric current which resulted in 28 Coulombs

cumulatively transferred to the electrode during the 1st feeding cycle, 235 Coulombs transferred during the 2nd feeding cycle, and 140 Coulombs transferred during the 3rd feeding cycle (data not shown). Importantly these figures are orders of magnitude greater than those that would result from the complete oxidation of the actually removed 1,2-DCA (i.e., maximum 1 Coulomb).

In order to facilitate the bioelectrochemical oxidation of 1,2-DCA, the soluble redox mediator AQDS was supplied during phase II (i.e., feeding cycles 4–6) to a final concentration of 0.5 mmol/L, while the electrode potential was maintained at +500 mV vs. SHE. The addition of AQDS resulted in the almost complete suppression of CH_4 production and in the formation of small amounts (up to 0.018 mmol/cycle) of ETH, which derived from the reductive dichloroelimination of 1,2-DCA, as well as in the accumulation of trace amounts of VC (<0.0005 mmol/ cycle, data not shown), which derived from the dehydrochlorination (i.e., a non-reductive pathway) of 1,2-DCA (Fig. 3.5a) (Aulenta, F., 2006b). During phase II, the average reductive dechlorination rate (based on ETH formation) was 10 ± 3 µmol/L d (Fig.3.4).
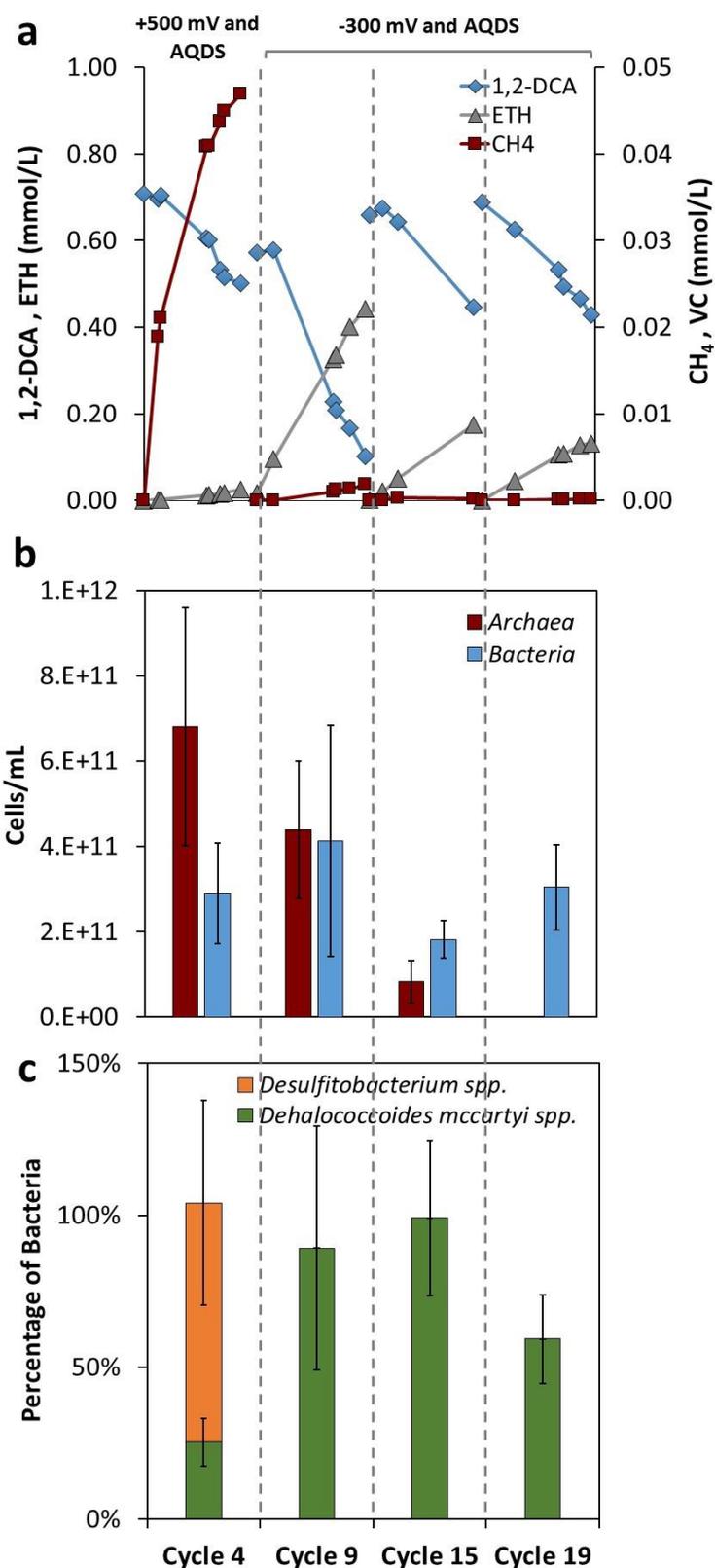
Figure 3.5 - (a) Time course of 1,2-DCA dechlorination, **(b)** *Archaea* and *Bacteria* concentration, **(c)** percentage of Bacteria binding to the *Dehalococcoides mccartyi* spp. and *Desulfitobacterium* spp. during representative feeding cycles.

The most likely reason for these results is that AQDS inhibited methanogenesis by serving as an energetically more favorable electron acceptor (compared to CO_2) during the

oxidation of available organic matter (Van Trump, J., 2009). Once reduced, AQDS was then likely used as an electron donor (in the bulk liquid) for the microbially catalyzed reductive dechlorination of 1,2-DCA to ETH.

Collectively, these results indicated that the inoculum harbored anaerobic dechlorinating microorganisms even though it originated from a fully aerobic activated sludge system. In order to take advantage of this metabolic potential, the electrode potential was poised at -300 mV vs. SHE during phase III (i.e., feeding cycles 7–10) so as to serve as a constant supply of electrons for the AQDS-mediated reductive dechlorination of 1,2-DCA.

The change in the operational conditions resulted in a 6.5-fold increase in the rate of dechlorination, which stabilized at 65 ± 5 µmol/L d (based on ETH formation) (Fig. 3.4). Under these conditions, the (cathodic) current flowing in the circuit was between 2 and 10 µA. Importantly, the removed 1,2-DCA was stoichiometrically converted to ETH, with negligible accumulation of toxic VC (<0.0001 mmol/cycle) (Fig. 3a). It is worth noting that these dechlorination rates are substantially higher that those obtained in a previous bioelectrochemical study, whereby hydrogen served as the electron donor for 1,2-DCA dechlorination (Leitão, P., 2015). This clearly indicates that AQDS is capable of effectively shuttling electrons to dechlorinating bacteria.

Starting at cycle 11 (i.e., the beginning of Phase IV), 1,2-DCA was replaced by 1,1,2-TCA (0.6 mmol/L, nominal concentration) as the terminal electron acceptor (Fig. 3.4). During this phase, the polarization of the electrode was maintained at -300 mV vs. SHE and AQDS continued to be supplied at a concentration of 0.5 mmol/L. Throughout this phase (i.e., from cycle 11 to cycle 13), very little 1,1,2-TCA removal was observed, as well as negligible formation of dechlorination products, clearly indicating that the culture was not able to thrive on this chlorinated compound (Fig. 3.4).

From cycle 14 onward (Phase V), the culture was switched back to 1,2-DCA as terminal electron acceptor (while keeping the electrode polarized at -300 mV and the AQDS concentration at 0.5 mmol/L). Reductive dechlorination immediately resumed, although at a substantially lower rate (i.e., 24 ± 1 µmol/L d) compared to that observed during Phase III. Also during this phase, 1,2-DCA was stoichiometrically dechlorinated to ETH with negligible accumulation of VC (Fig. 3.5a).

3.6.3 Dynamics of the microbial community

In order to gain a deeper insight into the dynamics of the microbial community as a function of the applied operating conditions, mixed liquor samples were removed from the bioelectrochemical reactor at the end of cycle 4, 9, 15 and 19 and analyzed by CARD-FISH using domain-specific oligonucleotide probes targeting *Archaea* and *Bacteria*, as well as probes targeting known dechlorinating bacteria (i.e., *Desulfitobacterium* species and *D. mccartyi* species).

At the end of cycle 4, (methanogenic) *Archaea* represented the main component of the microbial community accounting for approximately $6.8 \pm 2.8 \times 10^{11}$ cells/mL. These results agree with the fact that CH_4 production was the main observed metabolic process (Fig. 3.5b). The concentration of Archaea then gradually decreased to $4.4 \pm 1.6 \times 10^{11}$ cells/mL at the end of cycle 9, to $8.2 \pm 5.0 \times 10^{10}$ cells/mL at the end of cycle 15, and to undetectable levels at the end of cycle 19 (Fig. 3.5b). This finding appears to confirm that reduced AQDS is a thermodynamically ''targeting" electron donor capable of selectively stimulating the reductive dechlorination of 1,2-DCA over methanogenesis. This agrees with thermodynamic calculations predicting CH_4 generation coupled to oxidation of reduced AQDS to be an energetically unfavorable reaction (Van Trump, J., 2009). The most likely reason that methanogens thrived and then subsequently decreased during the experiments was that the organic matter associated with the inoculum was completed consumed over time.

As far as *Bacteria* are concerned, their concentration increased from $2.9 \pm 1.2 \times 10^{11}$ cells/mL (at the end of cycle 4) to $4.1 \pm 2.7 \times 10^{11}$ cells/mL by the end of cycle 9, when the highest dechlorinating activity was detected (Fig. 3.5b). A marked drop in concentration was observed at cycle 15 after the system was fed for three successive cycles on 1,1,2-TCA. This may be due to the fact that negligible dechlorinating activity, and therefore growth, was observed during this period. Notably, the lower 1,2-DCA dechlorination rates observed during Phase V (compared to Phase III) is in agreement with the lower concentration of *Bacteria* due to the lack of microbial growth on 1,1,2-TCA during Phase IV. A further increase in bacterial concentration was observed at cycle 19, after the culture had been switched back to 1,2-DCA and the dechlorinating activity resumed (Fig. 3.5b).

Remarkably, nearly all of *Bacteria* consisted of known 1,2-DCA dechlorinating species at cycle 4, namely *Desulfitobacterium* spp. (79 ± 34 % of total Bacteria) and *D. mccartyi* spp. (25 ± 8 %) (Fig. 3.5c) (Marzorati, M., 2007; Maymo-Gatell, X., 1999; Nijenhuis, I.,

2016). By cycle 9, *Desulfitobacterium* spp. could no longer be detected while *D. mccartyi* spp. remained the dominant bacterial species within the bioelectrochemical system until the end of the study. Although previously reported in the literature (Wang, S., 2015), the enrichment of *D. mccartyi*, a strictly anaerobic dechlorinating microorganism, from a fully aerobic municipal activated sludge is a somewhat surprising result which undoubtedly deserves further investigations. Certainly, it is apparent that reduced AQDS and 1,2-DCA turned out to be very selective growth substrates for this microorganism.
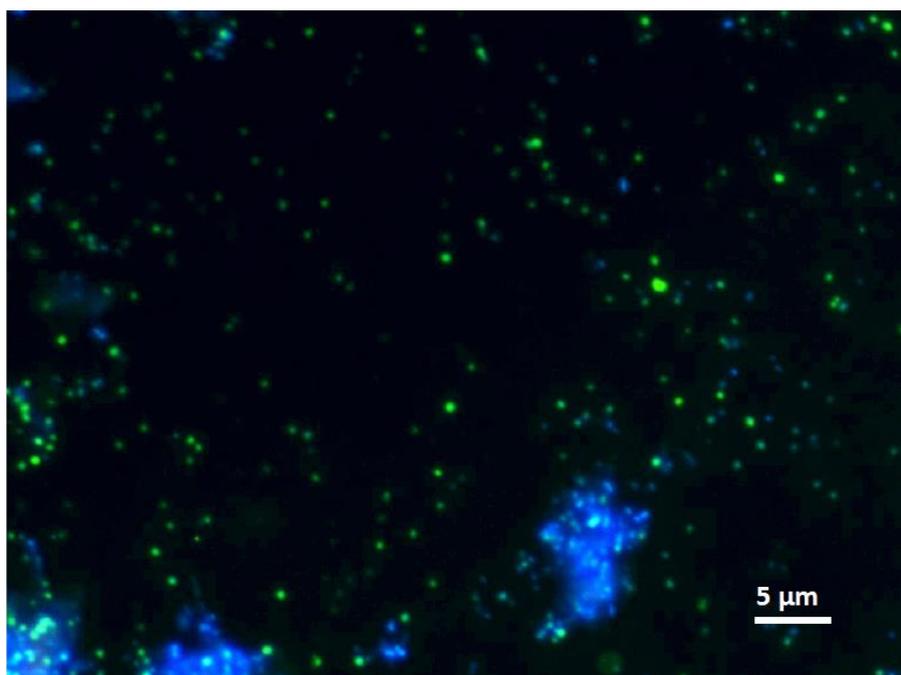
Figure 3.6 - Detection and quantification of *Dehalococcoides mccartyi* spp. (in green) by CARD-FISH in the mixed liquor of the BES at the end of cycle 19. DAPI-stained cells not binding to *the Dehalococcoides mccartyi* oligonucleotide probes appear in blue.

However, it is worth noting that while this microorganism accounted for nearly the totality of *Bacteria* at cycle 9 and 15 (89 ± 40 % and 99 ± 25 %, respectively), at cycle 19, it accounted for only 59 ± 15 % of total Bacteria, possibly indicating the enrichment of other bacterial species. Indeed, although the culture was still highly enriched in *D. mccartyi*, DAPI-stained cells not binding to the *D. mccartyi* probes, were also present and were observed in large aggregates (Fig. 3.6).

3.7 Conclusions

This study demonstrates that a microbial biocathode operated at a set potential of -300 mV vs. SHE and in the presence of the humic acid analogue AQDS, can stably (i.e., several weeks) and selectively dechlorinate 1,2-DCA to harmless ETH. Fill and draw operation of the BES resulted in the remarkable enrichment (up to 100% of total Bacteria)

of the obligate anaerobe *D. mccartyi*, in spite of the fact that a fully aerobic municipal activated sludge was used as inoculum. Finally, the results of this work are also important regarding the biogeochemical fate of 1,2-DCA in humics-rich contaminated aquifers.

3.8 Acknowledgements

3.9 References

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4 Grafting AQDS onto graphite electrodes using a commercial anion exchange membrane to promote the bioelectrochemical reductive dechlorination of 1,2-dichloroethane

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4.1 Highlights

- Described method is simple, fast, low-cost and reproducible.
- Enhancement of 1,2-DCA reductive dechlorination was achieved with immobilized AQDS and applied potential.
- Microorganisms seem to thrive in the modified electrode surface.
- Possibile future applications at a contaminated sites.

4.1.1 Keywords

Modified electrode; immobilized redox mediator; 1,2-DCA.

4.2 Graphical abstract
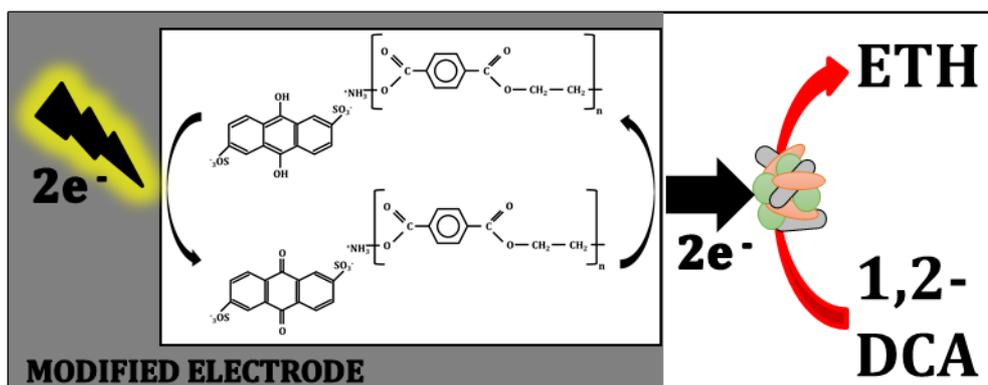
4.3 Abstract

In the present study we initially describe a simple, straightforward, reproducible and low cost method to immobilize Anthraquinone-2,6-disulfonate (AQDS) to a graphite electrode using a commercial anion exchange membrane. Voltametric experiments and experimental design allowed to quantify mid point potential and identify redox reactions taking place at the modified electrode. Finally, the modified electrode was applied in a lab scale batch 1,2-dichloroethane (1,2-DCA) remediation bioelectrochemical cell, carried out either at open circuit potential (OCP) or at -300 mV vs standard hydrogen electrode. System polarization enhanced the reductive dechlorination rate by 2 fold when compared with OCP dechlorination rates. Redox mediator remained attached at the electrode during the 90 days of the biotic experiment, with some changes in the current peak and position. Analysis perfomed at the end of the biotic experiment revealed the large biofilm formation at the surface of the modified electrode, which leads to assume that microorganisms seem to thrive in the conditions found at this location. The findings presented in this study can influence positevly *in situ* bioremediation since they can be applied to accelerate the reductive dechlorination process, eliminating the need of repeated injections of an AQDS solution, erradicanding possible problems in the dispersion of AQDS solution in the subsoil, reducing economic over costs and regulatory restrictions.

4.4 Introduction

Chlorinated aliphatic hydrocarbons (CAH), compounds of anthropogenic origin, are commonly found in the environment, due to improper usage, storage and disposal. This compounds are used worldwide in industrial applications as degreasing agents, solvents and as precursors for the production of other compounds. 1,2-dichloroethane (1,2-DCA) is mainly used for the production of vinyl chloride and as a solvent. Sites with underground contamination with this CAH revealed concentrations varying between 0.1 to 100 mg.L^{-1} (Klecka, G., 1998). Over the last years some studies have focused on this chlorinated solvent, not only because of the wide dispersion of this suspected carcinogen but also because bacteria that can degrade 1,2-DCA, to harmless end-products, exhibit low dechlorination rates (Leitão, P., 2015). Hence, the removal of 1,2-DCA remains problematic. Microorganisms capable of detoxify 1,2-DCA thus far are members from *Dehalococcoides* spp. (Maymo-Gatell, X., 1999; Grostern, A., 2006), *Dehalobacter* spp. (Grostern, A., 2006), *Desulfitobacterium* spp. (De Wildeman, S., 2003) and *Dehalogenimonas spp.* (Dillehay, J., 2014). The common approach for the clean-up of sites contaminated with CAHs is bioestimulation of autochthonous microorganisms present, by the addition of nutrients and electron donors (Majone, M., 2015). A major disadvantage of this approach is the need of continuously provide electron donors, which can lead to unwanted side reactions, increase of costs and longer remediation time needed to achieve remediation goals (Aulenta, F., 2007a).

A new approach has been pursuit to overcome this disadvantage: delivery of the needed electrons by the usage of potentiostatically controlled insoluble electrodes. Several research has been done on this topic with several CAH, namely tetrachloroethene (PCE) (Strycharz, M, 2008), trichloroethene (TCE) (Aulenta, F., 2009) and even with 1,2-DCA (Leitão, P., 2015). A previous study from our group has shown that 1,2-DCA can be bioelectrochemically degraded to harmless ethene, via dihaloelimination, without the addition of external electron donors (Leitão, P., 2015). To enhance dechlorinating rates, a later study examined the effect of adding a redox mediator, humic acid analogue anthraquinone-2,6-disulfonate (AQDS), to the cathodic chamber (Leitão, P., 2016). The supply of redox mediators does not have to be in large amounts to enhance biotransformation, because they are successively restored during electron flow between electron donor and the contaminant. Nevertheless the need of continuous addition of redox mediator as an electron shuttle to enhance dechlorination rates, poses as a limitation in real life applications to the process, because of possible problems in the dispersion of AQDS solution to the subsoil, economic implications and regulatory

restrictions. Some redox mediator immobilization procedures are already described in literature. Immobilization of metyl viologen (MV) assisted by a Nafion solution was achivied with a reproducibility of more than 80% and subsequently biotic batch tests were carried out for TCE and cis-DCE dechlorination (Aulenta, F., 2009). However these biotic tests were performed during a short period of time (8 hours) and dechlorination rates with the immobilized MV were lower than those obtained with soluble MV and with hydrogen production. Development of a TCE bioelectrochemical sensor was achieved with anthraquinone (AQ)-modified electrodes, carried out via spontenous reduction or via electrochemical reduction (Aulenta, F., 2011). In another study silver epoxy was used to immobilize a solid-phase humin on a graphite electrode for the bioelectrochemical enhancement of the pentachlorophenol reductive dechlorination (Zhang, D., 2014).

The present study aimed to eliminate the limitations associated with the addition of redox mediators, by developing a simple, straightforward, reproducible and low cost method to immobilize AQDS at a graphite cathode and subsequently test its application in a lab scale 1,2-DCA remediation bioelectrochemical cell. Modified electrodes were prepared and characterized by voltammetric techniques and biotic experiments were set up to evaluate the effect of the developed electrodes towards 1,2-DCA dechlorination.

4.5 Materials and methods

4.5.1 Electrodes modification

Two pre-treated (Gregory, K., 2004) graphite electrodes were modified: a fumasep electrode and a fumasep + AQDS electrode. Procedure for fumasep electrode consisted in dissolving a comercial anionic exchange membrane, fumasep® FAD (FuMA-Tech GmbH) in ethanol (0.25 % (w/v)) for ten minutes at 60 ˚C, to ensure complete dissolution. The pre-treated graphite electrode was completely immersed in this solution, for two hours at 60 ˚C. The fumasep electrode was air dried overnight in a vertical position and washed twice with deionized (DI) water and once with mineral media. Procedure for the fumasep + AQDS electrode was identical but with the further addition of a solution of AQDS, at a final concentration of 1 mM, to the initial fumasep solution and left to dissolve and react for one hour at 60 ºC. For the biotic tests the fumasep + AQDS electrode, had an AQDS solution with a final concentration of 0.5 mM.

4.5.2 Bioelectrochemical cell setup

The bioelectrochemical cell setup used in this study consisted of two gastight borosilicate glass bottles (with a total volume of about 270 mL per bottle) separated by a 3 cm^2 cross-sectional area Nafion^{TM} 117 proton exchange membrane (PEM). The PEM was boiled successively in H$_2$O$_2$ (3 % v/v), DI water, then in 0.5 M H$_2$SO$_4$ and DI water each for 2 h, and stored in DI water. The cathode and anode were graphite rods (6 mm diameter, Sigma Aldrich, Milano, Italy). The nominal surface area of the cathode (calculated taking only into account the part of the electrode that was immersed in the liquid phase) was 9.7 cm^2. The distance between the anode and the cathode was around 10 cm. For voltammetric characterization in the abiotic experiments the reference electrode was a KCl-saturated Ag/AgCl, +199 mV vs. standard hydrogen electrode, SHE; Amel S.r.l., Milano, Italy and for biotic experiments the reference electrode was KCl-saturated Ag/AgCl +201 mV vs. the standard hydrogen electrode (SHE), Metrohm, placed in the cathode chamber in a Lugin tube. The catholyte and anolyte consisted of anaerobic medium, as described below.

4.5.3 Bioelectrochemical experiments

4.5.3.1 Abiotic voltammetric characterization

Abiotic experiments were performed in anaerobic environment, under stirring and no stirring conditions by cyclic voltammetry (1, 5, 10, 20 and 50 mV/s) for pretreated graphite electrode in mineral media, pretreated graphite electrode in mineral media supplemented with 1 mM of AQDS, fumasep (0.25 % (w/v)) electrode in mineral media and fumasep + AQDS (0.25 % (w/v) + 1 mM) graphite electrode in mineral media.

Measurements were performed using IVIUMnSTAT, multichannel electrochemical analyser, Ivium Technologies (The Netherlands) and data processed with Ivium Soft.

To further confirm results, a sequence of steps were conducted, with stirring and with no stirring. A first set of CVs was performed with the fumasep + AQDS electrode in neat mineral media. Fumasep + AQDS electrode was replaced with a neat pre-treated graphite electrode maintaining the same mineral media and a new set of CVs was performed. The same neat pre-treated graphite electrode was maintained but catholyte was replaced with fresh mineral media supplemented with 1 mM AQDS and a new set of CVs was executed. Fumasep + AQDS electrode was placed in the cathodic chamber, media was replaced and another set of CVs was done.

4.5.3.2 Biotic experiments

To evaluate the effect of the fumasep + AQDS (0.25 % (w/v) + 0.5 mM) electrode in the bioelectrochemical 1,2-DCA reductive dechlorination with a dechlorinating culture, biotic bioelectrochemical experiments were performed. These experiments were operated as a fed and draw mode by anaerobically inoculating the cathodic compartment with 40 mL of a 1,2-DCA dechlorination culture. In one week cycles, anodic and cathodic chambers were flushed with N_2 to remove volatile compounds. A fixed liquid volume was replaced with fresh medium to maintain an average hydraulic retention time of 50 days and feed with 1,2-DCA at a nominal concentration of 0.12 mM. Liquid volume in the cathodic and anodic chamber was 150 mL each, pH was maintained at 7.50 and system placed in a thermostatic chamber at 25 °C, under magnetic stirring. The bioelectrochemical system was kept in the dark, to prevent undesired algae growth, which was shown to occur in preliminary experiments. Alternating on/off cycles were performed at a set cathode potential of -300 mV vs SHE or at open circuit, respectively.

Electrochemical measurements, including cyclic voltammetry's, and monitoring were performed using Autolab AUT50360 potensiostat and data processed with Nova software (version 1.10.4) from Metrohm Autolab B.V.

4.5.4 Strain, medium, and culture conditions

The 1,2-DCA dechlorinating culture, available from a previous bioelectrochemical study (Leitão et al., 2016), was enriched before the experiment start-up. The content of the cathodic chamber was anaerobically transferred to a serum bottle. This bottle was covered with aluminum foil, sealed with a Teflon-faced butyl rubber stopper and aluminum crimp seal, with a liquid and total volume of 95 and 120 mL, respectively. A dosage of 0.5 mM 1,2-DCA and 3.75 mM of H_2 (nominal concentrations) was used.

The medium contained the following components: NH_4Cl (0.5 g.L^{-1}), $MgCl_2\cdot6H_2O$ (0.1 g.L^{-1}), K_2HPO_4 (0.4 g.L^{-1}), $CaCl_2\cdot2H_2O$ (0.05 g.L^{-1}), trace metal solution (10 mL.L^{-1}) (Zeikus, J., 1977), vitamin solution (10 mL.L^{-1}) (Balch, W., 1979), and $NaHCO_3$ (15 mL.L^{-1}, 10 % w/v). All solutions were purged for at least 0.5 h with a N_2/CO_2 (70:30) gas mixture before use. The pH value of the medium was 7.5. Culture was maintained at 25 °C and at a pH of 7.00 under fill-and-draw conditions (weekly basis) by feeding with 1,2-DCA and H_2 at a cell retention time of 30 days and operated for over 80 days before usage in the present study.

4.5.5 Analytical methods

Volatile components (1,2-DCA, vinyl chloride (VC), ethene, methane) were quantified by injecting 50 µL of headspace (taken with a gas-tight syringe) into a Shimadzu GC-2014 gas chromatograph (2.4 m x 2.1 mm metal packed column 60/80 Carbopack B/ 1 % SP-1000; N_2 carrier gas 40 mL.min^{-1}; oven temperature 60 $^{\circ}$C with a ramp of 40 $^{\circ}$C.min^{-1} until 180 $^{\circ}$C hold for 1.25 min; flame ionization detector temperature 200 $^{\circ}$C).

Headspace concentrations were converted to aqueous-phase concentrations using Henry's law constants (Chen et al., 2012).

Volatile fatty acids (VFA), acetate and propionate were analyzed by injecting 1 µL of filtered (0.22 µm porosity) liquid sample, previously acidified with formic acid (10 % v/v), into a Shimadzu GC-2014 gas chromatograph (3 m × 2.1 mm stainless steel column packed with 60/80 mesh Carbopak C/ 0.3 % Carbowax 20 M/ 0.1 % H_3PO_4; N_2 carrier gas 40 mL.min^{-1}; oven temperature 120 $^{\circ}$C; injector temperature and Flame ionization detector temperature 200 $^{\circ}$C).

Total carbon, inorganic and non purgeable organic carbon were analyzed by a Shimadzu Total Organic Carbon Analyser (TOC-V CSN) with an oxygen flow of 150 mL.min^{-1} and a furnace temperature of 680 $^{\circ}$C. Total carbon was measured by injection of a preselected amount in the furnace. For the analysis of inorganic carbon a pre-treatment was performed with HCl 2 M and for non purgeable organic carbon, besides the acid pre-treatment, samples were purged with O_2 for 3 min. TOC and POC were calculated as follows by the measurements performed for TC, IC and NPOC:

$$TOC = TC - IC$$

$$POC = TOC - NPOC$$

4.5.6 Scanning electron microscopy/ Energy-dispersive X-ray spectroscopy analysis

Scanning electron microscopy (SEM)/ Energy-dispersive X-ray spectroscopy (EDS) was used to analyze liquid samples from the cathodic chamber and the surface of the fumasep+ AQDS electrode, at the end of the biotic experiments. Planktonic samples were pretreated by removing 5 mL of liquid content from the cathode chamber and centrifuging at 5000 rpm for 5 min. After removing the supernatant, the sample was fixed in 3 % (w/v) glutaraldehyde in cacodylate buffer (pH 7.2) for three hours at room temperature. The

combined mixture was centrifuged (5000 rpm, 5 min) and almost all of the supernatant was removed. A small quantity (250 µL) of the remaining solution was then mounted on a glass slide and placed at 50 ºC for 3 hours. Futhermore, the Fumasep + AQDS electrode was immerged in a petri dish containing 3 % (w/v) glutaraldehyde in cacodylate buffer (pH 7.2) for 3 hours at room temperature.

The following treatment steps, necessary to preserve the integrity of biological structures present, were similar to the fixed liquid sample and the fixed fumasep + AQDS electrode. Treatment consisted in dehydration with ethanol by a series of 50, 60, 70, 80, 90 and 2x 100 % (v/v), followed with HMDS in ethanol by a series of 50, 60, 70, 80, 90 and 2x 100 % (v/v), 10 minutes each at room temperature. Treated samples were stored in a desiccator in the dark until SEM analysis, for a maximum period of 24 hours in order to prevent moisture absorption/ adsorption. Planktonic treated samples were sputter-coated for 70 s at 15 mA current while fumasep + AQDS treated electrode was sputter-coated for 40 s at 15 mA with a palladium–gold thin film in argon atmosphere using the SPI Module Sputter Coater equipment.

All samples were viewed with a SEM/EDS system (FEI Quanta 400FEG ESEM/EDAX Genesis X4M, FEI Company, USA) in high-vacuum mode at 10 or 15 kV to observe size, morphology and distribution. X-ray microanalysis was performed in specific fields for elemental characterization.

4.5.7 Chemicals

All the chemicals were analytical grade or higher and used as received.

4.6 Results and discussion

4.6.1 Abiotic voltammetric characterization

Cyclic voltammetries were performed in abiotic mineral media with the neat electrode, neat electrode with 1 mM AQDS solution, fumasep electrode and fumasep + AQDS electrode (Figure 4.1a). Different profiles were obtained at each experimental set up tested. Voltammograms from the neat graphite electrode in mineral media displayed one redox peak that could be attributed to active species contained in the mineral medium (e.g., Fe^{2+}/Fe^{3+}). Neat graphite electrode in mineral media with AQDS in solution, at a concentration of 1 mM, revealed another redox peak identified as AQDS (Leitão et al., 2016). Fumasep graphite electrode in mineral media displayed voltammograms with high background current effect, diluting the contributions from active mineral species and from

the anion membrane. In the CV profile recorded for Fumasep + AQDS graphite electrode, redox peak from AQDS was still detectable and in similar potential values to those obtained in other experimental conditions and to values described in the literature.
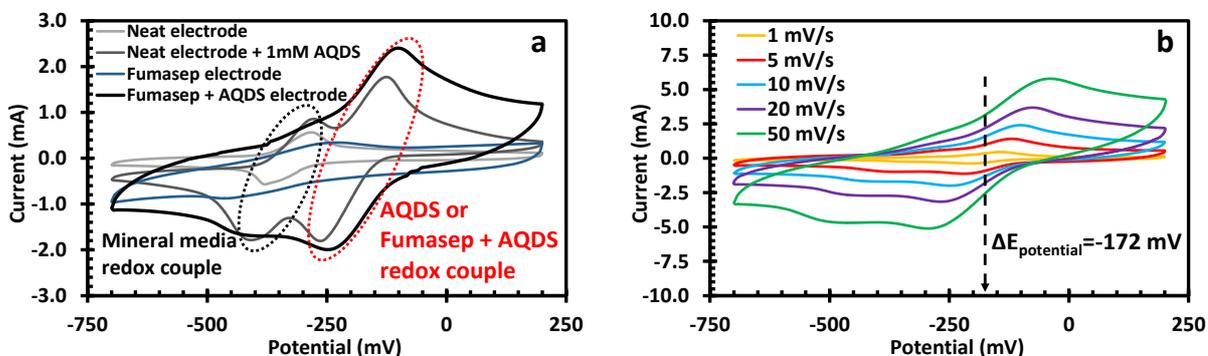
Figure 4.1 - (a) Cyclic voltammograms (10 mV/s) with no stirring performed with neat electrode in anaerobic mineral media (light grey line), neat electrode in anaerobic mineral media with 1 mM of AQDS in solution (dark grey line), fumasep electrode in anaerobic mineral media (blue line) and fumasep + AQDS electrode in anaerobic mineral media (black line). Dashed areas represent mineral media redox couple (black dashed area) and AQDS or Fumasep + AQDS redox couple (red dashed area). (b) Effect of the scan rate (1-50 mV/s) on cyclic voltammograms with no stirring of fumasep + AQDS electrode in anaerobic mineral medium. Formal potential of this complex was -172 mV.

Figure 4.1b shows the effect of scan rate (1–50 mV/s) on the cyclic voltammetry response of fumasep+ AQDS electrode. The midpoint redox potential (defined as the average of anodic peak potential, $E_{p,a}$ and cathodic peak potential, $E_{p,c}$) of surface-bound AQDS were in the range -169 to -172 mV, with stirring and no stirring, respectively. These potentials are of interest because they are sufficiently high to avoid interference from hydrogen evolution (E0' = -440 mV at pH 7.5), a main competitive reaction in aqueous media. Theoretical formal potential of AQDS at pH 7 is -184 mV vs. SHE. The difference in values obtained in this study to the theoretical one can be attributed to different pH conditions, to the contribution from the anionic exchange membrane and the state in which AQDS is present.
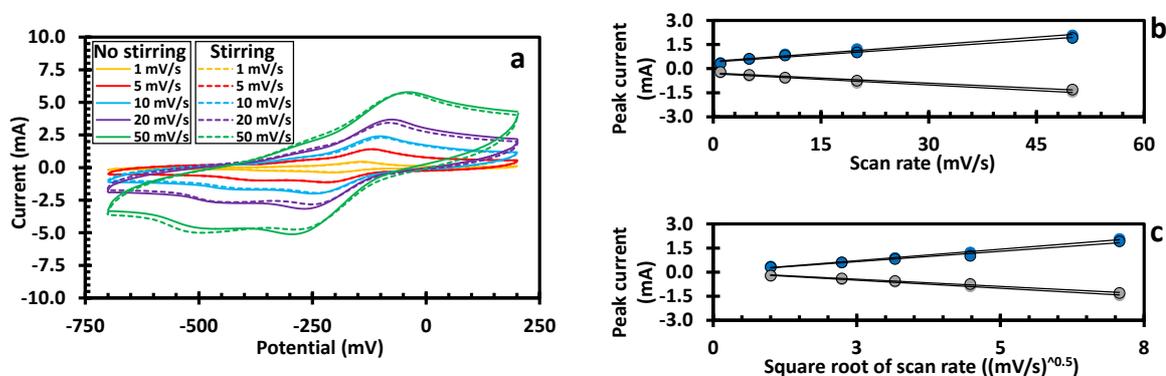
Figure 4.2 - (a) Effect of no stirring and stirring to the cyclic voltammograms profile (1-50 mV/s) to the fumasep + AQDS electrode in anaerobic mineral medium. (b) Dependency of anodic and cathodic fumasep + AQDS peak current on the scan rate. (c) Dependency of cathodic and anodic fumasep + AQDS peak current on the square root of the scan rate.

Data presented at fig. 4.2a indicates that the reactions were occurring at the surface of the electrode because no considerable change was observed in the fumasep + AQDS redox peak profiles at different scan rates with and without stirring. To further investigate the type of reactions occurring at the fumasep + AQDS electrode, anodic and cathodic peak currents were plotted as a function of the scan rate (fig 4.2b) and as a function of the square root of the scan rate (fig 4.2c), in no stirring and stirring conditions.

Anodic and cathodic peak currents exhibited a linear relationship with the scan rate but even a higher linear relationship with the square root of the scan rate, with no stirring and stirring (Table 4.1).

Table 4.1 - Correlation of anodic and cathodic Fumasep + AQDS peak current on the scan rate or square root (SQRT) of the scan rate, with no stirring and stirring.

			R^2 peak current vs scan rate (b)	R^2 peak current vs SQRT scan rate (c)
FUMASEP + AQDS	No stirring	Anodic	0.9766	0.9936
		Cathodic	0.9703	0.9964
	Stirring	Anodic	0.9795	0.9788
		Cathodic	0.9792	0.9917

A possible explanation for this behavior is that redox reactions were occurring both at the electrode surface and by diffusing processes. These results point out that the fumasep + AQDS complex film cannot be described neither as thin monolayer mechanisms nor by a film thicker than a monolayer mechanisms. Hence charge transport maybe achieved by physical diffusion through the film and/or by electron transport between a series of self-exchange reactions at the surface of the electrode.

The quantity of AQDS immobilized can be estimated according to the following equation:

$$\tau = \frac{Q_c}{nFA}$$

Qc is the electric charge from the area under the AQDS reduction peak (corrected for the baseline); n is the number of electrons exchanged per reactant molecule (n = 2); F is Faraday's constant; and A is the geometric (nominal) area of the electrode.

The quantity of AQDS immobilized at the electrode was in the range of 2.25 x 10^{-6} mol.cm^{-2} and an efficiency of 46 %. Values obtained by the previous expression were overestimated because redox processes were not confined to the electrode surface. Nevertheless the equation used is a good indicator to evaluate the overall immobilization process.

4.6.2 Effect of modified electrode in the 1,2-DCA dechlorination

The electrocatalytic activity to the reduction of 1,2-DCA by the bioelectrochemical electrode (i.e., the fumasep + AQDS electrode in contact with the dechlorinating culture) was characterized by cyclic voltammetry at a scan rate of 1, 5, 10, 20 and 50 mV/s, under stirring and no stirring conditions, and by chronoamperometry (alternating cycles at a set electrode potential of -300 mV with open circuit experiments).

Figure 4.3a presents the CV profiles evolution, at three different scan rates 1, 5 and 10 mV/s, during the biotic tests with the fumasep + AQDS (0.25 % (w/v) + 0.5 mM) electrode, before inoculation (cycle 0) and after inoculation (cycle 1, cycle 4 and cycle 11). At cycle 0 redox peaks are well defined (yellow line) but after inoculation with the dechlorinating culture, cycle 1 (red line), an increase in the cathodic peak current attributed to the mineral media and a shift in the potential of both redox peaks, is seen. At cycle 4 there is an increase in the cathodic peak current from the mineral media and an increase in distance between peaks. It is important to notice that AQDS peak is not particularly well identified at this voltammogram. CV profile from cycle 11 maintained similar configuration to the one of cycle 4 but with AQDS redox peaks clearly identified. When comparing CVs from cycle 0 and cycle 11 it is seen an increase in the media redox peak currents, a decrease in the AQDS redox peak currents and an increase in peak separation of the two redox couples. A probable explanation was that microorganisms and their metabolism altered the dynamic reactions taking place in the bioelectrochemical system (BES).
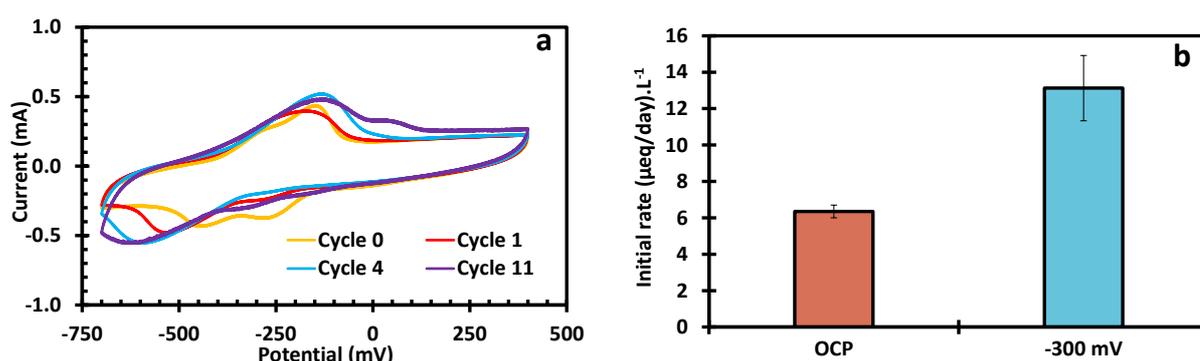
Figure 4.3 – (a) Evolution of cyclic voltammograms (10 mV/s) with no stirring, at four different cycles (0, 1, 4 and 11) in the biotic experiments with fumasep + AQDS electrode. (b) Effect of the cathode potential on the initial 1,2-DCA dechlorination rate, as a function of the two applied conditions, OCP and - 300 mV, in the last six cycles performed.

System polarization had a positive effect on the initial dechlorination rate, as can be seen in fig. 4.3b. At OCP conditions the average rate obtained was 6.35 ± 0.35 µeq/day.L^{-1}, two times lower than the value for the average rate obtained at the polarized experiments, 13.12±1.8 µeq/day.L^{-1}.

Our group previously examined the influence of the set cathode potential on the rate and efficiency of 1,2-DCA dechlorination (Leitão, P., 2015). These bioelectrochemical experiments were performed with a culture previously fed with 1,2-DCA as electron acceptor and hydrogen as electron donor, polarized at five different cathode potentials and OCP, without the addition of external redox mediators and using a pre-treated neat graphite electrode. 1,2-DCA dechlorination rate obtained in Leitão, P. (2015) at OCP and at -300 mV conditions was 4.54 ± 3.58 µeq/day.L^{-1} and 10.65 ± 3.9 µeq/day.L^{-1}, respectively. It should be noted that despite the lower dechlorination rate obtained, the culture used was previously enriched and that cycles performed at low potential led to the production of hydrogen that could have contributed for these results.

Another study from our group examined the effect of a wide range of operating conditions to lab-scale BES treatment, spanning from oxidative to reductive, both in the presence and in the absence of AQDS as a redox mediator (Leitão, P., 2016). The inoculum used consisted of activated sludge from a municipal wastewater treatment plant, the set cathode potential were +500 mV or -300 mV and as an electron acceptor 1,2-DCA or 1,1,2-TCA were used. In the last six cycles 1,2-DCA dechlorination rate at -300 mV with 0.5 mM AQDS in solution was 46.99 ± 4.98 µeq/day.L^{-1}. This high dechlorination rate was probably due to high biomass and diversity content of the inoculum, longer experiment time (almost 50 days longer) and concentration of AQDS was 0.5 mM and maintained at those levels by weekly amendments.

When comparing the results obtained in this study with those from the two previous studies, the addition of AQDS proved to be fundamental to increase dechlorination rates. Also grafting of AQDS at the electrode prevented the need of a constant supply of this redox mediator thru time, an important previous limitation of the technique. However, this grafted fumasep + AQDS electrode displayed lower 1,2-DCA dechlorination rates than the one obtained for AQDS liquid amendments.

Acetic and propionic acid were the only VFA detected. Concentration of acetic acid was 500 mg.L^{-1} at the beginning of the experiment but decreased to 200 mg.L^{-1}, value that remained stable after the seventh cycle. Propionic acid was detected at a concentration of 171 mg.L^{-1} at the beginning of the experiment, decreased to 40 mg.L^{-1} at cycle 6 (day 35) and to levels below the detection limit (<10 mg.L^{-1}) at the last cycle (Figure 4.4a).
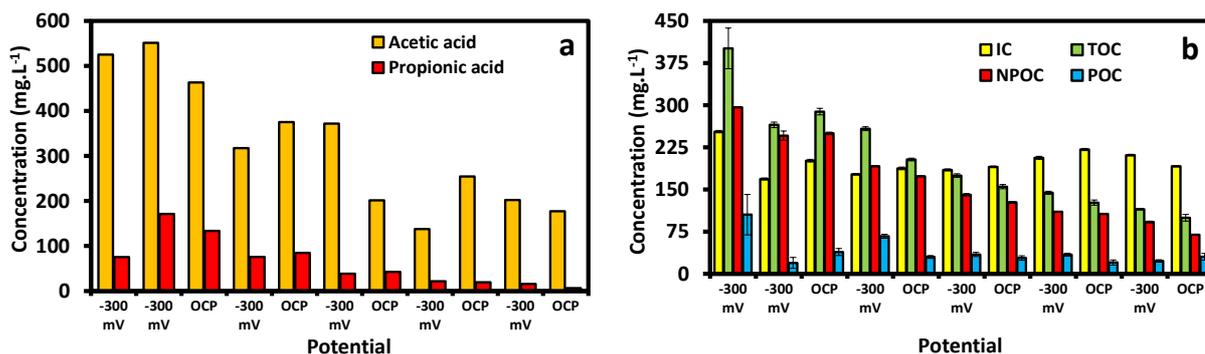
Figure 4.4 – Time course concentration of (a) acetic and propionic acid (b) inorganic carbon, total organic carbon, non purgeable organic carbon and purgeable organic carbon, at the different biotic cycles performed.

TOC analysis performed allowed to evaluate the trend of organic matter in liquid samples from the cathodic chamber (Figure 4.4b). Concentration of inorganic carbon remained stable during the different cycles, 199.3 ± 7 mg.L^{-1}. Total organic carbon concentration decreased from 375 mg.L^{-1}, at the beginning of the experiment, to the lowest value at the last cycle of less than 100 mg.L^{-1}. Taking into account these results biomass decay seems to be occuring. A possibility is that organic matter present in the liquid of the cathodic chamber at the beginning of the experiment decreased during the experiment because of biofilm formation at the surface of the electrode (discussed below at SEM/EDS analysis).

4.6.3 Scanning electron microscopy/ Energy-dispersive X-ray spectroscopy

Samples from the surface of the modified electrode and liquid from the cathodic chamber were analyzed by SEM. As presented in Figure 4.5 a) and b), the surface of the modified electrode was completely covered by a biofilm. Morphology of the microorganisms were mainly rod and coccoid. EDS analysis performed at specific sites (Figure 4.5 c)) revealed large amounts of carbon, and lower amounts of oxygen and sodium.

The liquid sample from the cathodic chamber was composed of rods but low in numbers (Figure 4.5 d)). Carbon, oxygen, silicon and sodium were the main elements detected by EDS at a rod microorganism. Since EDS beam, in this particular case, can penetrate at depths of 3 µm the background was also analyzed, revealing the composition of the graphite electrode, which can explain the large amount of carbon. The amount of carbon decreased substantially at EDS performed in a clear area. Since the liquid sample from the cathodic chamber was mounted in a glass slide, some elements detected are attributed to the glass slide.
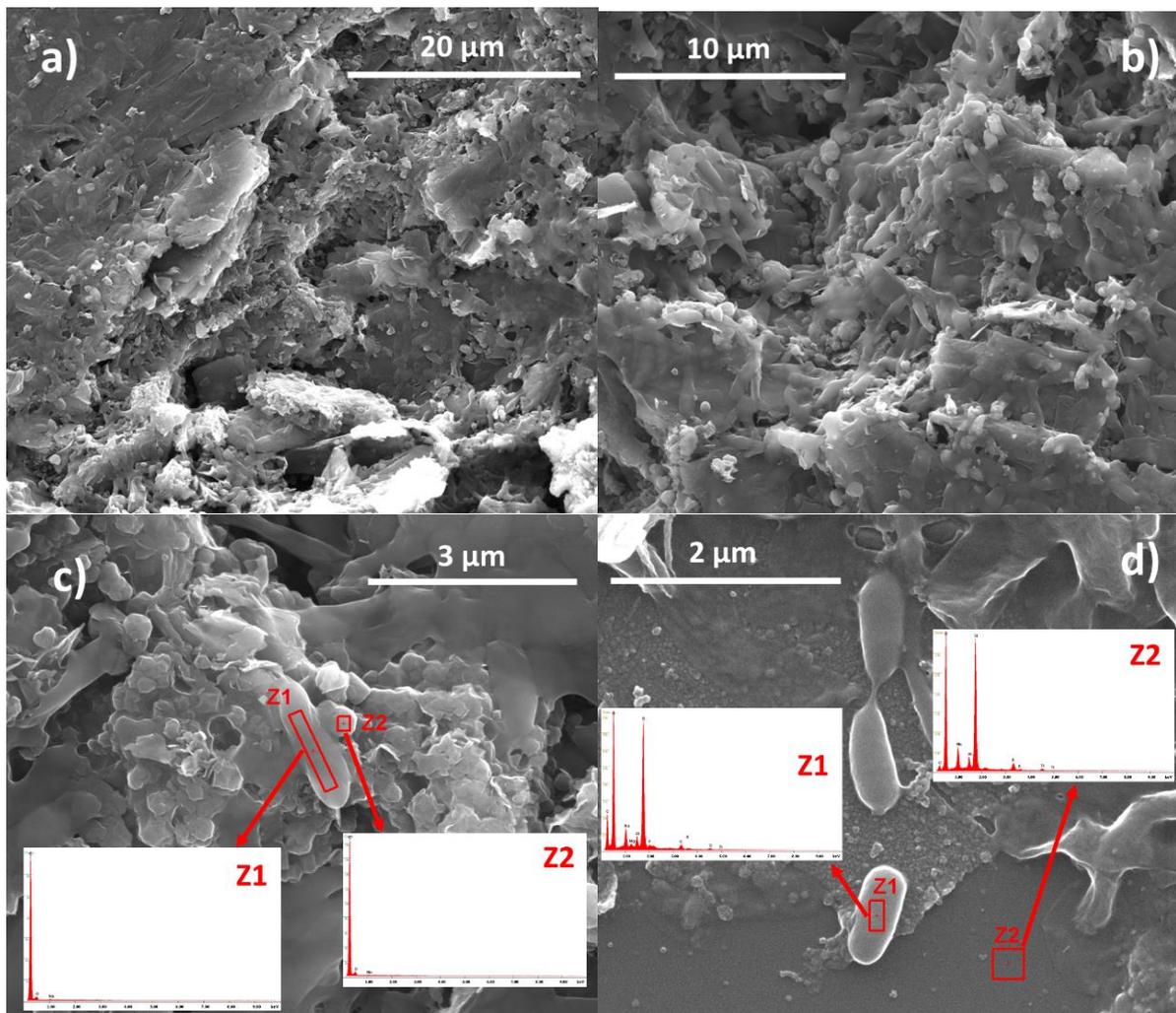
Figure 4.5 - - SEM micrographs and EDS spectra of the surface of the modified electrode (a), b) and c)) and from the liquid of the cathodic chamber (d)).

These results indicate that microorganisms thrive at the surface of the electrode. A possible explanation could be because it allows a solid surface to adhere and/or provides an easier access to the elements needed for halorespiration. A schematic representation of the bioelectrochemical apparatus and the mechanisms taking place are presented at figure 4.6.
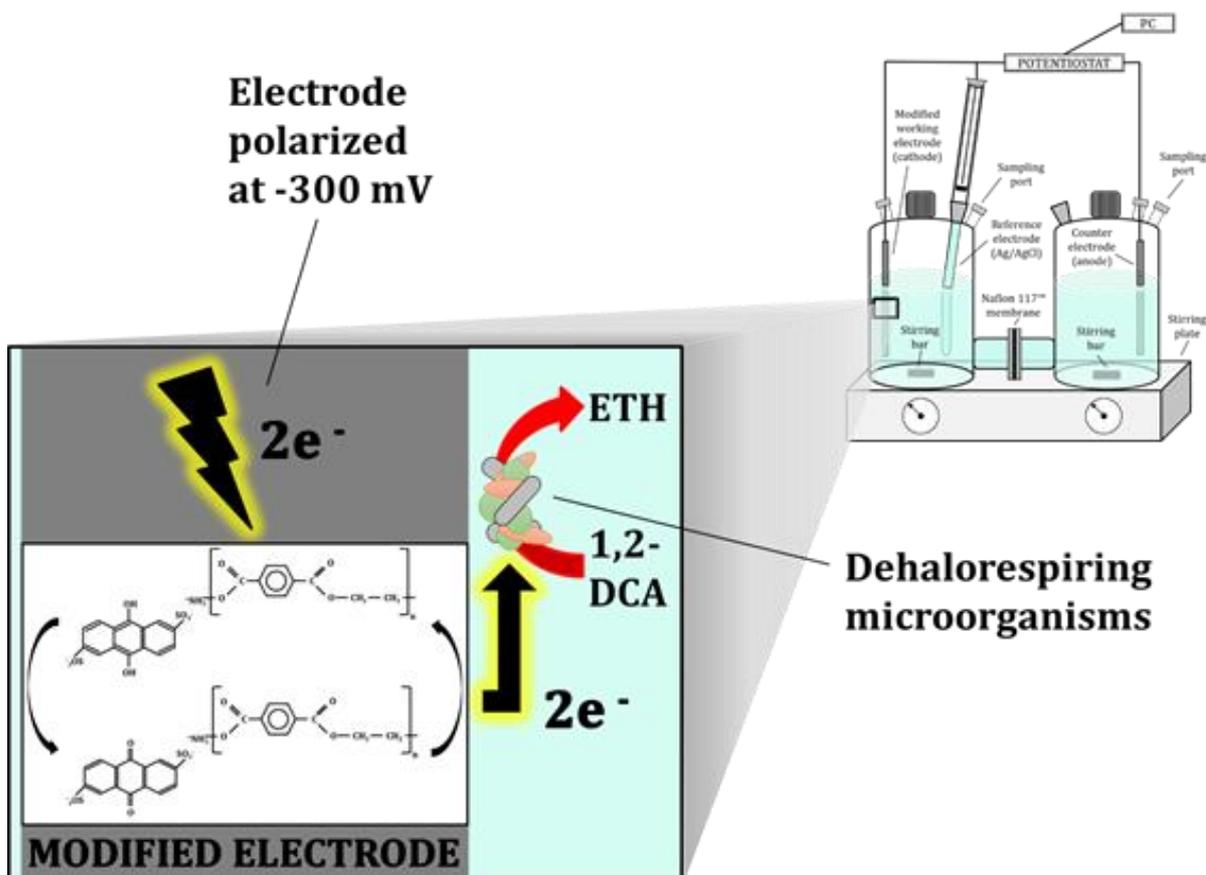
Figure 4.6 - Schematic representation of the BES setup and processes involved.

4.7 Conclusions

Fumasep anionic membrane allowed immobilization of the redox mediator AQDS at graphite electrodes. The method described is simple, fast, low-cost and reproducible. Batch experiments with a dechlorinating culture spiked with 1,2-DCA were performed with the fumasep + AQDS electrode potentiostatically controlled at -300 mV and at OCP. Measured reductive dechlorination rate at -300 mV cycles was 2-fold higher than the rate at OCP cycles, indicating a clear dechlorination enhancement in cycles with applied potential. Over time (> 90 days), AQDS remained stably attached to the cathode. These results suggest that AQDS can be effectively retained at the surface of a graphite electrode without losing electrochemical features. Microorganisms thrive at the surface of the modified electrode over the liquid state, probably because it provides a solid surface to adhere and form a biofilm and also because elements needed to perform catalytic reactions are more readily available. The fumasep + AQDS electrode could be applied to accelerate the bioremediation process at a contaminated site, eliminating the need of repeated injections of an AQDS solution, possible problems in the dispersion of AQDS solution to the subsoil, economic over costs and regulatory restrictions.

4.8 Acknowledgements

4.9 References

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5 Impact of magnetite nanoparticles on the syntrophic dechlorination of 1,2-dichloroethane

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5.1 Highlights

- Magnetite had a positive effect in 1,2-DCA dechlorination increasing by 3-fold dechlorination rates as well as a decrease by 20% decrease in the lag time for the onset of dechlorination.

- Direct correlation was established between the presence of Dehalococcoides mccartyi and dechlorination activity.

- Magnetite nanoparticles were not detected in the cell wall of the microorganisms suggesting a role for interspecies extracellular electron transfer.

- Future applications of magnetite conductive nanoparticles are envisioned as a syntrophic conductive 'bio-grid' for the cleanup of contaminated groundwater.

5.1.1 Keywords

Dechlorination; Magnetite; *Dehalococcoides mccartyi*; 1,2-Dichloroethane.

5.2 Graphical abstract
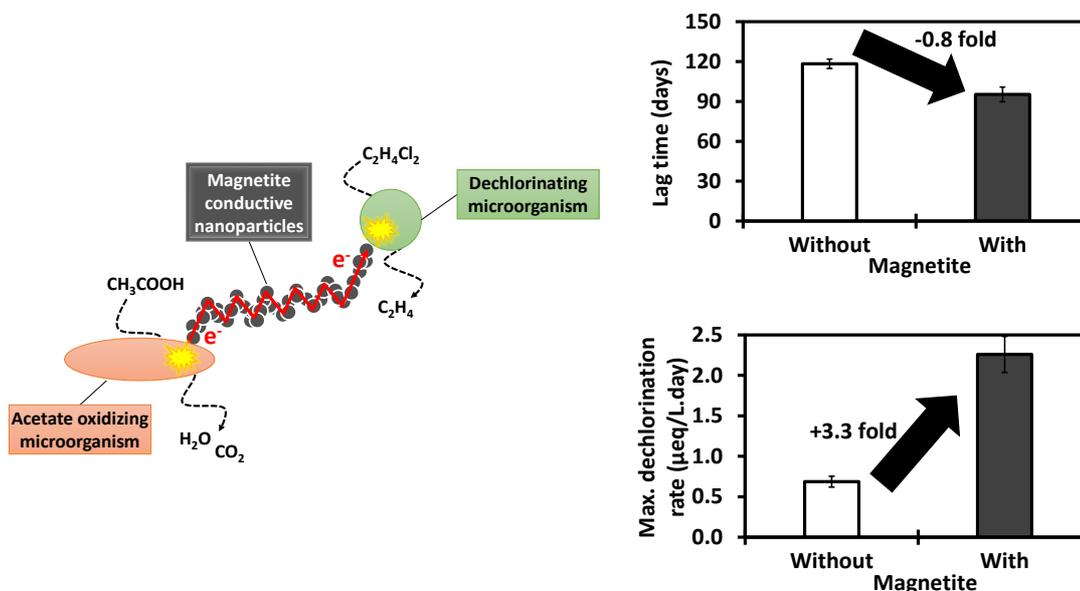
5.3 Abstract

In anaerobic environments microorganisms exchange electrons with community members and with soil and groundwater compounds. Interspecies electron transfer (IET) occurs by several mechanisms: diffusion of redox compounds or direct contact between cells and may be facilitated by conductive nanoparticles (NPs) possibly serving as an electron conduits. Our study examined the effect of magnetite (Fe_3O_4) NPs on the dechlorination of 1,2-DCA by a mixed-culture. The addition of NPs (170 mg/L total Fe) enhanced the 1,2-DCA dechlorination kinetics up to 3.3-times, while decreasing the lag time by 0.8 times (23 days) when compared to unamended microcosms. Dechlorination activity was correlated with the abundance of *Dehalococcoides mccartyi*, indicating a key role of this microorganism in the process. Given the widespread abundance of conductive minerals in the environment, the results of this study may provide new insights into the fate of 1,2-DCA and suggest new tools for its remediation by linking biogeochemical mechanisms.

5.4 Introduction

The chlorinated aliphatic hydrocarbon (CAH) 1,2-DCA is commonly used as a degreasing agent and as a precursor for the production of polyvinylchloride. As such, it is frequently detected in contaminated soil and groundwater due to improper handling, storage and disposal (Dinglasan-Panlilio, M., 2006).

CAHs, including 1,2-DCA, may be reduced chemically or biologically under anaerobic conditions. Microorganisms may serve as catalysts to carry out this transformation via reductive dechlorination or dihaloelimination into lesser or even non-chlorinated end products. Dehalorespiration is the growth linked process where microorganisms use an electron donor, such as hydrogen, and the CAHs as an electron acceptor (De Wildeman, S., 2003a). Several microorganisms have been shown to carry out these reactions and include those from the genus *Dehalococcoides* (Duhamel, M., 2007; He, J., 2003; Maymó-Gatell, X., 1999; Maymó-Gatell, X., 1997), Dehalobacter (Grostern, A., 2009) and *Desulfitobacterium* (De Wildeman, S., 2003b; Maes, A., 2006; Marzorati, M., 2007).

A variety of techniques have been developed for the remediation of CAHs. In-situ biostimulation of these dechlorinating populations is one technology which is commonly used due to its low cost and is more environmentally friendly when compared to other technologies (Zhang, S., 2016). This is usually done via the injection of a fermentable electron donor, such as lactate.

Recent evidence has shown that magnetite and other conductive minerals serve as electrical conduits to facilitate electron transfer between species. A specific example is the combination of *Geobacter sulfurreducens* and *Thiobacillus denitrificans*, which coupled acetate oxidation and nitrate reduction in the presence of magnetite nanoparticles (NPs) (Kato, S., 2012). In another study, electron transfer during ethanol oxidation by *G. sulfurreducens* was facilitated by activated carbon during the reduction of fumarate by *Geobacter metallireducens* and carbon dioxide by *Methanosarcina barkeri* (Liu, F., 2012; Morita, M., 2011). A recent study (Gacitúa, M., 2014) showed that H_2 production in a *Desulfovibrio paquesii* biocathode could be improved and stabilized by adding magnetite (NPs).

Nevertheless, limited information is available on the interactions between minerals present (or produced) in groundwater and dechlorinating communities. Some studies have suggested that iron and manganese minerals may inhibit CAH dechlorination by serving as more suitable electron acceptors (Lu, X., 2001; Zaa, C., 2010). Others demonstrated that

magnetite stimulated TCE reduction using acetate as an electron donor (Aulenta, F., 2013). Interestingly, in microcosms that contained magnetite both *Desulfitobacterium* spp. and *Desulforomonas* spp. were enriched but *Dehalococcoides mccartyi* was outcompeted (Aulenta, F., 2014).

Despite these findings, it is still unknown whether similar results may be obtained with other CAHs. Therefore, the goal of this work was to explore the impact of magnetite nanoparticles on 1,2-DCA dechlorination. Experiments were carried out to examine the impact of magnetite on dechlorination rates and lag time, as well as the interactions between different microbial community groups that are involved in the process.

5.5 Material and methods

5.5.1 Magnetite nanoparticles synthesis

Magnetite NPs were synthetized as previously described (Kang, Y., 1996). The procedure consisted of successively adding the following reagents and solutions with constant stirring: 0.85 mL of 12.1 M HCl, 25 mL of ultrapure deoxygenated water, 5.2 g of $FeCl_3$ and 2.0 g of $FeCl_2$. This solution was then added dropwise under vigorous stirring to a 250 mL 1.5 M NaOH solution. The magnetic NPs were isolated and the supernatant was decanted.

Afterwards, ultrapure deoxygenated water was added to the precipitate which was followed by centrifugation (4000 rpm) and decantation. This procedure was performed in triplicate. To neutralize the anionic charges present on the surface of the NPs, 500 mL of 0.01 M HCl was added to the precipitate under stirring. Afterwards, the solution was centrifuged (4000 rpm) and peptized with water. The magnetite NPs were stored at 4 °C until use.

5.5.2 Mixed dechlorinating culture

The dechlorinating culture used for these experiments was an electroactive culture enriched in a MES with 1,2-DCA as electron acceptor, a graphite rod as the electron donor, and AQDS as redox mediator (Leitão, P., 2016). The original inoculum used for these MES experiments was activated sludge taken from a treatment plant at Roma Nord, Italy. When MES experiments ended, the content of the cathode chamber (95 mL) was anaerobically transferred to 120 mL serum bottles which were then sealed with Teflon faced butyl rubber stoppers and aluminum crimp caps. This reactor was operated in a semi-continuous mode as follows: each week, the serum bottles were sparged with N_2 to remove any

remaining volatile compounds. Then, a liquid aliquot was anaerobically replaced with fresh anaerobic media, 1,2-DCA (0.05 mmol) and H_2 (0.8 mmol). The hydraulic retention time was maintained at 30 days. The reactor was operated in this manner for 4.5 hydraulic retention times (135 days) before the beginning of the experiments.

5.5.3 Microcosm experimental setup

Experiments were performed in 120 mL serum bottles with a total liquid volume of 90 mL. Five different experimental conditions were considered and are listed in Table 1. Each treatment was carried out in duplicate.

Table 5.1 - Experimental setup of the microcosms. Each treatment was performed in duplicate.

Microcosms / Component	Treatment A	Treatment B	Treatment C	Treatment D	Treatment E
Mineral Media	86 mL	88 mL	84 mL	84 mL	84 mL
Culture	---	2 mL	2 mL	2 mL	2 mL
Magnetite	4 mL	---	4 mL	4 mL	4 mL
1,2-DCA	4 µL	4 µL	---	4 µL	4 µL
Acetate	10 mM	10 mM	10 mM	---	10 mM

The mineral medium contained the following components: NH_4Cl (0.5 g.L^{-1}), $MgCl_2 \cdot 6H_2O$ (0.1 g.L^{-1}), K_2HPO_4 (0.4 g.L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.05 g.L^{-1}), trace metal solution (10 mL.L^{-1}) (Zeikus, J., 1977), vitamin solution (10 mL.L^{-1}) (Balch, W., 1979), and $NaHCO_3$ (15 mL.L^{-1}, 10 % w/v). The pH of the medium was 7.5.

After the addition of the solutions, the bottles were sealed with Teflon-faced butyl rubber stoppers and aluminum crimp caps. The bottles were then flushed with N_2 and 10 mL of CO_2 was added. All microcosms were incubated upside down, in the dark, at 25 ºC under mild agitation. The microcosms were operated in a semi-continuous fashion so that the hydraulic retention time was approximately 120 days.

5.5.4 Analytical methods

1,2-DCA, vinyl chloride (VC), ethene (ETH) and methane (CH_4) were monitored by gas chromatographic analysis of headspace samples (50 µL taken with a gas-tight, gas-locking syringe). A Shimadzu GC-2014 equipped with a flame ionization detector and a 2.4 m x 2.1 mm 60/80 Carbopack B/ 1 % SP-1000 column was used. The temperature was initially set at 60 ºC followed by a ramp of 40ºC per minute until 180 ºC which was held for 1.25 min. The carrier gas was N_2 (40 mL.min^{-1}) and the temperatures of the injector and the detector were 200 ºC. Headspace concentrations were converted to aqueous-phase concentrations using Henry's law constants (Gossett, J, 1987).

The iron load in solution was measured using an Oxford X-ray fluorescence X-MET7500 Instrument. Data was analyzed with the X-MET software by linear regression of iron standard solutions.

5.5.5 Biomolecular analysis

For CARD-FISH analysis, 500-µL samples were collected from the inoculum and from the microcosms at the end of cycles 2, 4 and 5 with a sterile syringe and immediately fixed in formaldehyde (2 % (v/v) final concentration) for 3 h at 4 ºC. Fixed samples were filtered through polycarbonate membrane filters (pore size 0.2 µm, diameter 47 mm, Millipore) by gentle vacuum (<0.2 bar) and were then stored at -20 ºC until further processing. CARD-FISH assays were performed to quantify members of *Archaea* (Arc915 probe), *Bacteria* (Eub338 I, II, III probes), *Dehalococcoides mccartyi* (Dhe1259 c and Dhe1259 t probes) and *Desulfitobacterium* spp. (Dsf440 probe plus Dsf475 helper probes) as previously described (Di Battista, A., 2012).

The probes were purchased from BIOMERS (http://www.biomers.net) and were labeled at the 50-end with HRP. Details of the oligonucleotide probes used are available at http://www.microbial-ecology.net/probebase. After oligonucleotide probe hybridization, total cell quantification was done by counterstaining the sample with VECTASHIELD® mounting medium containing 1.5 µg.mL^{-1} of DAPI (Vector Laboratories). Visualization was performed using an Olympus BX51 epifluorescence microscope (Olympus Portugal) equipped with filters sensitive to the applied fluorochromes. Quantification of fluorescent cells was done by counting random grids on filters. All images were acquired using the Olympus CellB (Olympus Portugal) software with a magnification of 1,000X. Error bars were calculated as standard deviations of cell counts using at least 5 microscopic grids for each filter.

5.5.6 Scanning electron microscopy/ Energy-dispersive X-ray spectroscopy analysis

Magnetite NPs that were added to the microcosms were analyzed by SEM/EDS. This was done by mounting 50 µL of synthetized magnetite NPs solution directly on aluminum stubs which were dried at 80 ºC for 24 hours.

Samples from treatments B and E were also analyzed by SEM/EDS; at the end of the experiment, a 5 mL sample was removed and centrifuged at 5000 rpm for 5 min. After removing the supernatant, the sample was fixed in 3 % (w/v) glutaraldehyde in cacodylate

buffer (pH 7.2) for 3 hours at room temperature. The combined mixture was centrifuged (5000 rpm, 5 min) and almost all of the supernatant was removed. A small quantity (250 µL) of the remaining solution was then mounted on an aluminum stub and dried at 50 ºC for 3 hours. Afterwards, the samples were dehydrated by immerging the stub with the fixed sample in 50 % and 100 % (v/v) (2x) ethanol solutions for 10 min each at room temperature. This was then followed by a series of HMDS in ethanol treatments at 50, 60, 70, 80, 90 and 2x 100 % (v/v) HMDS for 10 min each at room temperature. These dehydration steps were necessary to preserve the integrity of biological structures.

Stubs were then stored in a desiccator until SEM analysis for a maximum period of 24 hours in order to prevent moisture absorption/adsorption. The samples were sputter-coated for 60 s at 15 mA current with a palladium–gold thin film using the SPI Module Sputter Coater equipment.

All samples were analyzed with a SEM/EDS system (FEI Quanta 400FEG ESEM/EDAX Genesis X4M, FEI Company, USA) in high-vacuum mode at 10 or 15 kV to observe size, morphology and distribution. X-ray microanalysis was performed in specific fields for elemental characterization.

5.6 Results and discussion

5.6.1 Kinetics of 1,2-DCA dechlorination and methane production

The effect of magnetite NPs on dechlorination and methane production was evaluated in all microcosms. The concentration of iron included in the treatments with magnetite was approximately 170 mg Fe.L^{-1}. Figure 5.1 shows the results of dechlorination and methanogenesis rates for the different treatments for each cycle. Dechlorination was observed for treatments B, D and E, although there were differences in rates and lag times. Dechlorination was first observed during cycle 4 (day 95) for Treatment E but only during cycle 5 (day 118) for Treatments B and D. This corresponded to a 0.8-fold decrease between Treatments E and B (Figure 5.2A). It should be noted that VC represented less than 1% of the amount of 1,2-DCA that was removed (data not shown). Maximum dechlorination rates were 3.3 times higher in Treatment E when compared to Treatment B (Figure 5.2B) and 50 times higher than in Treatment D (data not shown). This very low dechlorination activity in Treatment D was likely sustained by the decay of organic matter that served as an electron donor.
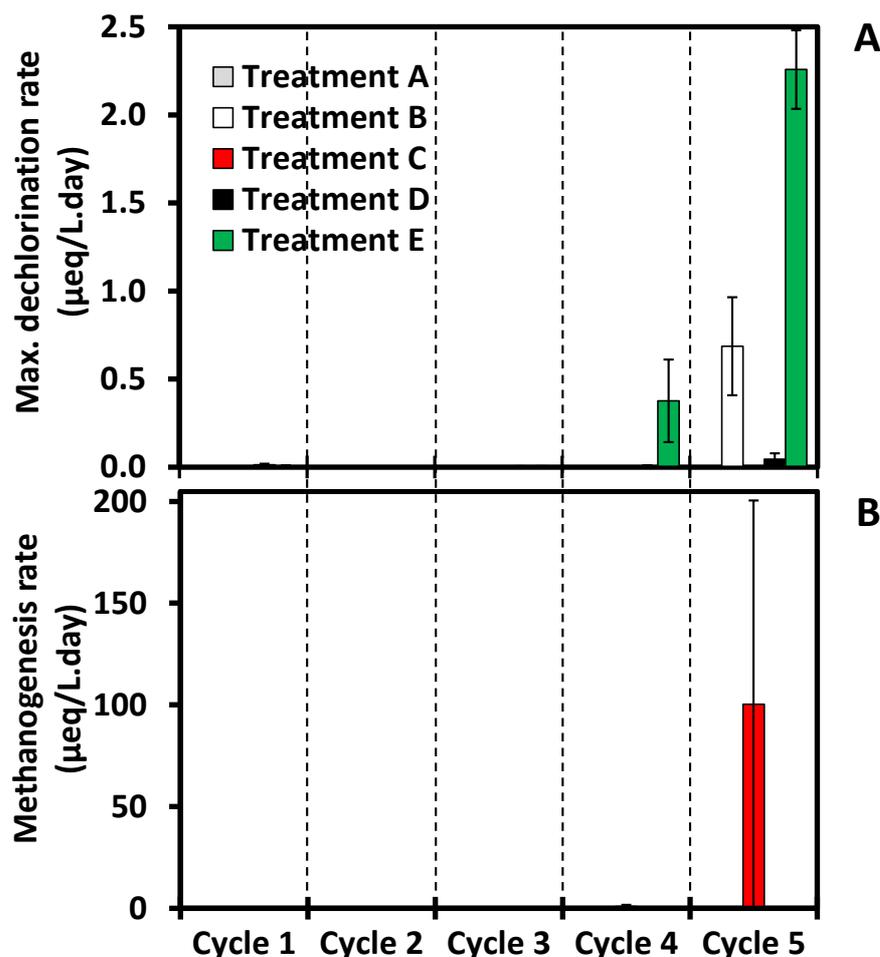
Figure 5.1 - Dechlorination rate (A) and methanogenesis rate (B) for the different experimental set-ups: Treatment A (not amended with the dechlorinating culture), Treatment B (not amended with magnetite), Treatment C (not amended with 1,2-DCA), Treatment D (not amended with acetate) and Treatment E (amended with all the components). Error bars represent the standard deviation of duplicates.

Overall, the dechlorination rates obtained in this study are comparable to those reported by others. Aulenta et al., 2013 showed that the TCE dechlorination rate increased by an average of 1.5 fold in the presence of magnetite during the first two feeding cycles. Although, the iron concentration was less than 10 mg.L^{-1}, 17 times lower than in the present study. Another study from the same group (Aulenta et al., 2014) tested the effect of filtered and untreated magnetite NPs using the same inoculum. TCE dechlorinating rates increased 4.5 times (filtered magnetite particles) and 9.5 times (untreated magnetite particles) when compared to treatments without magnetite. The inoculum used in those studies was a mixture of a TCE reducing culture and an acetate oxidizing culture (Aulenta et al., 2013). This acetate oxidizing culture was enriched in a MES and was highly efficient and oxidized more than 90 % of the added acetate at the anode (Villano et al., 2012), which may have contributed to the higher rates observed in that study compared to the present one. The inoculum used in this study was not previously fed with acetate.
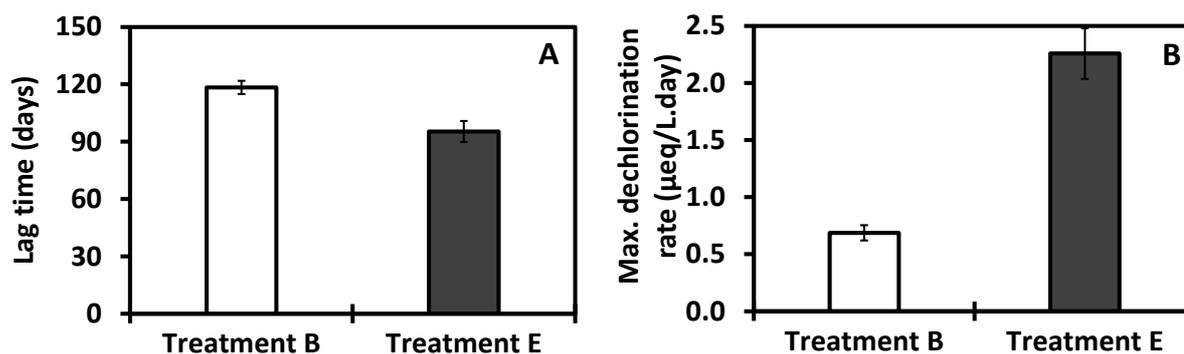
Figure 5.2 - A Dechlorination lag time (days) for microcosms that were not amended with magnetite (Treatment B) and those amended with all the components (Treatment E). B Maximum dechlorination rates (µeq/L.day) for microcosms that were not amended with magnetite (Treatment B) and those amended with all the components (Treatment E). Error bars represent the standard deviation of duplicates.

The highest methanogenic rate (100.30 µeq/L.day) was observed in cycle 5 for Treatment C, which did not contain 1,2-DCA. Niche adaptation of the microorganisms present might be responsible for this methanogenesis since reducing equivalents resulting from acetate oxidation could have been diverted from dechlorination towards methane production. Methanogenic rates for Treatments B and E were significantly lower than for Treatment C. The methanogenic rate observed in Treatment B was approximately 2.5 fold higher than the one obtained in Treatment E (0.0024 µeq/L.day and 0.00095 µeq/L.day, respectively).

5.6.2 Molecular and microscopy characterization of *Archaea* and *Bacteria*

Qualitative and quantitative analysis of *Archaea* and *Bacteria* domains using CARD-FISH of the original culture used to inoculate microcosms and of the microcosms of the treatments containing biomass at Cycles 2, 4 and 5 (Figure 5.3). The abundance of archaea and bacteria in the inoculum used was $7.88 \pm 1.31 \times 10^{10}$ cell numbers.mL^{-1} and $1.66 \pm 4.17 \times 10^{10}$ cell numbers.mL^{-1}, respectively (data not shown).
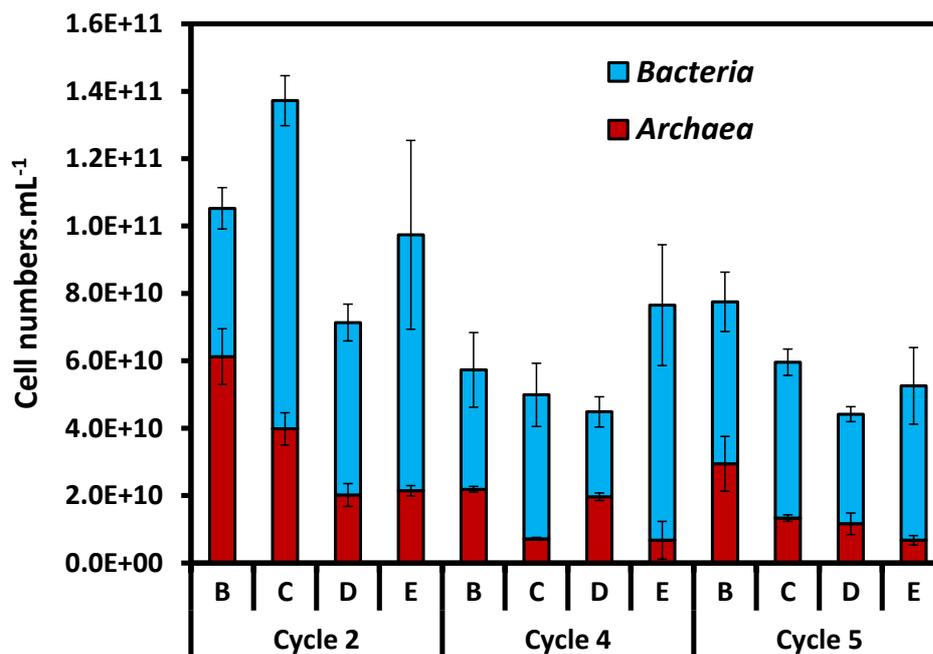
Figure 5.3 - Abundances of *Archaea* and *Bacteria* in microcosms not amended with magnetite (B), not amended with 1,2-DCA (C), not amended with acetate (D) and amended with all the components (E). Error bars represent the standard deviation of duplicates.

Archaea abundance was consistently higher for Treatment B (2.9, 3.1 and 4.4 fold in cycles 2, 4 and 5, respectively) when compared to Treatment E. Micrographs of Archaea counterstained with DAPI for treatments B, C, D and E are presented at fig. 5.4.
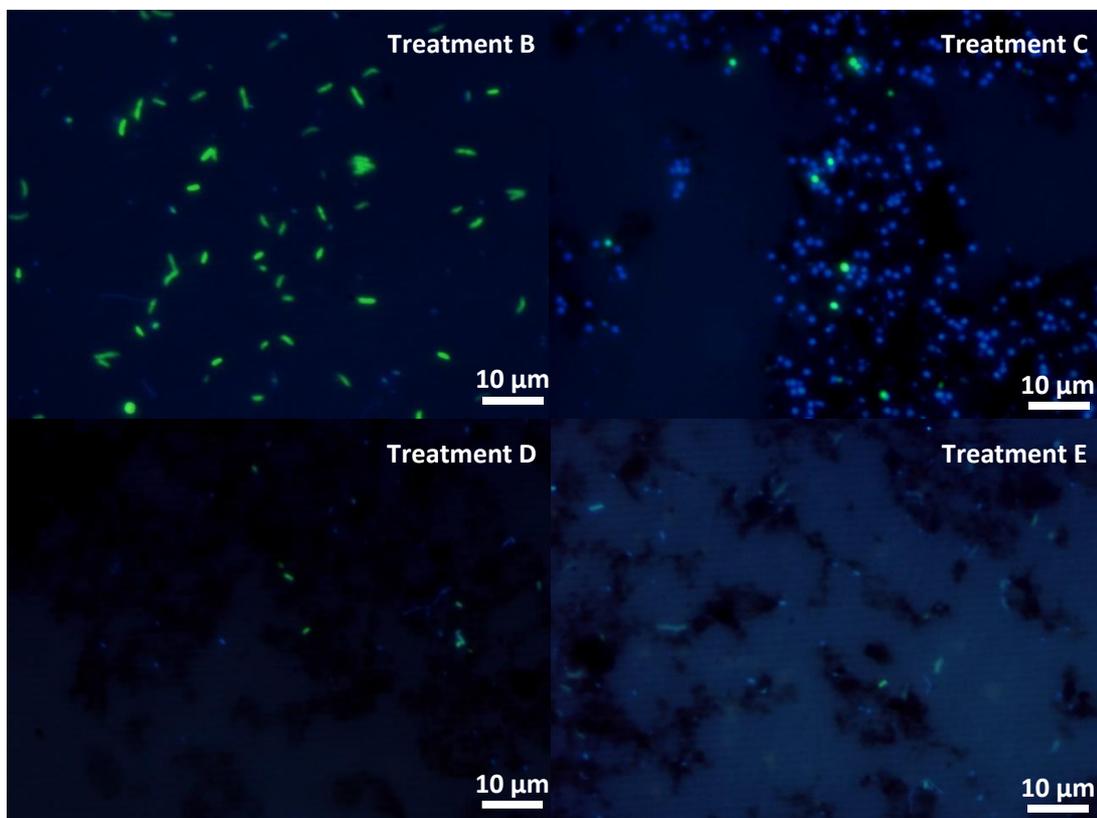
Figure 5.4 - Micrographs of Archaea (in green) with DAPI (blue) counterstaining for Treatments B, C, D and E.

Bacterial abundance in Treatment B initially decreased from cycle 2 to cycle 4 and increased in cycle 5 to levels similar to those found in cycle 2. The results for Treatment E showed that bacterial cell densities were lower in cycles 4 and 5 compared to cycle 2. For Treatment C, both archaea and bacteria decreased from cycle 2 to cycle 4 and then increased during cycle 5. This increase in archaea occurred at the time when methane production was observed. Interestingly, archaea cell abundance in Treatment C was lower than in Treatment B during cycle 5. This result suggests a more robust syntrophic partnership between acetate oxidizers and methanogens in the presence of magnetite without the competition from dechlorinating bacteria, which is consistent with the results of a previous study (Cruz Viggi et al., 2014). In addition, archaea cell numbers in Treatment E were lower than both Treatments B and C during this same cycle. This seems to indicate that the reducing equivalents from acetate oxidation were more efficiently transferred to the dechlorinators at the expense of methanogens. Archaeal and bacterial populations decreased throughout the experiment in Treatment D. This was likely due to the fact that because acetate was not added, syntrophic interactions between acetate oxidizing and halorespiring microorganisms were limited. Micrographs of Bacteria counterstained with DAPI for treatments B, C, D and E are presented at fig. 5.5.
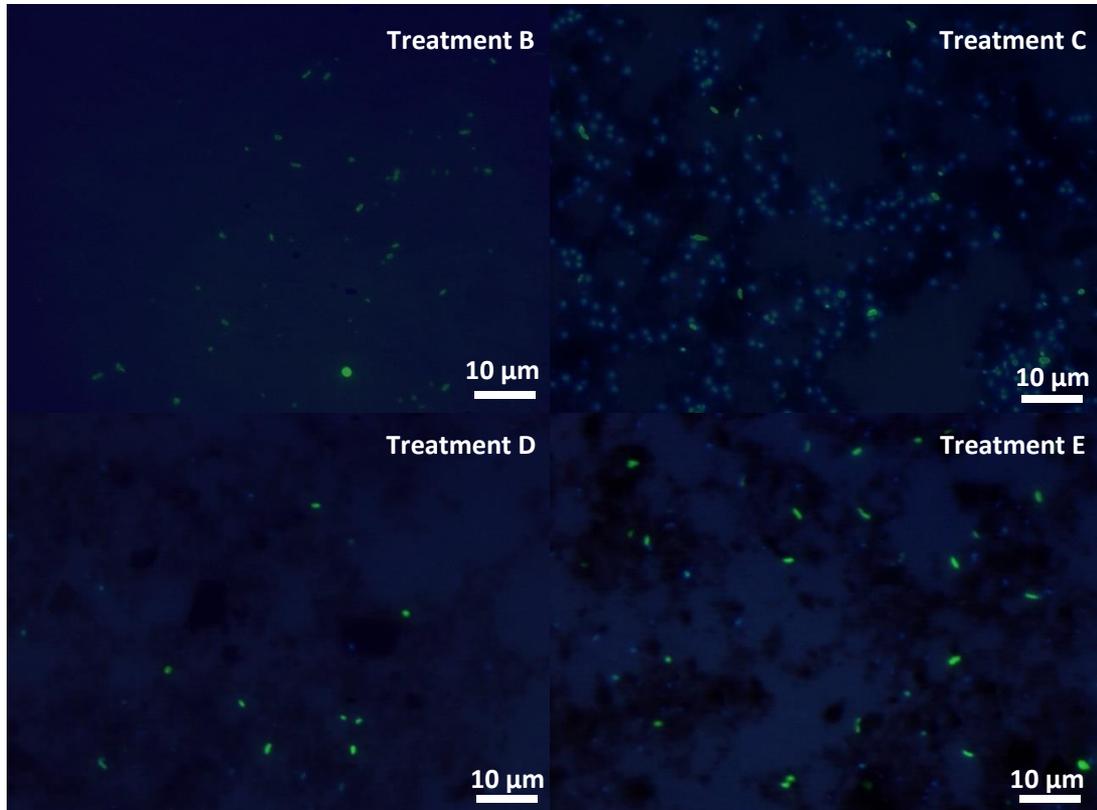
Figure 5.5 - Micrographs of Bacteria (in green) with DAPI (blue) counterstaining for Treatments B, C, D and E.

5.6.3 Molecular and microscopy characterization of dechlorinating microorganisms

CARD-FISH analysis was also performed with oligonucleotide probes targeting *Desulfitobacterium* spp. and *Dehalococcoides mccartyi* in the original culture used to inoculate microcosms and in treatments containing biomass (Treatments B, C, D and E) in cycles 2, 4 and 5. The abundance of *Desulfitobacterium* spp. and *Dehalococcoides mccartyi* in the inoculum was $7.75 \pm 1.16 \times 10^{10}$ cell numbers.mL^{-1} and $2.02 \pm 0.719 \times 10^{10}$ cell numbers.mL^{-1}, respectively.

Cell densities of these two genera and maximum dechlorination rates during cycles 2, 4 and 5 for Treatments B and E are shown in Figures 5.6 and 5.8, respectively, while the cell densities for Treatments C and D are summarized in Table 5.2.
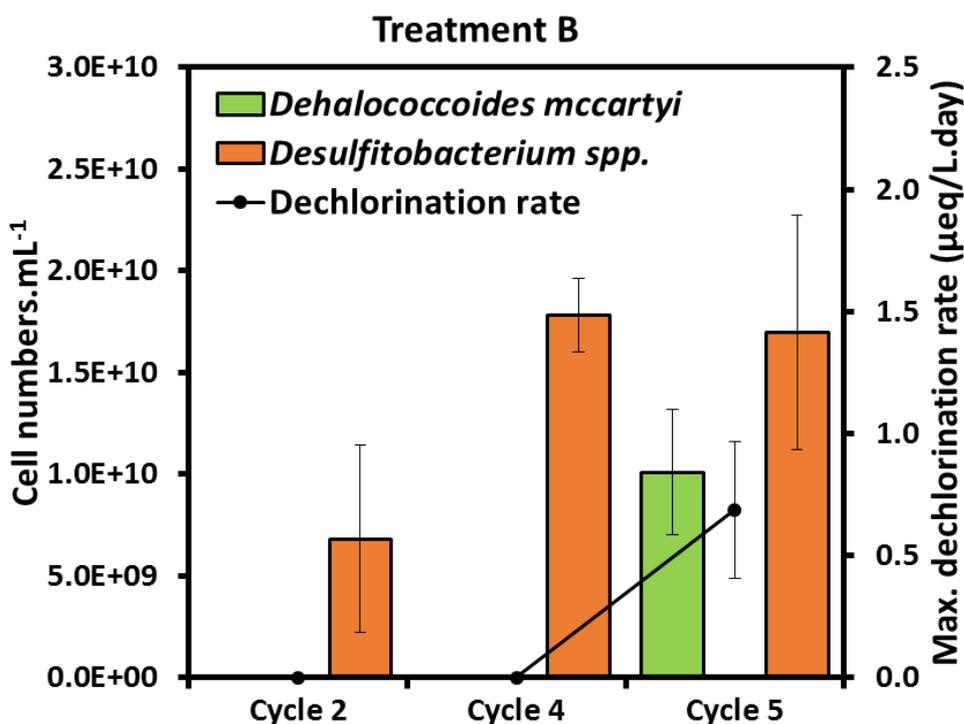
Figure 5.6 - Time course of *Dehalococcoides mccartyi* and *Desulfitobacterium* spp. cell densities and maximum dechlorination rates in microcosms not amended with magnetite (Treatment B). Error bars represent the standard deviation of duplicates.

For Treatment B. in cycle 2, the cell density of *Desulfitobacterium* spp. was $6.8 \pm 4.6 \times 10^9$ cells numbers.mL^{-1} and represented 12 % of total bacteria, while *Dehalococcoides mccartyi* was not detected. The cell density of *Desulfitobacterium* spp. increased to $1.78 \pm 0.18 \times 10^{10}$ cells numbers.mL^{-1} in cycle 4 and remained approximately the same in cycle 5 ($1.70 \pm 0.58 \times 10^{10}$ cells numbers.mL^{-1}). *Dehalococcoides mccartyi* was only detected in cycle 5 with a cell abundance of $1.10 \pm 0.31 \times 10^{10}$ cells numbers.mL^{-1} and the dechlorination rate was 0.69 µeq/L.day.

Micrographs of *Dehalococcoides mccartyi* and *Desulfitobacterium* spp. counterstained with DAPI in cycle 5 for Treatment B are represented in Figure 5.7. The relative abundances of *Dehalococcoides mccartyi* and *Desulfitobacterium* spp. in this cycle were 28.1 % and 42.1 % of the total bacteria, respectively.
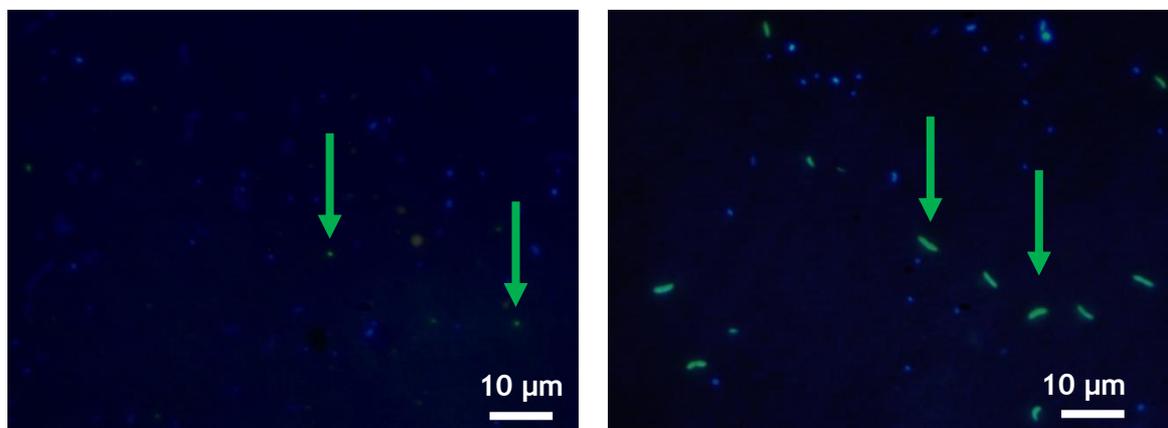
Figure 5.7 - Micrographs of *Dehalococcoides mccartyi* (green) with DAPI (blue) counterstaining (left) and *Desulfitobacterium* spp. (green) with DAPI (blue) counterstaining (right) for microcosms not amended with magnetite (Treatment B). Green arrows are used to enhance the visualization of some microorganisms.

Cell abundance of *Dehalococcoides mccartyi* and *Desulfitobacterium* spp. and maximum dechlorination rates for Treatment E are shown in Figure 5.8. The results obtained for cycle 2 are similar to those for Treatment B, where *Dehalococcoides mccartyi* was not detected while cell abundance of *Desulfitobacterium* spp. was $1.11 \pm 0.24 \times 10^{10}$ cells numbers.mL^{-1}. However, unlike Treatment B, the concentration of *Desulfitobacterium* spp. did not significantly change during the three cycles. In addition, the presence of *Dehalococcoides mccartyi* and 1,2-DCA dechlorination were observed at cycle 4. Rates and cell numbers of *Dehalococcoides mccartyi* continued to increase during cycle 5, with cell numbers similar to those of *Desulfitobacterium* spp. ($1.46 \pm 0.17 \times 10^{10}$ cells numbers.mL^{-1} and $1.37 \pm 0.25 \times 10^{10}$ cells numbers.mL^{-1}, respectively) with a dechlorination rate of 2.26 µeq/L.day.
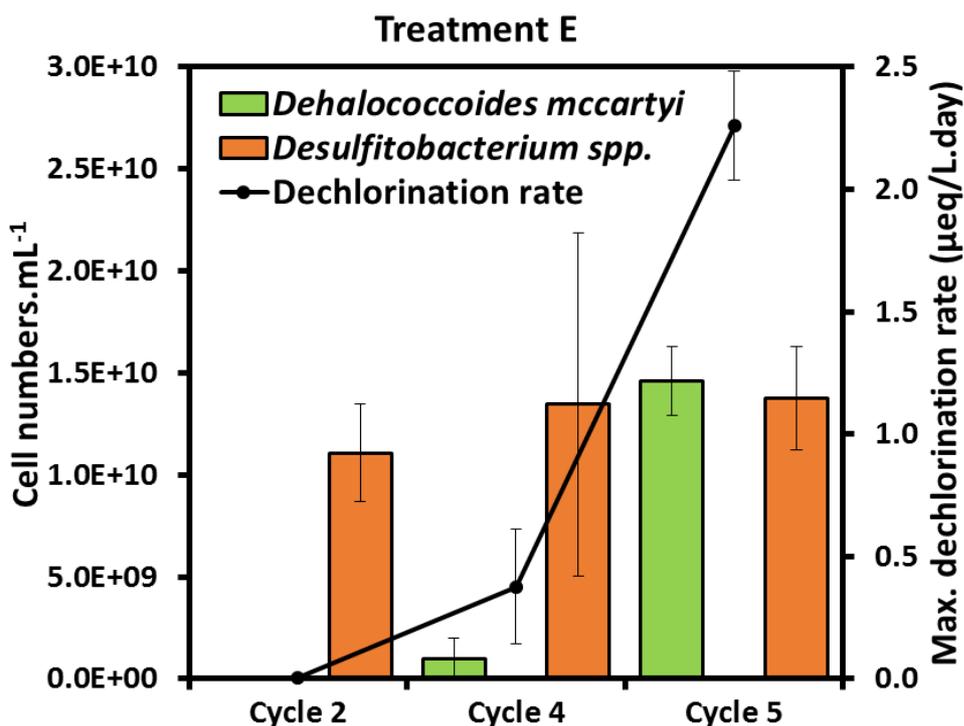
Figure 5.8 - Time course of *Dehalococcoides mccartyi* and *Desulfitobacterium spp.* cell densities and maximum dechlorination rates in microcosms amended with all the components (Treatment E). Error bars represent the standard deviation of duplicates.

Figure 5.9 shows micrographs of *Dehalococcoides mccartyi* and *Desulfitobacterium* spp. counterstained with DAPI in cycle 5 for Treatment E. The relative abundances of *Dehalococcoides mccartyi* and *Desulfitobacterium* spp. in this cycle were 33.5 % and 31.7 % of the total bacteria, respectively.
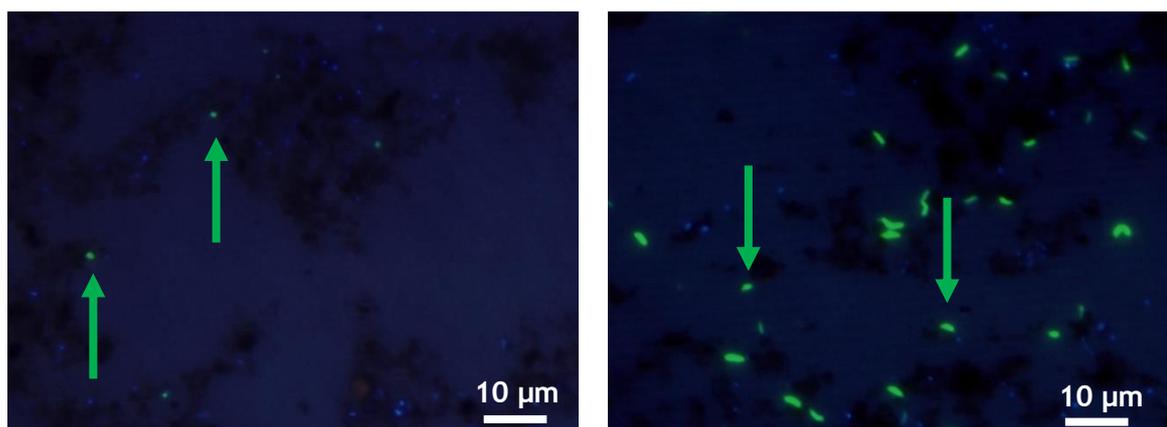
Figure 5.9 - Micrographs of *Dehalococcoides mccartyi* (green) with DAPI (blue) counterstaining (left) and *Desulfitobacterium* spp. (green) with DAPI (blue) counterstaining (right) for microcosms amended with all the components (Treatment E). Green arrows are used to enhance the visualization of some microorganisms.

Treatment C showed an increasing percentage of *Desulfitobacterium* spp. during the three cycles analyzed and the relative abundance during cycle 5 (Table 5.2) was approximately similar to those from Treatment B and E. In comparison, the relative

abundance of both *Desulfitobacterium* spp. and *Dehalococcoides mccartyi* were lower for Treatment D compared to the others.

Table 5.2 - Relative abundance of *Desulfitobacterium spp*. and *Dehalococcoides mccartyi* to total *Bacteria* in Treatments C and D in cycle 2, 4 and 5. Errors represent the standard deviation of duplicates.

Microcosms	Cycle	Dsf/EUB	Dhe/EUB
Treatment C	Cycle 2	14.2 ± 8.6 %	0.0 ± 0.0 %
	Cycle 4	30.0 ± 9.9 %	0.0 ± 0.0 %
	Cycle 5	32.9 ± 11.8 %	0.0 ± 0.0 %
Treatment D	Cycle 2	10.4 ± 2.8 %	0.0 ± 0.0 %
	Cycle 4	13.5 ± 2.8 %	0.0 ± 0.0 %
	Cycle 5	20.6 ± 4.9 %	1.9 ± 2.7 %

Taken as a whole, there is a clear correlation between *Dehalococcoides mccartyi* and dechlorination, even in Treatment D in which the relative abundance was only 1.9 %. In addition, the results also suggest that magnetite and 1,2-DCA played an important role in the earlier detection of *Dehalococcoides mccartyi* compared to other treatments. *Desulfitobacterium* spp. was already abundant in Treatments B through E at cycle 2 and its percentage increased over time. Since the microcosms were operated in a semi-continuous manner, this suggests that *Desulfitobacterium* spp. grew or survived on other substrates present or produced during the experimental period. Although, it is not yet known at this point what direct involvement it played in these tests but several studies have shown that *Desulfitobacterium* spp. and *Dehalococcoides mccartyi* are frequently found together (Duhamel & Edwards, 2007; Rouzeau-Szynalski et al., 2011; Villano et al., 2011).

5.6.4 Scanning electron microscopy/ Energy-dispersive X-ray spectroscopy

Analysis of the synthetized magnetite nanoparticles and samples of Treatment B (without magnetite) and Treatment E (amended with all the components) at the end of the experiment were performed by scanning electron microscopy/ energy-dispersive X-ray spectroscopy (SEM/EDS) in order to evaluate average size, shape and elemental composition in selected fields. Magnetite particle size ranged between 10 nm and 50 nm and was composed mainly of iron and oxygen (Figure 5.10).
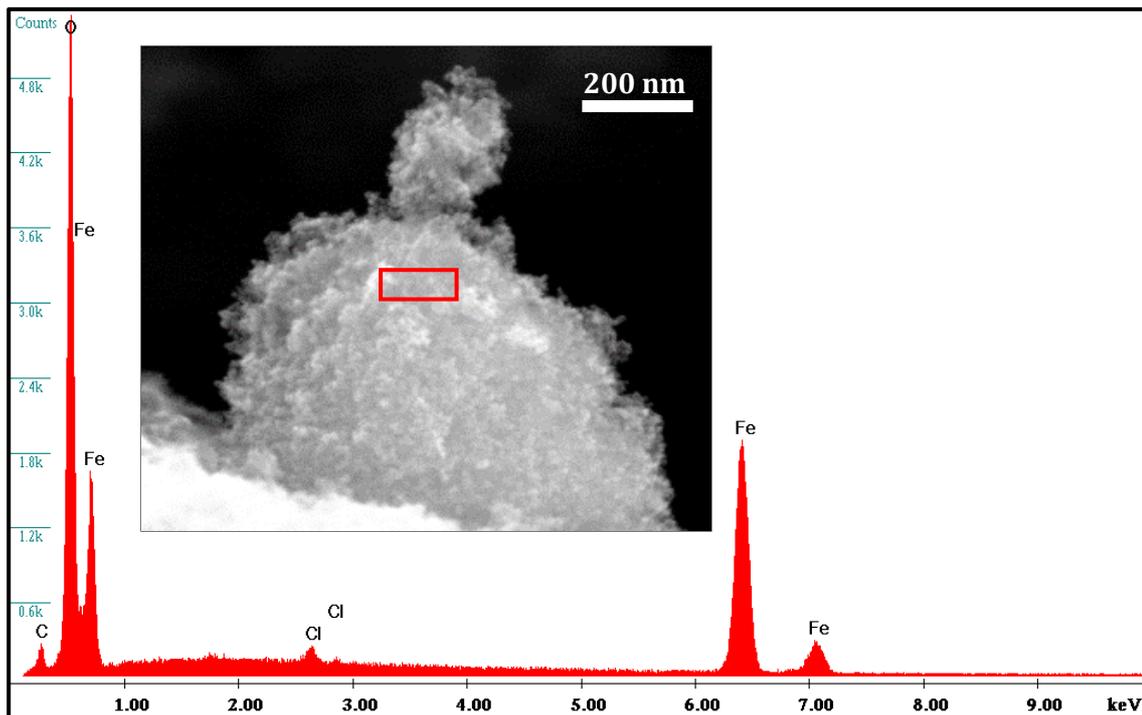
Figure 5.10 - SEM image of sample from neat abiotic synthetic magnetite nanoparticles and the EDS spectrum corresponding to the area outlined in red.

Micrograph and EDS spectra of Treatment B is shown in Figure 5.11. The image shows the presence of mainly rod shape microorganisms with a length of approximately 2 μm and a composition of carbon, oxygen, nitrogen and sodium, which are characteristics of living organisms.
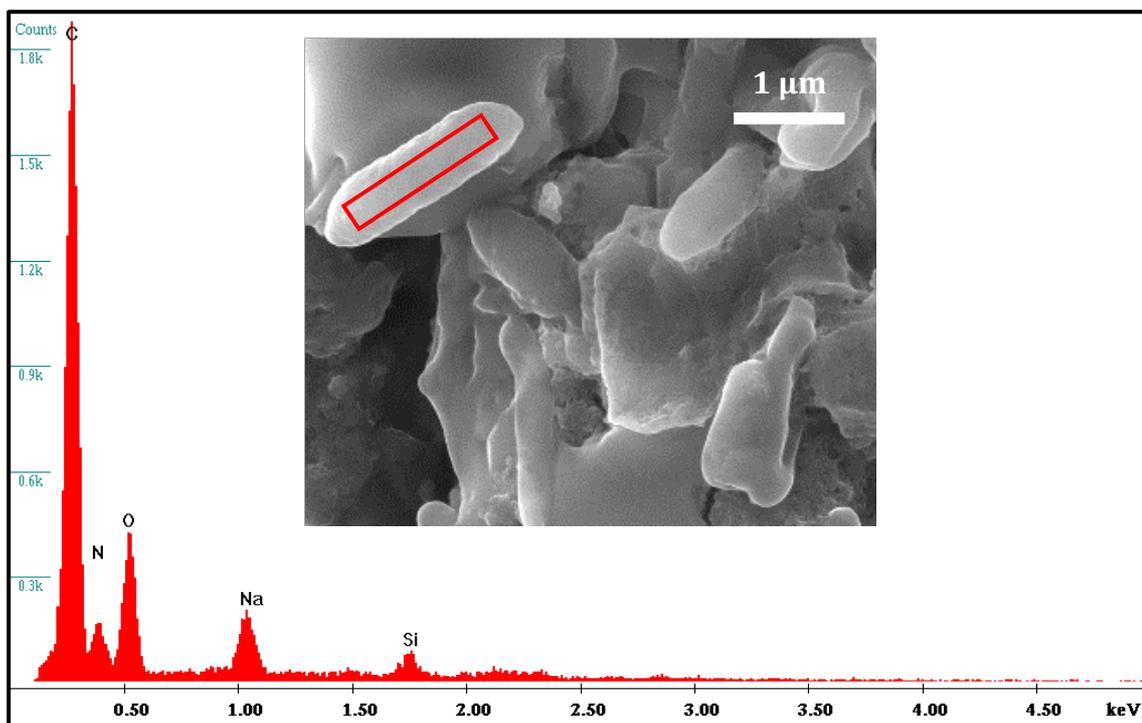
Figure 5.11 - SEM image of a sample from microcosms not amended with magnetite (Treatment B) and the EDS spectrum corresponding to the area outlined in red.

Three different EDS analyses were performed in the same field for Treatment E but in different areas: microorganism (Figure 5.12), magnetite NPs (Figure 5.13) and the aluminum stub (Figure 5.14). Based on the spectrum shown in Figure 5 the microorganism is mainly composed of carbon, oxygen, nitrogen and sodium, similar to the results obtained for Treatment B (Figure 5.11) and confirms that the EDS was performed on microbiological tissue. Aluminum was also detected and can be attributed to the material onto which the sample was mounted, as later confirmed by EDS analysis performed on the background (Figure 5.13). Iron precipitates were not observed and the presence of iron was not detected by EDS analysis (Figure 5.12). This suggests that the microorganisms do not incorporate magnetite into their cell wall.
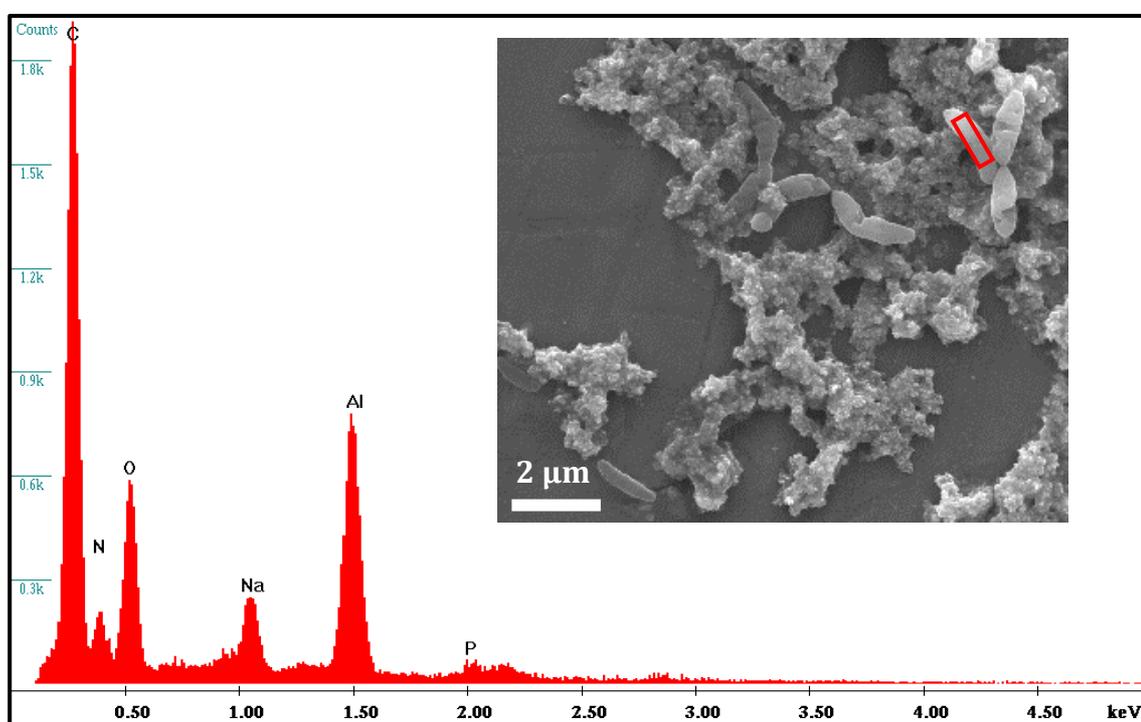
Figure 5.12 - SEM image of a sample from a microcosm amended with all the components (Treatment E) and the EDS spectrum corresponding to the area outlined in red.

The EDS spectrum shown in Figure 5.14 not only revealed high oxygen content, but also the presence of carbon, iron and sodium. Aluminum was the main element identified in a location where no visible material was present (Figure 5.13) and can be attributed to the stub used to mount the sample. Also, small amounts of carbon, oxygen, and copper were detected and were probably due to extracellular exudates expelled by microorganisms.
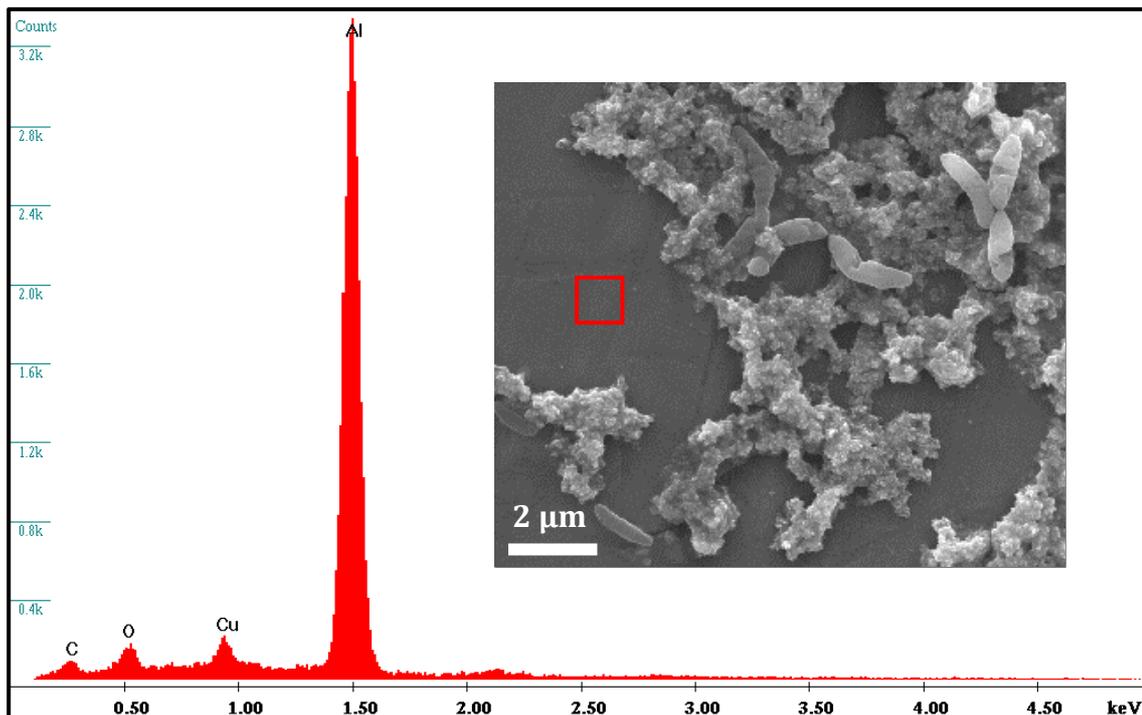
Figure 5.13 – SEM image of sample from microcosms amended with all the components (Treatment E) and the EDS spectrum corresponding to the area outlined in red.

Taken as a whole, results of Figures 5.12, 5.13, and 5.14 indicate that there is a close association between nanoparticles and microorganisms. When combined with the dechlorination results, this suggests that magnetite nanoparticles are serving as conduits for electron transfer between key trophic groups.
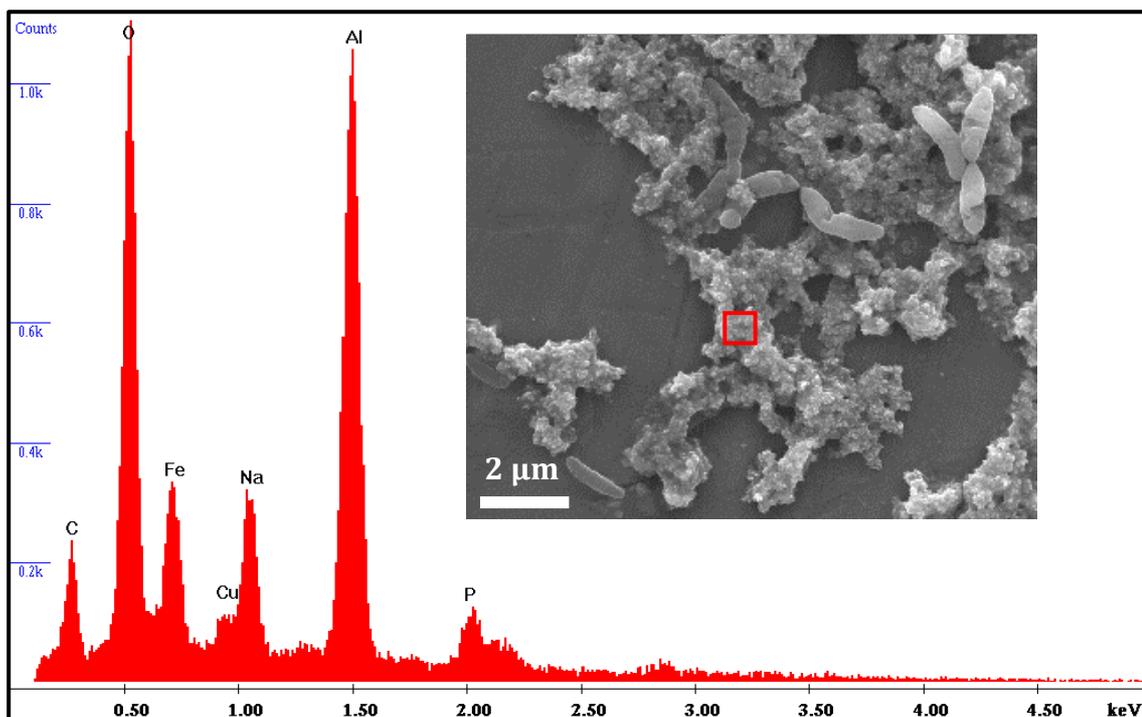
Figure 5.14 – SEM image of sample from microcosm amended with all the components (Treatment E) and the EDS spectrum corresponding to the area outlined in red.

5.7 Conclusions

This study examined the effect of magnetite NPs on 1,2-DCA dechlorination. The results demonstrated that the addition of magnetite to microcosms that contained a dechlorinating culture, 1,2-DCA and acetate had a positive influence when compared with the unamended ones. This included a 3-fold increase in dechlorination rates as well as a 20% decrease in the lag time for the onset of dechlorination. Microbial community analysis of *Archaea* showed that the concentration in the magnetite amended microcosms was lower than in unamended ones. Bacterial cell densities in magnetite-supplemented microcosms were higher than those in unamended ones. Based on the two genera of dechlorinating microorganisms analyzed, a direct correlation was established between the presence of *Dehalococcoides mccartyi* and dechlorination activity. Magnetite nanoparticles were not detected in the cell wall of the microorganisms. This suggests that this conductive mineral was not assimilated and that magnetite might only be used for interspecies extracellular electron transfer.

To the best of our knowledge, this is the first time that magnetite nanoparticles have been used to enhance 1,2-DCA dechlorination. These results may help to explain the fate of this CAH at a variety of sites, since conductive minerals are practically ubiquitous in the subsurface. Finally, future applications of these conductive NPs are envisioned as a syntrophic conductive 'bio-grid' for the cleanup of contaminated groundwater.

5.8 Acknowledgements

5.9 References

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6 Detection of *Dehalococcoides* spp. by peptide nucleic acid fluorescent in situ hybridization

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6.1 Highlights

- PNA-FISH can distinguish between two *Dehalococcoides* strains.

- Ability to be used in multiplex assays.

- Future application in monitoring and discrimination of *Dehalococcoides* populations, in laboratory cultures and at contaminated sites.

6.1.1 Keywords

Biodegradation; Chlorinated solvents; *Dehalococcoides*; Fluorescence in situ hybridization; Peptide nucleic acid.

6.2 Graphical abstract
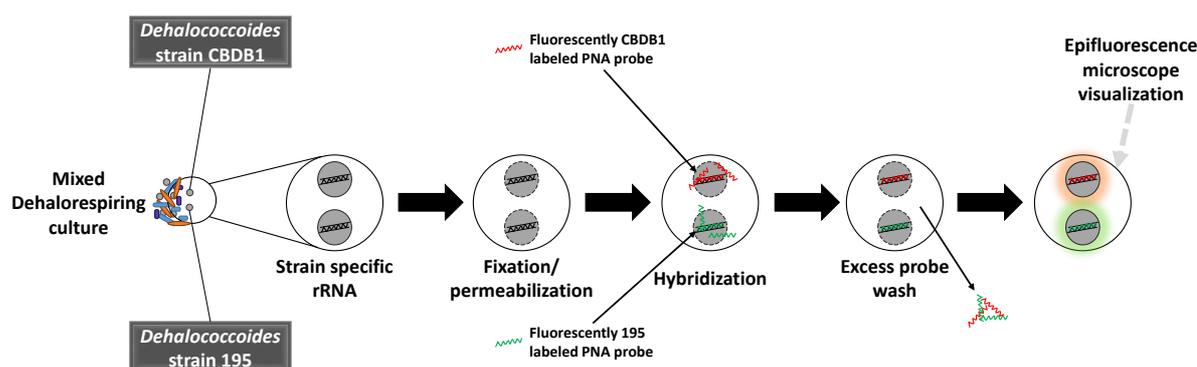
6.3 Abstract

Chlorinated solvents, including tetrachloroethene and thrichloroethene, are widely used industrial solvents. Improper use and disposal of these chemicals has led to a widespread contamination. Anaerobic treatment technologies that utilize *Dehalococcoides* spp. can be an effective tool to remediate these contaminated sites. Therefore, the aim of this study was to develop, optimize and validate peptide nucleic acid (PNA) probes for the detection of *Dehalococcoides* spp. in both pure and mixed cultures. PNA probes were designed by adapting previously published DNA probes targeting the region of the point mutations described for discriminating between the *Dehalococcoides* spp. strain CBDB1 and strain 195 lineages. Different fixation, hybridization and washing procedures were tested. The results indicated that the PNA probes hybridized specifically and with a high sensitivity to their corresponding lineages, and that the PNA probes developed during this work can be used in a duplex assay to distinguish between strain CBDB1 and strain 195 lineages, even in complex mixed cultures. This work demonstrates the effectiveness of using PNA fluorescence *in situ* hybridization to distinguish between two metabolically and genetically distinct *Dehalococcoides* strains, and they can have strong implications in the monitoring and differentiation of *Dehalococcoides* populations in laboratory cultures and at contaminated sites.

6.4 Introduction

Tetrachloroethene (PCE) and thrichloroethene (TCE) have been widely used as industrial solvents and dry cleaning fluids, and are common groundwater pollutants that are highly toxic and potentially carcinogenic. Under anaerobic conditions, PCE and TCE can be transformed into non-chlorinated ethene by dechlorinating bacteria. This process can occur naturally or through the addition of electron donor compounds that stimulate the growth of indigenous microorganisms (biostimulation) and/or exogenous microorganisms (bioaugmentation) that are able to degrade these compounds (Scow, K., 2005). The complete reductive dechlorination of chlorinated ethenes is performed by microorganisms of the genus *Dehalococcoides*. In this reaction, the chlorinated compound serves as the acceptor for electrons with hydrogen as the electron donor. In this growth linked process termed halorespiration, *Dehalococcoides* utilizes the chlorinated aliphatic compounds to obtain energy.

Dehalococcoides is notoriously difficult to isolate and grow, with doubling times of 1–2 days (He, J., 2003, 2005; Maymo-Gatell, X., 1997; Sung, Y., 2006) and complex nutrient requirements. *Dehalococcoides* spp., therefore, are normally cultured as a consortium with other microorganisms, such as acetogens and methanogens. *Dehalococcoides* spp. have been found in a variety of locations, and their presence has been linked to the complete dechlorination of PCE at contaminated sites in North America and Europe (Hendrickson, E., 2002). Although very few *Dehalococcoides* spp. have been isolated, some metabolic diversity has been identified between them. Originally, strain 195 was found to metabolize some chloroethenes (PCE, TCE, DCE and 1,1-Dichloroethene (1,1-DCE)), and the halogenated ethanes 1,2-DCA and 1,2-dibromoethane (Maymo-Gatell, X., 1997, 1999). Later research demonstrated the ability to metabolize higher chlorinated aromatics, such as hexachlorobenzene, pentachlorobenzene, 1,2,4,5-tetrachlorobenzene and 1,2,3,4-tetrachlorobenzene (Fennell, D., 2004). Conversely, strain CBDB1 (of the Pinellas subgroup lineage) was found to metabolize these same chlorinated aromatics, and others such as 1,2,3,5-tetrachlorobenzene, 1,2,4-trichlorobenzene and 1,2,3-trichlorobenzene (Adrian, L., 2000). In addition, recently, strain CBDB1 has been ascribed the ability to also metabolize the chloroethenes PCE and TCE (Marco-Urrea, E., 2011). Other members of the Pinellas subgroup (of which CBDB1 is a member) are able to metabolize lower chlorinated ethenes such as cis -DCE, 1,1-DCE and VC (He, J., 2003; Sung, Y., 2006). Table 6.1 shows the differences between the two lineages. Thus, the ability to distinguish between different lineages of these bacteria at contaminated sites may provide insights into the

potential fate of pollutants, the suitability of biostimulation remedial approaches and the need to perform bioaugmentation to reach remedial goals.

Table 6.1 - Comparison of different *Dehalococcoides* pure cultures and their ability to utilize different electron acceptors (adapted from Eaddy, A., 2008).

Compound	Strain 195 (Cornell)	BAV1 (Pinellas)	GT (Pinellas)	FL2 (Pinellas)	CBDB1 (Pinellas)
PCE	+	•	-	•	+
TCE	+	•	+	+	+
cis-DCE	+	+	+	+	-
trans-DCE	•	+	-	+	-
1,1-DCE	+	+	+	-	-
VC	•	+	+	•	-
CB	-	?	?	?	-
1,2-DCB	-	?	?	?	?
1,3-DCB	-	?	?	?	?
1,4-DCB	-	?	?	?	?
1,2,3-TCB	•	?	?	?	+
1,2,4-TCB	•	?	?	?	+
1,3,5-TCB	•	?	?	?	-
1,2,3,4-TeCB	+	?	?	?	+
1,2,3,5-TeCB	•	?	?	?	+
1,2,4,5-TeCB	+	?	?	?	+
PeCB	+	?	?	?	+
HCB	+	?	?	?	+

+ = Used as a terminal electron acceptor (TEA). • = Used cometabolically. – = Not used as a TEA. CB = Chlorobenzene; DCB = dichlorobenzene; HCB = hexachlorobenzene; PeCB = pentachlorobenzene; TCB = trichlorobenzene.

Because of the importance of these microorganisms in degrading chlorinated pollutants, molecular tools based on the detection of 16S rRNA gene sequences have been developed and widely used for qualitative and quantitative detection of *Dehalococcoides* spp. (Holliger, C., 1999; Maymo-Gatell, X., 1997; Rowe, A., 2008; Tas, N., 2009). In addition, other non-PCR-based molecular methods, such as FISH and CARD-FISH, have been used to distinguish between the different lineages of *Dehalococcoides* spp. (Dijk, J., 2008; Fazi, S., 2008; Yang, Y., 2003). The probes used for these FISH based tools take advantage of the fact that the two different lineages of *Dehalococcoides* spp. (strain 195 and Pinellas subgroup) can be distinguished from one another based on a conserved base difference in the 16S rRNA gene sequence.

FISH is based on the use of a fluorescently labeled nucleic acid probe (generally DNA) that is complementary to a sequence in the rRNA of the microorganism of interest (Almeida, C., 2009). After exposure to the sample containing the microorganism, the probe diffuses through the cell wall and reaches the rRNA, where it forms a stable duplex if the complementary sequence is present. A subsequent washing step ensures that only the fully complementary probes remain within the cell and are detected by either flow cytometry

or microscopy. Recently, PNA has been replacing DNA as a probe in FISH. The backbone of these synthetic nucleic acid molecules is neutrally charged, which means that PNA has more affinity for duplexes with DNA and RNA than DNA itself (Armitage, B., 2003). This higher affinity translates into shorter probes that can more easily diffuse through the microbial cell wall and hence increase the robustness of the FISH method. PNA-FISH has been successfully used for the detection of microorganisms in the food and health areas (Almeida, C., 2011; Azevedo, N., 2011; Guimaraes, N., 2007), but this improved methodology of detection has not yet been adapted to *Dehalococcoides* spp.

Because there are two different lineages of *Dehalococcoides* spp. that have different abilities to degrade pollutants, it is important to be able to identify which type of *Dehalococcoides* spp. is present in cultures or environmental samples to help predict the fate of pollutants or select the proper remedial treatments. Therefore, the aim of this work was to develop PNA-FISH probes and test them to determine whether this technique can be utilized to detect the two different lineages of *Dehalococcoides* spp. in both pure and mixed cultures.

6.5 Methods

6.5.1 Cultures

Pure cultures of *Dehalococcoides* strain CBDB1 and *Dehalococcoides mccartyi* strain 195 were grown at 30 °C in glass serum bottles (Adrian, L., 2000; Loffler, F., 2013; Marco-Urrea, E., 2011). Acetate (5 mM) was used as the carbon source for the pure cultures.

Mixed cultures SDC-9™ and Hawaii-05™ were chosen due to their application as commercial bioaugmentation cultures that have been used at a variety of sites because of their ability to utilize chloroethenes (Schaefer, C., 2009, 2010a, b; Vainberg, S., 2009). SDC-9 was enriched from a chlorinated solvent-contaminated site in southern California with PCE and sodium lactate (Vainberg, S., 2009). In 2005, Hawaii-05 was enriched from aquifer material from the Hickman Air Force Base with TCE and sodium lactate (Vainberg, S., 2009). Both cultures contain tceA and vcrA dehalogenase genes. They were grown at 28 °C in glass serum bottles with lactate (5 mM) as the carbon source (Vainberg, S., 2009).

The initial PCE concentration was approximately 100 µM for CBDB1, SDC-9 and Hawaii-05, and 200 µM for strain 195. Aliquots of cells were removed from the serum bottles after 50 % (CBDB1) and 100 % (195, SDC-9 and Hawaii- 05) of the initial PCE concentration was consumed.

6.5.2 Analytical methods

Concentrations of PCE and daughter products were analyzed using headspace samples (0.1 or 0.5 mL) injected onto a Shimadzu GC-2014 with flame ionization detection and a 1 % SP-1000 on a 60/80 Carbopack B column (Supelco). The column temperature program, and injector and detector temperatures have been described previously (Freedman, D., 1989). The production of PCE degradation daughter products (TCE, dichloroethenes (DCEs), vinyl chloride (VC) and ethene (ETH)) was used to confirm that the pure and mixed cultures were actively growing.

6.5.3 Chemicals

VC (99.5 %) and ETH (99.95 %) were obtained from Fluka. PCE (99 %), TCE (99.5 %), sodium lactate (60 % solution) and sodium acetate (99 %) were obtained from Sigma-Aldrich. Cis -DCE (97 %) and trans-DCE (99 %) were obtained from Acros.

6.5.4 Probe design

Specific PNA probes were designed by considering the position of a point mutation that has been used previously to distinguish between the strain 195 and Pinellas subgroups of *Dehalococcoides* (Berlier, J., 2003) and the predicted melting temperature (T_m) value of the possible PNA oligomers. The theoretical T_m was calculated based on thermodynamic parameters described previously by SantaLucia and Hicks, 2004. Once the probe sequence was selected, a search was made for the available 16S rRNA gene sequences (RDP-II), version 10 (http://rdp.cme.msu.edu/) to confirm the theoretical specificity and sensitivity of the probe against other microorganisms. For this analysis, only high quality sequences with more than 1,200 bp were selected. The specificity and sensitivity values were determined as previously reported (Almeida, C., 2010). The sequences were synthesized by PANAGENE (South Korea), purified by reverse-phase HPLC, and the N-terminus of the CBDB1 and 195 oligomers was connected to Alexa Fluor 594 and Alexa Fluor 488, respectively, via a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker.

6.5.5 Hybridization procedure

The PNA hybridization method used was developed based on the procedure reported (Azevedo, N., 2011; Perry-O'Keefe, H., 2001), but different fixation, hybridization and washing procedures were tested to optimize the method. Briefly, cell suspensions of the test cultures were prepared by harvesting cultures that were exponentially grown (fig. 6.1). Thirty mL of each diluted sample were then collected by centrifugation (30 min at

3200 g, Eppendorf centrifuge 5810, Eppendorf AG, Hamburg, Germany). In order to fix the cells, the pellet was resuspended in 1 mL of 4 % (w/v) paraformaldehyde for 1 hour at room temperature, followed by centrifugation. The fixed cells were then resuspended in 1 mL of 50 % ethanol (v/v) for at least 30 min at –20 °C. Then, 20 µL were placed into microscopy well slides and allowed to air dry.
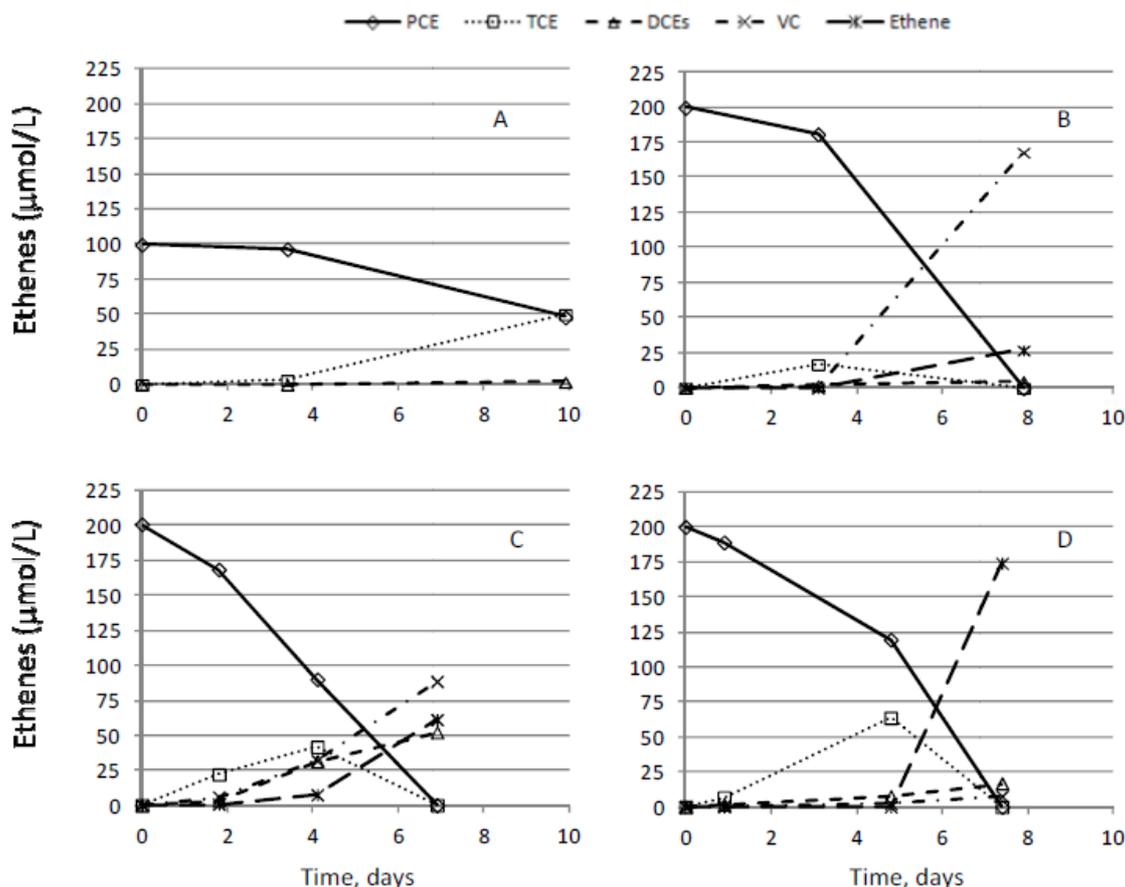
Figure 6.1 - PCE degradation for (A) *Dehalococcoides* **strain CBDB1, (B)** *Dehalococcoides* **strain 195, (C) SDC9™ and (D) Hawaii-05™. Aliquots from each batch reactor were removed at the last monitoring point.**

The tests slides were then covered with 20 µL of probe (400 nM) diluted in a hybridization solution that contained 10 % (w/v) dextran sulfate, 10 mM NaCl, 30 % (v/v) formamide, 0.1 % (w/v) sodium pyrophosphate, 0.2 % (w/v) polyvinylpyrrolidone, 0.2 % (w/v) Ficoll, 5 mM disodium Ethylenediaminetetraacetic acid (EDTA), 0.1 % (v/v) Triton X-100 and 50 mM TrisHCl. Control slides were covered with hybridization solution without the probe and, afterwards, both control and test slides were covered with coverslips and then placed in moist chambers and incubated for 90 min at 57 °C for the 195 lineage and 61 °C for CBDB1 lineage.

To perform the multiplex assay, 20 µL of fixed SDC-9 and Hawaii-05 cells were placed onto microscopy slides and allowed to air dry. Hybridization was performed using a solution with 20 µL of hybridization buffer (previously described) with 100 nM of each

respective probe covering each smear individually. Samples were sealed with coverslips and incubated for 90 min at 57 °C. Subsequent steps were performed as described below.

Following hybridization, coverslips were removed and slides were submerged in a pre-warmed washing solution (the temperature was similar to the one used for hybridization) containing 5 mM Tris base, 15 mM NaCl and 1 % (v/v) Triton X-100 (pH 10) for 15 min and then removed and allowed to air dry. Finally, 1 µL of DAPI was added and the slides were covered with one drop of non-fluorescent immersion oil. Slides were stored at 4 °C in the dark for a maximum of 24 h before microscopy.

6.5.6 Microscopy visualization

Visualization was performed using an Olympus BX51 epifluorescence microscope (Olympus Portugal SA, Porto, Portugal) equipped with a filter sensitive to the Alexa Fluor 488 signaling molecule attached to the 195 PNA probe (absorbance, 495 nm, emission, 519 nm) and a filter sensitive to the Alexa Fluor 594 signaling molecule attached to the CBDB1 PNA probe (excitation, 530–550 nm; barrier, 570 nm; emission long-pass filter, 591 nm). To ensure that the signal obtained was not related to auto fluorescence, all samples were visualized with other available filters. For every experiment, a negative control was performed simultaneously. For the negative control, all the steps described above were performed, but no probe was added during the hybridization procedure. All images were acquired using the Olympus CellB (Olympus Portugal) software with a magnification of ×1000.

6.6 Results and discussion

6.6.1 Probe development and application to pure cultures

PNA probes were designed by adapting previously published DNA probes (Yang, Y., 2003) targeting the 16S rRNA region of the point mutations described for detecting CBDB1 and 195 lineages of *Dehalococcoides* spp. Because the favorable size of PNA probes (related to their thermodynamic parameters) is between 13 and 16 bp, it was necessary to select a smaller sequence from the probe previously described. Consequently, the sequences 5-GTT CGC ACT GTT GC-3 and 5-GTT CAC ACT GTT GC-3 were selected and designated CBDB1 probe and 195 probe, respectively. The theoretical evaluation of the probes has shown high specificity and sensitivity values (table 6.2), since 55 of 56 *Dehalococcoides* sequences have been identified and only 1 non-target sequence has presented cross-hybridization with the probes. Additionally, melting temperatures and free

energy (ΔG) values for the CBDB1 and 195 probes were very similar, which might indicate a good performance in a multiplex assay.

Table 6.2 - Theoretical evaluation of the PNA probes.

Probe	Sequence (5'-3')	Target position (E. coli numbering)	kcal/mol ΔG	°C T_m	Sensitivity, %	Specificity, %
CBDB1	GTTCGCACTGTTGC	1,257 – 1,270	–17.92	71.30*	98.21	
195	GTTCACACTGTTGC	1,257 – 1,270	–16.36	68.13*	99.99	

* Considering a probe concentration of 1×10^{-4} M.

Initially, the development of a PNA-FISH procedure for the differentiation of *Dehalococcoides* spp. was found to be technically more challenging than PNA-FISH procedures developed earlier by our group to detect other microorganisms (Almeida, C., 2010; Guimaraes, N., 2007). This may be related to the slow growth rates of the microbes in pure cultures and low cell yields of *Dehalococcoides* spp. (Duhamel, M., 2006; Marco-Urrea, E., 2011; Maymo-Gatell, X., 1997), which resulted in low concentrations of cells in the test cultures and, possibly, low ribosomal content of the cells.

The first issue was solved by concentrating the cells by centrifugation and counterstaining the samples with DAPI. Centrifugation allowed having a higher cell concentration in each field of view in the microscope. This also increased the signal-to noise ratio by increasing the number of cells in the field of view relative to the occasional debris that might be present on the slide surface. This was particularly important while the method was not fully optimized and the fluorescent signal from the hybridization was faint. The number of *Dehalococcoides* spp. cells in each slide was assessed by counterstaining the sample with DAPI, a nonspecific dye that intercalates in the double-stranded DNA present in all cells. By comparing the signal obtained in the red and green filters sensitive to the probes against the signal obtained in the blue filter with DAPI, we were able to ensure that the hybridization was equally successful for all cells present in the sample.

The second issue that reduced detection sensitivity was related to the low ribosomal content of the cells, which has been reported previously (Fazi, S., 2008; Fletcher, K., 2011). To minimize this issue, we coupled the PNA probes with Alexa Fluor dyes. These dyes are well known for being more photostable and brighter than other commonly used fluorescent dyes operating at similar wavelengths (Berlier, J., 2003; Panchuk-Voloshina, N., 1999). Because growth phase also can affect the rRNA content of the cells, we also evaluated the effect of the growth phase of the microorganisms on hybridization. Samples from the two different pure culture strains of *Dehalococcoides* were taken on different

days and for different growth phases (fresh and old cultures). After optimization, the PNA-FISH method was able to detect cells in pure culture in all growth stages (Fig. 6.1). Negative controls without probes for the two different pure cultures of *Dehalococcoides* did not show fluorescence.
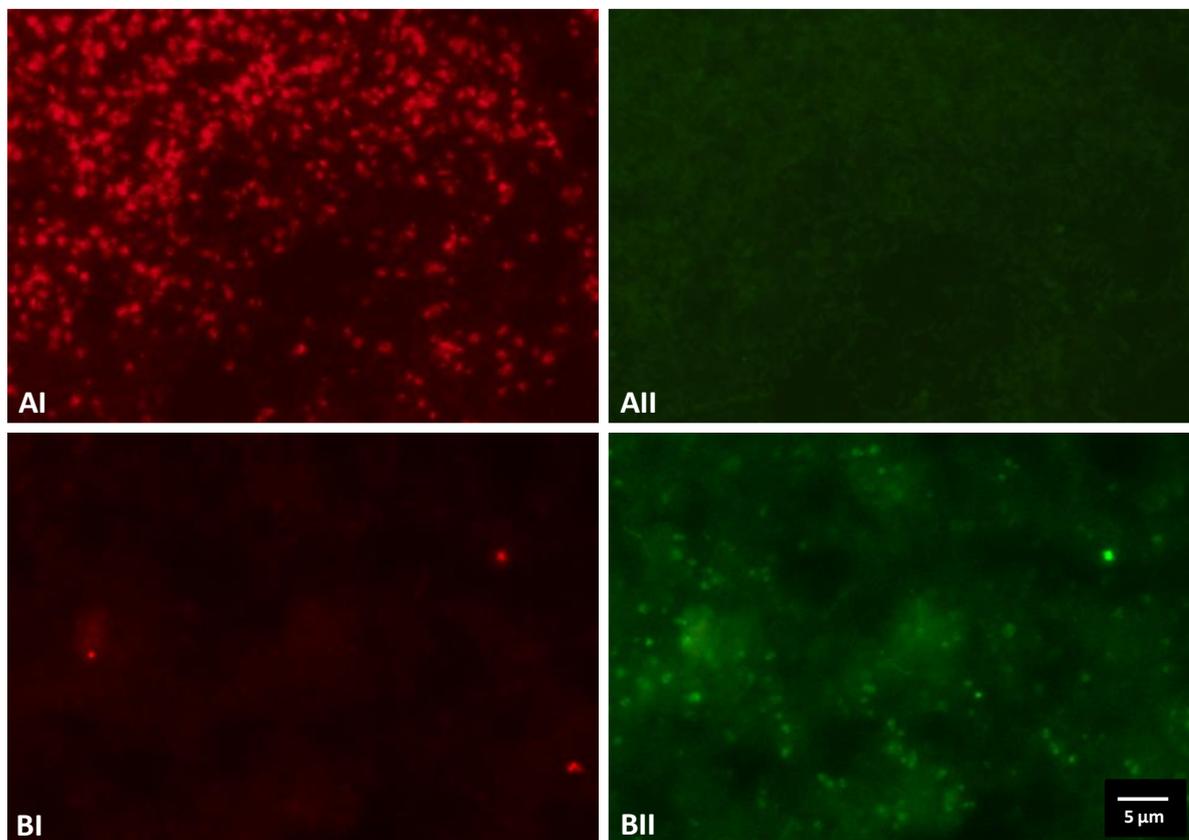
Figure 6.2 - Identification of *Dehalococcoides* spp. (CBDB1 and strain 195) by epifluorescence microscopy. A Detection of *Dehalococcoides* CBDB1 in pure culture using the CBDB1 PNA probe labeled with Alexa Fluor 594 (AI) and observation of the same field of view in the green channel that is not sensitive to the fluorochrome used (AII). B Detection of *Dehalococcoides* 195 in pure culture using the 195 PNA probe labelled with Alexa Fluor 488 (BII) and observation of the same field of view in the red channel (BI). The experiments were performed in parallel, and images were obtained with equal exposure times.

Other possible causes of low intensity of the fluorescence signal for the two strains tested could have been an insufficient penetration of the probe through the bacterial cell wall due to the thick cell wall of the Gram-positive *Dehalococcoides* spp. (Perry-O'Keefe H., 2001; Stender, H., 2003). By performing the experiment with PNA instead of DNA, we guaranteed that the diffusion of our probe would be easier, as PNA probes are electrically uncharged and smaller than DNA probes (Cerqueira, L., 2008; Pellestor, F. , 2004).

6.6.2 Development of a multiplex assay

The probes hybridized specifically to their corresponding strains meaning that the one base mismatch between the sequences was sufficient to discriminate between both strains using this technology. An important aspect was that the optimum temperature of

hybridization for the two probes differed in 4 °C. This was unexpected as there was only one base difference, but can be explained by the fact that the base pair for the 195 strain was formed between adenine and thymine (which creates two hydrogen bonds), whereas the CBDB1 base pair was between guanine and cytosine (which creates three hydrogen bonds). Nevertheless, the two probes will also work in a multiplex experiment if a hybridization average temperature of 57 °C is selected (Fig. 6.2). However, higher hybridization temperatures (59 °C and 61 °C) have proven to favor the binding of the CBDB1 probe.
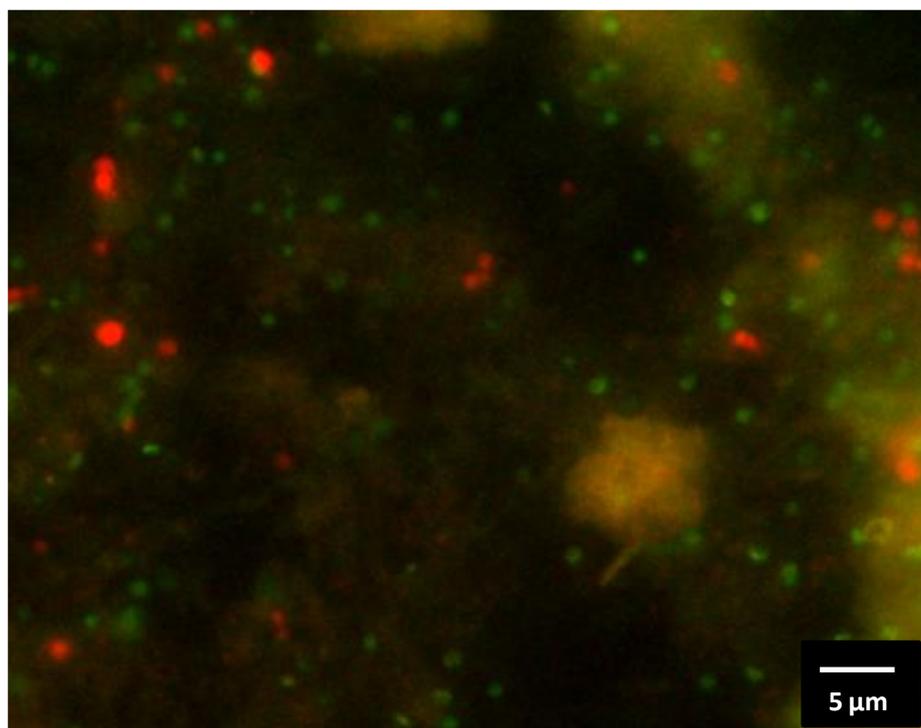
Figure 6.3 - Epifluorescence microscopy image of a multiplex experiment performed on the Hawaii-05™ showing the two lineages.

While the method is adequate to discriminate between these two strains of *Dehalococcoides* spp., the probes were also designed to minimize the detection of other microorganisms present in environmental samples (99.99 % specificity to *Dehalococcoides*, as indicated in table 6.2). A sequence search of the Ribosomal Database Project confirmed that the probes detect very low numbers of other microorganisms. One exception, however, was the complementarity observed between the 195 probe and the *Escherichia coli* strain W (Archer, C., 2011). Overall, because one strain is not representative of the whole species and because the morphology of *Dehalococcoides* spp. and *E. coli* are very different, the risk for a misidentification is rather low. This sequence complementarity was also observed for the DNA-FISH probes reported earlier by Yang and Zeyer, 2003.

6.6.3 Application of probes to mixed cultures

To further confirm the specificity and performance of the probe, we tested the PNA-FISH multiplex method on mixed cultures containing other microorganisms (e.g. acetogens and methanogens). Results showed that the probe only detected coccoid cells in these cultures. For the Hawaii-05 TM culture, both lineages of *Dehalococcoides* were observed (Fig. 6.3), whereas SDC-9 TM only contained the 195 lineage. Interestingly, 16S rRNA gene sequencing performed with SDC-9 suggested that the culture contains at least four different strains of *Dehalococcoides* (Trotsky, J., 2010), and the PNA-FISH testing performed here suggests that those strains may all be from the 195 lineage. For certain samples, auto fluorescence by other microorganisms could be observed, but the signal-to-noise ratio of the probe was still sufficient to discriminate between populations (Fig. 6.3).
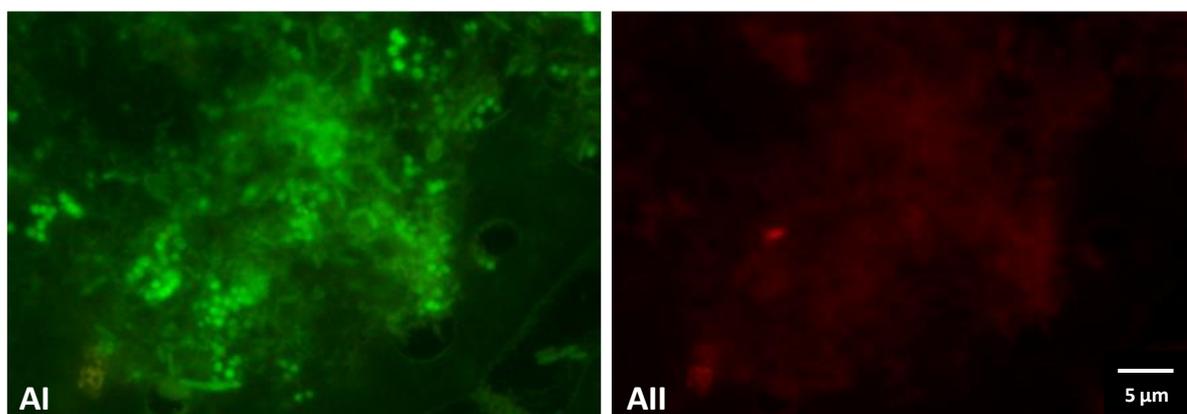
Figure 6.4 - Epifluorescence microscopy image of a multiplex experiment performed on the SDC-9 showing the detection of the CBDB1 lineage against a background of other cells (AI). This auto fluorescence was also detected in the red filter that was not sensitive to the fluorochrome attached to the probe (AII).

Both cultures exhibit metabolic behavior specific for their lineage. For example, SDC-9 and Hawaii-05 have the ability to metabolize a variety of chlorinated ethenes, including cis- DCE and VC, but neither culture is able to dechlorinate chlorobenzenes. As previously mentioned, the strain 195 and Pinellas subgroup lineages have metabolic similarities but also have some distinguishing features. Strain CBDB1 has the ability to metabolize a wide variety of chlorinated aromatics but is limited in its ability with chloroethenes. Conversely, other members of the Pinellas subgroup (FL2, GT and BAV1) have the ability to metabolize many of the lower chlorinated organics (table 6.1). Strain 195 is the only member of its lineage and demonstrates many of the same metabolic activities of the Pinellas subgroup, including the ability to metabolize chlorinated aromatics and ethenes.

6.7 Conclusions

This work demonstrates the effectiveness of using PNA-FISH to distinguish between two metabolically and genetically distinct *Dehalococcoides* strains. In previous works, PNA-FISH has been shown to surpass standard DNA-FISH in terms of robustness, and is now starting to be commercially available to detect microorganisms in clinical samples (Cerqueira, L., 2013). Hence, the method can have strong implications for the monitoring and discrimination of *Dehalococcoides* populations in laboratory cultures and at contaminated sites. Nevertheless, future work should be performed to compare the method developed here with FISH and catalyzed reporter deposition FISH to determine which method is most effective in determining the concentration of *Dehalococcoides* spp. and also the application of this method to field samples.

6.8 Acknowledgements

6.9 References

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7 Concluding Remarks

The main objective of this thesis was to remediate chlorinated ethanes through anaerobic biodegradation. As a mean to enhance anaerobic biodegradation two main approaches were explored: 1) replacement of the addition of electron donors with (graphite) electrodes (bioelectrochemical system - BES), in some cases facilitated by redox mediators and 2) improvement of the extracellular electron transfer reactions occurring in dechlorinating communities using conductive minerals.

For the successful establishment of the first approach it was necessary to enrich an electroactive culture capable of dechlorinating one of the target contaminants (1,2-DCA). Biomolecular analysis (CARD-FISH probes) targeting dehalorespiring microorganisms identified *Dehalococcoides* spp. as the nearly sole bacterial species within the system. Performed tests demonstrated the ability of the mixed culture to accept electrons from a graphite electrode in order to completely dechlorinate 1,2-DCA to innocuous ethene, via dihaloelimination, without the addition of redox mediators.

To evaluate the possibility of enhancing the dechlorination kinetics within the bioelectrochemical system, the impact of a redox mediator (AQDS) was tested. In order to test this hypothesis a bioelectrochemical system amended with AQDS (in solution) was initially tested. This redox mediator improved 1,2-DCA degradation kinetics to ethene, but without any evidence of dechlorination activity towards 1,1,2-TCA. Interestingly, a remarkable enrichment of the obligate anaerobe *Dehalococcoides mccartyi* was achieved in

the BES experiments taking as the starting point an inoculum originated from a fully aerobic municipal activated sludge.

At this stage it was hypothesized that the immobilization of the redox mediator on the electrode surface would have a similar dechlorinating potential and could simultaneously eliminate the need of repeated injections of AQDS, ultimately leading to a safer application of the process and reduced costs in future field applications. However, limited amount of adequate procedures for this immobilization were available and required significant development prior to any bioelectrochemical tests. Using a commercial anionic membrane, it was possible to develop a simple, fast, and reproducible method for the immobilization of AQDS on the graphite electrodes. Biotic experiments suggested that AQDS was effectively retained on the electrode for over 90 days without losing its catalytic activity. Although performance remained higher than without redox mediator addition, it was found to be inferior when compared to the use of AQDS in solution, which might indicate the need to further improve the immobilization technique.

The other approach tested in this thesis was the influence of a conductive mineral (magnetite nanoparticles) on 1,2-DCA dechlorination. Using acetate as the electron donor, the presence of magnetite decreased the lag time of dechlorination and increased the dechlorination rate. Based on the results obtained it is assumed that this mineral is used in interspecies extracellular electron transfer, facilitating catalytic electron transport, which has a beneficial effect on the microbial communities present and in particular on the ones that have dehalorespiring capabilities.

Finally, given the pivotal importance of accurate and precise usage of biomolecular techniques to properly monitor the dechlorination process, peptide nucleic acid (PNA-FISH) probes were developed, optimized and validated. Specifically designed to detect and distinguish between two *Dehalococcoides* spp. strains, results indicated that the PNA probes hybridized with high sensitivity to their corresponding lineages and could be used even in complex mixed cultures.

Assessing this work as a whole, the potential of bioelectrochemical systems for anaerobic biodegradation of 1,2-DCA has been demonstrated along with a set of recommended practical parameters for process optimization. Tools and techniques to increase efficiency and to improve monitoring capabilities of the process, such as AQDS addition and immobilization, the application of PNA-FISH, are also described. Furthermore, the potential of conductive minerals to enhance reductive dechlorination was also shown but not yet tested in a bioelectrochemical system.

Recommendations for further work include:

- Bioelectrochemical tests with other chlorinated ethanes, individually or as a mixture, hence mimicking the actual occurrence of these contaminants in the subsurface.
- Study of other dehalorespiring microorganisms, such as *Dehalogenimonas* spp. or *Dehalobacter* spp., to evaluate if they exhibit electroactive capabilities.
- Employ different redox mediators, immobilization techniques and conductive minerals. For example, the use of natural magnetite, in opposition to the synthetized magnetite nanoparticles used, or the impact of incorporating conductive minerals in a bioelectrochemical system.
- Study the effect of adding manganese nanoparticles (pyrolusite) on reductive dechlorination and the combination of magnetite and/or pyrolusite with immobilized redox mediators.
- Research concerning microbial remediation cells optimization, regarding among other aspects, anode and cathode material, distance between cathode and anode, BES configuration and proton exchange membrane.
- Test bioelectrochemical system technologies with a real groundwater contaminated with chlorinated ethanes, followed by test in pilot and field scales. This would give vital information regarding costs and remediation times, among other data, only quantifiable in these conditions, which is important when choosing the remediation technology to be applied in a contaminated site.

Other recommendations concerning the biomolecular techniques are 1) the application of the developed PNA-FISH procedure in real environmental samples, for example aquifer solid samples, 2) the comparison of the developed PNA-FISH method with other methods, e.g. FISH, CARD-FISH, should be performed to determine which is the most effective in determining the concentration of *Dehalococcoides* spp and 3) the development of PNA-FISH methods for other dehalorespiring microorganisms.

8   Engineering Implications

The work presented in this thesis demonstrated the lab scale feasibility to degrade 1,2-DCA from contaminated groundwater through the use of BES technology. However, and despite the increasing interest of the scientific community, there is limited information regarding the scale up of this technology from lab scale to field applications. This is without a doubt one of the greatest challenge facing future applications of the present work. Although electrochemical remediation has been successfully applied to the treatment of inorganic and organic pollutants in field applications it has yet to be used as a direct remediation mechanism but as a mean to move and concentrate contaminants for subsequent treatment by other techniques. Nevertheless, some similarities between electrochemical remediation and bioelectrochemical remediation might enable the transfer of some accumulated field knowledge to accelerate application of the latter.

This thesis contributed to further clarify the potential of BES technology as a substitute to traditional bioremediation techniques while also providing clear indications on how to maximize its use. Obtained parameters such as set cathode potential, while tested at labscale, are directly transferable to field applications, albeit with likely reductions in rate and yield of the reductive dechlorination.

The BES system was stable and consistent during several weeks, a predictability and reliability seldom found in remediation techniques. The graphite electrodes demonstrated to be a viable option for a conductive and non-corrosive carbon material that can be safely

used in field applications. These facts will make the implementation of BES more attractive to practitioners of field remediation projects.

The observed enrichment of a *Dehalococcoides* spp. culture capable of degrading 1,2 DCA (despite the use of an aerobic activated sludge as inoculum) indicates that, if properly prepared, the obtainment of capable cultures may not be a limiting factor for future applications.

Tests with magnetite nanoparticles demonstrated a significant increase in dechlorination rate opening up the possibility of its use in a syntrophic conductive 'bio-grid' for the cleanup of contaminated groundwater.

The biomolecular analysis used and developed as monitoring tools can easily be adapted to other laboratories as well as field testing conditions, enabling the monitoring of several remediation techniques which might involve bioremediation or natural attenuation.

Finally, the addition of AQDS as a redox mediator both in solution or immobilized on the electrode in the BES was used to increase the 1,2-DCA dechlorination rate. The results, however, provide higher dechlorination rates when using AQDS insolution as opposed to immobilized AQDS.

As a result, and for engineering applications, a compromise may be needed between the performance of reductive dechlorination by the different developed bioelectrochemical techniques (without redox mediator, with redox mediator in solution and with immobilized redox mediator) and associated costs and remediation goals. Without the addition of the redox mediator the reductive dechlorination rate was 10 μeq/d.L^{-1} while using the redox mediator in solution and immobilized redox mediator this rate was 46 μeq/d.L^{-1} and 13 μeq/d.L^{-1}, respectively. These rates suggest that a case-by-case analysis in the field is required when considering the application of these three options. For instance, in some cases, the seemingly inferior performance of immobilized redox mediator might justify its use in locations with low hydraulic conductivity whereas injection of AQDS in liquid form in other situations might enable a faster remediation process in urgent cases, regardless of the costs and technical difficulties.

A. Volatiles analytical methods

Volatile compounds were quantified using gas chromatography with a flame ionization detector (GC-FID) in headspace samples which were converted to aqueous-phase concentrations using Henry's law constants (Table A.1).

Table A.1 - CAHs values of molar mass and dimensionless Henry's constant (25 °C).

CAH	Molar mass (g/mol)	K_H (dimensionless)
1,1,2-TCA	133.40	0.0372
1,2-DCA	98.95	0.0560
VC	62.50	1.14
ETH	28.05	8.50
CH_4	16.04	30.35

Rome: Standards of 1,1,2-TCA, 1,2-DCA, VC, ETH and CH_4 were prepared in serum bottles or in the BES apparatus, with a defined liquid volume of water. The recipients were sealed with Teflon-faced butyl rubber stoppers and aluminum crimp caps and flushed with N_2/CO_2 (70:30 v/v) for 30 min. A given volume of volatile compound was added to each of the recipients to obtain a calibration curve in the range of the values expected in the experimental work. To reach equilibrium, standards were left shaking for a 3 hour period, in an upside down position in the case of the serum bottles. Headspace was analyzed by injecting 50 μL (taken with a gas-tight syringe) into a Perkin–Elmer GC 8500 gas chromatograph (2 m x 2 mm glass column packed with 60/80 mesh Carbopak B/1% SP-1000 Supelco; N_2 carrier gas 18 mL/min; oven temperature 190 ^0C; FID temperature 250 ^0C). Gas phase concentrations were plotted as a function of the peak area.

Porto: Standards of 1,2-DCA, VC, ETH and CH_4 were prepared in serum bottles or in the BES apparatus, with a defined liquid volume of water. The recipients were sealed with Teflon-faced butyl rubber stoppers and aluminum crimp caps and flushed with N_2 for 15 min and 10 mL of CO_2 was added. A given volume of volatile compound was added to each of the recipients to obtain a calibration curve in the range of the values expected in the experimental work. To reach equilibrium standards were left shaking for a 3 hour period, in an upside down position in the case of the serum bottles. Headspace was analyzed by injecting 50 µL (taken with a gas-tight, gas-locking syringe) into a Shimadzu GC-2014 gas chromatograph equipped with a FID and a 2.4 m x 2.1 mm 60/80 Carbopack B/ 1% SP-1000 column. The temperature was initially set at 60 ºC followed by a ramp of 40 ºC per minute until 180 ºC which was held for 1.25 min. The carrier gas was N_2 (40 mL/min) and the temperatures of the injector and the detector were 200 ºC. Gas phase concentrations were plotted as a function of the peak area.

The obtained calibration curves are indicated in Figures A.1 to A.9.

A.1 1,1,2-Tricloroethane

Peak area = 8133.6 x Concentration
R^2 = 0.9893
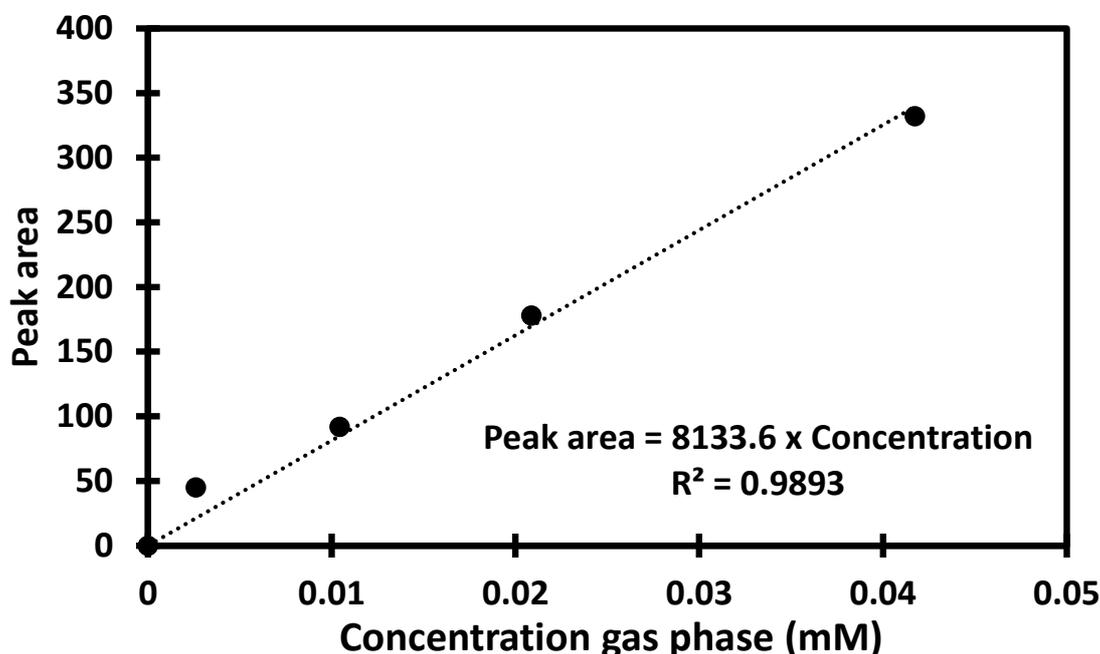
Figure A.1 - Calibration curve 1,1,2-trichloroethane Rome.

A.2 1,2-Dichloroethane
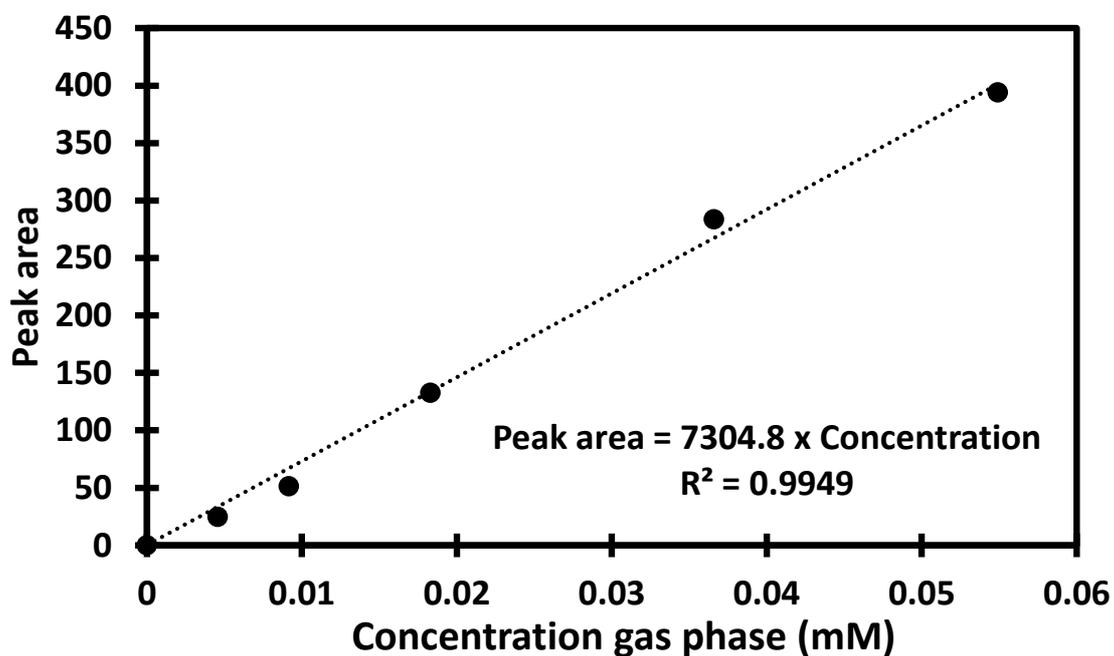
Figure A.2 - Calibration curve 1,2-dichloroethane Rome.
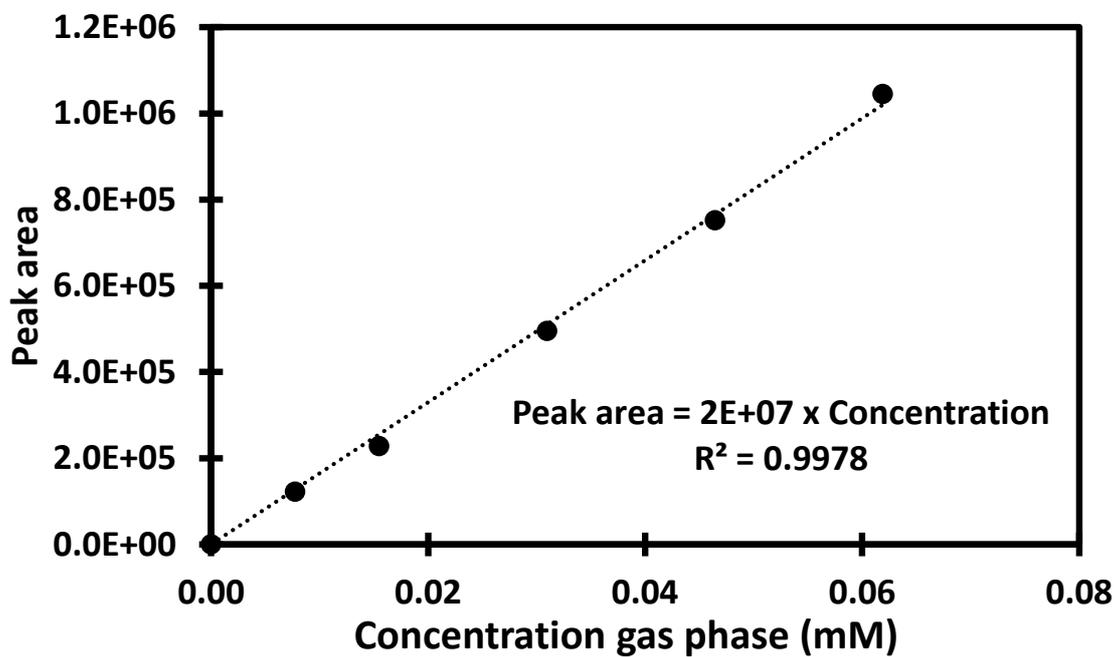
Figure A.3 - Calibration curve 1,2-dichloroethane Porto.

A.3 Vinyl chloride
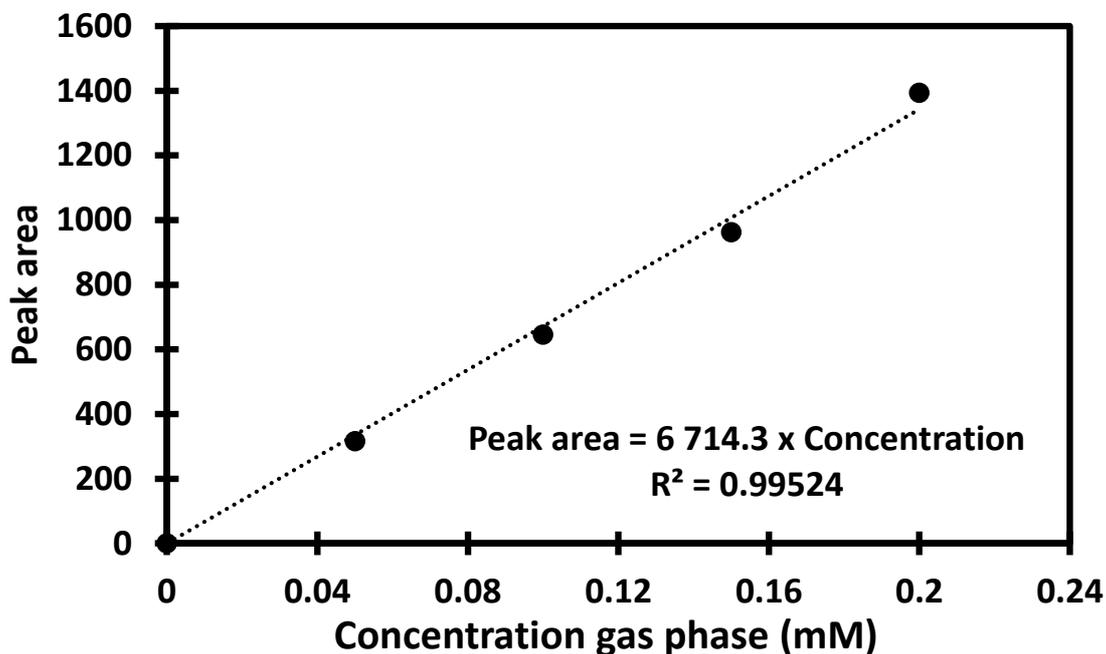
Figure A.4 - Calibration curve vinyl chloride Rome.
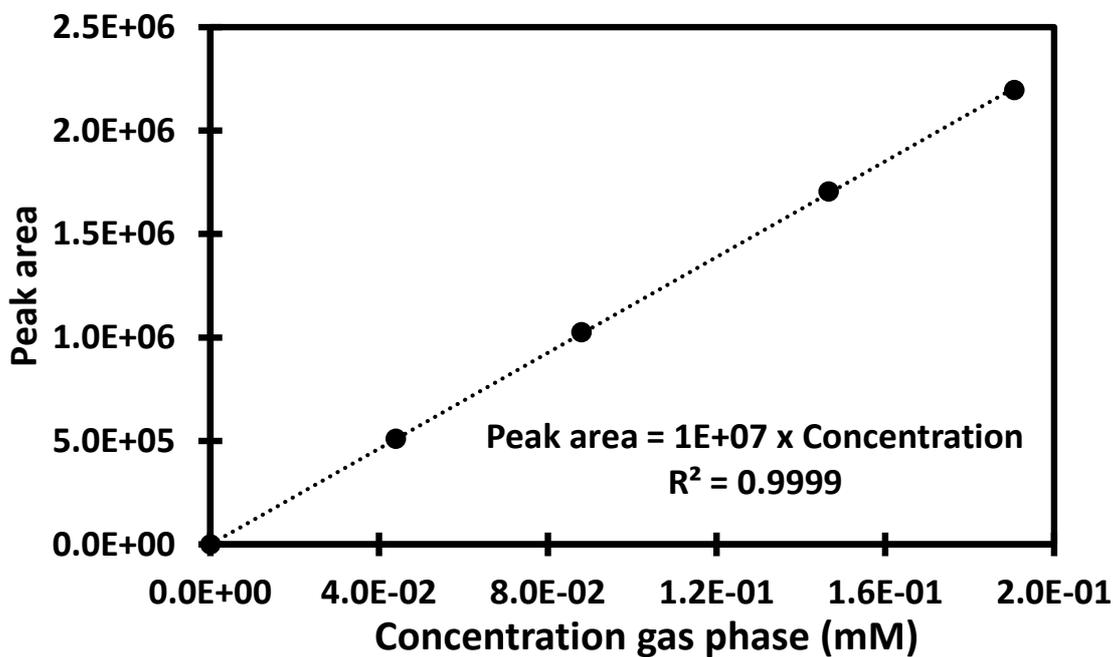
Figure A.5 - Calibration curve vinyl chloride Porto.

A.4 Ethene
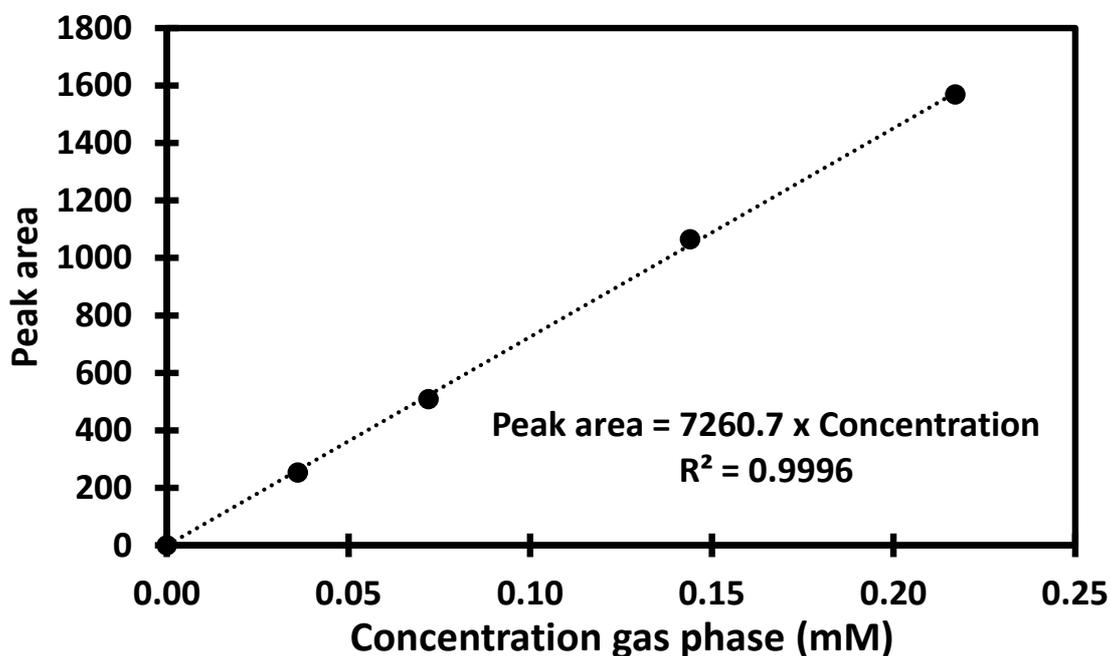
Figure A.6 - Calibration curve ethene Rome.

Peak area = 7260.7 x Concentration
$R^2 = 0.9996$
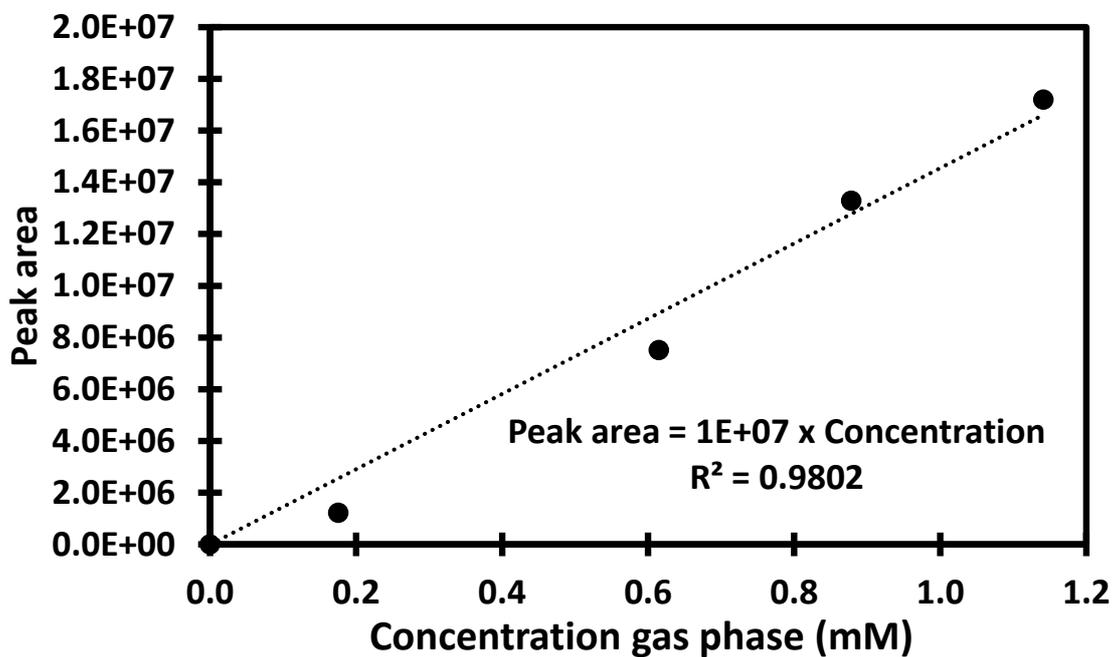
Figure A.7 - Calibration curve ethene Porto.

Peak area = 1E+07 x Concentration
$R^2 = 0.9802$
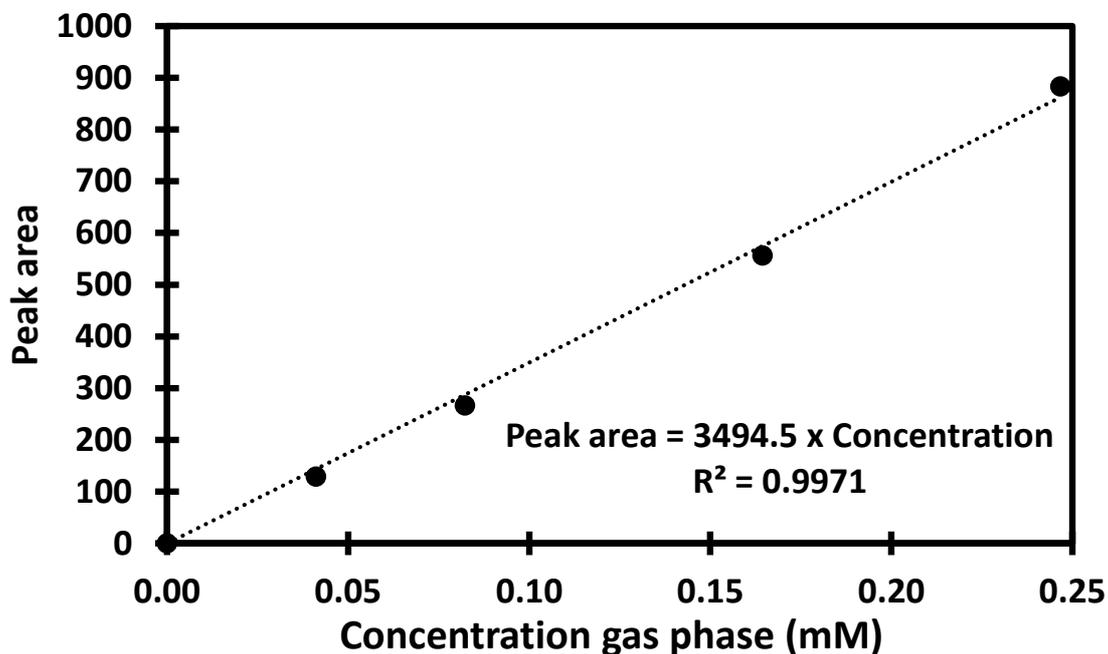
A.5 Methane

Figure A.8 - Calibration curve methane Rome.
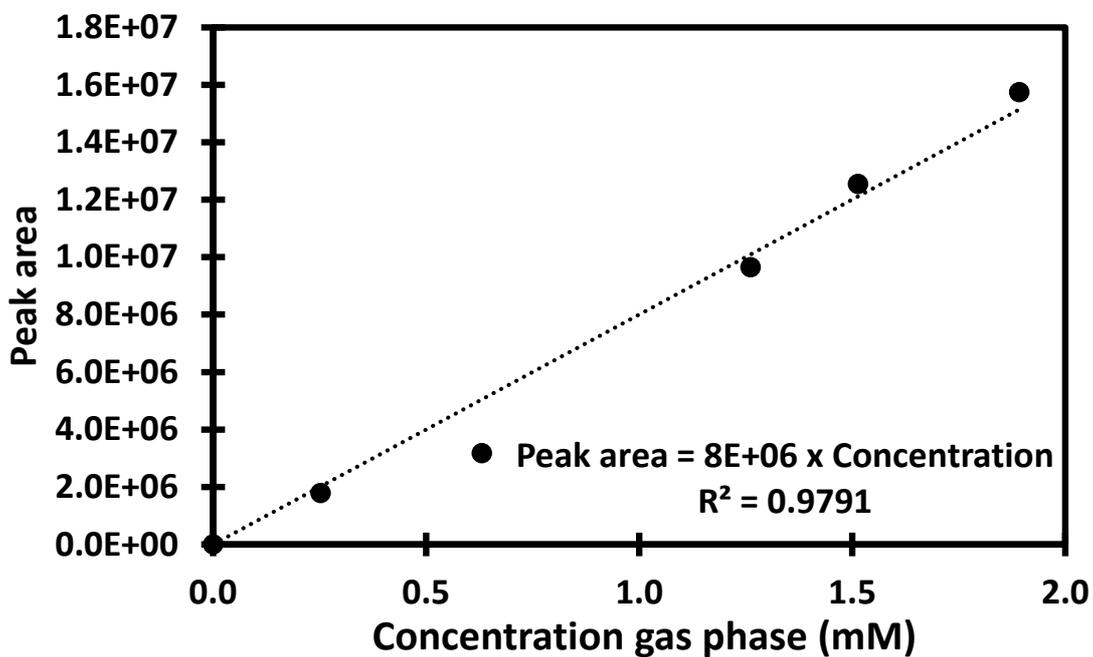
Figure A.9 - Calibration curve methane Porto.

B. Hydrogen analytical method

Standards of H_2 were prepared in serum bottles or in the BES apparatus, with a defined liquid volume of water. The recipients were sealed with Teflon-faced butyl rubber stoppers and aluminum crimp caps and flushed with N_2/CO_2 (70:30 v/v) for 30 min. A given volume of volatile compound was added to each of the recipients to obtain a calibration curve in the range of the values expected in the experimental work. To reach equilibrium standards were left shaking for a 3 hour period, in an upside down position in the case of the serum bottles. Headspace was analyzed by injecting 50 μL (taken with a gas-tight syringe) in a PerkinElmer Auto System gas chromatograph (4.6 m x 2.1 mm stainless steel column packed with 60/80 Carboxen-1000 support Supelco; N_2 carrier gas 40 mL/min; oven temperature 225 ^0C; TCD temperature 250 ^0C). Gas phase concentrations were plotted as a function of the peak area (Figure B.1). Hydrogen dimensionless Henry's constant at 25 ^{0}C is 52.436.

Paek area = 57.213 x Concentration
R² = 0.9995
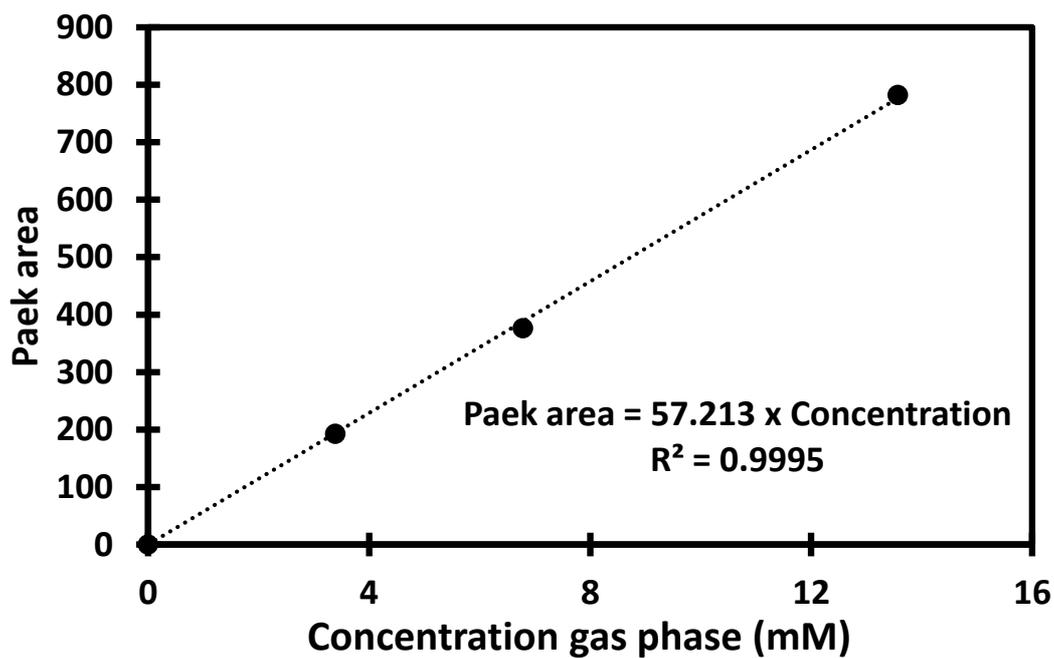
Figure B.1 - Calibration curve hydrogen Rome.

C. Volatile fatty acids analytical methods

Rome: Organic acids (acetate, propionate, butyrate, and isobutyrate) were analyzed by injecting 1 µL of filtered (0.22 µm porosity) liquid sample into a PerkinElmer Auto System gas chromatograph (2 m × 2 mm stainless steel column packed with 60/80 mesh Carbopak B-DA 80–120 4 % CW 20 M Supelco; N_2 carrier gas 20 mL/min; oven temperature 175 °C; injector temperature 200 °C; FID temperature 250 °C).

The obtained calibration curves are indicated in Figures C.1 to C.4.

Peak area = (9.5163 x Concentration) - 0.552
R^2 = 0.9998
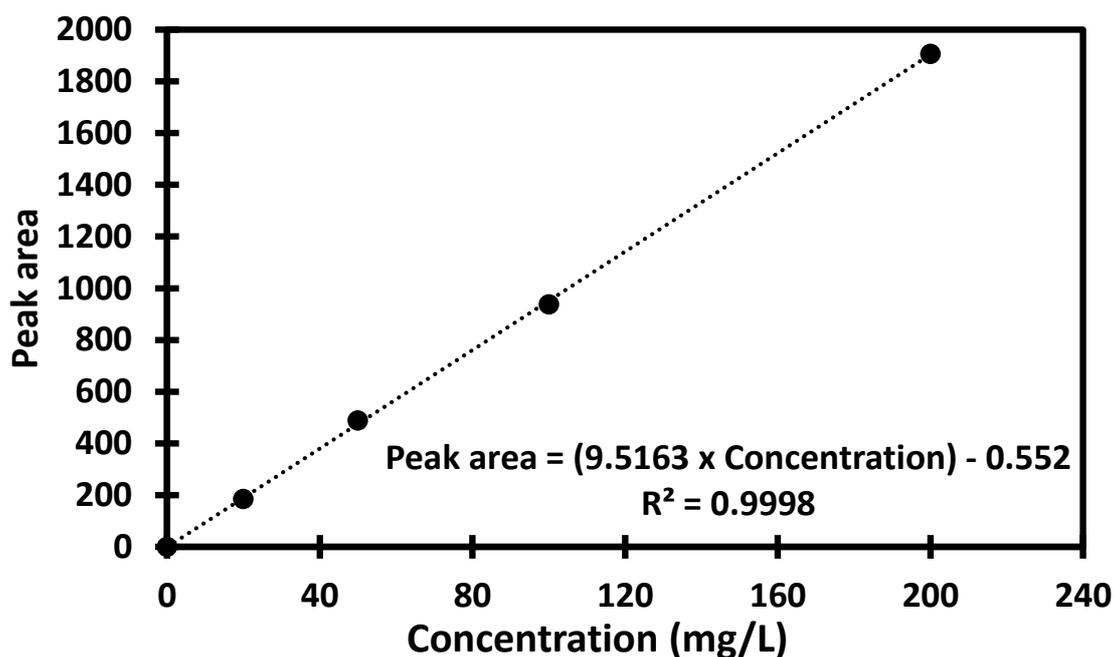
Figure C.1 - Calibration curve acetate Rome.
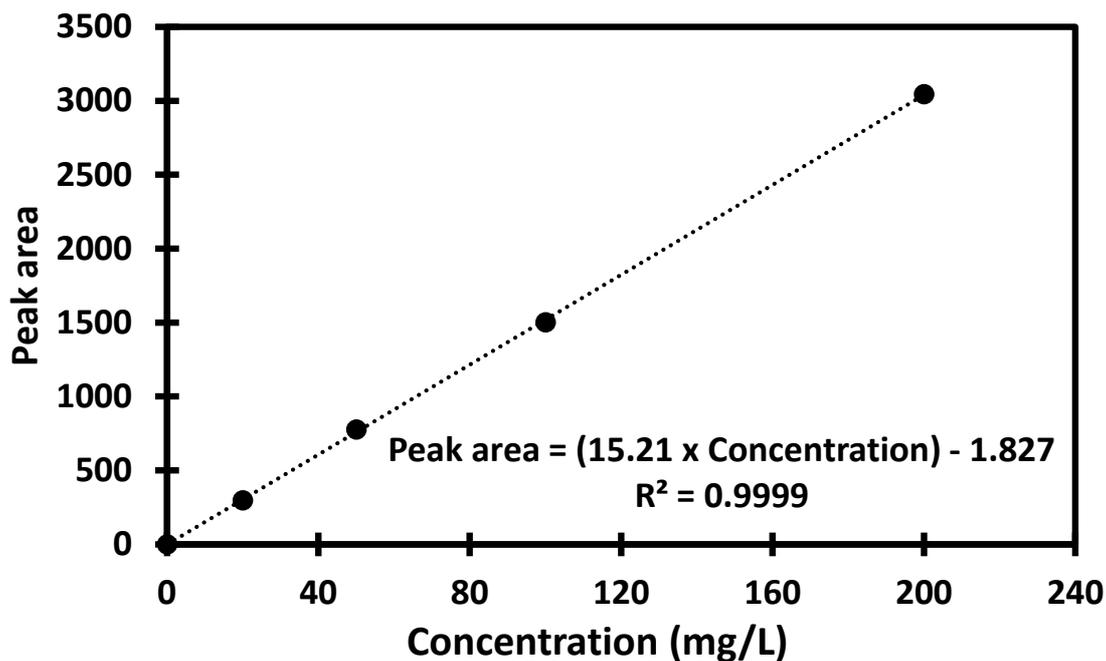
Figure C.2 - Calibration curve propionate Rome.
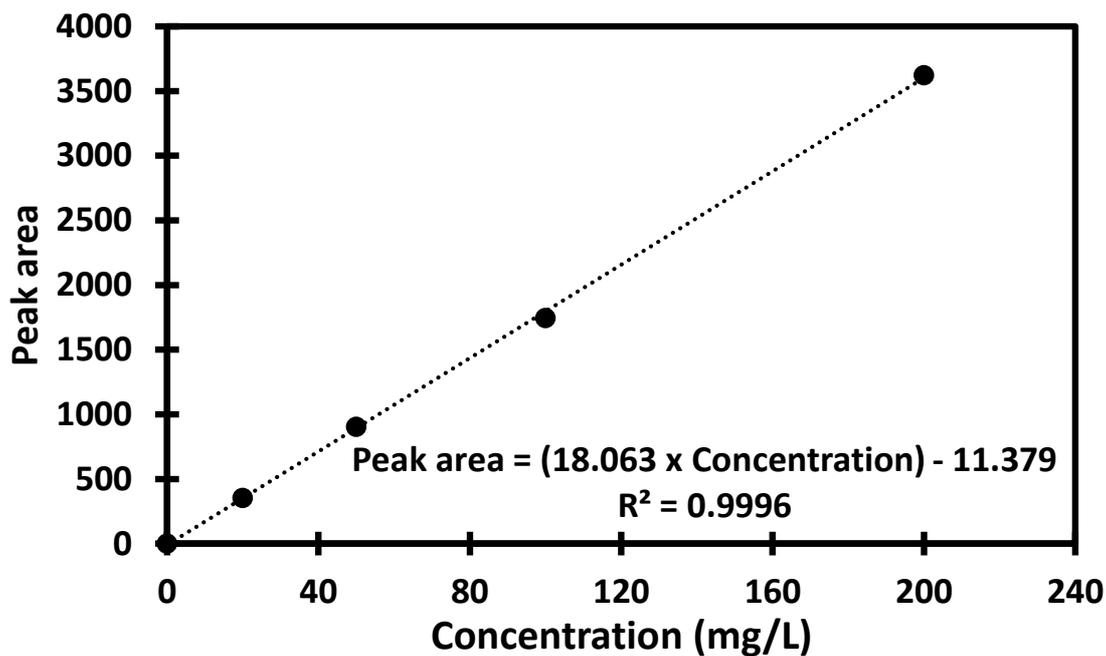
Figure C.3 - Calibration curve butyrate Rome.
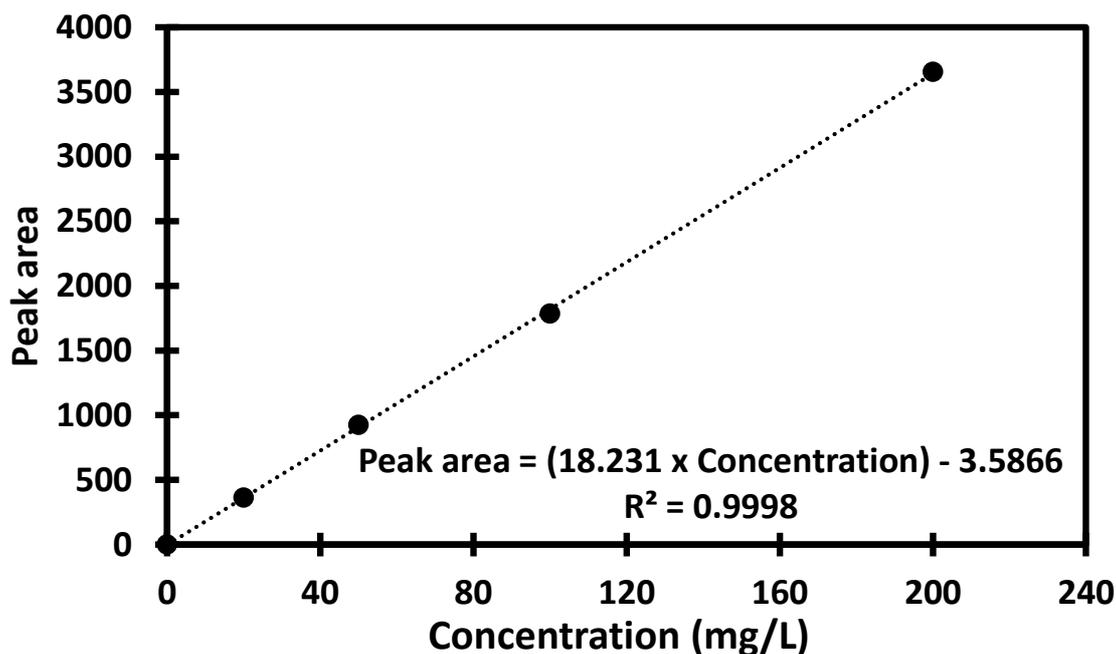
Figure C.4 - Calibration curve isobutyrate Rome.

Porto: acetate and propionate were analyzed by injecting 1 µL of filtered (0.22 µm porosity) liquid sample, previously acidified with formic acid (10 % v/v), into a Shimadzu GC-2014 gas chromatograph (3 m × 2.1 mm stainless steel column packed with 60/80 mesh Carbopak C/ 0.3 % Carbowax 20 M/ 0.1 % H_3PO_4; N_2 carrier gas 40 mL/min; oven temperature 120 °C; injector temperature and FID temperature 200 °C).

The obtained calibration curves are indicated in Figures C.5 to C.6.
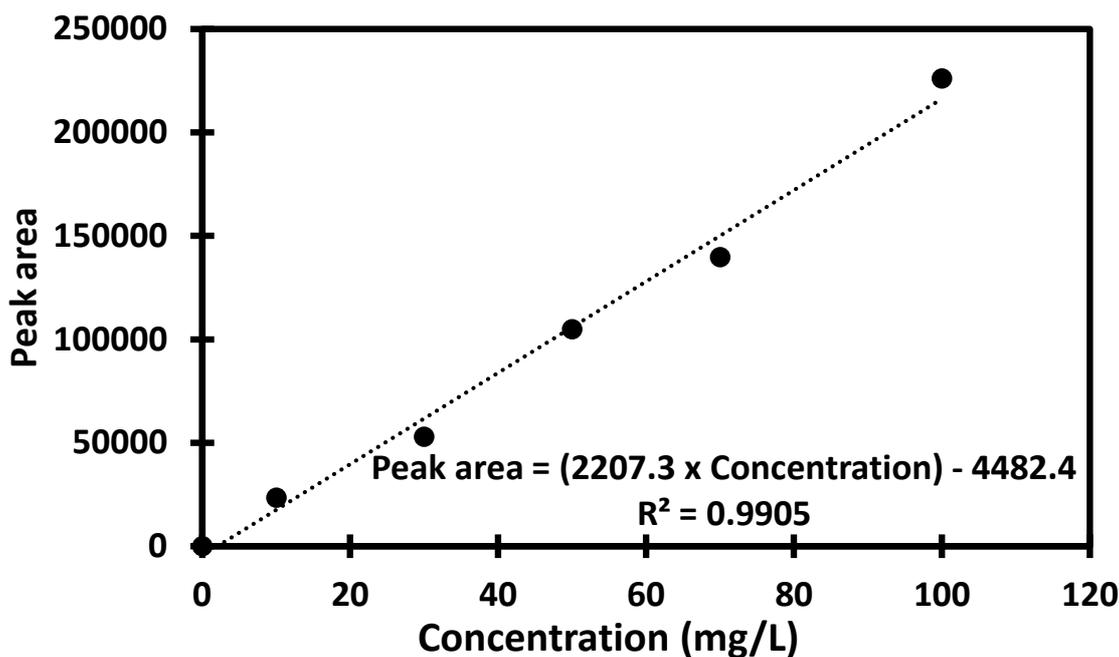
Figure C.5 - Calibration curve acetate Porto.
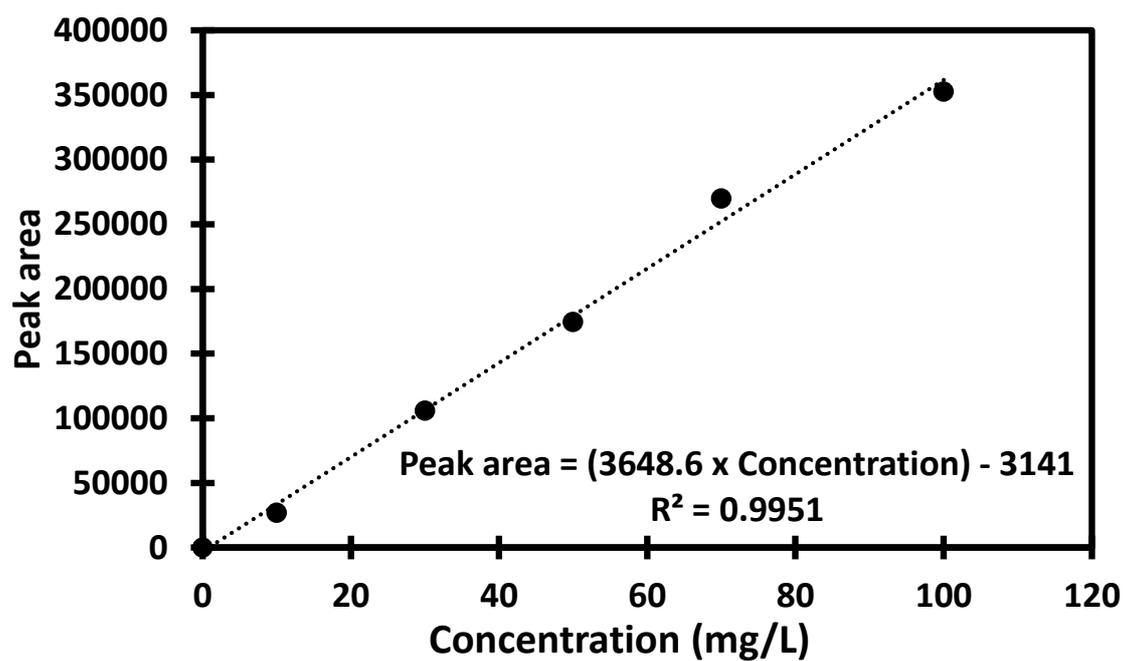
Figure C.6 - Calibration curve propionate Porto.

D. Medium composition

D.1 Trace metal solution

The composition of the trace metal solution is presented in table D.1.

Table D.1 - Trace metal solution composition.

Compound	Concentration ($g.L^{-1}$)
$N(CH_2COOH)_3$	4.5
$FeSO_4.7H_2O$	0.556
$MnSO_4\ H_2O$	0.086
$CoCl_2.6H_2O$	0.17
$ZnSO_4.7H_2O$	0.21
H_3BO_3	0.019
$NiCl_2$	0.02
Na_2MoO_4	0.01

pH was adjusted to 7.0 with a KOH 10 M solution.

D.2 Vitamin solution

The composition of the vitamin solution is presented in table D.2.

Table D.2 - Vitamin solution composition.

Compound	Concentration (mg.L^{-1})
Biotin	20
Folic acid	20
Pyridoxine hydrochloride	100
Thiamine hydrochloride	50
Riboflavin	50
Nicotinic acid	50
D-pantothenic acid	50
Cyanocobalamin	2
4-aminobenzoic acid	50

D.3 Mineral media solution

The composition of the mineral media solution is presented in table D.3.

Table D.3 - Mineral media solution.

Compound	Concentration
NH_4Cl	0.5 g.L^{-1}
$MgCl_2 \cdot 6H_2O$	0.1 g.L^{-1}
K_2HPO_4	0.4 g.L^{-1}
$CaCl_2 \cdot 2H_2O$	0.05 g.L^{-1}
Trace metal solution	10 mL.L^{-1}
Vitamin solution	10 mL.L^{-1}
$NaHCO_3$ (from 10% w/v stock solution)	15 mL.L^{-1}

The pH of the medium was 7.5.

E. Proton exchange membrane pretreatment, graphite electrode pretreatment and graphite electrode modification

E.1 Proton exchange membrane pretreatment

PEM Nafion™ 117 was cut with the appropriate size (3-cm^2 cross-sectional area) to fit the separation of the anode and cathode chamber of the BES. The membrane was pretreated by boiling in H_2O_2 (30%), DI water, 0.5 M H_2SO_4 and DI water, each for 2 h, and then stored in DI water prior to being used.

E.2 Graphite electrode pretreatment

Electrodes were graphite rods (6 mm diameter, Sigma Aldrich, Milano, Italy). The rod had an original length of 150 mm and was cut in half, so that the length was approximately 75 mm. A 1 mm hole was made with a drilling point, at 10 mm distance from the top of the rod.

New electrodes were soaked in 1 M HCl, DI water, 1 M HCl and DI water, for one day in each solution. Titanium wire (0.81 mm diameter, Alfa Aesar, Germany) was passed through the hole of the graphite rod and then through the butyl rubber stopper with a sufficient length to fit the neck of the BES. The nominal surface area of the cathode

(calculated by taking into account only the part of the electrode that was immersed in the liquid phase) was 9.7 cm^2.

Connections from the titanium wire to the potentiostat were made with cables supplied with the equipment.

After use in a BES experiment the electrodes were soaked in 1 M HCl, DI water, 1 M NaOH and DI water, for one day in each solution, to remove possible metal and biomass contamination.

E.3 Graphite electrode modification

E.3.1 Fumasep electrode

The procedure for the fumasep electrode consisted of dissolving an anionic exchange membrane, fumasep® FAD (FuMA-Tech GmbH) in ethanol (0.25 % (w/v)) for ten minutes at 60 °C, to ensure complete dissolution. The pre-treated graphite electrode was completely immersed in this solution, for two hours at 60 °C. The fumasep electrode was air dried overnight in a vertical position and washed twice with DI water and once with mineral media.

E.3.2 Fumasep + AQDS electrode

The procedure for the fumasep + AQDS electrode consisted of dissolving an anionic exchange membrane, fumasep® FAD (FuMA-Tech GmbH) in ethanol (0.25 % (w/v)) for ten minutes at 60 °C, to ensure total dissolution. AQDS at a final concentration of 0.5 or 1 mM was added to the previous solution and left to dissolve and react for one hour at 60ºC. The pre-treated graphite electrode was completely immersed in this solution, for two hours at 60 °C. The fumasep + AQDS electrode was air dried overnight in a vertical position and washed twice with DI water and once with mineral media.

F. Electrochemical analysis

Note: all potentials are in respect to the standard hydrogen electrode.

Rome: electrochemical measurements, including cyclic voltammetry and chronoamperometry, were performed using an IVIUMnSTAT, multichannel electrochemical analyser, and data was processed with Ivium Soft software version 2.161 (Ivium Technologies (The Netherlands)).

Cyclic voltammograms were recorded at different scan rates (from 1 mV/s to 20 mV/s or 50 mV/s) between 200 mV and -700 mV. Each measurement consisted of a 4 cycle analysis and the average cyclic voltammogram was calculated by the software. The midpoint potential (defined as the average of anodic peak potential, $E_{p,a}$ and cathodic peak potential, $E_{p,c}$) of the redox peaks in each average scan was calculated by automatically subtracting the baseline and manually marking the peaks. An average mid-point potential value of all scan rates performed was also calculated.

Chronoamperometric tests were carried out with different cathode potentials, namely OCP, -300 mV, -500 mV, -600 mV, -700 mV and -900 mV, and the current was registered as a function of time.

Porto: electrochemical measurements, including cyclic voltammetry and chronoamperometry, were performed using an Autolab PGSTAT204

potentiostat/galvanostat and data was processed with Nova software (version 1.10.4) from Metrohm Autolab B.V. (The Netherlands).

Cyclic voltammograms were recorded at different scan rates (from 1 mV/s to 50 mV/s) between 200 mV and -700 mV. Each measurement consisted of a 4 cycle analysis and the average cyclic voltammogram was calculated by the software

Chronoamperometric tests were carried out with different cathode potentials, namely OCP and -300 mV, and the current was registered as a function of time.

G. CARD-FISH

G.1 Solutions

G.1.1 Agarose 0.2% solution

Dissolve 0.2 g of low gelling point agarose in a final volume of 100 mL MQ water. Autoclave the solution (121 °C, 25 minutes).

G.1.2 Ethanol 96% solution

Dilute 96 mL of ethanol with 4 mL of MQ water to a final volume of 100 mL.

G.1.3 NaOH 10 M solution

In 70 mL of MQ water add 40 g of NaOH. Adjust to final volume of 100 mL.

G.1.4 HCl 10 M solution

In 15 mL of MQ water add 83.3 mL of HCl. Adjust to final volume of 100 mL.

G.1.5 EDTA 0.5 M

Dissolve 186.1 g of $Na_2EDTA.2H_2O$ in 700 mL of MQ water. Adjust pH to 8.0 with 10 M NaOH (±50 mL). Adjust to final volume of 100 mL. Autoclave the solution (121 °C, 25 minutes). Keep at RT.

G.1.6 TrisHCl 1 M solution

Dissolve 157.6 g of tris base in 800 mL of MQ water. Adjust pH to 7.4 with 10 M HCl. Add MQ to 1 L. Adjust to final volume of 100 mL. Autoclave the solution (121 °C, 25 minutes). Keep at RT.

G.1.7 TE solution

Into 70 mL of MQ water add 1 mL of TrisHCl 1 M solution and 0.2 mL of EDTA 0.5 M. Adjust pH to 8 with NaOH 10 M (some drops). Adjust to final volume of 100 mL. Autoclave the solution (121 °C, 25 minutes). Keep at RT.

G.1.8 Proteinase K 1 g/L solution

Dissolve 100 mg of proteinase K (34 U/mL-0.034 U/µL) with MQ water to a final volume of 100 mL. Store in aliquots at -20 °C.

G.1.9 HCl 0.01 M solution

In 49.95 mL of MQ water dilute 0.05 mL of HCl 10 M solution.

G.1.10 NaCl 5 M solution

Dissolve 146.1 g of NaCl in 500 mL of MQ water. Autoclave the solution (121 °C, 25 minutes). Keep at RT.

G.1.11 Maleic acid buffer 100 mM solution

Dissolve 0.58 g of maleic acid in 30 mL of MQ water. Add to the previous solution 1.5 mL of NaCl 5 M solution. Adjust to final volume of 50 mL.

G.1.12 Blocking reagent 10% solution

Dissolve 10 g of blocking reagent in 100 mL of maleic acid buffer final volume. Adjust pH to 7.5 with NaOH 10 M solution. Autoclave the solution (121 °C, 25 minutes). Store for several months at 4 ºC.

G.1.13 Triton X-100 0.05 % solution

Dilute 0.5 mL of triton X100 in 50 mL of MQ water final volume.

G.1.14 Hybridization buffer solution

In the following order add 1800 μL of NaCl 5 M solution, 200 μL of TrisHCl 1 M solution, 1 g of dextran sulfate and MQ water depending on stringency (see Table G.1). Heat the previous solutions at 48 °C for 30 minutes in a water bath. Allow to cool down and add formamide depending on stringency (see Table G.1), 1000 μL of blocking reagent 10 % solution and 5 μL of triton X100 0.05 % solution. Aliquot and store at -20 °C for several.

Table G.1 - Volume of formamide and MQ water for 10 mL of hybridization buffer, according to % of stringency.

% Stringency	Volume of formamide (μL)	Volume of MQ water (μL)
5	500	6500
10	1000	6000
20	2000	5000
30	3000	4000
35	3500	3500
40	4000	3000
50	5000	2000
55	5500	1500

G.1.15 Probe working solution

Dissolve oligonucleotides to 50 ng/μL. The greatest danger for dissolved oligonucleotides lies in nucleases. To minimize degradation by nucleases, it is best to store oligonucleotides in dry state. Oligonucleotides are remarkably stable, even when stored at 4 °C or room temperature. For longer storage, dry oligonucleotides should be stored at -20 °C. Repeated freeze/thaw cycles should be avoided.

G.1.16 SDS 20 % solution

Dissolve at 40 °C in water bath 100 g of SDS in 500 mL of MQ water. Filter the solution through an acrodisc with a pore size of 0.2 μm.

G.1.17 1xPBS solution

Dissolve 0.276 g of NaH_2PO_4, 1.424 g of Na_2HPO_4 and 7.597 g of NaCl with MQ water to a final volume of 1 L. Autoclave the solution (121 °C, 25 minutes). Store at 4 °C.

G.1.18 10xPBS solution

Dissolve 2.76 g of NaH_2PO_4, 14.24 g of Na_2HPO_4 and 75.97 g of NaCl with MQ water to a final volume of 1 L. Autoclave the solution (121 °C, 25 minutes). Store at 4 °C.

G.1.19 Amplification buffer solution

Add 5 g of dextran sulphate, 20 mL NaCl 5 M solution, 5 mL 10xPBS and 0.5 mL blocking reagent 10 % solution with MQ water to a final volume of 50 mL.

G.1.20 Dye-tyramide solution

This solution must be prepared in the hood and on ice. Add 5 μL of trimethylamine, with 0.5 mL of dimethylformamide and with 5 mg of tyramine HCl. In the dark, to 1 mg of Alexa 488 (Molecular probes Cat. Nr. A-20000, Life Technologies), add 100 μL of dimethylformamide and 25.2 μL of the first solution prepared. Incubate at room temperature in the dark for 6 to 12 hours, slowly rotating. Dispense in 50 μL eppendorfs 3.13 μL (0.025 mg). Dry the aliquots under vacuum at room temperature to be stored at -20°C (stable for years). To reconstitute aliquots to final concentration of 1 mg/L add 25 μL dimethylformamide.

G.2 Hybridization procedure in filters

To a given volume of sample add formaldehyde at a final concentration of 2 % for 3h at 4 °C. Add double the previous total volume of ethanol. Store at -20°C if sample is not immediately filtered. Periods longer than 2 days without filtering should be avoided. Filter the fixed sample through polycarbonate membrane filters (pore size 0.2 μm, diameter 47 mm, Millipore) by gentle vacuum (<0.2 bar). Store at -20 °C until further processing. Cut filter sections and number them. Place filter sections face up into glass slides, embedded in agarose 0.2 % solution and place for 10 min at 35 °C. Dehydrate for 1 min at RT in ethanol 96% solution. Air dry filters on blot paper. Prepare the lysozyme solution as indicated in Table G.2 depending on the number of filter sections.

Table G.2 - Quantity of reagents needed for permeabilization with lysozyme.

Number of filter sections	1 to 3	4 to 15	16 to 20
Mass of lysozyme (g)	0.01	0.02	0.10
EDTA 0.5 M solution (μL)	200	400	2000
TrisHCl 1 M solution (μL)	100	200	1000
Final volume with MQ water (mL)	1	2	10

Dip all the sections in lysozyme solution and incubate for 60 min at 37 °C. Wash for 1 min at RT with MQ water. Incubate for 25 min at 37 °C in a solution containing 18 μL of Proteinase K 1 g/L solution in 6 mL TE solution. Wash for 1 min at RT with MQ water. Incubate for 10 min at RT in 0.01 M HCl to bleach endogenous peroxidase. Wash with a lot of MQ water. Wash for 1 min at RT in ethanol 96 % solution. Air dry filters. Mix hybridization buffer solution according to the stringency described for probe being used and the following:

-for upto 15 filter sections: mix 200 µL of hybridization buffer (HB) and 2 µL of probe working solution;

-for upto 20 filter sections: mix 400 µL of hybridization buffer and 4 µL of probe working solution.

Place sections in the hybridization mix. Incubate ON at 35 ºC in the dark under rotation. Wash sections for 10 min at 37 ºC in pre-warmed (48 ºC) washing buffer according to Table G.3.

Table G.3 - Volume of NaCl 5 M and MQ water for 50 mL of washing buffer according to % of stringency.

% Stringency	Volume of NaCl 5M (µL)	Volume of H_2O (µL)
5	3150	44825
10	2025	45950
20	1350	46625
30	640	47335
35	420	47555
40	270	47705
50	90	47885
55	30	47945

Do not air dry sections after washing. Wash sections for 15 min at RT in 1xPBS solution with mid agitation. Dab sections on blotting paper, but do not let run dry. Prepare the substrate mix as follows: with 200 µL 1xPBS solution and 1 µL H_2O_2 30%. From the previous solution take 12 µL and add to 1200 µL of amplification buffer. From the previous solution take 1000 µL and add 1 µL of dye-tyramide solution. Place all sections in this last solution and incubate for 10 min at 37 ºC in the dark-rotating. Wash sections for 25 min at RT in 1xPBS with mid agitation, in the dark. Wash sections for 1 min at RT in MQ water. Wash for 1 min at RT in ethanol 96 % solution. Air dry filters. Counter stain sections with one drop of Vectashield-Citiflor-DAPI. Cover slides with coverslips and on top place non-fluorescent immersion oil.

G.3 Microscopy visualization

Rome: slides were examined by epifluorescence microscopy (Olympus, BX51) and the images were captured with an Olympus F-View CCD camera and processed and analyzed with AnalySIS software (SIS, Germany).

Porto: slides were examined using an Olympus BX51 epifluorescence microscope (Olympus Portugal) equipped with filters sensitive to the applied fluorochromes. All images were acquired using the Olympus CellB (Olympus Portugal) software with a magnification of 1000x.

H. Scanning electron microscopy/ Energy-dispersive X-ray spectroscopy procedure

All samples were analyzed with a SEM/EDS system (FEI Quanta 400FEG ESEM/EDAX Genesis X4M, FEI Company, USA) in high-vacuum mode at 10 or 15 kV. X-ray microanalysis was performed in specific fields for elemental characterization.

H.1 Non biological samples

Treatment of synthetized magnetite nanoparticles solution consisted of mounting 50 µL directly on aluminum stubs and dried for 24 hours at 80 ºC.

Stubs were stored in a desiccator until SEM analysis for a maximum period of 24 hours in order to prevent moisture absorption/adsorption.

H.2 Biological planktonic samples in stubs

5 mL of sample was removed and centrifuged at 5000 rpm for 5 min. After removing the supernatant, the sample was fixed in 3 % (w/v) glutaraldehyde in cacodylate buffer (pH 7.2) for 3 hours at room temperature. The combined mixture was centrifuged (5000 rpm, 5 min) and almost all of the supernatant was removed. A small quantity (250 µL) of the remaining solution was then mounted on an aluminum stub and dried at 50 ºC for 3 hours. Afterwards, the samples were dehydrated by immerging the stub with the fixed sample in 50 % and 100 % (v/v) (2x) ethanol solutions for 10 min each at room

temperature. This was then followed by a series of HMDS in ethanol treatments at 50, 60, 70, 80, 90 and 2x 100 % (v/v) HMDS for 10 min each at room temperature. These dehydration steps were necessary to preserve the integrity of biological structures.

Stubs were stored in a desiccator in the dark until SEM analysis for a maximum period of 24 hours in order to prevent moisture absorption/adsorption.

Samples were sputter-coated for 60 s at a 15 mA current with a palladium–gold thin film using the SPI Module Sputter Coater equipment.

H.3 Biological planktonic samples in glass slides

Planktonic samples were pretreated removing 5 mL of liquid content from the cathode chamber and centrifuging at 5000 rpm for 5 min. After removing the supernatant, the sample was fixed in 3 % (w/v) glutaraldehyde in cacodylate buffer (pH 7.2) for three hours at room temperature. The combined mixture was centrifuged (5000 rpm, 5 min) and almost all of the supernatant was removed. A small quantity (250 µL) of the remaining solution was then mounted on a glass slide and placed at 50 ºC for 3 hours. The following treatment consisted of dehydration with ethanol by a series of 50, 60, 70, 80, 90 and 2x 100 % (v/v), followed with HMDS in ethanol by a series of 50, 60, 70, 80, 90 and 2x 100 % (v/v), 10 minutes each at room temperature.

Treated samples were stored in a desiccator in the dark until SEM analysis for a maximum period of 24 hours in order to prevent moisture absorption/adsorption.

Planktonic treated samples were sputter-coated for 70 s at a 15 mA current with a palladium–gold thin film in argon atmosphere using the SPI Module Sputter Coater equipment.

H.4 Electrode with biological material samples

Fumasep + AQDS electrode was immersed in a petri dish containing 3 % (w/v) glutaraldehyde in cacodylate buffer (pH 7.2) for 3 hours at room temperature. The following treatment consisted of dehydration with ethanol by a series of 50, 60, 70, 80, 90 and 2x 100 % (v/v), followed with HMDS in ethanol by a series of 50, 60, 70, 80, 90 and 2x 100 % (v/v), 10 minutes each at room temperature.

Treated electrode samples were stored in a desiccator in the dark until SEM analysis for a maximum period of 24 hours in order to prevent moisture absorption/adsorption.

The fumasep + AQDS treated electrode was sputter-coated for 40 s at a 15 mA current with a palladium–gold thin film in argon atmosphere using the SPI Module Sputter Coater equipment.

I. Magnetite synthesis

The procedure for synthetizing magnetite nanoparticles (NPs) consisted of dissolving, with constant stirring, 5.2 g of $FeCl_3$ and 2.0 g of $FeCl_2$ in 0.85 mL of HCl 12.1 M. This solution was then added dropwise under vigorous stirring to a 250 mL 1.5 M NaOH solution. The magnetic NPs precipitate was isolated using a magnetic field. The supernatant was purified by centrifugation (4000 rpm) and decantation and was then suspended in 250 mL of MQ deoxygenated water. This procedure was performed in triplicate.

Surface anionic charge neutralization was performed to eliminate repulsion charges between nanoparticles. This was done adding 500 mL of HCl 0.01 M to the nanoparticles suspension under stirring. After homogenization, the solution was centrifuged (4000 rpm) and peptized with water. The magnetite NPs were stored at 4 ^{0}C until use.

J. Iron calibration curve

The iron concentration in solution was measured using an Oxford X-ray fluorescence X-MET7500 Instrument. Data was analyzed with the X-MET software by linear regression of iron standard solutions. The experimental values used are presented in Table J.1 and the calibration curve is shown in Figure J.1.

Table J.1 - Experimental conditions for the analysis of iron concentration in standards and samples analysed.

Element:	Fe(1, 4)
Lower(keV):	6.33
Upper(keV):	6.47
Peak(keV):	6.40
Voltage(kV):	40.00
Current(uA):	10
Counting time(s):	60
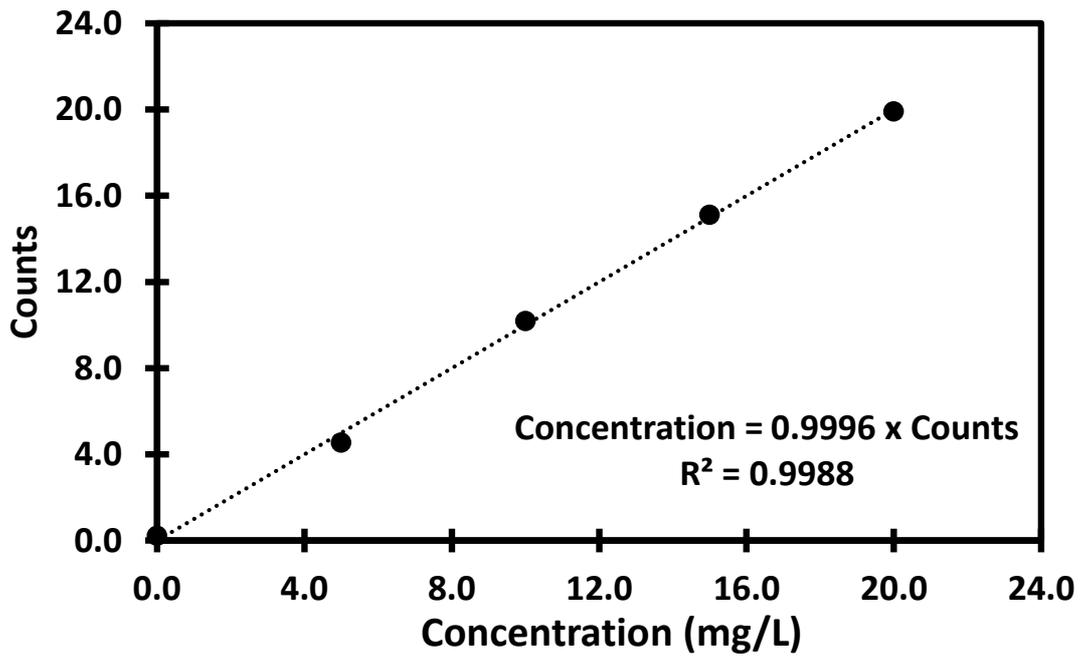
Figure J.1 – Calibration curve iron.

Samples with an iron concentration outside the range of the calibration curve were diluted in order to be included in the concentration range.

K. Total organic carbon calibration curve

Total carbon, inorganic and non purgeable organic carbon were analyzed by a Shimadzu Total Organic Carbon Analyser (TOC-V CSN) with an oxygen flow of 150 mL/min and a furnace temperature of 680 °C. Total carbon was measured by injection of a preselected amount in the furnace. For the analysis of inorganic carbon a pre-treatment was performed with HCl 2 M and for non purgeable organic carbon, besides the acid pre-treatment, samples were purged with O_2 for 3 min. TOC and POC were calculated as follows from the measurements performed for TC, IC and NPOC:

$$TOC = TC - IC$$

$$POC = TOC - NPOC$$

The obtained calibration curves are indicated in Figures K.1 to K.3.
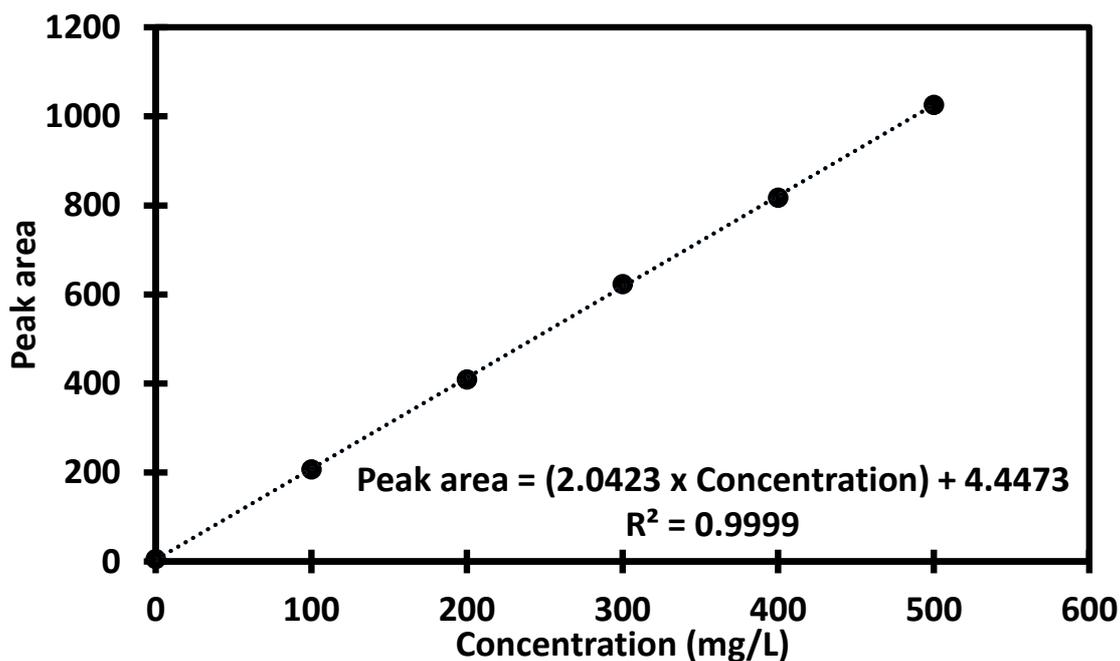
Figure K.1 - Calibration curve total carbon.

Peak area = (2.0423 x Concentration) + 4.4473
R² = 0.9999
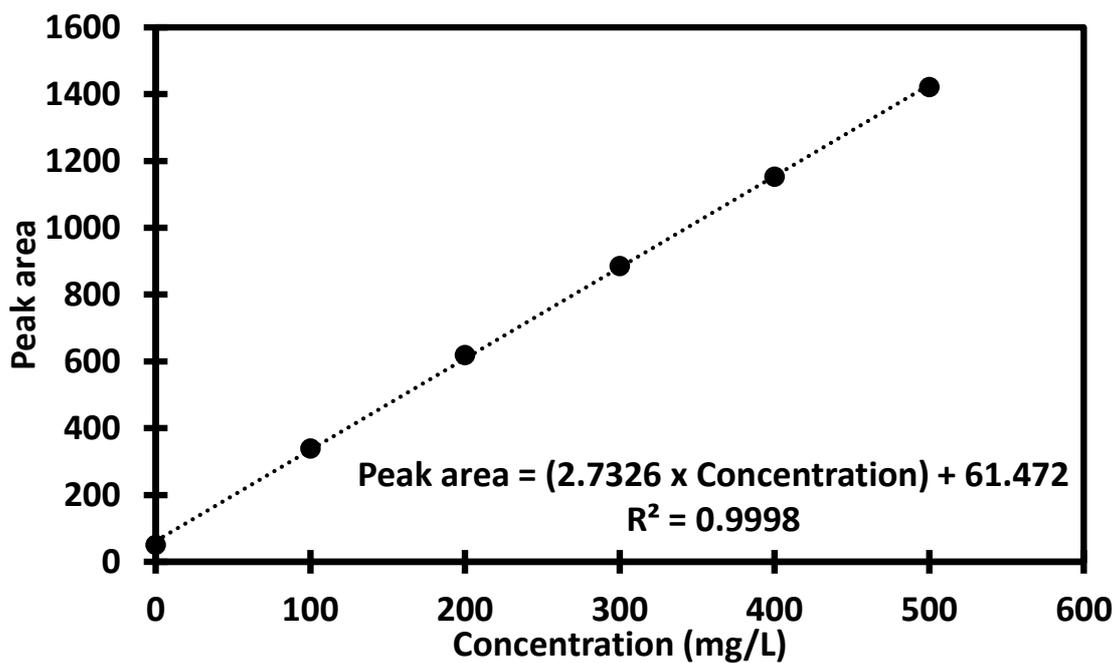
Figure K.2 - Calibration curve inorganic carbon.

Peak area = (2.7326 x Concentration) + 61.472
R² = 0.9998
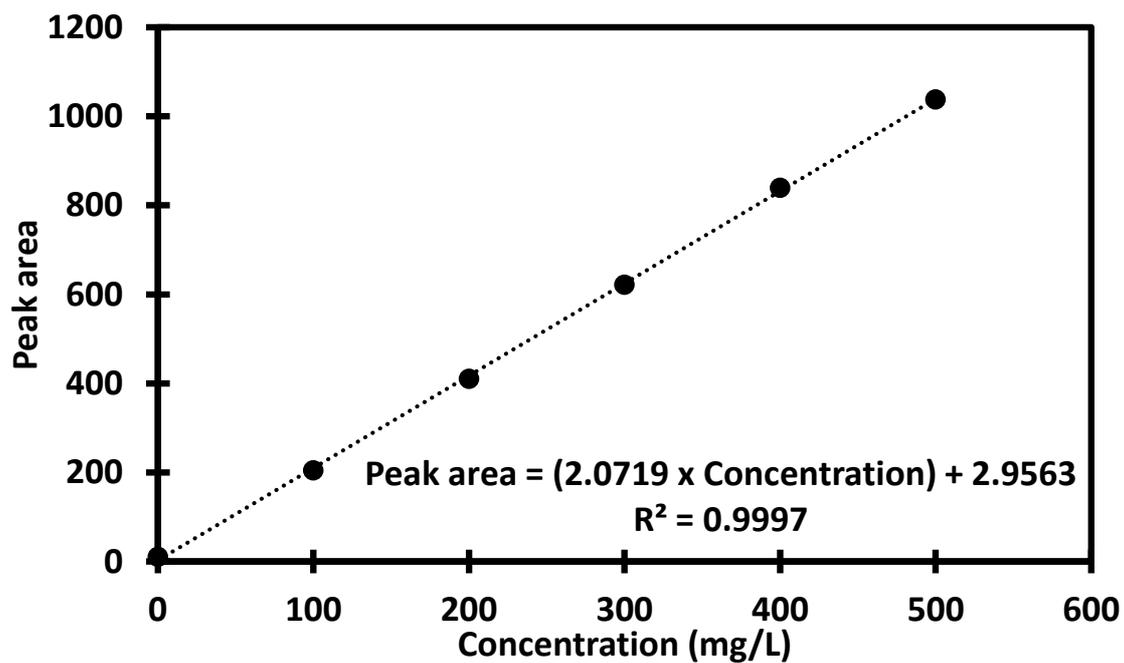
Figure K.3 - Calibration curve non purgeable organic carbon.

L. PNA-FISH

L.1 Solutions

L.1.1 Probe solution

Add 1 mL of acetonitrile to 8 mL of ultrapure water. Acetonitrile must be handled with care. In the hood add 100 μL of trifluoracetic acid to the previous solution and fill the rest of the volume with ultrapure water. Filter the solution through an acrodisc with pore size of 0.2 μm and store at 4 °C. Solution can be stored for a period of one year.

L.1.2 3xPBS solution

Weigh 4.8 g of sodium chloride, 0.120 g of potassium chloride, 0.486 g of dibasic sodium phosphate and 0.120 g of potassium hydrogenphosphate. Add MQ water to a final volume of 200 mL and Autoclave the solution (121 °C, 25 minutes).

L.1.3 NaOH 2 M solution

In 70 mL of MQ water add 9 g of NaOH. Adjust to final volume of 100 mL.

L.1.4 HCl 1 M solution

In 70 mL of MQ water add 8.33 mL of HCl. Adjust to final volume of 100 mL.

L.1.5 Paraformaldehyde 4% solution

Heat 65 mL of DI water at 60 °C and in the hood add 4.0 g of paraformaldehyde. Keep the solution under heating and agitation and add NaOH 2 M dropwise until the solution turns clear (± 1 to 2 min). Remove the heating source and add 33 mL of the 3xPBS solution. Adjust with HCL 1 M the pH to 7.2 and adjust the volume to 100 mL with MQ water. Filter the solution through an acrodisc with a pore size of 0.2 μm. Cool rapidly at 4°C, in the cooler or with ice.

L.1.6 Ethanol 50% solution

Dilute 50 mL of ethanol with 50 mL of MQ water to a final volume of 100 mL.

L.1.7 Hybridization solution

Add 1 g of dextran sulphate, 0.0058 g of sodium chloride, 500 μL formamide, 0.01 g of sodium pyrophosphate, 0.02 g of polyvinylpyrrolidone, 0.02 g of ficoll, 0.02 g of disodium EDTA, 0.01 mL of triton X-100 and 0.079 g of TrisHCl. Add MQ water to a final volume of 10 mL and adjust the pH to 7. Filter the solution through an acrodisc with a pore size of 0.2 μm and store at 4 °C.

L.1.8 Washing solution

Weigh 0.303 g of tris base, 0.438 g of sodium chloride and measure 500 μL triton X-100. Add MQ water to a final volume of 500 mL, adjust the pH to 10 and autoclave the solution (121 °C, 25 minutes). The solution must be prepared fresh and should not be used after 1 to 2 weeks after preparation.

L.2 Probe aliquots procedure

The probe arrives lyophilized and must be stored at -20 °C. All of the following steps must be performed in the dark, as any other step that involves probe manipulation.

L.2.1 Original probe aliquot

Add to the probe the necessary volume of probe solution so that the final concentration is 100 μM (for example 25 nmol of probe add 250 μL of probe solution). Store the solution at -20 °C wrapped in aluminum foil.

L.2.2 Stock probe aliquot

Add 40 µL of the original probe aliquot to 960 µL of MQ water. Store stock probe aliquots (4 µM) at -20°C wrapped in aluminum foil.

L.2.3 In use probe aliquot

Add 50 µL of the stock probe aliquot to 950 µL of hybridization solution. To prepare a mixture of two probes, add 50 µL of each stock probe aliquot to 900 µL of hybridization solution. Store in use probe aliquots (200 nM) at 4 °C wrapped in aluminum foil.

L.3 Hybridization procedure in slides

Take 1 mL of sample and centrifuge for 15 minutes at 4000 rpm to create pellets. Dispose of almost all of the supernatant. Usually reject 0.8 mL of supernatant and suspend the rest in the remaining liquid volume. Put 20 µL into each of the slide wells (8 mm of diameter). Allow to air dry, or in the incubator at 55 °C for 10 minutes or alternatively at the flame, but carefully. Cover the surface of the sample with 30 µL of 4 % paraformaldehyde solution and let it rest for 15 minutes. Add 30 µL of ethanol 50 % so that it covers the surface of the well. Let it rest for 10 to 15 minutes. Discharge as much as possible of the excess liquid. In samples add 20 µL of the in use probe aliquot (200 nM) and in the negative controls add 20 µL of hybridization solution. Cover the slides with coverslips and place them in a moist chamber. Incubate for 1 hour and 30 minutes at 57 °C for 195 probe, at 61 °C for CBDB1 probe and for the multiplex assay at 59 °C. Fill the coplin jar with washing solution and pre-heat it in the incubator. Remove the slides from the moist chambers and the coverslips from the slides. Place the slides in the pre-warmed washing solution and the coplin jar in the incubator for 30 minutes. Remove the slides from the coplin jar and allow them to air dry. Cover the slides with one drop of non-fluorescent immersion oil and visualize at the microscope. Slides can be stored at 4 °C in the dark for a maximum of 24 h before microscopy.

L.4 Microscopy visualization

Visualization was performed by using an Olympus BX51 epifluorescence microscope (Olympus Portugal SA, Porto, Portugal) equipped with a filter sensitive to the Alexa Fluor 488 signaling molecule attached to the 195 PNA probe (absorbance, 495 nm, emission, 519 nm) and a filter sensitive to the Alexa Fluor 594 signaling molecule attached to CBDB1 PNA probe (excitation, 530 to 550 nm; barrier, 570 nm; emission long-pass filter, 591 nm). To ensure that the signal obtained was not related to autofluorescence, all samples were visualized with other available filters. For every experiment, a negative control was

performed simultaneously. For the negative control all steps described above were performed, but no probe was added during the hybridizations procedure. All images were acquired using the Olympus CellB (Olympus Portugal) software with a magnification of ×10.