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Bacterial community dynamics in a novel 1,2-DCA dechlorinating anaerobic consortium

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To those who trusted in me,

You know who you are…

CONTENT

SUMMARY

1,2-dichloroethane (1,2-DCA) is a chlorinated solvent, mainly produced as intermediate for the synthesis of polyvinylchloride (PVC) the third-most widely used plastic polymer. Its production worldwide sums up to more than 20 million tons per year and due to its relatively high solubility in water, it tends to partition in groundwater aquifers, constituting a permanent source of contamination, being a typical persistent organic pollutant (POP).
A groundwater aquifer composed of two different layers (upper and lower layer) separated by a layer of clay, near to Ferrara (Italy) has been the object of this study, in which we investigated the potential of the indigenous microbial community to carry over remediation of pollutant, following amendment with electron donors suitable to stimulate reductive dehalogenation of 1,2-DCA.

In chapter 4, we investigated response of lower aquifer microbial community to lactate biostimulation, in terms of population shift, through *16s rRNA* gene libraries. Contextually we investigated the functional diversity of the putative reductive dehalogenase (RD) genes retrieved in the aquifer, prior and after the amendment. The population variation was moderate in terms of Shannon index and community organization shifts. However the structure of the community changed nearly completely, showing emerging co-dominating population, in which no known 1,2-DCA degraders were present. Functional genes analysis showed some redundancy of the function as well, since four distinct, but highly similar among each other, RD gene sequences were retrieved and compared with sequences found in the upper aquifer by Marzorati and colleagues (2007). A high identity percentage (99%) among these sequences and the three of the upper aquifer was found. These sequences clustered with WL rdhA1 sequence previously retrieved in a 1,2- DCA degrading co-culture dominated by *Dehalobacter* sp.WL (Grostern & Edwards, 2009). In spite of the presence of the already know RD specific for DCA, no

known dechlorinators were detected. This suggest that the diversity associated to reductive dehalogeantion is wider than how we imagined.

In chapter 5, we investigated the response of the upper aquifer communities, proceeding from two distinct piezometers, to amendment with various electron donors, with the aim of obtaining an enriched dechlorinating community and characterize it through transfers. Total diversity and functional genes were monitored through transfers through different techniques: 16S rRNA clone libraries, Illumina MySeq and functional genes PCR-DGGE. Eventually, the enriched culture was monitored through flow cytometry to follow the population growth during 1,2-DCA degradation. The best response, in terms of conservation of dechlorinating activity through transfers, was observed following amendment with a mixture of acetate and formate, which eventually led to a co-dominated community in which a putative dechlorinating Unc. *Geobacter* sp. grew together with a *Pseudomonas aeruginosa* strain, with an apparent mutual benefit. The putative dechlorinator Unc. *Geobacter* sp. closed-characterized-relatives appeared to be *G. psychrophilus* and *G. chapellei*, while highest identity was found with an Unc. *Geobacter* sp. retrieved during previous studies on the same aquifer. To date this is the first *Geobacter* sp. associated to dichloroelimination of 1,2-DCA.

In the third and final part, we intended to investigate the mechanisms of interaction between the dominating species of the previously enriched culture, stressing the role of *P. aeruginosa* secondary metabolites, namely pyocyanin (PYO) and rhamnolipids (RLs), whose positive effect in exoelectrogenic activity of *P. aeruginosa*, as well as of other bacteria, has been described in several studies. At this purpose, we set up different parallel replicates in which various combinations of *P. aeruginosa* metabolites were tested to verify their effect on dechlorination rate as well as on population growth. Results obtained showed a beneficial effect of RLs and PYO addiction to culture, but not of PYO alone. Moreover PYO was detected in all microcosms after 1,2-DCA degradation, suggesting RLs concentration as the limiting factor for PYO function as putative electron shuttle.

CHAPTER 1

Organohalides general description

Halogenated organic compounds, also known as organohalides (OHs), is one of the largest and most widespread class of chemicals present in the environment and they are an environmental issue since decades (Bellar *et al*., 1974; Goldberg *et al*., 1975). Organohalides include all the organic compounds in which one or more substituent bound to the carbon skeleton is a halogen atom in its halide form (oxidation state -1), namely F^-, Cl^-, Br^-, I^-.

The broad spectrum of application, coupled with the frequent misuse caused a unparalleled release of these compounds into the environment, often causing heavy undesired impacts, especially if we think about the toxicity for a wide range of life forms, as well as to the recalcitrance to degradation and penchant to accumulation in soils, waters and into the atmosphere (Häggblom & Bossert, 2003). Finally, it is worth mentioning another unpleasant feature, like the ability of some of these compounds, such as chlorofluorocarbons (CFCs), to deplete the ozone layer that protects the Earth from detrimental UV radiation produced by the Sun (Manzer, 1990). Thus, OHs are considered typical persistent organic pollutants (POPs) and a serious threat to the agricultural and hydrogeological reservoirs integrity, in facts a relatively small amount of some of the most toxic representatives of this class of compounds might contaminate a groundwater aquifer enough to make it unusable for human consumption, e.g. 2,3,7,8-Tetrachlorodibenzodioxin (2,3,7,8-TCDD) legal limit in USA is 5×10^{-9} µg/L (http://www3.epa.gov/). Still, before we could realize the real extent of the threat, thousands of tons of OHs, mainly chlorinated organic compounds (COC), by far the most abundant among this class of compounds, were released into the environment every year. Just about 30 years ago, only in Europe, factory emissions of COC were estimated at about 9000 tons per year in water and 50000 tons per year in air. Ever since the use of COC has been regulated and emissions drastically decreased throughout the end of the XX

century and nowadays they are estimated about 40 tons per year in water and 1000 tons per year in air, as by Euro Chlor report of 2010 (http://www.eurochlor.org/). Still, in most cases, it is a priority to deal with the contamination accumulated in the environment across the whole past century, since the COC were first produced.

Natural occurrence and formation

When the environmental contamination of OHs started to be recognized as an emerging issue, this class of compounds was considered exclusively of anthropogenic origin. Still, through the the last decades, more than 3000 naturally occurring OHs were identified to date, disclosing a natural halogen cycle in which microorganisms are the key players involved both in organication and mineralization of halogens (De Jong *et al*., 1994; Giese *et al*., 1999; O'Hagan & Harper, 1999). For this reason, we can talk of a real halogen cycle in which halogens are bind to organic carbons on one side and released to their inorganic form on the other side.

Among the natural occurring organohalides the most abundant are brominated and chlorinated ones, whereas iodinated ones are less frequent, and fluorinated ones are very rare, despite fluorine being the most abundant halogen present in the earth crust (O'Hagan & Harper, 1999).

Biogenic sources

OHs producing organisms are widely spread across different taxa, from both eukaryotic and prokaryotic kingdoms. In facts, they include bacteria, fungi, lichens, marine sponges, worms, insects and mammals. The marine environment harbors a huge variety of OHs producing organisms: from the sponges of the phylum *Porifera*, able to produce a pool of brominated compounds such as bromophenols and bromoindoles (Norte *et al*., 1999), to the marine microalgae like Kelp, that produce brominated, chlorinated and iodinated metabolites (Giese *et al*., 1999).

In terrestrial environments there are insects like the grasshopper *Romalea microptera* (Eisner, 1971) and arachnids like the tick *Amblyomma americanum*, (Berger, 1972) which are both able to produce chlorophenols, respectively as a defense and as sexual pheromones. Eventually, the fungi phylum, comprehends a big variety of OHs producing organisms in terrestrial environments, moreover some of them can further process naturally occurring OHs to yield dimeric compounds like chlorinated phenoxyphenols, dibenzo-*p*-dioxins and dibenzofurans (Öberg *et al*, 1990), partially explaining the finding of such highly toxic compounds in pristine environments.

Geogenic sources

Further studies elucidated other possible ways in which OHs are formed as reaction products in nature, without any biological process. Geological events like volcanic eruptions provide temperature and pressure conditions apt to promote the reaction of halide salts with organic matter, thus forming compounds such as chloromethanes, tetrachloroethane (TCA), trichloroethene (TCE), or chlorobenzenes, that have been extensively detected in lava samples. Similarly, OHs can be generated during combustion of organic matter in the presence of halides, e.g., burning of fresh wood during forest fires, or burning of municipal solid waste can typically produce such reactions (Jordan *et al*., 2000).

Anthropogenic production and industrial uses

Since the end of the XVIII century the reaction of organic substrates with chloride gas or with sodium hypochlorite were studied and the resulting products attracted increasing attention due to their peculiar chemical-physical features. In facts, as an increasing amount of OHs, mainly chlorinated ones, was synthetized, their use became more and more common, thus promoting industrial mass production. By consequence, a heterogenic, huge variety slowly started to diffuse throughout the environment by different pathways.

Chlorine disinfection of drinking water, since its first adoption in 1908, brought about a substantial eradication of water-borne diseases, but with the drawback of generating several organochlorides (OCs), especially chlorometane and chloroform, as undesired byproducts, due to the reaction of humic acids present in the water with chlorine.

Chlorine bleaching of pulp for the production of paper is another critical point through which OCs reached the environment until relatively few years ago, when processes were revised to achieve a lower environmental impact. Still, until then several hundred of OCs were identified in bleaching plants effluents, ranging from lowly chlorinated organic acids to dioxins and dibenzofurans, including a poorly classified class of compounds collectively known as chlorolignin (Häggblom & Bossert, 2003).

Another important class of OHs that heavily contaminated the environment includes chlorinated solvents. Use of chlorinated solvents as degreasers and cleaning agents spread dramatically, together with their misuse and bad disposal practice, all through the past century and it's not a long time since when stringent guidelines on their use and disposal were eventually introduced, limiting their flow to the environmental compartment. Until then, several millions of tons per year of these solvents like trichloroethylene (TCE) and tetrachloroethylene (PCE) were produced and leaked through the environment accumulating, together with their metabolites dichloroethylene (DCE) and vinyl chloride (VC), in various compartments such as groundwater and seawater (Häggblom & Bossert, 2003). Moreover, VC together with dichloroethane (1,2-DCA), knew a mass production, being the key intermediates for the synthesis of the plastic polymer polyvinylchloride (PVC). According to market reasearches PVC global demand has risen since 2000 from 22.2 millions tons (mt) to 32.3 mt in 2011 and is expected to top 49 mt by 2020 (data by Companies and Market - http://www.companiesandmarkets.com/).

Finally, it is worth mentioning another field in which OHs knew a broad application since a long time: that is biocides. Nowadays, among the most used biocides there are many halogenated compounds, mostly chlorinated (Barbash & Resek, 1996). For their very purpose, these compounds imply a great environmental concern for two principal causes: in the first place, they are all toxic for some class of organisms, be them microorganisms, plants, or insects and they may have either a specific or a very broad target; in the second place, their introduction into the environment is deliberate and wanted, as well as needed, sometimes, as it has been the case of dichlorodiphenyltrichloroethane (DDT) which greatly contributed to the eradication of malaria from many regions in Africa and southern Europe. In contrast, the detrimental effects on wildlife of chlorinated pesticides, such as DDT, were huge and nearly led to the extinction of the bald eagle and the peregrine falcon in North America. These events prompted the development of the environmental movement in the United States and led to regulation of chlorinated pesticides by the federal government, eventually a ban on DDT was declared in 1972 (Jackson, 2004).
Countless other OHs found their way to the environment through just as many applications they were given. Their physical-chemical properties and the consequent behavior in the environment will be discussed into the next section.

Chemical physical features and environmental fate

The unique chemical features of the COC due to the presence of the halogen substituents caused an increasing synthesis followed by large-scale industrial production of such compounds that proved useful for a variety of applications including as solvents, degreasers, biocides, pharmaceuticals, plasticizers, hydraulic and heat transfer fluids, intermediates for countless chemical synthesis processes and other industrial applications.

Moreover, other OHs are generated as by-products during combustion, water disinfection and chlorine bleaching of pulp for paper production (Häggblom & Bossert, 2003).

OHs result by substitution of at least a hydrogen atom bound to a carbon (C-H) by a halide atom (C-X) with X standing for any of the elements in the halogen group. They can be classified by their halide substituent(s) and by their carbon skeleton. The presence of one or more halogen substituents is indeed the principal feature influencing the behavior of OHs that acquire their peculiar properties due to the presence of this very functional group. One of the main features shared by almost all these compounds is their great stability and persistence to both biotic and abiotic degradation (Häggblom & Bossert, 2003). An important difference among the halogenated compounds and their alkyl counterparts is the stronger polarity of the C-X bond compared to the C-H bond, the strength and polarity of this bond decrease as the atomic weight of the halogen substituent increase. Thus, polarity will be higher for F and it will decrease through Cl, Br and, finally, I. This effect is due to the higher electronegativity of the halogen atoms compared to hydrogen. In spite of this, OHs are generally hydrophobic, and so they are scarcely soluble in water, ranging from few g/L for chlorinated solvents to few mg/L for polychlorinated compounds with some little intra class variation. The liquid ones (e.g. chlorinated solvents) have a low solubility in water, yet they are more soluble than the non-halogenated compounds from which they originate, and differently from the latter, they are denser than water, so they tend to form a separate lower phase when mixed with an aqueous solution (Pankow, J. F., & Cherry, 1996). Lowly halogenated compounds are generally very volatile, having a high vapor pressure, this feature tends to disappear increasing the number of halogen substituents. Highly halogenated compounds in contrast are all strongly hydrophobic and have mostly a solid form and a low volatility. These very chemical physical features determine the behavior of OHs into the environment, since on them depends their mobility in terrestrial, aquatic and atmospheric

compartments. The volatile OHs are widely distributed into the atmosphere and undergo to the so called “global distillation effect”, which distributes volatile OHs through volatilization and condensation cycles eventually concentrating most of these compounds at higher and colder latitudes (Simonich *et al*., 1995). This process similarly applies to semivolatile compounds such as polichlorobiphenils (PCBs), DDT and chlorinated dioxins, whose small particles behave like volatile OHs into the atmosphere. On the other hand, chlorinated solvents, like chloroalkanes and chloroalchenes, are moderately soluble in water, in which they tend to diffuse, hereafter their generally high vapor pressure makes them volatilize for what concerns surface water, following the fate of volatile OHs, as described above, while their high density makes them very likely to accumulate in deep sea, as well as at the bottom layers of groundwater aquifers, forming the so called dense non aqueous phase liquid (DNAPL) zones (Pankow, J. F., & Cherry, 1996). Resistance to both chemical and biological degradation is one of the qualities that determined the success of OHs in countless industrial applications, but at the same time, it is the main reason behind the environmental problems related to halogenated compounds, together with their general toxicity to various life forms. Nevertheless, there is a variety of microorganisms possessing the potential to catalyze the dehalogenation reaction, cleaving the halogen-carbon bond through different mechanisms, following the environmental niche they occupy and the nature of the organohalide substrate, thus allowing the detoxification of such compounds by metabolic or cometabolic reactions (references are reported in detail below). It is worth highlighting that enzymes capable of degrading OHs, originally evolved to allow the consumption of natural OHs and the ability to degrade also anthropogenic OHs could have arisen when the xenobiotics fortuitously entered already existing pathways and were degraded through promiscuous enzymatic activities (Copley, 2009). Another important factor that could explain the current variability of dehalogenation pathways in the microbial pool is genetic rearrangement through either selective transfer, amalgamation or mutation granted

the rise of new pathways for the degradation of the recent xenobiotics (Häggblom & Bossert, 2003).

The main pathways through which dehalogenation can be achieved are listed below.

1. Reductive: reductive dehalogenases (RDs) catalyze the reduction of the C-X bond to a C-H bond. It is typical for compounds such as chlorinated and brominated solvents, chlorophenols and PCBs. Bacteria able to reductively dehalogenate has been found among the genus *Dehalococcoides* (Grostern & Edwards, 2006; Maymó-gatell *et al*., 1999), *Dehalobacter* (Holliger *et al*., 1998), *Desulfitobacterium* (De Wildeman *et al*., 2004), *Dehalogenimonas* (Yan *et al*., 2009), *Dehalospirillum* (Scholz-Muramatsu *et al*., 1995), *Geobacter* (Sung *et al*., 2006), *Desulfomonile* (Mohn & Kennedy, 1992), *Desulfuromonas* (Sung *et al*., 2003), *Desulfovibrio* (Sun *et al*., 2000), *Corynebacterium* (Nakamura *et al*., 1992), *Rhodopseudomonas* (Kamal & Wyndham, 1990) and *Thauera* (Song *et al*., 2001). Moreover strains able to reductively degrade the recalcitrant PCBs has been recovered among the genus *Alcalegenes*, *Arthrobacter*, *Corynebacterium*, *Dehalococcoides*, *Janibacter* and *Pseudomonas* (Borja *et al*., 2005).
2. Oxygenolytic: mainly aromatic halides are degraded in aerobic environments through a NADH catalized reaction where the halogen atom is removed by the aromatic ring and replaced by a phenolic group –OH. This reaction has been studied in species from the genus *Arthrobacter* (Marks *et al*., 1984), *Burkholderia* (Fetzner *et al*., 1989), *Pseudomonas* (Engesser & Schulte, 1989), *Sphingobium* (Orser *et al*, 1993), *Rhodococcus* and *Mycobacterium* (Häggblom *et al*., 1988); moreover oxygenolytic degradation of trichloroethane has been reported in *Pseudomonas putida* (Nelson *et al*., 1988)

3. Hydrolytic: observed on chloroaromatic compounds, chlorinated pesticides and chloroalkanes; depends on ATP hydrolysis and Co-enzyme A mediation and has been reported in species belonging to genus *Pseudomonas* (Klages, 1980; Souza *et al*., 1996), *Xanthobacter* (Janssen *et al*., 1989) and *Rhodococcus* (Cook & Hütter, 1986)
4. Thiolytic: it is a reductive reaction catalyzed by glutathione and has been reported in genus *Hyphomicrobium* (Kohler-Staub & Leisinger, 1985) and *Sphingobium* (McCarthy *et al*., 1997).

The high catabolic potential for OHs presented by microorganism pinpoints biological remediation as a highly versatile and promising tool to cope with environmental issues related to this kind of contaminants, so it is of great importance the development of instruments and strategies to investigate such potential and to find the keys to exploit its full power. The study of these features is an important field of microbial ecology.

CHAPTER 2

Microbial ecology

Ecology is a science focused mainly on the study of the distribution, abundance and dynamics of organisms in a given environment and on the factors influencing such patterns. Similarly, Microbial Ecology is the discipline studying the same mechanisms (distribution, abundance and dynamics) shaping the communities of microorganisms and all the environmental and evolutionary factors involved. It is a relatively recent discipline, since researchers started to focus on the factors driving microbial evolution not before the 1960s. Its very purpose is the understanding and description of the mechanistic relationships between habitat features, evolutionary pressure, microbial diversity, biochemical processes and genetic controls underlying to them (Hughes & Bohannan, 2004). The understanding of microorganisms' behavior in a given environment is a key factor for the knowledge of such environment. Although their generally small dimensions, their number, estimated on Earth, spanning from 4 to 6 x 10^{30} (Whitman *et al.*, 1998) is huge and their total biomass can approximately reach the global biomass of all eukaryotes (Madsen, 2005). Bacteria are virtually ubiquitous, with immense metabolic resources that makes them able to respond to environmental changes promptly, in facts some of them can modify their surroundings to generate more suitable conditions to their metabolism; on the other hand they are very small and fragile so, in turn, they are very likely to be strongly influenced by the surrounding environment. So if it is true that a given microorganism can be found everywhere, it is equally true that it may not have the ability to grow in most environments, because they lack some growth factor, so that it cannot be detected. In contrast, the same microorganism can be dominant in a different niche either because its

physiological needs are fulfilled, or because competitors are absent or in such numbers that prevents them to prevent its growth (Epstein, 2013).
An economic law (Pareto's law) was used to describe microbial systems, as well as several other phenomena that fall beyond its primary purpose, briefly declined like this: in a dominated community, the 20% of the microbial community (meant as number of species) controls the 80% of the resources, and consequently constitutes 80% of total population, lesser values, instead, indicate an even community, in which there is no clear predominance of any population (Dejonghe *et al.*, 2001). This implies that, following the habitat, only few species are dominating, while the others are present in low numbers and metabolic activity as if they were in storage. Environmental changes, modifying the abiotic matrix can favor the latent microbes over the dominant ones and overturn the previous equilibrium. Abiotic factors influencing microbial growth are temperature, humidity, pH, salinity, presence of essential nutrients (such as carbon and nitrogen sources, electron donors/acceptors, inorganic cofactors), light, redox potential and pressure. All these features eventually head the evolution of a microbial system towards a direction difficult to predict, due to the number of factors involved and to the relatively scarce knowledge of most environmental microorganisms' metabolisms. Microbial ecologists' ultimate aim is to elucidate all of the mechanisms underlying this complex dynamics, analyzing microbial diversity, functional diversity and redundancy, as well as interaction among species and community emergent properties (Konopka, 2009). Moreover, considering the microscopic scale in which microbial systems cope, further issues emerge: such as the heterogeneity of the environment which typically has gradients of substrates, temperatures, humidity, thus making very difficult the interpretation of data that consequently lack homogeneity (Konopka, 2009).
To date the progressive emancipation of microbiologist from culture dependent methods, helped by the emersion of new low cost/high throughput techniques, is greatly enhancing the sampling resolution that can be achieved (Ercolini, 2013).

Microbial communities

Communities are defined as multi-species assemblages, in which organisms live together in a contiguous environment and interact with each other. This concept was first introduced for plants and animals, and was then extended to the microbial world as well. Microbial communities definition implies a light revision of the concept, since it could be difficult to define a "contiguous environment" at the microscale level, so microbes strongly interacting with each other in a microenvironment comprise a local community (Konopka, 2009). Nevertheless, the distribution of microorganisms and of physical-chemical parameters in a given environment is typically patchy; the patchwork of local communities has been termed as a phenomenological community that is a range of macroscale habitats in which the assemblage of microbes persists in spatial association (Konopka, 2009). It has been suggested as well the definition of indexical community, defined as the set of populations that directly interact with a key population or with a given biogeochemical phenomenon (Sterelny, 2006).

The first interaction among microbes that comes to mind is the competition for nutrients, in which microorganisms deploy different strategies, e.g. secretion of siderophores,

extracellular enzymes, or specific nutrients carriers (Sowell *et al*., 2009; Miethke & Marahiel, 2007). Still, even at our current state of knowledge, there are many more kind of interactions, which we briefly describe hereafter; interaction with superior organisms will not be treated, since it exceeds the purposes of this work.

Syntrophy or cross feeding is the process which allows two or more species to live off the products of each other and it has been described in a variety of works (McInerney *et al*., 2009; Narihiro *et al*., 2015), it can happen through different forms, such as sequential utilization, or chemical modification of the environment. The formation of consortia, mats and biofilms is very common among microbes, since it has been estimated that about 99% of the whole microbial biomass lives in

biofilms and/or aggregated forms (Cattò *et al*., 2015). In particular, biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and extracellular DNA (Høiby *et al*., 2011). Despite the increasing body of studies on this subject, molecular mechanisms and drivers leading to biofilm formation and conservation remain unclear in most cases. Biofilms can be considered a resistance form in which spatial distribution of cells directly modulates their metabolism, creating gradients of nutrients and oxygen demand conferring resistance to antibiotics through an active response induced by starvation rather than mere metabolic flux reduction and slowing down of cell replication (Nguyen *et al*., 2011).

Another possible interaction among microorganism is horizontal gene transfer (HGT), through the so-called genomic islands, discrete DNA segments capable of integration into the chromosome of the host, excision, and transfer to a new host by transformation, conjugation or transduction (Juhas *et al*., 2009). Typically, genomic islands carry genes related to antibiotic resistance, virulence factors and alternative catabolic pathways.

Taxonomic diversity is a key feature when describing microbial communities, an inventory of diversity within a discrete sample has two important components: taxon richness and the relative abundance of different taxa in the community. The analysis of 16S rRNA gene sequences directly from environmental samples (Tringe and Hugenholtz, 2008) has greatly enriched our understanding of global microbial diversity and an even stronger boost derives from the increasing employment of new high throughput sequencing methods that are gradually overcoming the cost related problems deriving from a thorough sampling of a given environment. In facts, undersampling is becoming gradually less and less frequent with the growth of pyrosequencing employment.

In addition to taxonomic diversity, two even more important parameters to define a microbial community are functional diversity and functional redundancy. Higher levels of redundancy for ecological functions should be related to the reliability

with which an ecosystem will continue to deliver services in the face of environmental changes (Naeem, 1998). It is very common, in facts, that specific ecological functions are distributed across a wide range of taxa. In some cases it is relatively easy to analyze diversity at functional level, since some functional genes share a conserved region even in evolutionary distant taxa, as it is the case for sulfite reductase *dsrA* (Miletto *et al*., 2007) and for nitrite or nitrous oxide reductases for nitrate reduction (Mills *et al*., 2008). In contrast, there are other important functions such as extracellular electron transfer, in which key functional metabolites like extracytoplasmic multiheme cytochromes produced by metal reducing bacteria such as *Geobacter*, *Anaeromyxobacter* and *Shewanella*, are poorly conserved, preventing the phylogenetic screening of such functions in most cases, apart from few exceptions (Dantas *et al*., 2013).

Resistance, in microbial ecology, is function of the capability of a system to not show a significant loss of function following an environmental stress; resilience, instead, measures the ability of such system to restore functionality after an acute stress effect. (Pimm, 1984) Stability of a community could be related to both parameters: depending on one side on the resistance of the community to a stress, and on the other on the resilience of the system if considered in a longer-term perspective. Eventually, stability, primarily relates to the conservation of the function rather than to the population sizes of the constituent (Tillman, 1999).

Interspecies electron transfer

A very powerful driver that strongly contributes to the modelling of microbial communities is the electron flow within the system. In facts, microbes evolved a wide variety of metabolic fluxes whose ultimate function is to harness the energy deriving from oxydoreductive reactions and make it available for their functionality. In facts, this kind of syntrophic interaction is highly widespread across microorganism, especially in environments where low energy metabolisms prevail like in anoxic oligotrophic aquifers, sediments or sludge (McInerney *et al*.,

2009). Extracellular transfer of reducing equivalents has been described in a broad range of bacteria, generally defined exoelectrogens, from distant taxa and growing on different metabolisms and three main mechanisms has been described through which they perform this task:

- ✓ Transfer of reducing equivalents through fermentation and production of H_2 and small organic acids such as acetate and formate (Bryant *et al*., 1977)
- ✓ Direct extracellular electron transfer or direct interspecies electron transfer (DIET), carried over mainly by dissimulatory Fe(III) reducing bacteria which have shown the ability to transfer electron from respiratory chain either through electrical conductive pili, called nanowires, or through membrane bound cytochromes (Lovley and Phillips, 1988; Bond and Lovley, 2003)
- ✓ Extracellular electron transfer (EET) through redox active soluble mediators acting as electron shuttles (Pham *et al*., 2008)

It is worth mentioning that this process, not only has an important ecological role, but also has several potential application in bioenergy production, and bioremediation of POPs and heavy metals (Liu & Logan, 2004; Strycharz *et al*., 2008; Prakash *et al*., 2010) Microbial fuel cells (MFC) and microbial electrolysis cells (MEC) belong to a field that researchers are increasingly investigating, due to the appealing possibility of exploiting microbial metabolism to exploit otherwise unusable low-grade waste materials such as soils and sediments, wastewater and agricultural waste streams to produce either electric energy (MFC), or value added chemicals such as biofuels or hydrogen (MEC). In MFCs, microorganisms form a biofilm on the surface of the anode and oxidize organic material and then they transfer electrons to the anode of the fuel cell, thus generating an electric current. On the other hand, in MECs, oxidation of organic matter produces CO_2, protons

and electrons in the anodic semi-chamber, then protons osmotically leak through a semipermeable membrane to the cathodic semi-chamber where they either are reduced to H^2 (Logan *et al*., 2008), thanks to a low voltage applied current, or concur to hydrogenation of CO_2 by methanogens to form CH_4 (Cheng *et al*., 2009). More than 30 electrically active bacteria strains has been isolated to date in MFC experiments only, including *Geobacter* (Bond and Lovley, 2003; Ishii *et al*., 2008;), *Shewanella* (Kim *et al*., 1999), *Pseudomonas* (Rabaey *et al*., 2004). In addition, a number of other exoelectrogens have also been discovered and identified, such as *Acidiphilium* sp. (Borole *et al*., 2008), *Arcobacter butzleri* (Fedorovich *et al*., 2009), *Aeromonas hydrophila* (Pham *et al*., 2003) ,*Aeromonas* sp. (Chung & Okabe, 2009), *Bacillus subtilis* (Nimje *et al*., 2009), *Clostridium butyricum* (Park *et al*., 2001), *Desulfuromonas acetoxidans* (Bond *et al*., 2002), *Desulfobulbus propionicus* (Holmes et al., 2004), *Desulfovibrio desulfuricans* (Zhao *et al*., 2008), *Escherichia coli* (Zhang *et al*., 2006), *Enterobacter cloacae* (Nimje *et al*., 2011), *Geothrix fermentans* (Bond and Lovley, 2005), *Geopsychrobacter electrodiphilus* (Holmes *et al*., 2004), *Klebsiella pneumoniae* (Zhang *et al*., 2008), *Ochrobactrum anthropic* (Zuo *et al*., 2008), *Rhodoferax ferrireducens* (Chaudhuri & Lovley, 2003), *Rhodopseudomonas palustris* (Xing *et al*., 2008), *Thermincola* sp. (Wrighton *et al*., 2008), *Tolumonas osonensis* (Luo *et al*., 2013), *Kocuria rhizophila* (Luo *et al*., 2015).

The description of all these species' physiology and metabolism goes beyond the purpose of this review so we recommend referring to cited bibliography for further insight, while we will proceed to the description of the different mechanisms of electron transfer adopted by microorganisms, bringing practical examples from literature, when needed.

One of the most typical and studied interactions is the one occurring among fermentative eubacteria and methanogenic archaea, which cooperate in transforming organic compounds, such as volatile fatty acids (VFA, including butyrate, propionate, and acetate) into methane through a two-step mechanism.

First organic matter is fermented through a variety of catabolic pathways eventually yielding reducing equivalents in form of formate, acetate and H_2 by mean of fermentative bacteria then, these same byproducts are used by methanogens that eventually generate CH_4. (Rotaru *et al*., 2012, 2014) Below two examples of the occurring semi reactions are reported.

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO^-3 + H^+ + 3H_2 \qquad \Delta G^{\circ'} = +76.1 \text{ kJ}$$

$$3H_2 + 3/4HCO_3^- + 3/4H^+ \rightarrow 3/4CH_4 + 9/4H_2O \qquad \Delta G^{\circ'} = -101.7 \text{ kJ}$$

Remarkably, the first reaction is energetically unfavorable as a catabolic reaction and only proceeds when the concentrations of the products (especially H_2) remain very low, this means that H_2 scavenging by hydrogenotrophic methanogens is necessary for maintaining the process. In the same way, acetoclastic methanogens can scavenge acetate through an energetically favorable reaction (Thauer, Jungermann, & Decker, 1977):

$$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4 \qquad \Delta G^{\circ'} = -31.0 \text{ kJ}$$

The flux of reducing equivalents from fermenters to methanogens is considered the limiting step in methanogenesis (De Bok *et al*., 2004) and, according to Fick's diffusion law, is inversely proportional to the distance between the H_2 producing and the H_2 consuming cell. For this reason, it is very frequent the formation of aggregates including both actors in methanogenic communities (Skiadas *et al*., 2003) Interestingly, in studies conducted on defined co-cultures, co-aggregation was observed among fermenters and methanogens, which did not produce aggregates in pure culture, as it is the case of. the propionate-oxidizing bacterium *Pelotomaculum thermopropionicum* and hydrogenotrophic methanogen *Methanothermobacter thermautotrophicus* (Ishii *et al*., 2005), in the same study

was reported that the formation of aggregates was more likely to happen with lowly energetic substrates, such as propionate, rather than with highly energetic ones, such as ethanol.

H_2 mediated electron transfer was long considered the only way for microorganism to acquire/donate reducing equivalents from/to each other, but a growing body of thermodynamically discording data prompted the uprising of new studies which disclosed a completely different paradigm of electron transfer in microbiology, namely direct interspecies electron transfer (DIET) through electric currents.

An increasing amount of studies has been performed to elucidate the mechanisms and the actors of this interaction; the great majority of these studies is focused on a peculiar genus of dissimilatory iron reducing bacteria belonging to the subphylum of the *δ-proteobacteria*, namely *Geobacter*, whose peculiar physiology will be treated in a dedicated paragraph.

Studies conducted on *G. sulfurreducens* and *G. metallireducens*, proved their ability to generate currents in microbial fuel cells (MFC) and to perform EET (Lovley & Phillips, 1988; Bond & Lovley, 2003). Further studies pinpointed the role of outer membrane c-type cytochromes and electrically conductive pilus-like structures, called nanowires in *G. sulfurreducens* (Rotaru *et al*., 2012; Lovley, 2012). In this microorganism the nanowires were reported to be responsible for the electron flow to other bacteria and to insoluble iron oxides.

Among the electron accepting bacteria, on the other hand, there are nitrate reducers like *Thiobacillus denitrificans* (Kato, 2012), and methanogenic archaea, such as *Methanosaeta harundinacea* (A.-E. Rotaru *et al*., 2014) and *Methanosarcina barkeri* (A. E. Rotaru *et al*., 2014). The latter two, in particular, are methanogenic archaea and the cited studies evidenced their ability to receive electrons through DIET, in addition to the classic reducing equivalents transfer through H_2 and 1C/2C organic acids. In addition, ability to accept directly exogenous electrons has been reported in *G. sulfurreducens* (Summers *et al*., 2010). In the same study as above, conducted on a co-culture of *G. metallireducens and G. sulfurreducens,* the

latter proved to be independent from electron transfer via H_2 or formate. In facts, a *G. sulfurreducens* strain with deletion of the genes *fdnG* and *hybL* (respectively formate dehydrogenase and uptake hydrogenase) was able to perform fumarate reduction with ethanol as sole electron source together with *G. metallireducens,* whereas the former is unable of ethanol oxidation and the latter is unable of fumarate reduction and vice versa.

Another species of bacteria that has been thoroughly studied for its putative ability to form conductive nanowires is the γ-proteobacteria *Shewanella oneidensis* (strain MR-1). In facts, some studies pinpointed its pilus like structures and cytochromes as responsible for its ability, shared with *G. sulfurreducens,* to reduce insoluble ferric oxides (Gorby *et al*., 2009). In the same study, deletion and insertion mutant for, respectively, two decaheme *c*-type cytochrome (MtrC and OmcA) and their related type II secretion system (GSPD), revealed a great impairment in ferrihydrite reduction rate, and current production. Nevertheless, a recent paper elucidated the substantial differences among the putative conductive pili produced by *S. oneidensis* and the ones produces by *G. sulfurreducens* (Lovley & Malvankar, 2014). In particular, an immunofluorescence based study stated that the extensions produced by *S. oneidensis*, are not pili and do not contain pilin, but are rather structures originating from the outer membrane and periplasm (Pirbadian *et al*., 2014). On the other hand, *G. sulfurreducens* as well expresses a multiheme *c*-type cytochrome, named OmcS, but its denaturation has no impact on pili conductivity (Malvankar *et al*., 2011). The difference among the two kind of filaments produced by *Shewanella* and *Geobacter* becomes clear since in the latter conductivity can be related directly to the pili chemistry, while in the former it is dependent on cytochromes. Further experiments, where heterologous expression of *P. aeruginosa*'s pili in *G. sulfurreducens* was obtained highlighted an impairment in current production, even if OmcS cytochrome were unexpectedly expressed on heterologous pili (Liu *et al*., 2014). This seem to happen because, even if the N-terminal sequences of *Pseudomonas aeruginosa* and *G. sulfurreducens* protein

PilA are highly conserved, the carboxyl terminus of the *G. sulfurreducens* is substantially truncated. An ancestor of *G. sulfurreducens* probably evolved this feature, positioning aromatic aminoacids residues close enough for pi-pi overlapping of the aromatic rings, that is the alleged feature behind the metal like conductivity exhibited by *G. sulfurreducens'* pili (Malvankar *et al*., 2011; Vargas *et al*., 2013).

On the other hand, *G. sulfurreducens'* cytochrome appear to work as capacitors, allowing the bacteria to accumulate reducing potential in the periplasm and outer membrane. Its electron-accepting capacity, determined with two independent methods, was estimated to be c. 1.6×10^{-17} mol. electrons per cell (Esteve-Núñez *et al*., 2008). This capacitance can, in the absence of an extracellular electron acceptor, potentially support enough inner membrane electron transfer/proton pumping for G. sulfurreducens to satisfy its maintenance energy requirements for 8 min or to swim several hundred cell lengths before all the electron-accepting capacity of the hemes is exhausted (Esteve-Núñez *et al*., 2008).

An independent set of experiments furtherly shed light on *S. oneidensis* metabolism, indicating that this bacterium would rather use soluble redox mediators, such as riboflavin or flavin mononucleotide (FMN), than DIET to perform extracellular electron transfer. All evidences collected during the experiment, conducted in an electrochemical cell, highlighted the dependence of current production at the anode on the medium, since when this was replaced by fresh anaerobic medium current production immediately decreased of about 70%, and was only slowly restored during the following 72 hours, consistent with the secretion of some soluble redox mediator (Marsili *et al*., 2008). Moreover, cell free supernatant retrieved at different times was tested in cyclic voltammetry to find out that the peaks at -0.21 V (vs standard hydrogen electrode) acquired a higher intensity with the proceeding of the incubation of the bacteria (Marsili *et al*., 2008).

Another experiment, on the other hand, highlighted the role of Mtr respiratory pathway in *S. oneidensis*: this is composed by several cytochromes and multiheme

proteins, namely OmcA, MtrC, MtrA, MtrB, and CymA, and deletion mutants unable to express these proteins showed impairment in electron transfer to electrodes, with worst performance by strain *ΔOmcA*, at only 50% current production compared to wild type strain MR-1. All mutants appeared unable, in contrast to MR-1, to form biofilm around the electrode (Coursolle, Baron, Bond, & Gralnick, 2010). Interestingly, in *G. sulfurreducens*, cytochromes, while apparently not directly involved in biofilm formation, have an important role in current production, in particular deletion mutants of *OmcZ* gene showed an increased resistance to electron transfer to electrodes in the OmcZ-deficient strain (Reguera *et al*., 2005; Richter *et al*., 2009). On the other hand, these functions both appear to be dependent on nanowires formation as well (Reguera *et al*., 2006).

Studies on *S. oneidensis* allow us to proceed to another important and not yet fully investigated mechanism through which bacteria perform extracellular electron transfer (EET). In facts, syntrophic cooperation via IET is also facilitated by electrically conductive substances, including insoluble mineral particles, carbon materials, magnetite nanoparticles (Aulenta *et al*, 2013; Kato, 2012; Cruz Viggi *et al*., 2014; Kouzuma *et al*., 2015)), as well as organic soluble mediators such as riboflavin, FMN (Marsili *et al*., 2008; Coursolle *et al*., 2010), pyocyanin (Pham *et al*., 2008), humic acids and synthetic analogs like anthraquinone-2,6-disulfonate (AQDS) (Lovley *et al*., 1998).

The studies cited above showed that in this heterogeneous group all compounds share the ability to facilitate EET, cycling from reduced to oxidized form and back, thus working as electron shuttles either between different bacterial species, or between bacteria and insoluble electron acceptors, or, eventually, between bacteria and poised electrodes.

The potential applications for these molecules are yet to be fully explored and range from biological synthesis of fuels and value added intermediates to xenobiotics and heavy metals bioremediation enhancement (Aulenta *et al*., 2013; Borch *et al*., 2005; Cruz Viggi *et al*., 2014; Dos Santos *et al*., 2004); not forgetting

the large number of experiments conducted to evaluate the impact of electron shuttles in MFC and MEC (Shen *et al*., 2014; Wu *et al*., 2014).

The unique physiology of *Geobacter* genus

At this time of the argumentation, it is worth describing the peculiar features of the most studied genus among the electrically active bacteria: the *Geobacter* genus. *Geobacter* species, during the last 30 years, have attracted an ever-increasing attention by several researchers and they are now known to play an important biogeochemical role in a diversity of natural environments. Namely, the following microbial processes were first identified in studies with *Geobacter* species:

- ✓ Oxidation of organic compounds to CO_2 with Fe(III) or Mn(IV) as the terminal electron acceptor (Lovley, 1991)
- ✓ Conservation of energy from organic matter oxidation coupled to Fe(III) or Mn(IV) reduction (Lovley, 1991)
- ✓ Production of extracellular magnetite from metabolic Fe(III) reduction (Lovley, 1991)
- ✓ Anaerobic oxidation of aromatic hydrocarbons (Lovley *et al*., 1993)
- ✓ Microbial reduction of U(VI) and Co(III) (Lovley *et al*., 1993; Caccavo *et al*., 1994)
- ✓ Utilization of humic substances as an electron acceptor for respiration (Lovley *et al*., 1996)
- ✓ The potential for an expressly poised electrode to serve both as an electron donor or acceptor to support microbial respiration coupled to, respectively, reduction of metals and organic acceptors, or oxidation of organic matter (Gregory *et al*., 2004; Bond and Lovley, 2003)
- ✓ Use of cytochromes as capacitors to allow respiration when suited exogenous electron acceptors are not available (Esteve-Núñez *et al*., 2008)
- ✓ Extracellular electron transfer via microbial nanowires presenting a metallic-like long-range conduction ability (Reguera *et al*., 2006)

- ✓ Production of conductive biofilms with conductivities comparable to that of synthetic polymers (Nevin *et al*., 2008)
- ✓ The potential for interaction with syntrophic partners via direct interspecies electron transfer (DIET) (Summers *et al*., 2010)
- ✓ Potential for bioremediation of chlorinated solvents by using electrodes as sole reducing equivalents donors (Strycharz *et al*., 2008)

Geobacter genus is the main one of the family of Geobacteraceae, which belongs to the phylum *Proteobacteria,* class *δ-Proteobacteria,* and order *Desulfuromonadales.* In turn, the species of the genus can be divided into three different clades, namely: subsurface clade I, subsurface clade II and metallireducens clade (Rotaru *et al*., 2011), with the members of the first two being the most represented in Fe(III) reducing environments (Holmes *et al*., 2007).

The hallmark feature of *Geobacter* species is their ability to couple the oxidation of organic compounds to the reduction of Fe(III), either in soluble or insoluble forms, which places them in a key position in the anaerobic microbial food chain across all environments in which Fe(III) reduction is the main terminal electron accepting process (Rotaru *et al*., 2011).

Several independent studies, relying on cultivation independent molecular analysis, have generally found that *Geobacter* species are the most abundant Fe(III) reducing microorganisms in environments in which such reaction is actively happening. Pure culture isolates were recovered from a diversity of enviroments, further molecular and/or enrichment studies have detected *Geobacter* in diverse environments such as aquifers contaminated with petroleum, groundwater contaminated with landfill leachate or organic acids, contaminated soils, aquatic sediments, uranium-contaminated subsurface sediments amended with electron donors to promote metal reduction (Rotaru *et al*., 2011).

To date more than 30 pure culture isolates belonging to *Geobacter* genus are available. All of them are Gram-negative rods able to oxidize acetate and to reduce

Fe(III). Moreover, most of them can reduce Mn(IV), U(VI), elemental sulfur, and humic substances or their analog anthraquinone-2,6-disulfonate (AQDS). Many isolates have the ability to use other short chain organic acids, ethanol, or hydrogen as an electron donor.

The two most thoroughly studied *Geobacter* species have been *G. metallireducens* and *G. sulfurreducens.* The former is the first *Geobacter* species recovered in pure culture and many of the novel physiological features listed above were discovered in studies conducted on this species.

Some *Geobacter* species have been isolated in studies focused on various physiological properties such as the ability to use aromatic compounds as energy source or potential for remediation of chlorinated solvents, as it is the case of the TCE/PCE dechlorinating bacterium *G. lovleyi* (Sung *et al*., 2006). *G. thiogenes* is the only other known dechlorinating *Geobacter,* reducing trichloroacetic acid (De Wever *et al*., 2000).

As mentioned above, *Geobacter* species can use a variety of electron acceptors for their respiratory metabolism with Fe(III) as the main one. *Geobacter* species has shown ability to grow on various forms of ferric iron, either chelated or insoluble, like, respectively, ferric citrate or poorly crystalline iron oxides (Coppi *et al*., 2007; Lovley *et al*., 1993). In addition, the metabolic versatility of this genus allows its members to use a number of alternative electron acceptors, in facts *Geobacter* species can use graphite electrodes to carry out oxidation of organic matter coupled to growth (Bond and Lovley, 2003). *Geobacter* species can use various metals as electron acceptors, as well, when iron is not available, like the previously mentioned Mn(IV) (Lovley *et al.*,1991), but several other less abundant metals can serve to accomplish this purpose like U(VI) (Caccavo *et al*., 1994; Sung *et al*., 2006), Co(III) (Caccavo *et al*., 1994), V(V) (Ortiz-Bernad *et al*., 2004), Tc(VII) (Lloyd *et al*.,2000), Cr(VI) (Lovley *et al*., 1993), Np(V) (Lloyd *et al*.,2000), Pu(IV) (Boukhalfa *et al*., 2007), Ag(I) and Hg(II) (Lovley *et al*., 1993). Moreover, researchers reported the ability of some *Geobacter* species to use elemental sulfur

reduction, like *G. sulfurreducens* (Caccavo *et al.*, 1994), some other species of *Geobacter* are expected to perform this low specificity reaction, but they often fail in laboratory experiments, probably due to sensitivity to sulfide (Kaden *et al*., 2002). Eventually, *Geobacter* species were reported to use a poorly charachterized class of compounds: humic acids as electron acceptors, or electron shuttles for usually inaccessible crystalline iron oxides reduction, as well as their synthetic analog Anthraquinone-2,6-disulfonate (AQDS) (Lovley *et al.*, 1996). To conclude the overview on the possible electron acceptors available for *Geobacter* species, we remind the phenomenon, described above, of the DIET, in which *Geobacter* species show the unique feature which allows them to discharge electrons from respiratory chain to a syntrophic microbial partner via microbial nanowires (Summers *et al*., 2010).

The pili like structures thoroughly studied in *G. sulfurreducens*, proved essential for efficient iron oxides reduction and DIET, presenting a conductivity comparable to synthetic organic polymers such as polyaniline (Lovley, 2015), but recent studies reported ability of *G. sulfurreducens* strain KN400, with deletion of the gene for PilA, the structual protein of pilin, proved able to adapt to grow on iron oxides with a reduction rate comparable to wild type. This was probably due to increased release of *c*-type cytochrome PgcA in medium to serve as electron shuttle (Smith *et al*., 2014). The great number of extracellular and membrane bound cytochromes that *Geobacter* species are able to express cover a number of different functions, including that of a capacitor, enabling *G. sulfurreducens* to store reducing potential when suitable electron acceptors are not available (Esteve-Núñez *et al*., 2008).

As for their electron sources, *Geobacter* species are known to commonly use organic acids, mainly relying on acetate, (Champine, J. E., & Goodwin, S. 1991) which is commonly oxidized via the TCA cycle and whose metabolism related genes are highly conserved, from acetate transporters to TCA enzymes, even in species isolated in geographically far sites. In contrast, genes expressing

cytochromes, in spite of being usually largely represented in the available genomic sequences of this genus, with an average of about 80 different cytochromes per species, are by far less conserved with just about 14% of these sequences conserved through all available genomes (Butler *et al*., 2010). This marked functional redundancy, along with the great variability pinpoint this genus as a key player within a very specific and yet very diffused biogeochemical pathway: anaerobic oxidation of organic matter coupled to iron and other metals reduction in subsurface sediments and aquifers. In a variety of different conditions, these bacteria specialized to thrive with the most thermodynamically favorable electron acceptor available by channeling the electron flow through the most convenient cytochrome chain, and thus playing an important role in electron flow across anaerobic environments.

In addition to acetate and short fatty acids, like propionate (Aklujkar *et al*., 2009; Aklujkar *et al*., 2010), some *Geobacter* species can use other electron donors: for example *G. sulfurreducens* was reported to use a NiFe Hydrogenase to scavenge reducing equivalents from hydrogen (Coppi *et al*., 2004) and this 4 subunits enzyme is conserved in some other species *like G. uraniireducens, G. bemidjiensis, G. lovleyi, "G. remediphilus"* and *"G. andersonii"* (Butler *et al*., 2010).

Some *Geobacter* species were seen to use expressly poised electrodes to reduce substrates such as nitrate, U(VI) (Gregory & Lovley, 2005), fumarate (Gregory *et al*., 2004) and chlorinated solvents (Strycharz *et al*., 2008).

Moreover, *G. metallireducens* was the first microorganism that was observed to grow through anaerobic oxidation of aromatic compounds (Lovley *et al*., 1993) and a number of fellow isolates was observed to share this feature and was reported to grow exploiting the oxidation of benzene, toluene, phenol, benzoate, *p*-cresol, benzyl alcohol, through the formation of a benzoyl-CoA complex (Holmes *et al*., 2011; Kunapuli *et al*., 2010;. Prakash *et al*., 2010; Straub & Buchholz-Cleven, 2001).

In addition to their peculiar metabolism, to the ability of store electric potential in their cytochrome net and to the production of conductive nanowires, *Geobacter* genus members generally present a very complicated signalling net, formed by an unusually large number of two-componenets systems and chemotaxis receptors, linked to a sophisticated system of transcription factors that can regulate response to environmental variations, from availbility of metals (O'Neil *et al*., 2008) to oxidative stress response when, for example, oxygen leakage occurs in subsurface sediments (Lin *et al*., 2004). All these features allow *Geobacter* members to rank among the highest scores in bacterial IQ tests (Rotaru *et al*., 2011). Bacterial IQ is a value than derives from the ratio between the number of signalling proteins genes present (histidine kinases, methyl-accepting chemotaxis receptors, Ser/Thr/Tyr protein kinases, adenylate and diguanylate cyclases and c-di-GMP phosphodiesterases) in the genome and the length in bp of the genome itself. In other word it could be defined the frequence of signalling proteins sequences throughout each bacterial genome and basically it refects the ability to interact in mixed community and adaptability of the microorganisms. Interestingly, there is a marked gap between the high IQs of environmental bacteria and the low ones ranked by endoparasites, reflecting the great versatility that is needed to thrive in ever varying environmental conditions compared to the usually stable conditions observable into specific host cells (Galperin *et al*., 2005).

Finally, to end the introduction on microbial ecology, we will briefly describe the techniques traditionally used to explore microbial diversity, together with the emerging techniques.

Analytical techniques

Microbial ecologist acquired through the years an increasing pool of techniques suitable to their work. Classical culture-dependent methods have been gradually integrated by molecular screening of communities through PCR-dependent and -independent methods that provided a great insight to this field. It is worth remembering that is crucial for microbial ecologists' purposes the emancipation from culture-dependent techniques, since it is known that the great majority of bacteria cannot be cultured as isolates (Epstein, 2013). The development of the new high throughput sequencing methods furtherly boosted researchers' possibilities to study microbial interactions in nature at different levels, from the study of the mere taxa distribution to their response to a given stimulus, at genetic (metagenome), transcriptomic (metatranscriptome), proteomic (metaproteome) and metabolic (meta-metabolome) level.

Analysis of 16S r RNA acquired increasing importance through the years, because of its unique features, such as its universal distribution among prokaryotes, its particular profiles that joints highly conserved sequences to variable parts, constituting a sort of barcode, low rate of horizontal gene transfer (HGT). At the same time, databases storing all retrieved ribosomal sequences, such as RDP (https://rdp.cme.msu.edu), NCBI (www.ncbi.nlm.nih.gov), EzTaxon (eztaxon-e.ezbiocloud.net) became accessible on the net, providing an essential tool to microbial ecologist. Here we report some examples of PCR analysis of 16S rRNA.

- ✓ Denaturant gradient gel electrophoresis (DGGE) is a fingerprinting technique that exploits differences in G+C content to discriminate 16S amplicons obtained with universal primers along a polyacrylamide gel casted with a denaturing gradient. A different G+C content corresponds to a different migration of the band and, thus diverse environmental samples express specific band profiles in which every band matches with a different microbial species (Muyzer *et al*., 1999).

- ✓ Automated ribosomal interspace sequence analysis (ARISA) is a fingerprinting technique that focus on the length polymorphisms of the spacer region comprised between 16S and 23S rRNA genes. In this case PCR products are separated by capillary electrophoresis (Ranjard *et al*., 2001).
- ✓ Gene libraries are used to create collections of clones containing a different DNA fragment each, usually whole 16S sequences. This technique is very informative, since it allows sequencing of whole 16S genes, but at the same time is more expensive and time consuming than all other approaches described here (Head *et al*., 1998).
- ✓ Length heterogeneity PCR (LH-PCR) studies the length polymorphism of the first two variable regions of the 16S rRNA gene using universal bacterial primers. Amplicons are then separated by capillary electrophoresis (Ritchie *et al*., 2000).
- ✓ Quantitative PCR (qPCR) is a PCR reaction allowing quantification of starting nucleic acid template, be it DNA, cDNA or RNA. It works through the measure of fluorescence emitted by a labelled reporter molecule, which increases proportionally to the accumulation of amplicons produced after each thermal cycle (Van Raemdonck *et al*., 2006).

Next generation sequencing (NGS) is the trending technique in microbial ecology at the moment and an increasing amount of works suggests its appealing potential for high-throughput sequencing of microbiomes in every given environment: from human associated microbes (Costello *et al*., 2009; Grice *et al*., 2009), to deep sea (Sogin *et al*., 2006) and soil (Roesch *et al*., 2007) bacteria. The most modern and automated traditional Sanger based sequencers are capable of sequencing up to 1 kb for 96 individual specimens at a time. In contrast, new high-throughput sequencing devices based on different chemistries and detection techniques can potentially generate tens of millions of sequencing reads in parallel. This massively

parallel throughput sequencing capacity can generate sequence reads from fragmented libraries of a specific genome and allows previously impossible tasks for microbial ecologists (Shokralla *et al*., 2012). During the last ten years, different platforms have been developed and the depth to which they can scan microbial diversity is increasing every year. From Roche 454 which could generate 10 Mb of 100 bp reads and raised its performance to 1000 Mb of 800 bp reads through years, to Illumina and Solid platforms able to generate a huge amount of reads, spanning between 100000 and 1000000 Mb of shorter sequences, respectively 100 and 75 bp, and other platforms which will be briefly described hereafter.

- ✓ Roche 454 was the first commercially available NGS platform since 2005. This release initiates a series of downstream reactions to produce light by the action of the enzyme luciferase. The amount of generated light is directly proportional to the number of nucleotides incorporated (Margulies *et al*. 2005) and is registered by a CCD sensor, when PCR amplicons fixed on micro beads are washed by a flow of reactants containing each one a single nucleotidic species repeatedly. Reaction is carried over in a picotiter plate containing millions of microwells in which a single amplicon bead fits, thus allowing the detection of a huge amount of sequences at a time. To date 3 types of this platform are available, namely Roche 454 GS FLX, Roche 454 GS FLX+ and Roche 454 GS Junior able to generate up to 500, 700 and 35 Mb of 500, 800 and 450 bp reads respectively. It is progressively surpassed by newer and more performing platforms
- ✓ Illumina, introduced as Solexa in 2007 soon became the instrument of choice for whole genomes sequencing. Illumina technology uses a ligation of library sequences with specific adapters compatible with oligos covalently bound in the interior of flow cells. Then, amplification is performed by synthetizing a library amplicon cluster through incubation with isothermal polymerase and nucleotides. Finally, polymerase and

fluorescent-labelled nucleotides with chemical modifications on their 3' OH are provided alternatively to the clusters and fluorescence is measured for each nucleotide species incorporated, fluorescent modified nucleotide is chemically removed and polymerase reaction is allowed to proceed further. The sequence of each cluster is computed and subjected to quality filtering to eliminate low quality reads (Shendure & Ji, 2008). Today, four versions of Illumina sequencers are commercially available. The HiSeq 2000, HiSeq 1000 and Genome Analyzer IIx have sequencing outputs of up to 600, 300 and 95 Gb, respectively. Moreover, MiSeq platform can generate up to 150 bp sequencing reads with a total throughput of 1.5–2 Gb per run. In 2012, Illumina introduced HiSeq2500 platform as an upgrade of HiSeq2000. This new platform can generate up to 120 Gb of data in 27 h, enabling researchers to sequence a whole genome in 24 h. In 2014 this platform was furtherly improved in 2014 with the release of HiSeq3000 and 4000 able to generate up to, respectively, 750 and 1500 Gb of 150 bp reads in about 80 h (Reuter, Spacek, & Snyder, 2015). In 2014 Illumina introduced NextSeq 500 as well as a fast benchtop sequencer for individual labs able to generate 120 Gb of reads in about 30 h using a simplified technology which allows to shorten analysis without impairing the output quality (Reuter *et al*., 2015).

- ✓ Sequencing by Oligo Ligation Detection (SOLiD) was introduced by Applied Biosystems (Life Technologies) in 2007, and, unlike the previously described platforms uses sequencing-by-oligo ligation technology. Oligos immobilized on 1 mm magnetic beads enable the generation of up to 100 and 250 Gb of sequences, for the 5500 system and the 5500xl system, respectively, with a length inferior to the 100 bp (Mardis *et al*., 2008).
- ✓ Life Technologies Ion Torrent was introduced in 2010 by Life Technologies as a postlight sequencing technology. This system relies on

the real time detection of hydrogen ion concentration, released as a byproduct when a nucleotide is incorporated into a strand of DNA by the polymerase action. (Mellmann *et al*., 2011). The three basic ion chips, namely 314, 316 and 318 can generate up to 10 Mb, 100 Mb or 1 Gb respectively. The new Ion Proton chips furtherly increase sequence output. In facts Ion Proton I chip with 165 million wells yields about 100 fold more than the Ion 314 chip and Ion Proton II chip with 660 million wells increases the potential output about 10 times more. Reads are about 200 bp long.

All PCR techniques are very useful, but when used on multiple templates, like it typically happens for microbial ecology approaches, present biases due to slight differences in primer binding energy on different templates (Kanagawa, 2003; Acinas *et al*., 2005). Recent studies confirmed this biases in next generation sequencing techniques as well (Pinto & Raskin, 2012). This bestow a great importance to non-PCR related techniques, as powerful tool to validate molecular data obtained through PCR methods and as a further point of view for studying microorganisms *in situ.*

Non-PCR related techniques used in microbial ecology

- ✓ Fluorescence in situ hybridization (FISH) is a powerful cytogenic technique able to detect and localize specific DNA sequences related to a peculiar microbial group or function. In facts, fluorescent probes are used to bind sequences with high affinity and then are revealed through confocal fluorescence microscopy or flow cytometry (FC) (Wagner *et al*., 1996).
- ✓ Flow cytometry is a very versatile technique that joints a cell separation device to a laser beam with fluorescence detector able to perform multiparametric analysis on cell morphology and viability, nucleic acids content, physiological activity, and several parameters more that fall far

beyond the purposes of microbial ecology. Moreover, it can be coupled to fluorescent probes, as reported above, to link morphological and physiological characterization to targeting of fingerprint sequences (Rigottier-Gois *et al*., 2005). Another useful application of FC is cell sorting, which prompted the rise of single cell genomics, allowing the separation of a single cell from a mixed sample. (Gasol *et al*., 1999)

- ✓ Phylo Chip is a high density microarray able to harbor hundreds of thousands of oligonucleotidic probes which, coupled to dedicated hybridization scoring algorithms, has proven repeatedly as an efficient tool to disclose microbial diversity and to pinpoint previously undescribed candidate taxa (De Santis *et al*., 2005).

Single molecule sequencing systems (SMS) are NGS platforms that use single strands of DNA as templates, skipping the PCR step.

- ✓ HeliScope introduced in 2008, was the first commercially available SMS. The library construction step exploits single-stranded DNA fragments of one DNA molecule at a time. In this platform, there is no use of amplification: the sequencing happens through polymerase cycles coupled with four fluorescently labelled nucleotides (Harris *et al*., 2007). This technology allows the production of up to 28 Gb of short 35 bp sequences.
- ✓ Pacific Biosciences introduced single molecule real time (SMRT) in 2010 (Korlach *et al*. 2010). As above, there is no need for amplification of templates and sequencing proceeds by synthesis. Unlike other technologies, the terminal phosphate carries the fluorescent marker, causing light emission with the nucleotide incorporation (Gupta, 2008). This way, the speed of nucleotide incorporation as well as sequence quality are enhanced. In facts SMRT can yield up 75 Mb of more than 1.5 kb long reads in about

30 min, which is remarkable compared to both PCR based NGS platforms and HeliScope.

- ✓ Oxford Nanopore Technology, is a platform which relies on the transition of DNA or individual nucleotides through a small channel (nanopore) on a synthetic membrane. Sequencing is performed by measuring characteristic changes in current that are induced as the bases flow through the pore, leaded by a molecular motor protein. This platform allows reading in both direction, increasing quality of output. The first instrument Based on this technology is MinION, a USB-powered portable sequencer, which Oxford Nanopore Technologies released in early 2014. A single 18 h run can produce about 100 Mb of reads, with median and maximum lengths of about 6 kb and more than 60 kb, respectively (Quick *et al*., 2014).

CHAPTER 3

Chlorinated solvents remediation

After a speculative section, needed to introduce the environmental problems related to halogenated compounds, and their widespread application; in addition to the disciplines and techniques devoted to the study of such phenomena from the microbiological point of view, the argumentation shifts on a more specific and applicative field, which is, in synthesis, the true core of this manuscript: **the (bio-)remediation of chlorinated solvents**, with particular focus on 1,2-DCA removal from groundwater.

Why a bioremediation approach?

1,2-DCA production per year worldwide is around $16x10^6$ tons and as seen before this solvent tend to partition in groundwater anoxic aquifers, making them unsuitable for human use (Grostern & Edwards, 2009).

Drinkable water availability is becoming an emerging issue, especially in countries with constant demographical growth where it is also necessary considering the increasing consumption of water for food production. Moreover, the detrimental effects on wildlife and on public health given by chlorinated compounds exposure are studied and documented since more than half a century (Williams, 1959; Stewart *et al*., 1961; Bailey *et al*., 1969; Traiger & Plaa, 1974). For all these reasons, remediation of water is becoming an important issue all over the world and an increasing number of researchers is focusing on the countless facets of this extremely branched matter.

Traditional physical/chemical treatments to eliminate chlorinated pollutants, such as solvent vapor extraction, are typically expensive and extremely energy consuming. In contrast, a bioremediation approach presents a number of advantages that makes it generally preferable over a traditional one: the possibility

to perform the treatment in situ, avoiding costs and hazards deriving from removal of waste; the effective transformation of organic pollutants to inorganic carbons and water or, at least, to safer compounds; the low environmental impact, avoiding removal of soil and aquifers dewatering through pumping. On the other hand, bioremediation processes could be very complex and require extensive monitoring, to avoid undesirable reaction to occur; furthermore varying physical and biochemical parameters from one site to another can result in a very different removal efficiency, implying a challenging backstage work before reaching the point of field trial (Alvarez & Illman, 2005). Nevertheless, bioremediation strategies are generally more effective and less impacting; moreover, the complete remediation of the site by traditional techniques is very unlikely to be achieved, because of the penchant of chlorinated solvents for accumulating in the lower part of aquifers forming the previously mentioned DNAPL. They tend to accumulate into the deepest part of aquifers accumulating on impermeable layers such as clay, eventually saturating the sediment. Because of this, DNAPL are difficult to find and to treat, and can constitute a permanent pollution source, keeping the levels of chlorinated solvents throughout the surrounding groundwater over the legal limits for drinking water in spite of the dilution caused by the water flowing through the aquifer (Vogel *et al*., 1987). Researchers (Freeze and McWhorter, 1997) have demonstrated the extreme difficulty of removing chlorinated solvents found in the DNAPL phase, especially when solvents have saturated the sediments or porous rocks. Limitations on DNAPL removal can make complete remediation impossible at many sites.

Another important feature that allows this kind of substances to accumulate throughout various ecological niches, is their resistance to degradation by microorganisms that, even when possessing the metabolic potential to degrade halogenated compounds to non-toxic products efficiently, are typically found in low cells numbers and with few energetic resources that would not enable them to grow coupling dehalogenation to energy conservation. This situation dramatically

extends the time requested for the natural attenuation of such compounds. Just in the early 1990s, biostimulation approaches, following microcosm studies, began to apply to organohalides polluted groundwater or landfills in order to obtain a faster remediation of such areas by the activity of resident microbial populations following proper amendment, we recommend referring to bibliography for an insight on early bioremediations trials (Semprini *et al*., 1992; McCarty, 1993).
Up to now, constant efforts are being made to screen for new microorganisms and enzymes that could perform reductive dechlorination of solvents when opportunely stimulated and an important number of them has been identified, in particular among three main phyla: *Chloroflexi*, *Firmicutes* and, to a lesser extent, *Proteobacteria*. Reductive dechlorination of solvents, both alkanes and alkenes, happens through two main pathways: dehydrochlorination and dichloroelimination where, respectively, one Cl and two Cl atoms are substituted by H and released as chloride ions (Smidt & de Vos, 2004). Compared to studies on chlorinated ethenes, there is considerably less literature regarding reductive dechlorination of chlorinated ethanes, like 1,2-DCA, but this reaction has been reported in a number of papers describing mixed cultures and isolates able to grow using 1,2-DCA as final electron acceptor. In particular, it is worth remembering *Dehalococcoides ethenogenes* strain 195, that has been reported dechlorinating 1,2-DCA to ethene and, to a minor extent, to vinyl chloride (VC), proving able to use both dichloroelimination and dehydrochlorination (Maymó-Gatell *et al*., 1999).
Desulfitobacterium dichloroeliminans
strain DCA1 is one of the first isolated bacteria known to completely dechlorinate vicinal dichloroalkanes, including 1,2-DCA, by dichloroelimination (De Wildeman *et al*., 2003). Various unc. *Dehalobacter* and *Dehalococcoides* strains were associated to 1,2-DCA degradation in mixed culture experiments (van der Zaan *et al*., 2009; Grostern & Edwards, 2006), such as the multiple *Dehalobacte* strains detected in the WL 1,2-DCA dechlorinating mixed culture (Grostern & Edwards, 2009). More recently, 1,2-DCA, as well as other chlorinated alkyls dechlorination

has been described in *Dehalogenimonas* genus, a deep branch of the phylum *Chloroflexi*, with the most closely related genus being *Dehalococcoides*, with less than 90% identity of 16s (Yan *et al*., 2009). Both *D. lykanthroporepellens* strain BL-DC-9 and *D. alkenigignens* strain IP3-3 proved able to grow exploiting dechlorination of 1,2-DCA, as well as other chlorinated substrates (Maness *et al*., 2012; Bowman *et al*., 2013; Dillehay *et al*., 2014), moreover, Dillehay and colleagues demonstrated the preferential dechlorination of 1,1,2-trichloroethane (1,1,2-TCA) over 1,2-dichloropropane (1,2-DCP) and.1,2-DCA by both strains. 1,2-DCA, in particular, was not degraded until low level of 1,1,2-TCA were achieved.

Recent studies on 1,2-DCA remediation at lab scale, but simulating a permeable reactive barrier, exploited the synergetic effect of Zero valent iron (ZVI) and poly-hydroxybutyrate (PHB), to enhance remediation of 1,2-DCA by creating proper conditions for halorespiring bacteria to grow after PHB fermentation and relative VFA and H_2 production (Baric *et al*., 2014). ZVI in fact, has previously proved able to enhance abiotic degradation of several xenobiotics such as vinyl chloride, but not 1,2-DCA, while on the other hand, apparently concurred in creating a proper environment for dechlorinating bacteria (Wei *et al*., 2012).

Poly-hydroxyalkanoates (PHA) are solid slow releasing substrates which are proving as promising biostimulation agents for remediation of chlorinated solvents, even without addition of ZVI, since they are relatively low cost substrates that are slowly depolymerized by microorganisms, supporting long term degradation with a single application. In particular poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) has proved an interesting substrate for different reasons, since it can be can be produced at low-cost from renewable sources and several microorganisms shown ability to hydrolyze PHBV to 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV) in both terrestrial and aquatic habitats. PHBV been tested more recently in similar a permeable reactive barrier experiment, resulting in promotion of 1,2-DCA degradation to ethene through 50 days with an incoming

flow 0.1 mmol/L of chlorinated solvent, after inoculum with an acetate/H_2 fed dechlorinating enrichment culture (Mannino & Ceccarelli, 2014).

A novel paper used a bioelectrochemical cell with a polarized graphite electrode as an electron donor for the reductive dechlorination of 1,2-DCA by a mixed culture, the reaction was shown to happen by means of H_2 electrochemical production. The highest rate of 1,2.DCA degradation was registered when electrode was set at -900mV, when H_2 generation was highest, consistent with the increase of *Dehalococcoides* putative population, identified by CARD-FISH. On the other hand, 1,2-DCA degradation was observed as well at -300mV, with no H_2 production, but with a huge increase in coulombic efficiency, suggesting that the dechlorinating reaction was happening through direct electron transfer from electrode to cells, without H_2 mediation, lamentably CARD-FISH failed to identify about 40% of the population from the electrode biofilm, and only the remaining 60% was attributed to *Dehalococcoides* genus (Leitão *et al*., 2015).

In other studies heavy metals inhibitory effect on 1,2-DCA dechlorination by microorganism has been investigated and researchers reported dose dependent impairment of dechlorination rate by the following metallic ions, ordered from the most inhibiting to the less: Hg^{2+} As^{3+} Cd^{2+} Pb^{2+} (Arjoon *et al*., 2013), highlighting a possible critical point for the feasibility of a bioremediation strategy in complex polluted environments.

For soil compart to date, no significant biological approaches for chlorinated solvents are reported, since phytoremediation approaches proved limited by uptake and mass exchange problems, even if plants were enhanced by heterologous expression of bacterial genes for haloalkane dehalogenase (DhlA) and haloacid dehalogenase (DhlB) (James & Strand, 2009).

On the other hand, successful treatments by thermal desorption or mechanical aeration of contaminated soils in China has been described recently (Gao & Jiang, 2012; Ma *et al*., 2015).

Case study

Since more than ten years, our research group has carried on a collaboration with ENI spa, to evaluate the feasibility of a bioremediation approach on a historically 1,2-DCA contaminated groundwater aquifer next to Ferrara in Northern Italy. The site presents an inferior and a superior aquifer, separated by a clay layer, through which the solvent leaked until the deepest part of the basin. Concentration of 1,2-DCA, measured at spatially scattered piezometers spans from 100 to nearly 6000 ppm, whereas the legal limits for human use of water are below 500 ppb, thus more than 10000 times lower than the highest concentration measured at piezometer E_{up} (5932 mg/L). Remediation potential for this site have been investigated by previous works (Marzorati *et al*., 2005, 2007, 2010). In these works the response of the microbial community to *ex situ* biostimulation with typical electron donors for dechlorinating reactions, such as lactate, formate, acetate and cheese whey, was evaluated and microbial diversity following amendment was screened by PCR-DGGE and ARISA (Marzorati *et al*., 2005). Subsequently, the dehalogenase cluster retrieved in one of the best performing cultures was completely sequenced and showed a high identity with sequence from *Desulfitobacterium dichloroeliminans* strain DCA1. Identity measured was, 98%, 99%, 97% and 99% respectively, for the genes *dcaA*, *dcaB*, *dcaC and dcaT*, on the other hand similarity of aminoacidic sequence ranked, 98%, 99%, 96% and 99% respectively, for the subunits *dcaA dcaB*, *dcaC* and *dcaT* (Marzorati *et al*., 2007). Finally, the microbial diversity of the preciously set up enriched cultures was investigated by means of a 16s rRNA library, after lactate amendment. Up to more than 90% of sequences were related to *Clostridiales* order, with the great majority of them belonging to the *Peptococacceae* family, in fact, the three most represented sequences were related to, ordered by the most abundant to the less one, *Desulfitobacterium dichloroeliminans* (97% identity), *Dehalobacter* sp. WL (99% identity) and *Desulfitobacterium metallireducens* (96% identity). All of the three were previously described as solvents dechlorinators, as for the most abundant

population found that did not belong to the *Firmicutes* phylum, the *δ-Proteobacterium Trichlorobacter thiogenes* (later renamed *Geobacter thiogenes*) (98% identity), accounting for about 3-4% of the OTUs (normalized by the average 16s rRNA genes copy number of each genera) (Marzorati *et al*., 2010). In the same work, a library for screening the functional diversity of reductive dehalogenases (RDs) was built as well, and two main enzymes were identified. Their peptidic sequence was eventually aligned with known sequences from *Dehalobacter* and *Desulfitobacterium* genera, finding a substantial similarity for each of the RDs groups found to RDs from these genera, except for two boxes in which most of the mismatching aminoacids concentrate.

AIM OF THE WORK

Chlorinated solvents constitute a severe threat to our water reservoirs and their remediation by chemical-physical methods has proved often unfeasible. For this reason is extremely important to extend our knowledge of the microbial processes that lead to degradation of these pollutants. This, in facts, is the only way to enhance the strategies for promoting more efficient bioremediation processes.

For this reason our main task has been to describe the response the indigenous microbiota of a 1,2-DCA polluted groundwater aquifer to amendment with various known electron donors, suitable for reductive dechlorination. The findings described in this work are preliminaries to a pilot scale test on field, which will hopefully prompt the complete recovery of a long lost aquifer. The focus of this work has been in a first phase the monitoring of the response of the microbial community from the low layer of the aquifer to lactate amendment, in terms of total microbial population composition shifts and in terms of functional diversity of 1,2-DCA specific reductive dehalogenases. The aim of this preliminary screening was to assess potential for bioremediation in the aquifer as well as to individuate known potential 1,2-DCA among the resident microbiota.

The second part focused on the description of the microbial diversity in upper aquifer and to obtain enriched dehalogenating cultures and investigate both their response to different electron donors, both in terms of 1,2-DCA degradation rate, conservation of activity through transfers and populations proportions shifts; functional diversity was screened as well. Total populations of both *Eubacteria* and functional genes analysis was carried over with a variety of approaches and with samples proceeding from spatially scattered piezometer of the previously described aquifer, to test for the better strategy to apply on field.

Several molecular techniques were tested to find the most suitable in order to follow the variation of microbial population following *in vitro* biostimulation and, in a second phase, apt for screening during *in situ* biostimulation. The enriched

communities that showed higher rates of dechlorination and higher stability throughout transfers have been furtherly studied, to elucidate the drivers that stood behind the segregation of such communities and possibly unveil new strategies for improvement of current groundwater bioremediation processes. Moreover, a side benefit not initially seeked, has been the stabilization of an enriched 1,2-DCA community that could be a powerful bioaugmentation agent.

Finally, we wanted to elucidate the dynamics that brought to the formation of the most stable dechlorinating community, which outcompeted all the others screened, both in terms of dechlorination rate and in terms of conservation of activity through transfers. The chapter 6 of this work tries to shed a light on the role of secondary metabolites of a non- dechlorinating *P. aeruginosa* strain in promoting the conservation of function through transfers, via some interaction with the putative 1,2-DCA dechlorinating Unc. *Geobacter* sp., identified in the previous work. In other words, we hypothesized that a sort of syntrophic reaction established between the two dominating species of the enrichment culture and that it was driven by limiting metabolites produced by *P. aeruginosa* known to allow exoelectrogenic activity. In order to verify this hypothesis shifts in dechlorination rate and population growth following amendment with hexogenous *P. aeruginosa* metabolites were measured.

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CHAPTER 4

Diverse reductive dehalogenases are associated to *Clostridiales*-enriched microcosms dechlorinating 1,2-dichloroethane

INTRODUCTION

Chlorinated compounds are among the major global environmental contaminants (Stringer & Johnston, 2001). A large number of compounds of this class of chemicals have been produced in big quantities for several applications in industry and agriculture such as biocides, flame-retardants, solvents and intermediates for the production of polymers (e.g. PVC) (Stringer & Johnston, 2001; Ruder, 2006). Their widespread diffusion and use resulted in the massive release in the environment, with consequent concerns for human health due to the persistence, tendency to bio-accumulate and proven toxicity (Hughes *et al*., 1994; Ruder, 2006). Due to the physicochemical properties most halogenated compounds are recalcitrant to aerobic dehalogenation and tend to accumulate in anoxic ecosystems (e.g. soils and groundwater aquifers). For this reason many of the research efforts of the last decades, aimed at defining efficient remediation approaches, were focused on the investigation of anaerobic degrading potential of microbial cultures enriched/isolated from typical anoxic environments. Chlorinated solvents in these conditions can undergo biologically mediated degradation through either oxidative, fermentative and reductive processes (Smidt & de Vos, 2004).

Particular interest has been focused on the third kind of biodegradation process, since several studies have highlighted the high dechlorinating performances of pure and mixed microbial cultures through reductive dehalogenation (Field &. Sierra-Alvarez, 2004; Macbeth *et al*., 2004; Marzorati *et al*., 2006, 2010; Hirschorn *et al*., 2007; Arjoon *et al*., 2013). The peculiarity of this process is that the chlorinated molecule is the terminal electron acceptor of the membrane-bound electron-

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transport-chain coupled to the generation of energy in the form of ATP (Smidt & de Vos, 2004).

Among the wide variety of chlorinated solvents, 1,2-dichloroethane (1,2-DCA) is considered one of the major pollutant, being one of the most widespread contaminating groundwater worldwide and being classified as a possible human carcinogenic agent by many environmental agencies (Ruder, 2006). 1,2-DCA can undergo either partial or complete detoxification in anoxic conditions through three different mechanisms: dichloroelimination, reductive hydrogenolysis and dehydrochlorination (Field &. Sierra-Alvarez, 2004). Among these, only the first mechanism leads to the production of the harmless end-product ethylene, while the other two generate molecules which toxicity is even higher than 1,2-DCA, in particular the carcinogenic vinyl chloride (VC). Key enzymes involved in this anaerobic dehalogenating metabolism are the reductive dehalogenases (RDs), a class of cobalamin-dependent oxygen-sensituve enzymes, usually associated to the membranes and capable of replacing halogen atoms with hydrogen ones from the carbon backbone of the molecules (Holliger *et al*., 1999; Smidt & de Vos, 2004). Different studies have unveiled details about structure and function of some enzymes belonging to this class (van de Pas *et al*., 1999; Neumann *et al*, 2002; Maillard *et al*., 2003). Only recently, novel RDs sequences were correlated with 1,2-DCA dechlorination to ethene in a 1,2-DCA dehalogenating enrichment culture containing a *Dehalobacter* sp. WL (rdhA1, rdhA2 and rdhA3) (Grostern &. Edwards, 2009), and *in situ* in the upper water layer of a double layer aquifer contaminated by 1,2-DCA (RD54) (Marzorati *et al*, 2007). The enrichment culture setup from the upper layer of the aquifer (culture 6VS) contained both *Dehalobacter* and *Desulfitobacterium* spp. In addition to the two just cited representatives of the phylum *Firmicutes*, only few other bacterial strains have been identified so far as capable of detoxify 1,2-DCA to ethylene via dichloroelimination. Maymó-Gatell *et al*. (1999) and Ritalahti *et al*. (2003) were the first to report the ability of two *Chloroflexi* representatives, respectively

Dehalococcoides ethenogenes strain 195 and *Dehalococcoides sp.* strain BAV1 to grow on 1,2-DCA as electron acceptor producing ethylene as the main end-product. A peculiarity of the species of this genus is their capability to grow exclusively on chlorinated compounds as electron acceptor. Other representatives of the phylum *Chloroflexi* with the ability to grow on 1,2-DCA described recently are two strains of the genus *Dehalogenimonas*: *D. lykanthroporepellens* (Moe *et al*., 2009) and *D. alkalygenes* (Bowman *et al*, 2012), both characterized by the ability to degrade high concentration of 1,2-DCA up to 8.7 mM (Maness *et al*., 2012).

In the present work the dechlorinating bacterial microbiome in the lower layer of the same aquifer investigated by (Marzorati *et al*, 2007) has been characterized in terms of structure and functionality, before and after the supplement with lactate. We have investigated i) the response of the indigenous microbial community to lactate treatment, ii) the key microbial dehalogenating bacteria, and iii) the RDs involved in the dehalogenation process.

MATERIALS AND METHODS

Preparation of enrichment cultures

Evaluation of biodegradation of 1,2-DCA was carried out in anaerobic microcosms set up with groundwater collected from the lower layer (from 14 m to 40 m deep) of an aquifer previously studied in the northern Italy (Marzorati *et al*, 2005, 2007, 2010), heavily polluted exclusively by 1,2-DCA since more than 30 years. Concentration of the contaminant in the lower aquifer was about 197 ± 23 mg l^{-1} and it was maintained the same during preparation of anaerobic cultures. Other chlorinated ethanes and ethenes were not detected. Thirty-ml triplicate microcosms were assembled in 50-ml vials under an atmosphere of 80% N_2, 15% CO_2 and 5% H_2 in the anaerobic glove-box Simplicity 888 (Plas-Labs, USA). Culturing medium consisted of a 1:200 dilution of a trace elements solution (12.8 g l^{-1} nitrilotriacetic acid, 1.35 g l^{-1} $FeCl_3 \cdot 6\ H_2O$, 0.1 g l^{-1} $MnCl_2 \cdot 4\ H_2O$, 0.024 g l^{-1} $CoCl_2 \cdot 6\ H_2O$, 0.1 g

l^{-1} $CaCl_2 \cdot 2\ H_2O$, 0.1 g l^{-1} $ZnCl_2$, 0.025 g l^{-1} $CuCl_2 \cdot 2\ H_2O$, 0.01 g l^{-1} H_3BO_3, 0.024 g l^{-1} $Na_2MoO_4 \cdot 2\ H_2O$, 1 g l^{-1} NaCl, 0.12 g l^{-1} $NiCl_2 \cdot 6\ H_2O$, 0.026 g l^{-1} $Na_2SeO_3 \cdot 5\ H_2O$), a supplementary salt solution (43 mg l^{-1} NH_4Cl, 0.5 g l^{-1} KH_2PO_4, 0.2 g l^{-1} $MgCl_2 \cdot 6\ H_2O$, 0.01 g l^{-1} $CaCl_2 \cdot 2\ H_2O$), 0.05% (w/v) yeast extract, 0.5 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid/NaOH (Hepes/NaOH) solution pH 7.0, cysteine 1 mM and vitamin B_{12} 50 mg l^{-1}. Lactate at final concentration of 5 mM was used as the only carbon source and electron donor (De Wildeman *et al*., 2003). Control microcosms were prepared by incubating parallel vials containing the same culturing medium with filter-sterilized groundwater samples. All microcosms were sealed with teflon-faced septa and aluminum crimp seals, and static incubated in the dark at 23°C.

Concentration of 1,2-DCA and of its possible degradation products, ethane and VC, was evaluated by the injection of 500-µl samples of headspace of the microcosms in a Gas Chromatograph/Flame Ionization Detector (GC/FID) Agilent 7694 equipped with a DB624 column (J&W Scientific, Folsom, CA). The temperature of the oven and of the detector were set at 80 and 200°C respectively. 1,2-DCA limit of detection was 1.0 µg l^{-1}.

Genomic DNA isolation

Groundwater and microcosm samples, respectively 30 and 1.5 ml (samples withdrawn from replicate cultures were pooled together for a total final volume of 4.5 ml), were filtered using Sterivex filters (Millipore, Milan, Italy). Total genomic DNA was extracted from the filtered bacterial cells by incubating the filter with 2 ml of a lysis solution containing 1 mg ml^{-1} lysozyme, 1% (w/v) sodium dodecyl sulphate and 0.5 mg ml^{-1} proteinase K, and purified as previously described by Murray *et al*., 1998.

PCR amplification of bacterial and archaeal *16S rRNA* and RD genes

Bacterial *16S rRNA* gene was amplified from the groundwater metagenome using universal primers 27f and 1492r (Wen *et al*., 2012) with the following reaction concentrations in a final volume of 50 µl: 1X PCR buffer, 1.5 mM $MgCl_2$, 0.12 mM dNTPs, 0.3 µM of each primer, 1 U of Taq polymerase. Thermal protocol used was the following: initial denaturation at 94°C for 5 minutes, followed by 5 cycles consisting of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes and subsequently by 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes. A final extension at 72°C for 10 minutes was performed.

PCR with specific primers for *Archaea* was attempted in order to investigate the *16S rRNA* diversity of this group of prokaryotes. A first step was carried out using universal archaeal forward primer 21f and 1492r, using the same reaction mix and thermal protocol presented elsewhere (Van der Wielen *et al*., 2005). Since the first PCR step did not give any amplicon, a second round of PCR using primers PARCH 340F and 934R was attempted, as previously described by Cytryn *et al*. (2000). However, also this second amplification attempt did not resulted in any PCR product.

A 2000 bp region of the reductive dehalogenase gene cluster previously identified by Marzorati and colleagues (2007), was amplified using primers PceAFor1 (5'-ACGT GCA ATT ATT ATT AAG G-3') and DcaBRev (5'-TGG TAT TCA CGC TCC GA-3'), in order to construct a gene library of the functional genes encoding for the RD specific for 1,2-DCA degradation. The reaction mix was prepared as follows: 1X PCR buffer , 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 µM each primer, and 1 U of Taq polymerase in a final volume of 25 µl. The thermal consisted of an initial denaturation at 94°C for 3 minutes, followed by 31 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 1 minute, extension at 72°C for 2 minutes and subsequently a final extension at 72°C for 7 minutes.

16S rRNA and RD genes libraries

Cloning reactions were performed with pGEM cloning kit (pGEM-T Easy Vector Systems, Promega, Milan, Italy) following the instructions of the manufacturer. Sixty ng of PCR product were used for each cloning reaction, maintaining a molar ratio insert:vector of 3:1. A PCR assay was performed on white positive colonies to amplify the insert using primers T7 (3'-CTA ATA CGA CTC ACT ATA GGG-5') and SP6 (3'-ATT TAG GTG ACA CTA TAG AAT A-5'). PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions.

16S rRNA gene phylogenetic and RDs Diversity Analyses

Clones from bacterial *16S rRNA* and RD genes libraries were sequenced respectively with primers 27F, and PceAFor1, using the ABI Prism BigDye terminator cycle sequencing kit (Applied Biosystems, Milan, Italy) and an ABI 310 automated sequencer (Applied Biosystems). Sequences were edited with software Chromas Lite version 2.01. Sequences of the *16S rRNA* bacterial libraries were checked for chimeric PCR products using DECIPHER on-line software tool (Wright *et al*., 2012) and non-chimeric sequences were then used to define operational taxonomic units (OTUs) at 99% of similarity (OTU99) using DOTUR Schloss *et al*., 2009). Shannon Diversity Index (*H'*) was calculated using software PAST version 3.02 (Hammer *et al*., 2001). The sequences of the OTU representatives were analysed using the Basic Local Alignment Search Tool (BLAST) of the on-line GenBank database (Altschul *et al*., 1990) and by the CLASSIFIER Match Tool version 2.6 of Ribosomal Database Project II (RDP II) (Wang *et al*., 2007). Pareto-Lorenz distribution curves (PL curves) (Lorenz. 1905), Marzorati *et al*., 2008) were constructed based on the *16S rRNA* gene clone library results, in order to graphically evaluate the community organization (*Co*) of the bacterial consortia as described elsewhere (Merlino *et al*., 2013).

Identification of the closest relative match for the RDs libraries was carried out comparing the sequences with BLAST. Sequences of functional gene libraries were used to construct Neighbor-Joining phylogenetic tree, with bootstrap of 1000 repetitions and computing the evolutionary distances through the Kimura's two-parameter model using software MEGA version 5 [35]. Alignment of amino acids sequences of the functional genes deducted from the nucleotide sequences of the RDs libraries was carried as described elsewhere (Regeard *et al*., 2004)in order to identify characteristic amino acid residues conserved in all RDs.

Nucleotide Sequence accession numbers

Nucleotide sequences of all clones identified in this study were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the accession numbers FM210335, and FM204948 to FM204979 for bacterial *16S rRNA* genes, and FM204931 to FM204934 for RDs sequences.

RESULTS AND DISCUSSION

Structure and Diversity of the bacterial community before and after the biostimulation

A triplicate series of anaerobic microcosms with a concentration of 1,2-DCA of 197±23 mg l^{-1} was set up using groundwater from the lower layer of a double aquifer contaminated by 1,2-DCA analogously to the experiments previously run for the upper layer of the same aquifer system (Marzorati *et al*., 2010). Following the addition of 5 mM lactate, all the microcosms readily degraded 1,2-DCA in 15 days, with an average dechlorination rate of 13.1±1.9 mg l^{-1} day^{-1}. Ethane accumulated as the only end product while the toxic intermediate VC was always below the detection limit, suggesting that degradation of 1,2-DCA occurred only via dichloroelimination (De Wildeman *et al*., 2003). The analogous biostimulation treatment with groundwater from the upper layer (Marzorati *et al*., 2010) gave

considerably higher degradation rate of 69.4±2.2 mg l^{-1} day^{-1}. It can be speculated that this almost-four times statistically significant difference (as determined by the Student t-test with $p < 0.000001$) between the two layers was possibly due to differences in the enriched dechlorinating species.

The bacterial diversity of the community before (t_0) and after (t_1) the biostimulation treatment was evaluated by establishing *16S rRNA* gene clone libraries. Differently from what observed previously on the upper layer of the aquifer (Marzorati *et al*., 2010), PCR with specific primers for *Archaea* did not result in any amplicon nor before or after lactate amendment, even after a second round of PCR using nested primers. This suggests that in the lower aquifer *Archaea* are not implicated in the dechlorination process.

The bacterial libraries were made of 91 clones each. Chimera check allowed excluding 6.0% of all the sequences obtained, lowering the number of clones to 89 and 82 for t_0 and t_1 respectively. Good coverage of the dominant OTUs was confirmed with rarefaction analysis of the clone libraries (Figure 1). The diversity of the bacterial communities was evaluated by means of two parameters: i) Shannon index (*H'*), which allowed describing the species richness and ii) Evenness index, used to describe the relative abundance among species within the communities. Shannon index, which accounts for both abundance and evenness of the species present, was 3.33 in the lower aquifer respect to 1.91 in the upper one, indicating that the lower aquifer hosted greater species diversity than the upper one before the treatment. At t_1 after lactate amendment the Shannon index in the lower aquifer decreased (2.88 vs. 3.33), while in the upper aquifer it remained almost unchanged (1.81 vs. 1.91). The small *H'* variation in the lower aquifer suggests that relatively limited changes in the biodiversity of the bacterial community occurred after the biostimulation treatment.

The PL curves, used as a graphical estimator of the *Co* (Lorenz, 1905; Marzorati *et al*., 2008), confirmed the little bacterial diversity change in the lower aquifer, in response to the biostimulation treatment (Figure 2). *Co* curves at t_0 showed a

situation were 20% of the OTUs represented about 48% of the total abundance of clones. After the lactate treatment this proportion grew to 58%, indicating that both communities were characterized by a relatively moderate organization. It can be speculated that the bacterial community of the lower aquifer was characterized by a slight dominance both before and after the biostimulation treatment and sudden changes in the environmental conditions, as those determined by the supplement of lactate, would change the dominant species but not influence the overall *Co* and evenness structure of the community.

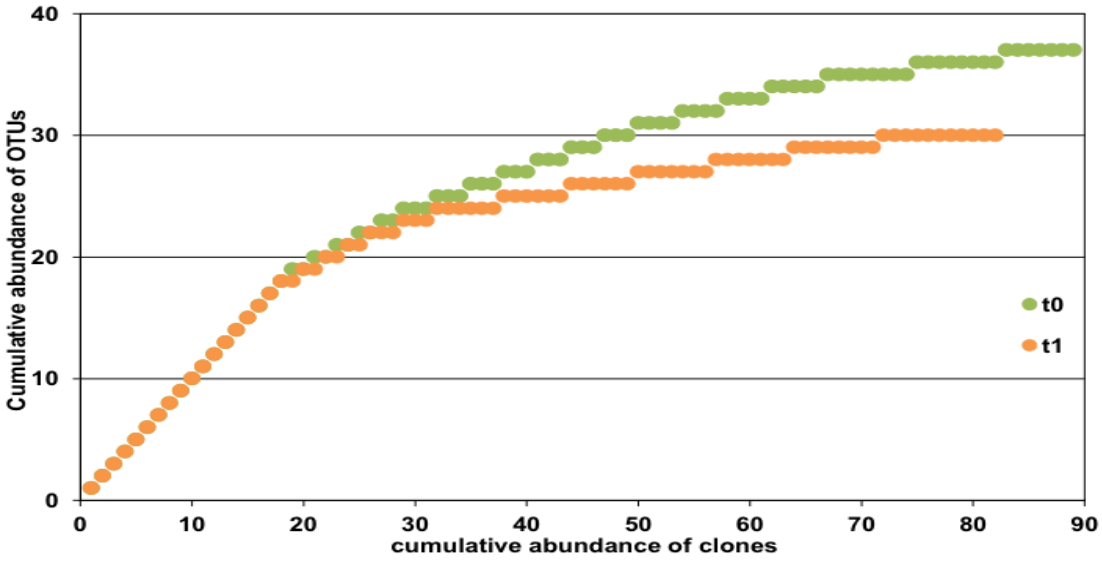


Figure 1 - Rarefaction curves calculated for the bacterial 16S rRNA gene clone libraries, before (t_0) and after (t_1) the biostimulation treatment with lactate.

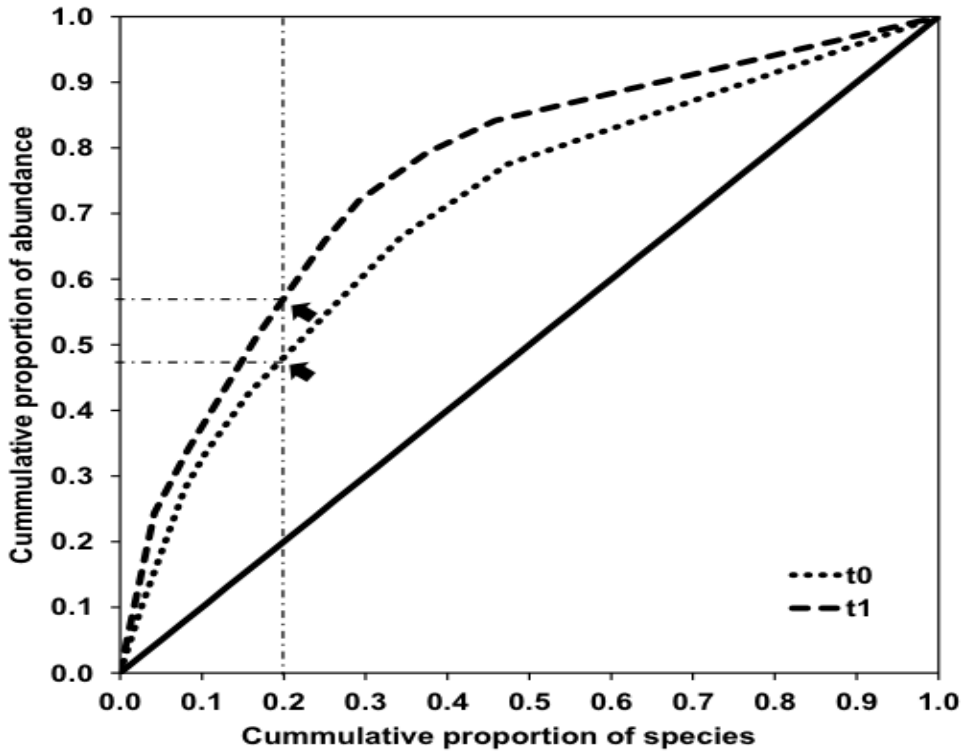


Figure 2 - Pareto-Lorenz distribution curves representation of the Community organization (Co) of the microbial communities before (t_0, dotted line) and after (t_1, dashed line) the treatment with lactate. The continuous line represents the perfect evenness. Black arrows indicate the OTU cumulative proportion of abundances corresponding to for an OTU cumulative proportion of 20%.

The 171 clones obtained in the two libraries were grouped in 60 distinct OTUs. A summary of the representatives of each OTU identified through BLAST and CLASSIFIER is presented in Table 1, together with the number of clones of each OTU occurring before and after the biostimulation treatment. Thirty-eight of the 60 OTUs were detected before the lactate amendment and 24 after it, with only two OTUs detected both at t_0 and at t_1, respectively affiliated to an uncultured *Clostridiales* and to *Sulfuricurvum sp*. The bacterial community at t_0 was characterized by a wider diversity, with dominating sequences belonging to *Proteobacteria* phylum (Table 1, Figure 3): in order of abundance *δ*- (38 clones describing 15 OTUs), *β*- (26 clones describing 11 OTUs) and *ε-Proteobacteria* (15 clones describing 4 OTUs). Within the *δ-Proteobacteria*, all the sequences were closely related to genus *Geobacter* (97-100% identity), the majority of which were affiliated to uncultured *Geobacter sp.* and *Geobacter thiogenes* (15 clones each). Species of the genus *Geobacter* were commonly found in freshwater sediments and subsurface environments (Lovley *et al*., 2004). Previously, De Wever and colleagues (De Wever *et al*., 2000) described the ability of *Geobacter thiogenes* to dechlorinate trichloroacetic acid. Another representative of the *Geobacter* clade, *G. lovley* (6 clones) a known tetrachloroethene-dechlorinating bacterium (Sung *et al*., 2006), was also identified. Within the *β*- and *ε-Proteobacteria* groups the most represented phylotypes were closely related with *Hydrogenophaga taeniospiralis* (11 clones) and *Sulfuricurvum kujiense* (10 clones). These two genera are environmental microorganisms typically detected in contaminated freshwater ecosystems (Kodana *et al*., 2004). For instance, *H. pseudoflava* was identified by Liang and colleagues (Liang *et a*l., 2012) in a TCE-degrading consortium enriched from TCE-contaminated aquifer sediments and groundwater. A psychrotrophic *H. pseudoflava* strain IA3-A was isolated from polychlorinated biphenyls-contaminated soil and grew on biphenyl as sole carbon and energy source (Lambo & Patel, 2006). Both genera *Hydrogenophaga* and *Sulfuricurvum* were recently

enriched and associated to NO_3^--reduction in a membrane biofilm reactor inoculated with wastewater sludge and treating perchlorate (Zhao *et al*., 2011).

The biostimulation with lactate determined a remarkable change of the diversity within the bacterial community. A lower diversity (24 OTUs) was observed and phylotypes related to *Firmicutes*, *Bacteroidetes* and *β-Proteobacteria*, not detected at t_0, became dominant: i.e. representatives of genera *Acidaminobacter* (20 clones), *Parabacteroides* (21 clones) and *Malikia* (13 clones) were strongly enriched (Table 1, Figure 3). Conversely, *Geobacter*, *Hydrogenophaga* and *Sulfuricurvum*, the phylotypes dominating the consortium before the treatment were not detected in the library after the treatment. A similar shift of diversity was previously observed in the upper layer microcosms (Marzorati *et al*., 2010). However, while in the upper layer of the aquifer phylotypes of known 1,2-DCA dehalogenating genera of the *Clostridiales* (*Desulfitobacterium* and *Dehalobacter*) were enriched after the biostimulation with lactate, none of the genera enriched in microcosms from the lower layer has been so far associated to reductive dechlorination of 1,2-DCA. Among the phylotypes enriched in the lower layer microcosms, the only characterized representative of genus *Acidaminobacter*, *A. hydrogenoformans*, has been described as a fermentative species whose growth is enhanced by co-cultivation with a hydrogen-consuming partner, such as in our study could be a microbe able to couple the H_2 consumption with 1,2-DCA reductive dechlorination (Narihiro *et al*., 2010). Interestingly, another phylotype enriched at t_1 was related to an uncultured *Clostridiales* bacterium (12 clones) and, noteworthy, the only reductive dehalogenases specific for 1,2-DCA identified so far were previously associated only to 2 genera belonging to *Clostridiales* order, *Desulfitobacterium* (Marzorati *et al*., 2007) and *Dehalobacter* (Grostern & Edwards, 2009). Taken together these data indicate that in the lower aquifer the lactate amendment enriched different phylogenetically distant taxa previously not associated with 1,2-DCA dechlorination, suggesting that novel reductive dechlorinators may mediate such a process.

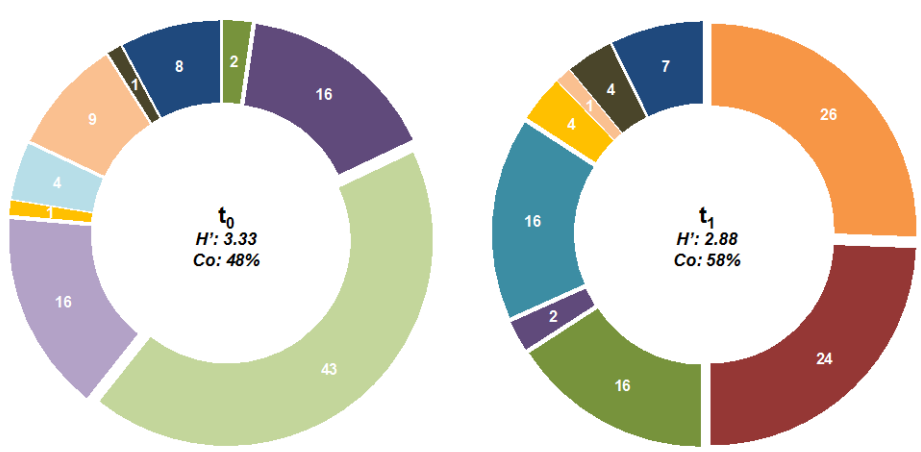
OTU	Clones		Basic Logic Alignment Search Tool - GenBank			CLASSIFIER Match Tool - Ribosomal Database Project II		
	t_0	t_1	Closest described relative	Acc. n°	% Identity	Phylogenetic group	Closest classified relative	% certainty[A]
1	3	0	*Geobacter thiogenes*	NR_028775	98.96	Deltaproteobacteria	*Geobacter*	100
2	3	0	*Geobacter thiogenes*	NR_028775	98.78	Deltaproteobacteria	*Geobacter*	100
3	8	0	*Geobacter thiogenes*	NR_028775	99.09	Deltaproteobacteria	*Geobacter*	100
4	3	0	Unc. bacterium	AM410013	97.34	Deltaproteobacteria	*Geobacter*	100
5	1	0	Unc. *Geobacter sp.*	FM204959	98.63	Deltaproteobacteria	*Geobacter*	100
6	1	0	Unc. *Geobacter sp.*	EU266833	98.62	Deltaproteobacteria	*Geobacter*	100
7	3	0	Unc. *Geobacter sp.*	AY752765	98.56	Deltaproteobacteria	*Geobacter*	100
8	1	0	Unc. *Geobacter sp.*	AY752765	98.42	Deltaproteobacteria	*Geobacter*	100
9	1	0	Unc. *Dehalobacter sp.*	HM748813	99.43	Clostridia	*Acetobacterium*	100
10	4	0	Unc. *Sulfurimonas sp.*	KF851122	98.34	Epsilonproteobacteria	*Sulfuricurvum*	100
11	4	0	*Ferribacterium sp. 7A-631*	KF441656	99.75	Betaproteobacteria	*Ferribacterium*	93
12	1	0	Unc. *Gallionellaceae* bacterium	EU266776	96.48	Proteobacteria	Betaproteobacteria	100
13	1	0	Unc. *Rhodocyclaceae* bacterium	JQ279024	98.83	Betaproteobacteria	Rhodocyclaceae	98
14	1	0	Unc. *Rhodocyclaceae* bacterium	HQ003471	97.64	Betaproteobacteria	Rhodocyclaceae	100
15	0	1	*Acinetobacter baumannii*	KJ958271	99.60	Gammaproteobacteria	*Acinetobacter*	100
16	0	1	*Pseudomonas putida*	GU396283	98.97	Gammaproteobacteria	*Pseudomonas*	100
17	0	7	Unc. *Bacteroides sp.*	AB529592	99.44	Bacteroidia	*Parabacteroides*	99
18	0	1	Unc. *Bacteroidetes* bacterium	FJ535139	98.31	Bacteroidia	*Parabacteroides*	100
19	0	1	Unc. *Bacteroides sp.*	JQ624314	99.75	Bacteroidia	*Parabacteroides*	100
20	0	1	Unc. *Bacteroidetes* bacterium	DQ676360	98.97	Bacteroidia	Porphyromonadaceae	99
21	0	1	Unc. *Bacteroides*	FM204969	99.88	Bacteroidia	*Parabacteroides*	99
22	0	1	Unc. *Acidaminobacter*	HM217344	98.61	Clostridia	Clostridiales Incerta Sedis XII	91

23	0	6	Unc. *Acidaminobacter*	HM217344	98.78	Clostridia	Clostridiales	100
24	0	5	Unc. *Acidaminobacter*	HM217344	99.46	Clostridia	Clostridiales Incerta Sedis XII	80
25	2	0	Unc. *Hydrogenophaga sp.*	HM124825	99.67	Betaproteobacteria	*Hydrogenophaga*	100
26	3	0	*Hydrogenophaga taeniospiralis*	AY771764	98.02	Betaproteobacteria	*Hydrogenophaga*	100
27	0	7	*Malikia spinosa*	NR_040904	99.86	Betaproteobacteria	*Malikia*	100
28	1	0	Unc. *Hydrogenophaga sp.*	DQ413154	98.70	Betaproteobacteria	*Hydrogenophaga*	100
29	1	0	Unc. Elusimicrobia	GU236016	94.55	Elusimicrobia	*Elusimicrobium*	98
30	0	2	*Hydrogenophaga taeniospiralis*	AY771764	98.75	Betaproteobacteria	*Hydrogenophaga*	95
31	8	0	*Hydrogenophaga taeniospiralis*	AY771764	99.06	Betaproteobacteria	*Hydrogenophaga*	98
32	0	6	*Malikia spinosa*	NR_040904	99.73	Betaproteobacteria	*Malikia*	88
33	1	0	Unc. *Acidovorax*	AM084039	99.04	Betaproteobacteria	Comamonadaceae	100
34	3	1	Unc. *Dechloromonas*	JN679130	98.95	Betaproteobacteria	Rhodocyclaceae	100
35	1	0	Unc. *Gallionella*	FJ391502	98.72	Proteobacteria	Betaproteobacteria	100
36	0	2	*Vogesella indigofera*	NR_040800	99.60	Betaproteobacteria	*Vogesella*	100
37	0	1	*Shewanella putrefaciens*	JN019028	99.87	Gammaproteobacteria	*Shewanella*	100
38	9	0	*Sulfuricurvum kujiense*	CP002355	99.22	Epsilonproteobacteria	*Sulfuricurvum*	100
39	1	0	Unc. *Arcobacter*	JQ861849	97.96	Epsilonproteobacteria	*Arcobacter*	93
40	2	0	*Geobacter metallireducens*	NR_075011	98.31	Deltaproteobacteria	*Geobacter*	100
41	3	0	Unc. *Geobacter*	EU266817	99.76	Deltaproteobacteria	*Geobacter*	100
42	2	0	Unc. *Geobacter*	EU266841	99.16	Deltaproteobacteria	*Geobacter*	100
43	5	0	*Geobacter lovleyi*	NR_074979	99.03	Deltaproteobacteria	*Geobacter*	100
44	1	0	*Geobacter*	NR_028775	97.48	Deltaproteobacteria	*Geobacter*	100
45	1	1	Unc. *Firmicutes* bacterium	HQ003641	98.70	Clostridia	Clostridiales Incerta Sedis XII	100
46	0	20	Unc. *Firmicutes* bacterium	HQ003641	99.45	Clostridia	*Acidaminobacter*	86
47	0	1	Unc. *Clostridium*	FM204998	100.0	Clostridia	*Clostridium XlVa*	100
48	0	3	*Acetobacterium malicum*	NR_026326	99.53	Clostridia	*Acetobacterium*	100
49	1	0	Unc. bacterium	AB759668	95.24	Bacteria	Firmicutes	100
50	2	0	Unc. rumen	AB615047	94.24	Lentisphaeria	*Victivallis*	97
51	2	0	Unc. *Cytophaga*	EU809766	99.35	Lentisphaeria	*Victivallis*	97
52	1	0	Denitrifying	FJ802233	98.54	Ignaviabacteria	*Ignavibacterium*	91
53	0	8	Unc. *Bacteroides*	FJ862827	99.18	Bacteroidia	*Parabacteroides*	100

54	0	3	*Macellibacteroides fermentans*	NR_117913	99.08	Bacteroidia	*Parabacteroides*	99
55	0	1	Unc. *Bacteroidetes* bacterium	FJ535139	94.64	Bacteroidia	Porphyromonadaceae	88
56	0	1	Unc. *Bacteroidetes* bacterium	DQ676360	99.30	Bacteroidia	Bacteroidales	99
57	1	0	Unc. *Prolixibacter sp.*	JQ723616	97.85	Bacteria	Bacteroidetes	100
58	1	0	Unc. *Geobacter*	JQ086897	98.72	Deltaproteobacteria	*Geobacter*	100
59	1	0	*Geobacter lovleyi*	NR_074979	99.37	Deltaproteobacteria	*Geobacter*	100
60	1	0	*Sulfuricurvum kujiense*	NR_074398	99.29	Epsilonproteobacteria	*Sulfuricurvum*	100

Table 1: Summary of the OTU representatives, identified in the 16S rRNA gene libraries.
[A] – Confidence threshold of the RDPII CLASSIFIER Tool is 80%.

a



b

Genus	t_0	% t_0	t_1	% t_1
Parabacteroides	0	0.00	21	25.61
Acidaminobacterium	0	0.00	20	24.39
Unc. *Clostridiales* sp.	2	2.25	13	15.85
Hydrogenophaga	14	15.73	2	2.44
Malikia	0	0.00	13	15.85
Geobacter	38	42.70	0	0.00
Sulfuricurvum	14	15.73	0	0.00
Acetobacterium	1	1.12	3	3.66
Ferribacterium	4	4.49	0	0.00
Unc. *Betaproteobacteria* sp.	8	8.99	1	1.22
Bacteroides	1	1.12	3	3.66
Others	7	7.87	6	7.32
Total Clones	**89**		**82**	

Figure 3 - a) Pie-charts illustrating the percentages of clones, identified in the bacterial communities at t0 and at t1, grouped in phylotypes at genus level; H', Shannon Index and Co, community organization (Evenness Index); b) table showing the abundance and the percentages of clones grouped in phylotypes at genus level.

Reductive dehalogenase gene libraries

The reductive dehalogenase diversity in the lower aquifer was investigated in response to lactate biostimulation to evaluate whether reductive dehalogenating functional redundancy could be associated to the diversity pattern depicted by the *16S rRNA* gene libraries. In previous works a complete sequence of one RD gene cluster specifically adapted to 1,2-DCA was obtained from microcosms of the upper layer of the aquifer (Marzorati *et al*., 2007). Four genes (*dcaB*, *dcaC* and *dcaT*) of the identified RD cluster presented high nucleotide identity (above 98%) with the RDs specific for chlorinated alkenes, but only 94% and 90% nucleotide and amino acid identities in the gene coding for the main catalytic subunit of the reductive dehalogenase (*dcaA*). The sequence differences were associated to dechlorination of 1,2-DCA since *Desulfitobacterium dichloroeliminans* strain DCA1, capable of dechlorinating 1,2-DCA but not chlorinated ethenes, showed the same amino acid signatures in the two sole RDs identified in the genome (Marzorati *et al*., 2007).

Using the same RD-targeting PCR approach of Marzorati *et al*. (2007) a total of 17 clones were obtained after the treatment, representing four different RDs. Figure 4 shows their phylogenetic relationship with known RDs. The RD sequences found in the lower aquifer layer were grouped in one cluster together with those previously identified in the upper aquifer layer (Marzorati *et al*., 2010). The percentage of similarity among the newly identified RDs was between 100-99% and shared 99% nt identity with WL rdhA1, one of the three RDs identified by Grostern and Edwards (2009) in a 1,2-DCA degrading co-culture where the main representative was *Dehalobacter sp.* WL. It has been previously shown that the 53% of the total amino acid diversity of *dcaA* RDs (RD-54 and RD-DCA1) respect to *pceA* RDs specific for tetrachloroethene (PCE; RDs from *Dehalobacter restrictus* strain DSMZ 9455T, *Desulfitobacterium sp.* strain Y51 and *Desulfitobacterium hafniense* strain PCE-S) (Suyama *et al*., 2002; Maillard *et al*., 2003, 2005), was mainly localized in in two small regions (blocks A and B, Figure

5) that represent only 19% (104 amino acids over 551) of the total *dcaA* residues. These two regions of hyper-variability were proposed to be involved in the recognition of 1,2-DCA or in general in the substrate specificity of RDs (Marzorati *et al*., 2007). The alignment of the RDs identified in the lower aquifer layer with the above-indicated homologs it was possible to identify the two mentioned hyper-variable regions overlapping with blocks A and B (Figure 5). The alignment permitted to identify amino acids specifically associated to: i) PceA of the PCE-RDs (black residues in a light grey background); ii) DcaA of the group I, specific for WL rdhA1 and for the reductive dehalogenases enriched from the lower aquifer layer (white residues in a light grey background); iii) DcaA of the group II proposed to be specific for 1,2-DCA RDs from *Desulfitobacterium* (black residues in a dark grey background); iv) all the RDs within the groups I and II but not conserved in the PCE-specific RDs (white residues in a black background).

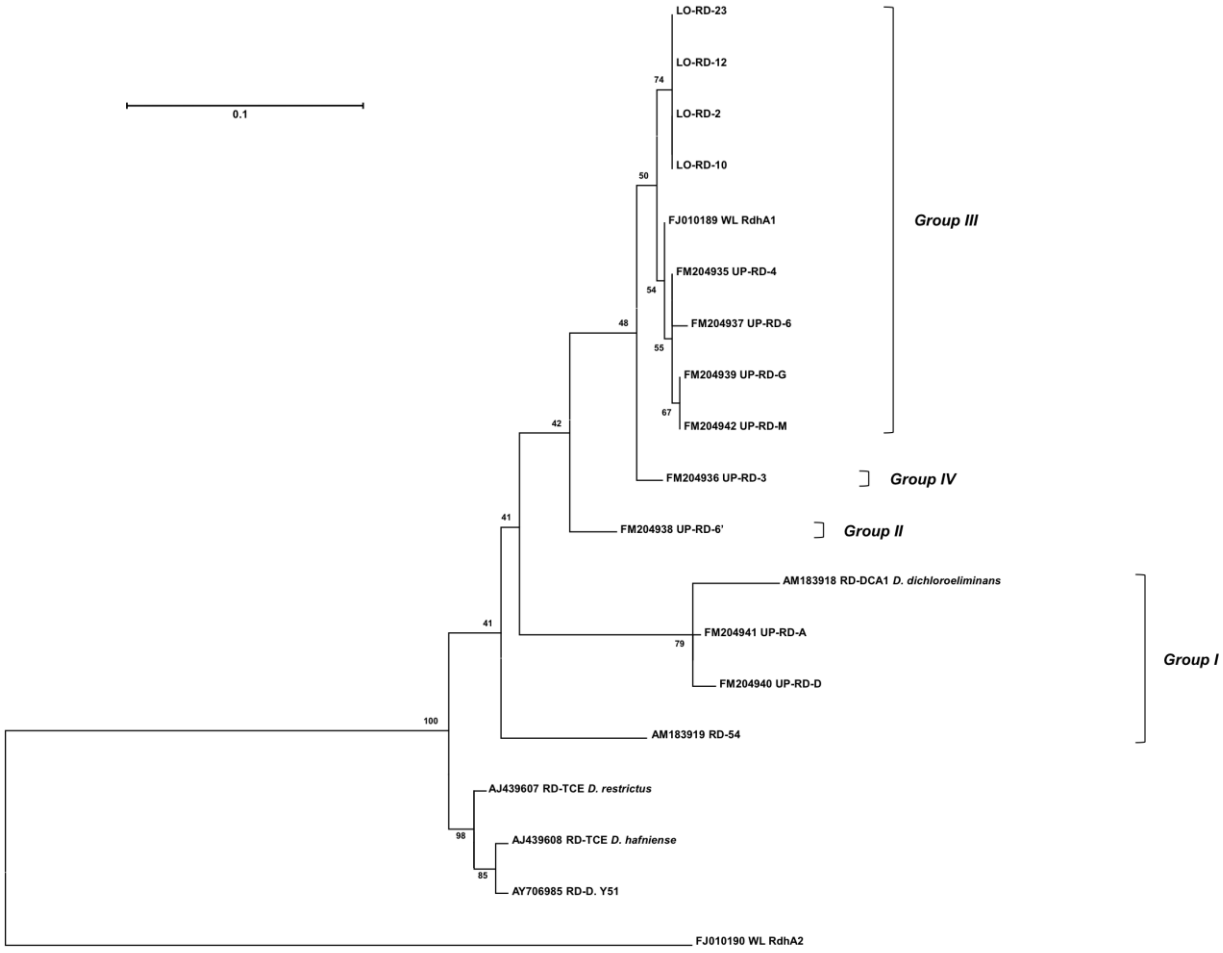


Figure 4 - Neighbour-joining tree with branch length to assess the relationship between DcaA of the new RDs identified in the lower aquifer (LO-RD-X) with those previously characterized from the upper aquifer (RD-54 (Marzorati et al., 2007) and UP-RD-X, (Marzorati et al., 2010)) and from D. dichloroeliminans strain DCA1 (RD- DCA1, (Marzorati et al., 2007)). Other A subunits of PceA of Dehalobacter restrictus strain DSMZ 9455T (RD-TCE D. restrictus - AJ439607), Desulfitobacterium hafniense strain TCE1 (RD-TCE D. hafniense - AJ439608), Desulfitobacterium sp. strain Y51 (RD-D. Y51 - AY706985), WL rdhA1 (FJ010189) and WL rdhA2 (FJ010190) are also reported. The numbers at each branch point represent percentage of bootstrap calculated from 1000 replicate trees. The scale bar represents the sequence divergence.

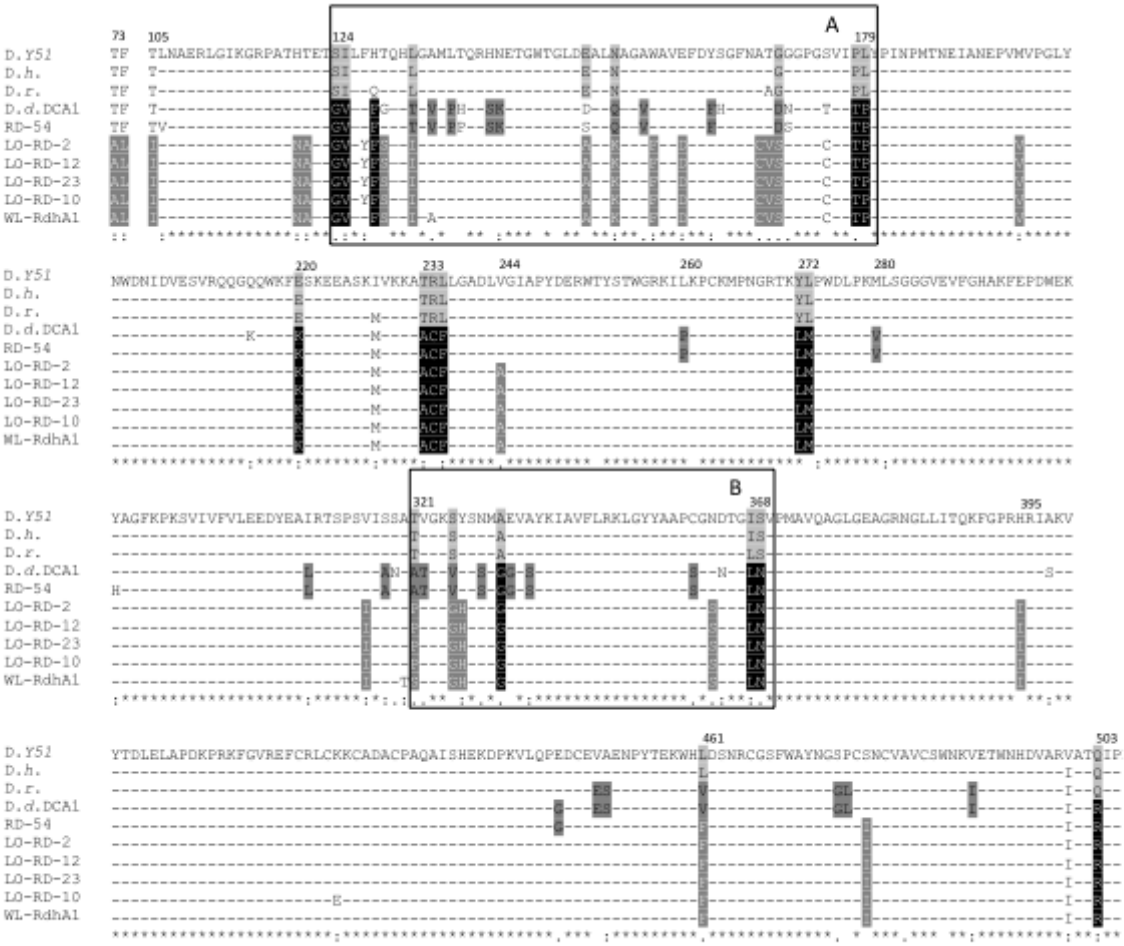


Figure 5 - Amino acid alignment of the DcaA proteins of the new identified RDs, with those previously identified in the groundwater (RD-54 - AM183919) and in D. dichloroeliminans strain DCA1 (D.d. DCA1 - AM183918), with PceA of Desulfitobacterium sp. strain Y51 (D. Y51 - AY706985), D. hafniense strain TCE1 (D.h. - AJ439608) and D. restrictus strain DSMZ 9455T (D.r. - AJ439607) and with the WL rdhA1 (FJ010189). Black line (blocks A and B) rectangles indicate two amino acid stretches where resides 53% of the total amino acid diversity between DcaA and PceA (Marzorati et al., 2007). Within the blocks A and B of the selected RDs sequences, as well in other smaller regions of the DcaA subunit, it was possible to identify aminoacids specific for: i) PceA of the PCE-RDs (black residues in a light gray background), ii) DcaA of the groups I, specific for WL rdhA1 and for the reductive dehalogenases identified in the low aquifer after the lactate treatment (white residues in a light gray background), iii) those of the group II proposed to be specific for 1,2-DCA RDs from Desulfitobacterium (black residues in a dark gray background), iv) and finally those common to all the RDs within the groups I and II but not conserved in the PCE-specific RDs (white residues in a black background). Asterisks, colons and dots below the alignment indicate an identical position in all the proteins, a position with a conservative substitution and a position with a semi-conservative substitution, respectively.

CONCLUSIONS

By comparing the diversity of bacteria and RDs in the two aquifer layers following biostimulation with lactate it can be argued that the RDs linked to 1,2-DCA reductive dechlorination, despite diverse, are structurally conserved. However, they can be associated to different bacterial carriers selected by the environmental conditions of the specific aquifer, indicating their plasticity to adapt to different cellular scaffolds and machineries.

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CHAPTER 5

Characterization of a *Geobacter* enrichment culture that dechlorinates 1,2-dichloroethane to ethylene

ABSTRACT

In order to investigate the microbial potential for bioremediation in a 1,2-dichloroethane (1,2-DCA) contaminated aquifer, we set up enriched dechlorinating cultures to evaluate the effect of different electron donors in promoting dichloroelimination of 1,2-DCA. All enriched cultures were screened for functional diversity by dehalogenase targeted PCR-DGGE, revealing the enrichment of two different reductive dehalogenases (RDases), with high similarity to the ones previously retrieved in this aquifer. Total microbial diversity was screened, as well, in particular thhe most enduring and performing enrichment was screened through 16S rRNA libraries, ILLUMINA 16S rRNA libraries and qPCR. Two dominating population were identified: an Unc. *Geobacter* sp. and a P. aeruginosa strain JCM 5962. Only P. aeruginosa could be isolated and proved unable to perform dichloroelimination. Phylogenetic analysis of the putative dechlorinating *Geobacter* strain were carried over and confirmed its affiliation to *Geobacter* genus, but without matching any previously identified species. Eventually, qPCR analysis highlight a positive correlation between the number of *Geobacter* 16S rRNA copies and the number of RD copies. All these data combined strongly suggest that Unc. *Geobacter* sp. is a novel putative 1,2-DCA dechlorinating bacteria and the first described in its genus.

INTRODUCTION

1,2-dichloroethane (1,2-DCA) is regarded as one of the top priority pollutants in many industrialized countries. The European Pollutants Release and Transfer Register (EPRTR, http://prtr.ec.europa.eu/) accounts for 64 facilities in the 27 UE member Countries still producing and releasing 1,2-DCA in the environment (data updated to 2013). In particular the estimated total amount of 1,2-DCA released in water basins is 6.29 tons/year, the highest among the other top priority chlorinated solvents, namely trichloroethylene (TCE), perchloroethylene (PCE) and vynil chloride (VC). The widespread distribution of 1,2-DCA is due mainly to its use as intermediate in the manufacture of polyvinylchloride plastic and as processing aid for the production of a number of different fine chemicals (Henschler, 1994; Huanga *et al*., 2014). As many other anthropogenic chlorinated compounds, 1,2-DCA accumulation in the environment poses severe concerns for human health due to the proven toxicity (Hughes *et al*., 1994; Shiyan *et al*., 2015).

1,2-DCA tends to accumulate in anoxic ecosystems, especially in groundwater aquifers (Holliger *et al*., 1990; Marzorati *et al*., 2005), due to its high water solubility (8000 mg l-1) and recalcitrance to degradation (Vogel *et al*., 1987; Barbee, 1994). Moreover, even if it the ability of some strains to degrade chlorinated compounds in aerobic conditions was previously demonstrated (Hage and Hartmans, 1999), it is also known that aerobic degradation pathways of halogenated compounds seem to be not favorable, because of associated redox stresses induced by the concomitant endogenous production of reactive oxygen species (Nikel *et al*., 2013). For all these reasons, anaerobic microbially-mediated degradation through reductive dehalogenation is regarded as a feasible and effective strategy for the remediation of sites impacted by 1,2-DCA (Klečka *et al*., 1998; Marzorati *et al*., 2005; Merlino *et al*., 2015).

The utilization of 1,2-DCA as electron acceptor in reductive dechlorination processes was already previously reported in many studies. The ability of

Dehalococcoides ethenogenes strain 195 to reduce 1,2-DCA mainly to ethylene through the oxidation of H2 was described by Maymó-Gatell and colleagues (1999). Subsequently, the ability to grow using 1,2-DCA as a terminal electron acceptor has been described for few others strains: Dehalococcoides sp. strain BAV1 (He *et al*., 2003), Desulfitobacterium dichloroeliminans strain DCA1 (De Wildeman *et al*., 2003), Dehalobacter sp. WL (Grostern and Edwards, 2009) and Dehalogenimonas lycanthroporepellens (Yan *et al*., 2009; Moe *et al*., 2009). Moreover, Marzorati and colleagues (2007) identified the gene dcaA coding for the reductive dehalogenase (RDase) specifically adapted to the degradation of 1,2-DCA, included in the frame of the RD gene cluster RD-54 together with other 4 open reading frames (ORF) named dcaB, dcaC, dcaT, and orf1, suggested to code for a set of proteins involved in the RD of 1,2-DCA (Marzorati *et al*., 2007).
So far, the discovered microbial diversity associated to the degradation of 1,2-DCA has been limited compared to others chlorinated compounds, such as for instance TCE and PCE (Hug *et al*., 2013; Merlino *et al*., 2015). This is even more evident considering the high abundance of organochlorines of natural origin (Gribble, 2010; Krzmarzick *et al*., 2012), the plasticity of the gene clusters associated to reductive dehalogenation pathways (McMurdie *et al*., 2009) and the potential emergence of new enzymatic activities from the initially inefficient promiscuous activity of already existing enzymes which catalyze similar reactions in presence of anthropogenic chlorinated pollutants (Copley, 2009). Thus, we believe it is of capital importance to identify and isolate new bacterial strains with the ability to degrade 1,2-DCA in order to: i) deepen the knowledge about how widespread is the 1,2-DCA-degradation potential in the environment, ii) help designing more efficient bioremediation approaches and iii) in general to improve the understanding of the ecological importance of microbial dehalogenation in the global halogen cycle.
In the present study, we report the achievement of a 1,2-DCA dechlorinating simplified consortium in which we speculate about the ability of an uncultured

Geobacter sp. to reductively dechlorinate 1,2-DCA-dehalogenating, able to couple growth to the dichloroelimination process. The enrichment of the dehalogenating consortium was followed with the aim of 16S rRNA gene ILLUMINA libraries and RDase specific PCR-DGGE and flow cytometry. The co-culture was also characterized by the enrichment of a non-dehalogenating *Pseudomonas aeruginosa* sp.

MATERIALS AND METHODS

Establishment of anaerobic enrichment cultures

Water was collected from two selected piezometers (p8 and p16) of the upper layer of an aquifer in the North of Italy heavily contaminated by 1,2-DCA for more than 30 years (Marzorati *et al*., 2005; Marzorati *et al*., 2007; Marzorati *et al*., 2010; Merlino *et al*., 2015). Water samples were collected in sterile amber glass bottles with no head-space to minimize oxygen contamination and, after transportation in the laboratory, stored at room temperature (RT) in the dark for until the set-up the anaerobic microcosms (MCs). Water samples were purged with N2 to remove all the 1,2-DCA before to be used as inoculum for the enrichment cultures. In an anaerobic glove-box Simplicity 888 (Plas-Labs, USA) under an atmosphere of N2, different series of triplicate primary MCs (transfer 0, t0) were set up amending 50 ml of groundwater from each well with different electron donors, namely lactate 2 mM (L) and acetate plus formate 1 mM each (AF), a mix of seven B vitamins (100 mg l-1 Vitamin B12, 80 mg l-1 p-aminobenzoic Acid, 20 mg l-1 D(+)-biotin, 200 mg l-1 Nicotinic Acid, 100 mg l-1 Calcium Pantothenate, 300 mg l-1 Pyridoxine hydrochloride and 200 mg l-1 Thiamine hydrochloride dehydrate; Seven Vitamin Solution, SVS), cysteine 1 mM (to maintain reducing conditions) and 0.5 mM Hepes/NaOH pH7 [4- (2-hydroxyethyl)piperazine-1-ethanesulfonic acid solution] (to keep the pH in a range of 6.8–8.2). The choice of using formate and acetate together was taken since it was previously shown that none of them could support

the growth and the 1,2-DCA dechlorination alone, since the former seems to be used only as electron donor and the latter as a carbon source, while lactate was proved to be used both as electron donor and carbon source (De Wildeman *et al*., 2003). On the other hand to evaluate the effect on the dehalogenating activity of the resident microbiome of any other organic molecules possibly present in the groundwater which could be used as carbon source and/or electron donor, series of MCs with no addition of any electron donor and carbon source (w/o-a, without amendment) were also set-up. Control MCs were prepared by setting up parallel vials containing the same culturing medium with filter-sterilized groundwater samples. All the MCs were sealed with thick black butyl-rubber stoppers and aluminum crimp seals and static incubated in the dark at 26°C, immediately after the addition of 1,2-DCA as the only electron acceptor (from 100 ppm up to 300 ppm). When almost complete depletion of 1,2-DCA was observed, in order to achieve the selective enrichment of the actively dechlorinating microorganisms, 5 ml of each culture were transferred in new microcosms containing the same components of the original culture plus a synthetic culturing medium consisting of a 1:200 dilution of a trace elements solution (12.8 g l-1 nitrilotriacetic acid, 1.35 g l-1 FeCl3·6 H2O, 0.1 g l-1 MnCl2·4 H2O, 0.024 g l-1 CoCl2·6 H2O, 0.1 g l-1 CaCl2·2 H2O, 0.1 g l-1 ZnCl2, 0.025 g l-1 CuCl2·2 H2O, 0.01 g l-1 H3BO3, 0.024 g l-1 Na2MoO4·2 H2O, 1 g l-1 NaCl, 0.12 g l-1 NiCl2·6 H2O, 0.026 g l-1 Na2SeO3·5 H2O) and a supplementary salt solution (43 mg l-1 NH4Cl, 0.5 g l-1 KH2PO4, 0.2 g l-1 MgCl2·6 H2O, 0.01 g l-1 CaCl2·2 H2O).

Evaluation of the biodegradation of 1,2-DCA

Concentrations of 1,2-DCA and presence of the two possible product of its biodegradation, ethylene and vinyl chloride (VC), were evaluated by the injection of 500-µl samples of headspace of the microcosms in a Gas Chromatograph/Flame Ionization Detector (GC/FID) Fractovap GI (Carlo Erba, Italy) equipped with a

Carbopack B packed column. The carrier gas used was N2 and the temperatures of injector, oven and detector were set respectively at 220 °C, 160 °C and 250 °C.

Isolation of the Genomic DNA

Samples of groundwater and of cultures form the microcosms, respectively 30 and 10 ml (one samples from each replicate of the enrichment cultures was taken for DNA isolation), were filtered through 0.22 µm polycarbonate filters (Millipore, USA) in order to collect the microbial cells. Filters were stored at -20 °C in 1.5-ml Eppendorf tubes until nucleic acids extraction. Total genomic DNA was isolated from the filtered bacterial cells using the NucleoSpin Tisue XS kit (Macherey-Nagel, USA), following the manufacturer's instructions.

Construction of the bacterial 16S rRNA gene clone library and identification of the *Geobacter* strain

Bacterial 16S rRNA gene was amplified from the groundwater metagenome using universal primers 27f and 1492r (Wen *et al*., 2012) using 2 µl of genomic DNA isolated from enrichment cultures actively dechorinating, with the following reaction concentrations in a final volume of 50 µl: 1X PCR buffer, 1.5 mM MgCl2, 0.12 mM dNTPs, 0.3 µM of each primer, 1 U of Taq polymerase (Invitrogen, USA). Thermal protocol consisted in an initial denaturation step at 94°C for 4 minutes, followed by 35 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. A final extension step at 72°C for 10 minutes was then performed.

Ligation reactions were performed using TOPO-TA Cloning Kit (Invitrogen, USA). Following manufacturer instructions, 4 µl of the 1.5 Kb PCR products resulting from the whole 16S rRNA gene amplification were mixed with 1 µl of linearized vector pCR® II-TOPO and 1 µl of the specific saline buffer. Ligation reactions were incubated for 30 minutes at RT. Two µl of the resulting product were used to transform 50 µl aliquots of competent E.coli JM109 (Promega, USA)

cells through heat shock reactions: plasmids and cells were mixed in ice and left in incubation for 30 minutes; then the mixes underwent thermal shock at 42°C for 45 sec, followed by 2 minutes in ice. Cells were then incubated while shaking at 37°C for 1 hour with SOC medium (composition of this medium is specified in the instruction manual of the TOPO-TA Cloning Kit). Finally, 100 µl of cells were plated in 4 replicates on LB medium (Amersham, USA) containing 100 µg/ml ampicillin and 40 µg/ml X-Gal and put in incubation at 37°C overnight (ON). Ninety white clones were selected and used to inoculated 96-wells microtiter plates containing 100 µl of liquid LB with 15% glycerol and ampicillin, left in incubation at 37°C ON. A small aliquot of the culture from each well was retrieved and DNA was extracted by boiling of the cells for 8 minutes. Five µl of each sample were then used as template for further PCR amplification with M-13 primer set, specific for amplifying the insert of the pCR® II-TOPO plasmid. Reactions were carried over in 50 µl volume with the following conditions and thermal protocol: 1.50 mM MgCl2, 0.20 mM dNTPs, 0.30 µM of each primer, saline buffer 1x and Taq polymerase 1.00 Unit per reaction (Invitrogen). The first denaturation step was at 94°C for 5 min, followed by 35 cycles with denaturation step at 94°C for 1 minute, annealing step at 55°C for 1 minute and elongation step at 72° for 1 minute and 30 seconds, final elongation step was at 72°C for 10 minutes. All successfully amplified clones were then sequenced in outsourcing by Sanger method at Macrogen (Korea). Sequences were edited with software Chromas Lite version 2.01. Sequences were analysed using the Basic Local Alignment Search Tool (BLAST) of the on-line NCBI database (Altschul *et al*., 1990), by the CLASSIFIER Match Tool version 2.6 of Ribosomal Database Project II (RDP II, Wang *et al*., 2007) and with the BLASTN program of the Ez-Taxon, an on-line database which contains only taxonomically relevant sequences of type strains with validly described names (Chun *et al*., 2007).

Evaluation of the diversity of 1,2-DCA specific RDase genes

In order to evaluate the diversity of the genes coding for RDases specific for the reductive dehalogenation of 1,2-DCA, a portion of the dcaA gene previously identified by Marzorati and colleagues (2007) was analysed through PCR-DGGE, amplifying a ≈600 bp region with primers DH3F (5'-ATT GGG AGA AGC ATG CAG GT-3'), with a 5'-end of a 40-bp GC clamp, and DH3R (5'-GAC CAC CGT TAT AGG CCC AGA-3'). The reaction mix was prepared as follows: 1X PCR buffer , 2.0 mM MgCl2, 0.2 mM dNTPs, 0.3 µM each primer, and 1.5 U of Taq polymerase in a final volume of 50 µl. The thermal protocol consisted of an initial denaturation at 94 °C for 4 minutes followed by 10 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 61 °C for 1 minute with a decreasing ramp of 0.5 °C/cycle and extension at 72 °C for 1 minute, and subsequently by other 25 cycles as above, with the exception of the annealing temperature that was set at 56 °C. A final extension step at 72°C for 10 minutes was then performed.

Amplification products were loaded onto 7.0 % (w/v) polyacrylamide gels (0.75 mm) containing a denaturant gradient of 40-60 % (where 100 % denaturant contained 7 M urea and 40 % formamide). Electrophoresis was run in 1 TAE buffer using a D-Code electrophoresis system (BioRad, USA) at 90 V and 60 °C for 18 hours. Gels were stained with SYBR(R) Green I Nucleic A (Invitrogen, USA) and documented with the GelDoc 2000 system (BioRad, USA) by using the Diversity Database software (BioRad, USA). Relevant DNA bands were excised from the gels and eluted in 50 µl of 10 mM Tris-HCl pH 8.0 for at least 3 hours at 37 °C. Five microliters of DNA from each band was PCR re-amplified. The mix used was the same as described above, while the thermal protocol was the following: initial denaturation at 94 °C for 5 minutes followed by 35 cycles consisting of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute and subsequently a final extension step at 72 °C for 10 minutes.

The PCR products were sequenced in out-sourcing (Macrogen, Korea) and the sequences obtained were compared to the on-line databases as described above.
To further investigate the diversity of the different dcaA genes selected in the MCs series, the complete sequences were obtained and compared. A first PCR with the primer-set PceAFor1/DcaBRev (Marzorati *et al*., 2007), giving a product of 1944 bp and covering all the sequence of the gene dcaA and a portion of the downstream gene dcaB, was merged with the PCR products obtained with the specifically designed primers DcaA1196-F (5'-ACG TGT CAA TGG GAT GAT G-3') and DcaA1574-R (5'-TGG TAC TTT GCA TCC ACC TT-3'), annealing respectively upstream and downstream of the primer PceAFor1 and giving a product of 405 bp covering the initial portion of the dcaA gene. The PCR with the primer-set PceAFor1/DcaBRev was performed as already described elsewhere (Marzorati *et al*., 2007). The concentration of the reagents used for the amplification with the primer-set DcaA1196-F/ DcaA1574-R were the following: 1.50 mM MgCl2, 0.12 mM dNTPs, 0.40 µM of each primer, saline buffer 1x and Taq polymerase 1.00 Unit per reaction (Invitrogen). The thermal protocol consisted in an initial denaturation step at 94 °C for 5 minutes followed by 35 cycles consisting of denaturation at 94 °C for 45 seconds, annealing at 59 °C for 45 seconds, extension at 72 °C for 1 minute and a final extension step at 72 °C for 10 minutes.

Phylogenetic analysis

The sequences of the 16S rRNA gene of the *Geobacter* strain and the sequences obtained from the re-amplification of the 1,2-DCA RDases PCR-DGGE were used to construct phylogenetic trees with bootstrap of 1000 repetitions, using software MEGA version 6 (Tamura *et al*., 2011) For *Geobacter* the tree was constructed using the Maximum Likelihood method through the Kimura's two-parameter model, while the tree for the sequences of the 1,2-DCA specific RDases was built using the Neighbor-Joining method computing the evolutionary distances through the Kimura's two-parameter model.

Quantitative PCR characterization of the dehalogenating consortia

Quantitative PCR (qPCR) assays were performed on the thermocycler I-Cycler (Biorad, USA) in 96-wells plates. Reaction mixes were set-up using the "Brilliant II SYBR® Green QPCR Master Mix" (Invitrogen, USA). Reaction mix, containing 5.0 µl of template DNA, were set-up as follows: 1X Brilliant II SYBR® Green, 0.12 µM of each primer. The number of copies of the dcaA gene and the 16S rRNA gene of different phylogentic groups, such as total Bacteria, *Geobacter*, *Pseudomonas aeruginosa*, were targeted. To quantify the copies of dcaA gene the primer-set DH3F/DH3R was used (see above, Marzorati *et al*., 2007), while the primers used for the phylogenetic groups analysed are listed in Table 1.

Phylogenetic group	Primer-set (sequence 5' to 3')	Reference
Total Bacteria	Bac357-F CCTACGGGAGGCAGCAG Bac907-R CCGTCAATTCCTTTGAGTTT	Favia et al., 2007
Geobacter	Geo564-F, AAG CGT TGT TCG GAW TTA T Geo840-R, GGC ACT GCA GGG GCT AAT A	Cummings et al., 2003
P. aeruginosa	Pse435-F, ACT TTA AGT TGG GAG GAA GGG Pse686-R, ACA CAG GAA ATT CCA CCA CCC	Bergmark et al., 2012

Table 1: list of the primer-sets used for the qPCR assays targeting the 16s rRna gene

Thermal protocol used consisted in initial denaturation at 94 °C for 3 minutes, follewd by 50 cycles at 95 °C for 30 seconds, X °C (X = 55 °C for DH3F/DH3R, 58 °C for Bac357-F/Bac907-R, 56 °C for Geo564F/Geo840R, 57 °C for Pse435-F/Pse686-R) for 1 minute, 72 °C for 1 minute. To verify the specificity of the reaction, a final analysis of the melting curves of the PCR products was performed with the following thermal protocol: denaturation at 95 °C per 1 minute, cooling at 50 °C for 1 minute and subsequent warming up to 95 °C with an increase of temperature of 0.5 °C per cycle (91 cycles, 10 seconds/cycle). Data were analysed and threshold cycles (Ct) calculated with the aim of the software I-Cycler version

3.0 (Biorad, USA). Standard curves were generated for every quantification assay using serial dilutions of plasmidic standards in a range of concentrations of 10^2-10^8 copies per 5µl of standard used as template. Plasmidic standards were set-up through PCR amplification with the same primer-sets used for the assays and cloning of the amplicons, with the aim of the TOPO-TA Cloning Kit (Invitrogen, USA). DNA isolated from isolates of *Desulfitobacterium dichloroeliminans* DCA1 (AM183918) and of *Asia* sp. (AM404260), from our enrichment cultures and our isolates of *P. aeruginosa* were used to generate the standards respectively for *Geobacter*, the *P. aeruginosa* clade, the *dcaA* gene and the total *Bacteria*. The gene copy numbers obtained from the qPCR assays were then converted to number of cells. Conversion factors of 4.0 copies/cell, 2.7 copies/cell and 4.0 copies/cell were considered respectively for *Bacteria*, *Geobacter* and the *P. aeruginosa* clade, as reported in the Ribosomal RNA Database (rrnDB, http://rrndb.mmg.msu.edu/search.php), while it was assumed a conversion factor of 1 copy/cell for what concerns the *dca*A gene.

Isolation, identification and evaluation of the dehalogenating ability of *P. aeruginosa*

The *P. aeruginosa* strain enriched in the dehalogenating consortia was isolated. Initially, 100 µl of undiluted liquid culture withdrawn from enrichment microcosm cultures were plated on R2A agar medium and left in static incubation at 37 °C ON. Grown colonies were subsequently restriked two times in order to obtain pure colonies, which were then used to inoculate liquid cultures of R2A, shaken incubated at 37 °C ON, used to perform further analysis. The ON grown liquid cultures were used to isolate genomic DNA by boiling of the cells for 8 minutes. Subsequently, the *Pseudomonas* strain was identified through amplification of the *16S rRNA* gene, sequencing in outsourcing to Macrogen (Korea) and comparison against on-line databases as already described above.

In order to evaluate the putative ability of the *Pseudomonas* strain to perform reductive dehalogenation of 1,2-DCA, a series of triplicate anaerobic dehalogenating microcosms were set up as already described above, with the only exception that as inoculum 100 µl of 37 °C ON aerobically grown pure *Pseudomonas* culture were used. Aerobic dehalogenating microcosms were also set up as described above, but without reducing the medium by any means. Potential dehalogenating activity was followed by GC-FID for 3 weeks.
Finally, in order to have a conclusive confirmation of the putative role of *P. aeruginosa* in the dehalogenation process, the genomic DNA isolated from the pure cultures was used as template for the amplification of the DH3F/DH3R-included-region of the *dcaA* RDase gene using the same mix and protocol of the re-amplification of the bands excised from the acrylamide gel of the *dcaA* RDase gene PCR-DGGE.

Flow cytometric characterization of the enriched consortia

The microbial populations of the enrichment cultures were enumerated and characterized by flow cytometry (FCM) with a Flow Cytometer Accuri C6 (BD Biosciences, USA), using always fresh samples both untreated and after incubation at 37 °C for 10 minutes with 1X Sybr Green I (Invitrogen, USA).

RESULTS AND DISCUSSION

Degradation kinetics of 1,2-DCA

Five series of anaerobic enrichment cultures, namely 8L, 8AF, 8w/o-a, 16L and 16AF, were set up with the addition of 1,2-DCA as the only electron acceptor and of different organic acids as carbon and energy sources. The extent of degradation of 1,2-DCA and the generation of ethylene and VC, the two possible end-products of dehalogenation in anaerobioc conditions, were followed for all the cultures in order to evaluate the dehalogenation potential of the resident microbiome of the

heavily 1,2-DCA-contaminated site the groundwater was taken from and to assess the possible increase in the dechlorination rates at subsequent stages of enrichment. The cumulative degradation kinetics curves obtained for each series, starting from the primary microcosms t_0, are shown in Figure 1. In all the series of MCs set up, the only end-product detected through HS GC-FID was ethylene (data not shown). Only little peaks corresponding to the retention time of VC were sometimes identified in the actively dechlorating cultures as well as in the corresponding negative controls, suggesting the abiotic nature of the generation of VC (data not shown). These data confirmed that the prevalent dehalogenation process taking place in the enrichment MCs was the dichloroelimination, in which the two neighboring chlorine substituents are simultaneously removed and the aliphatic single C-C bond is converted into a double C=C bond (Smidt and de Vos, 2004). The degradation performances varied among the different enrichment series. In most of the nutrient conditions tested the dehalogenating activity was lost after few transfers. The series 8w/o-a (panel E, Figure 1) showed the slowest performances and the dehalogenating activity was lost after 3 transfers. The two series 16L and 16AF, showed comparable degradation performances throughout 6 consecutive transfers (panels B and D, Figure 1). The best performances were obtained with the series 8L and 8AF (panels A and C, Figure 1). However, after 7 consecutive enrichment steps the series 8L (panel A, Figure 1), similarly to the others, lost the dehalogenating activity, while the series 8AF maintained good degradation performances that were stably kept for about 230 days and 26 consecutive transfers. The panels of Figure 1, show how the loss of degradation capability is not sudden, but gradual with a progressive decrease of the dechlorinating performance transfer-after-transfer. It can be speculated that the decrease could be caused by the gradual loss of nutrient factors or signal molecules important for the activity of the 1,2-DCA degrading bacteria. These kind of molecules could be present in higher concentrations in the groundwater used as inoculum, and transfer-by-transfer could be diluted to concentrations too low or even lost and thus not

sustaining anymore the metabolic activity of the degraders (Nichols *et al.*, 2008). Indeed, transferring 10% of the active degrading culture, already after the 4th enrichment step the groundwater used as initial inoculum resulted 10000 times more diluted. Another possible explanation could be the gradual loss throughout the subsequent transfers of helper bacteria, which may produce specific growing factors or signal molecule vital for the survival and metabolic activity of the degraders (Kaeberlein *et al.*, 2002; D'Onofrio *et al.*, 2010). However, the growing conditions imposed in the microcosms may not be favorable to the proliferation of such helper bacteria, leading as a result to a gradual "wash out" from the culture transfer-after-transfer.

The 1,2-DCA dehalogenation kinetics of the series 8AF stabilized on an average of ≈15 ppm/day (100 ppm of 1,2-DCA degraded in 7 days), but also reached peaks of ≈30 ppm/day (≈300 ppm in 9 days). These degradation capabilities are comparable with the performances previously obtained with other enrichment cultures and co-cultures, such as the one described by Merlino and colleagues (2015) inoculated with the groundwater taken from the lower layer of the same contaminated aquifer and able to degrade 1,2-DCA at a rate of 13 ppm/day in presence of lactate as only carbon and energy source, as well as the co-cultures of *Dehalobacetr* sp. and *Acetobacterium* sp. fed with only acetate which gave performances of 15 ppm/day (Grostern and Edwards, 2009). However, previous works focused on the same upper layer of the aquifer analysed in this study, always gave higher perfomances: the enrichment cultures set up by Marzorati *et al*. (2005) and Marzorati *et al*. (2010) fed with lactate gave performances up to 53 ppm/day and 80 ppm/day respectively.

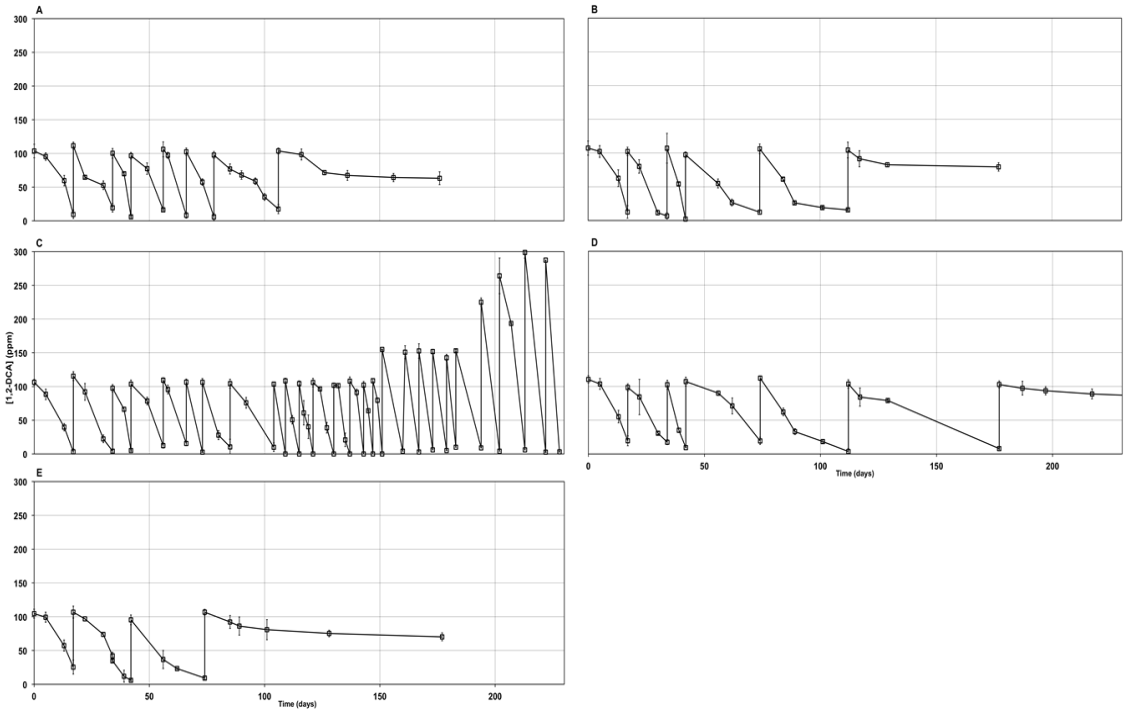

Figure 1 – Degradation kinetics of 1,2-DCA of the different series of MCs set up. *A*: 8L; *B*: 16L; *C*: 8AF; *D*: 16AF; *E*: 8w/o-a. When almost complete depletion of 1,2-DCA was achieved, 10% of the culture was transferred in a new microcosm and immediately spiked with 1,2-DCA.

Analysis of the enrichment process of the bacterial consortium

The evolution of the bacterial community harbored inside the series 8AF was characterized through ILLUMINA *16S rRNA* gene libraries, in order to evaluate the success of the enrichment process of 1,2-DCA-dehalogenating bacteria. From the histograms of Figure 2 it is possible to appreciate the change of the community between the primary microcosms (t_0) and the subsequent transfers. The community resulted to be drastically reshaped already at transfer t_1 by the anaerobic conditions aimed at favoring the reductive dehalogeantion of 1,2-DCA. Indeed if the bacterial consortium of the t_0 was characterized by the presence of 10 main genera, after 1 transfer, the presence of only one main population of *Geobacter* could be observed. However, from transfer t_2 the bacterial consortium seemed to be always dominated by a co-culture: the most abundant representative of the genus *Geobacter*, was indeed accompanied by *Desulfovibrio* representative and later on by a *Pseudomonas* representative. It is important to highlight that no presence of known 1,2-DCA-dehalogenating genera among the ones identified was detected. These data were also confirmed by a preliminary evaluation of the bacterial composition of the samples achieved through *16S rRNA* PCR-DGGE (data not shown). More importantly, among the three main genera identified in the consortia through the ILLUMINA analysis, different representatives were previously associated to the reductive dehalogenation of chlorinated compounds but never specifically to 1,2-DCA dechlorination. The strains *G. lovleyi*, for instance, was previously recognized as able to dehalogenate PCE and TCE using H_2 and acetate as electron donors (Sung *et al.*, 2006). Wagner and colleagues (2012) demonstrated how the ability of *G. lovleyi* to dechlorinate PCE and TCE seems to have been acquired though horizontal gene transfer of genomic islands containing the specific RDase homologues. More recently, the ability of this strain to use a poised electrode as the sole electron donor for the dehalogenation of PCE was also described (Strycharz *et al.*, 2008). Moreover, another strain of the genus *Geobacter*, *G. thiogenes*, was associated to the reductive dechlorination of trichloroacetic acid (TCA) (De Wever

et al., 2000). Another example of the importance of the genus *Geobacter* in the reductive dehalogenation processes is the involvement of *G. sulfurreducens* in the indirect dehalogenation of carbon tetrachloride (TC). Indeed, if on one hand *G. sulfurreducens* was shown to not be able to use directly chlorinated componds as terminal electron acceptors (Sung *et al.*, 2006), it was proven that in iron-reducing conditions and in presence of electron shuttle molecules, abiotic dechlorination of TC could occur by the interaction with *Geobacter*-biogenically generated iron species (Maithreepalaa and Doong, 2009). Representatives of the genus *Pseudomonas*, were only recently associated to the reductive dechlorination of dioxins (Tua *et al.*, 2014). Previously few representatives of this genus were identified as able to degrade different classes of chlorinated molecules in aerobic conditions (Field *et al.*, 2007; Adebusoye *et al.*, 2007) and in particular only one strain, *Pseudomonas* sp. DCA1 was shown to be able to grow in presence of O_2 on 1,2-DCA as sole carbon and energy source (Hage and Hartmans,1999). For what concern *Desulfovibrio*, representatives of this clade of sulfate-reducers were described as able to couple growth to reductive dehalogenation of 2-chlorophenol (Sun *et al.*, 2000) and of 2,4,6-Tribromophenol (Boyle *et al.*, 1999). However it can be speculated, from the results of the ILLUMINA analysis, that in the series 8AF the putative dechlorinating bacterium belongs to the genus *Geobacter* considering i) the constant presence and ii) the always higher proportion of its population in all the transfers of the actively dechlorinating consortium. We thus hypothesise that *Geobacter* established a positive cooperation in the beginning with *Desulfovibrio* and later on with *Pseudomonas*, favoring the stable dechlorination of 1,2-DCA throughout 25 subsequent transfers. Similar kind of positive interactions among different bacteria were previously described. For instance, some *Desulfovibrio* representatives were described several times in dehalogeanting co-cultures, in which was hypothesized their role in supplying H_2 and establishing a syntrophic interspecies-hydrogen-transfer with the reductive dehalogenating bacteria and achieving, in this way, a successful dechlorination process (Drzyzga *et*

al., 2002; Men *et al.*, 2012). Moreover an enriched 1,2-DCA dehalogenating consortium with similar structure to the one we obtained in this study, was composed by a *Dehalobacter* sp. coexisting together with an *Acetobacterim* sp. in actively dechlorinating enrichment cultures, where the growth of the *Dehalobacter* strain was positively correlated with the depletion of 1,2-DCA. The putative ability of *Acetobacterium* sp. to degrade 1,2-DCA was excluded by specific dehalogenating MCs inoculated with the isolated bacterium, but different attempts to obtain a pure culture of *Dehalobacter* were unfruitful and the role of the *Acetobacterium* sp. inside the co-culture was not elucidated. However, it seemed clear that the presence of the *Acetobacterium* sp. was somehow important to maintain the dehalogenation capability of 1,2-DCA of the co-culture (Grostern and Edwards, 2009). Taken together all these data suggest, similarly to what already stated above, that the coexistence of two different bacteria with the former helping the latter supplying nutrient factors or signal molecules could be a plausible explanation for the establishment of a successful dehalogeantion of 1,2-DCA. Following the same concept, it is plausible to speculate that this positive interaction between a dechlorinating microorganism and a helper was not established in the series 8L, 16L, 16AF and 8w/o-a. Interestingly, the analysis of the bacterial community selected at transfer t_2 of the series 8w/o-a showed a consortium completely dominated by only two strains: a *Geobacter* and a *Pseudomonas* representatives (data not shown), suggesting that the kind of cooperation established between *Geobacter* and *Pseudomonas* could occur in the natural environment without the supply of any kind of energy and/or carbon source. However, we cannot speculate about the factors influencing the shift of the bacterial consortium enriched in the series 8AF with the gradual disapperence of *Desulfovibrio* in favor of the *Pseudomonas* representative.

The data obtained from the ILLUMINA analysis confirmed that all the sequences affiliated to the *Geobacter* genus were included in one only Operational Taxonomic Unit (OTU), as well as for the sequences affiliated to the *Pseudomonas*

genus. However, due to the length of the sequences retrieved from the ILLUMINA, it was not possible to identify to the species level the two main population of the consortium.

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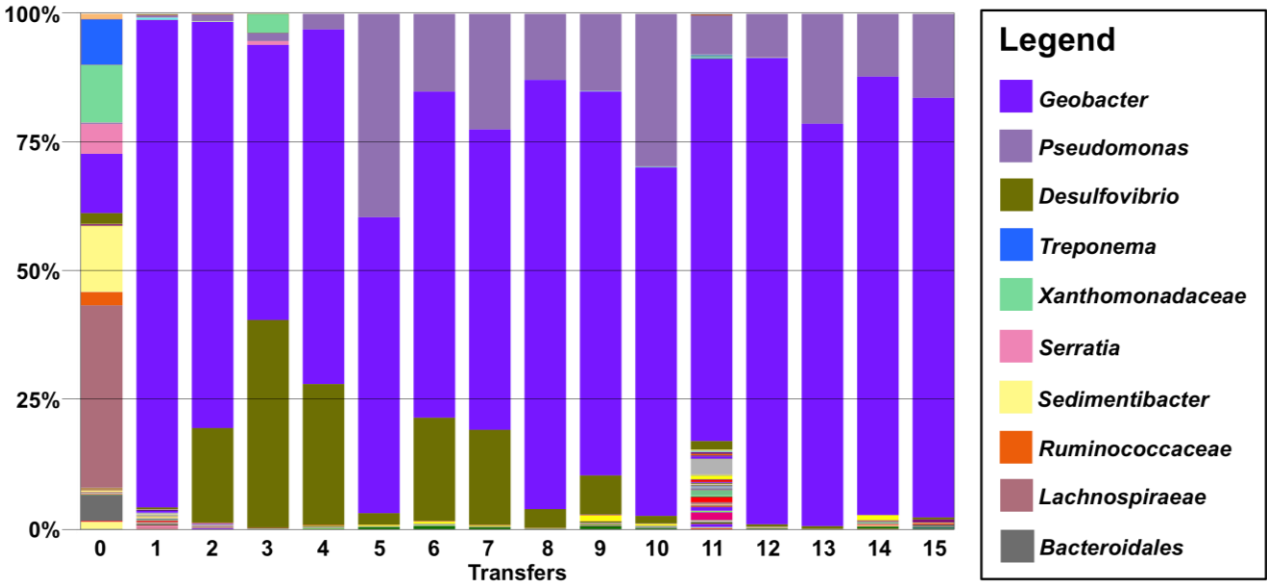

Figure 2 – Taxonomic affiliation at the Genus level carried out on 15 consecutive transfers of the actively dechlorinating consortium of the series 8AF. In the legend are reported the main Genera (when not available, Family and Order were reported) identified in the samples analysed

Enrichment of the RDases specific for the reductive dehalogenation of 1,2-DCA

During the the first steps of enrichment of the bacterial consortia, the selection of RDase genes associated to the dechlorination of 1,2-DCA through PCR-DGGE on the gene *dcaA* was evaluated, with the aim of investigating the functional redundancy of the enriched microbiomes towards 1,2-DCA dehalogenation and to evaluate the eventual selection of specific RDases. In Figure 3A it is shown the polyacrylamide gel obtained, which allows appreciating the presence of two close bands (bands 1,2,6 and 7) in the metagenomic DNA isolated from both the groundwaters used as inoculum for the set-up of the MCs. However, the figure suggests that the different conditions of enrichment used (different groundwater as

inoculum and different electron donors), resulted in the selection and enrichment of only one of the two enzymes: in particular the series 16L, 16AF and 8w/o-a showed the selection of the upper band, while the series 8L and 8AF selected for the lower band. The analysis of the sequences allowed identifying two slightly different sequences associated to previously described 1,2-DCA-specific RDases. To further investigate the differences in the *dcaA* sequences identified in the different MCs series, the whole *dcaA* gene sequences (≈1600 bp) were obtained through the combining of the amplicons of two PCR performed with PceAFor1/DcaBRev (1944 bp) and DcaA1196-F/ DcaA1574-R (405 bp).
The phylogenetic relationship between the whole *dcaA* gene sequences enriched in our MCs with the sequences of A-subunits of other already characterized RDases was evaluated and is graphically represented in the tree shown in Figure 3B. As previously described by Marzorati and colleagues (2010), the RDases cluster in four distinct groups. The RDase enriched in the MCs of the series 8L and 8AF was affiliated to group I, while the one enriched in the MCs series 16L, 16AF and 8w/o-a to group III. The analysis of the sequences associated to those two bands confirmed the presence of two different versions of 1,2-DCA specific RDases. The one clustered in group I was found to be closely related to the RDase previously identified in *D. dichloroeliminans* strain DCA1 (RD-DCA1) and in the same contaminated site (RD-54) (Marzorati *et al.*, 2007). On the contrary, the other RDase enriched in the series 16L, 16AF and 8w/o-a and belonging to group III, was found to be closely related to the RDases WL rdhA1 and WL rdhA3 identified by Grostern and Edwards (2009) in dehalogenating co-cultures containing *Dehalobacter* sp. and *Acetobacterium* sp.

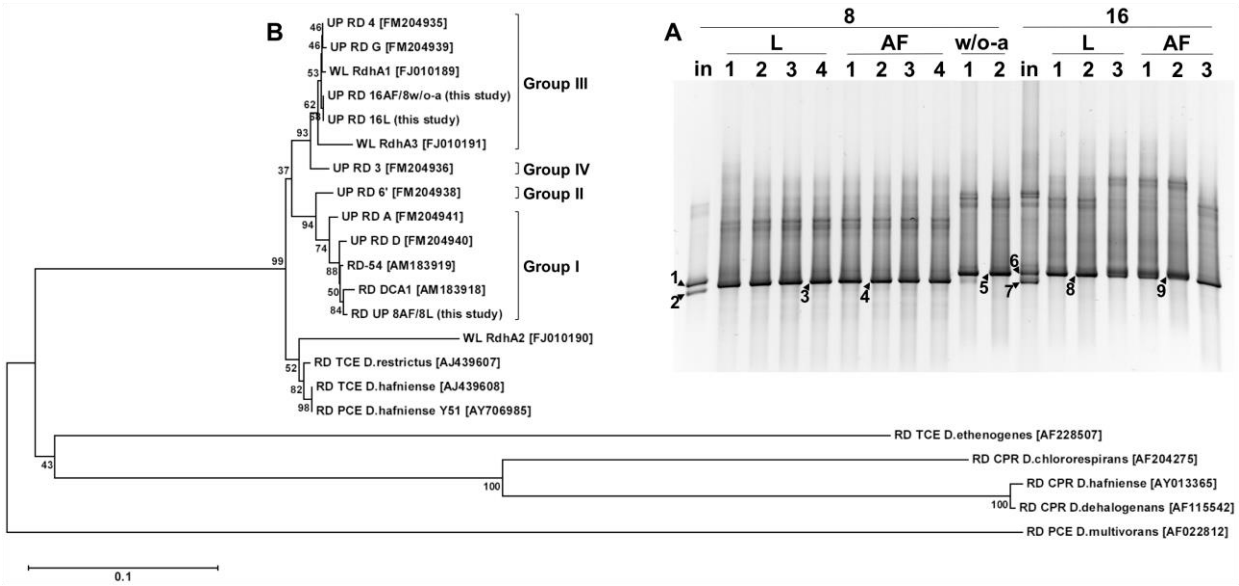

Figure 3 – A) PCR-DGGE of the ≈600-bp fragment amplified with the primer-set DH3F/DH3R. This is a magnification of the portion of the polyacrylamide gel comprised between 45% and 55% of the denaturant gradient. 8 and 16: indicate the series of MCs initially inoculated respectively with water from wells 8 and 16; in: DNA isolated from the original groundwaters used as inoculum for the MCs; L, AF and w/o-a: electron donors added to the MCs, respectively lactate, acetate+formate and no addition of carbon and energy source; 1-4: number of each transfer culture analysed. Black arrows and numbers indicate the bands excised from the polyacrilamide gel. B) Neighbour-joining tree of the RDase gene sequences to assess the relationship between the dcaA genes identified in the enrichment cultures with previously characterized RDases genes. The tree was built with the complete sequences of the RDase genes, and not the shorter sequences obtained from the PCR-DGGE. The complete sequences were obtained as described in Materials and Methods section. Together with the DNA sequences of the dcaA genes identified in this study (Up RD 16AF/8w/o-a, UP RD 16L, UP RD 8AF/8L), the genes coding for the catalytic subunits of RDases specific for 1,2-DCA described in other studies were used, such as the sequences obtained from the same contaminated aquifer (RD-54) (AM183919) and all the UP RD X (from FM204935 to FM2049) (Marzorati et al., 2007 and 2010), the dcaA gene identified in D. dichloroeliminans strain DCA1 (RD-DCA1, AM183918) (De Wildeman et al., 2003) the WL RdhA1 (FJ010189), WL RdhA2 (FJ010190) and WL RdhA3 (FJ010191) genes identified in the a 1,2-DCA dechlorinating co-culture containing Dehalobacter sp. (Grostern and Edwards, 2009), as well as the catalytic subunits of RDases specific for other chlorinated compounds: pceA of Dehalobacter restrictus strain DSMZ 9455T (RD TCE D. restrictus, AJ439607), pceA of Desulfitobacterium hafniense strain TCE1 (RD TCE D. hafniense, AJ439608), pceA of Desulfitobacterium hafniense strain Y51 (RD PCE D. hafnienese Y51, AY706985), tceA of Dehalococcoides ethenogenes (RD TCE D. ethenogenes, AF228507), cprA of Desulfitobacterium chlororespirans clone 1256 (RD CPR D. chlororespirans, AF204275), cprA of Desulfitobacterium hafniense strain DCB-2 (RD CPR D. hafniense, AY013365), cprA of Desulfitobacterium dehalogenans (RD CPR D. dehalogenans, AF115542) and pceA of Dehalospirillum multivorans (RD PCE D. multivorans, AF022812).

Identification of the *Geobacter* strain

A clone library was constructed using the genomic DNA isolated from the enrichment cultures as template, in order to assess the identity of the *Geobacter* strain enriched, since the data obtained from the ILLUMINA *16S rRNA* libraries did not provide sufficient resolution to get to the species level. A total of 70 clones were analysed, since a low diversity was expected due to the fact that the starting sample proceeded from enriched consortia. Among the 70 clones analysed, 65 yielded good quality sequences. According to NCBI database 64 out of 65 sequences, were related to the same Uncultured *Geobacter* sp. (FM204962) with an identity percentage ranging from 90.8% to 99.7% with an average identity percentage of 98.2%. These results were confirmed by the comparison with RDP II. All putative Uncultured *Geobacter* sp. sequences identified by BLAST, resulted to belong to *Geobacter* genus above the 80% confidence interval. The matches obtained with the Ez-Taxon database, substantially confirmed the above results. The sequences were all affiliated to the same genus by EZ-Taxon with identity percentages always in the range of 92.56% and 97.76%, confirming the chance that the strain enriched in the dehalogenating consortia is yet uncultured. The *Geobacter*-related sequences at the species level formed 3 different groups: 37 of them had an average percentage identity of 97.03% (ranging from 95.02% to 97.76%) with *G. psycrophilus* P35 (AY653549), 23 of them with an average identity of 96.80% (ranging from 92.56% to 97.45%) with *G. chapellei* strain 172 (U41561) and only one with a 97.28% identity with *Pelobacter propionicus* DSM2379 (CP000482), another representative of the *Geobacter* genus. The phylogenetic relation of the retrieved *Geobacter 16S rRNA* gene clones among themselves and between other known *Geobacter* isolates was assessed building a phylogenetic Maximum Likelihood tree (Figure 4), showing that the sequences belonging to the *Geobacter* strain enriched in the dehalogenating MCs form a cluster phylogenetically distinct from all the already known representative of the genus and confirming as the closest described relative *G. psycrophilus.*

Figure 4 - Phylogenetic tree of the Geobacter 16S rRNA gene sequences retrieved from the clone libraries. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 67 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 894 positions in the final dataset. Accession numbers of the sequences used are indicated in brackets.

Evaluation of the degradation potential for 1,2-DCA of *P. aeruginosa*

The strain co-enriched in the dehalogenating consortia together with the Uncultured *Gobacter* sp. was isolated on R2A agar and after 3 consecutive restrikes, DNA was extracted and the *16S rRNA* gene amplified. Sequences obtained allowed the identification through BLAST search of the strain as *Pseudomonas* sp. Mexd310, with an identity of 97.5%. Alignment of the sequence against the EZ-Taxon database confirmed the affiliation to the *Pseudomonas* clade, even tough to a different strain, namely *P. aeruginosa* JCM 5962 (BAMA01000316) with an identity of 100%. Moreover the analysis of the ILLUMINA data also confirmed the presence of only one OTU associated to the genus *Pseudomonas*, even tough the length of the retrieved sequences did not allow to get down to the species level. The belonging of the isolate to the *P. aeruginosa* clade is however also suggested by the green/light-blue color of the pure cultures grown both on agar and liquid R2A, due to the fluorescent phenazine molecule PYO typically produced by strains belonging to this species. Production of PYO by our isolate was confirmed by extraction with chloroform. PYO production by *P. aeruginosa* in the conditions described above was determined at about 1.16±0.08 µg/ml (data not shown).

Pseudomonas is a widely studied and ubiquitously distributed opportunistic pathogen that inhabits soil, freshwater, marine and coastal environments, as well as plant and animal tissues (Govan and Deretic, 1996; Hardalo and Edberg, 1997). Its wide distribution is mainly due to the versatile aerobic and anaerobic metabolisms (Arai, 2011). For instance, the already cited PYO was found to be involved in different cellular processes, such as virulence and competition with other microbes (Mavrodi *et al.*, 2013), intercellular signaling (Dietrich *et al.*, 2006) and survival in oxygen-depleted conditions (Wang *et al.*, 2010; Arai, 2011; Mavrodi *et al.*, 2013). Moreover, representatives of the genus *Pseudomonas,* and in particular some strains of the *P. aeruginosa* clade, were previously identified both in pure cultures and in complex communities involved in the direct degradation of environmental contaminants (Romero *et al.*, 1998), as well as indirectly involved in these

biodegradation processes through the production of the class of biosurfactant molecules rhamnolypids, which favored the degradation activity by other microbes of the same community (Arino *et al.*, 1998).

In order to assess the dehalogenating potential of *P. aeruginosa* in the enriched co-cultures, two series of aerobic and anaerobic dehalogenating microcosms were set-up. 1,2-DCA was measured during 3 weeks and no depletion, compared to the abiotic control, was observed in both the aerobic and anaerobic triplicates (Figure 1S). These results suggest that the *P. aeruginosa* strain, enriched together with the *Geobacter* strain, does not posses any potential for dehalogenation of 1,2-DCA neither under reductive nor oxidative conditions. The inability to perform anaerobic reductive dehalogenation was finally confirmed by the lack of PCR product using the *dcaA*-specific primer-set DH3F/DH3R (Figure 2S).

Evaluation of the quantitative correlation between *Geobacter 16S rRNA* and *dcaA* genes copy numbers

To verify the direct involvement of the *Geobacter* strain in the reductive dehalogenation of 1,2-DCA quantitative PCR assays targeting both the *16S rRNA* gene of *Geobacter* and the gene *dcaA*, were performed using as templates genomic DNA isolated from subsequent transfers of the series 8AF immediately before to transfer 10% of the actively dechlorinating cultures (Figure 5).

From the results, it is not possible to appreciate a constant increase in the estimated number of the cell of *Geobacter*, which vary in the range of 1.0E+06 to 1.0E+08, as would be expected from an active dechlorinting population. We speculate that this is due to the fact that the transfer of 10% of the active dehalogenating consortium was always done with a syringe. Thus the transferred volume was not precise and this could have influenced the initial ammuount of cells in the transfers, reflected by the fluctuations of the quantification by qPCR. However, the results suggest a direct correlation between the estimated cell number/ml of *Geobacter* with the copy number/ml of the gene *dcaA*. Indeed, both the number of *Geobacter*

cells and of *dcaA* copies detected in the different transfers are always overlapping, suggesting that each cell of *Geobacter* carries a single copy of the *dcaA* gene.

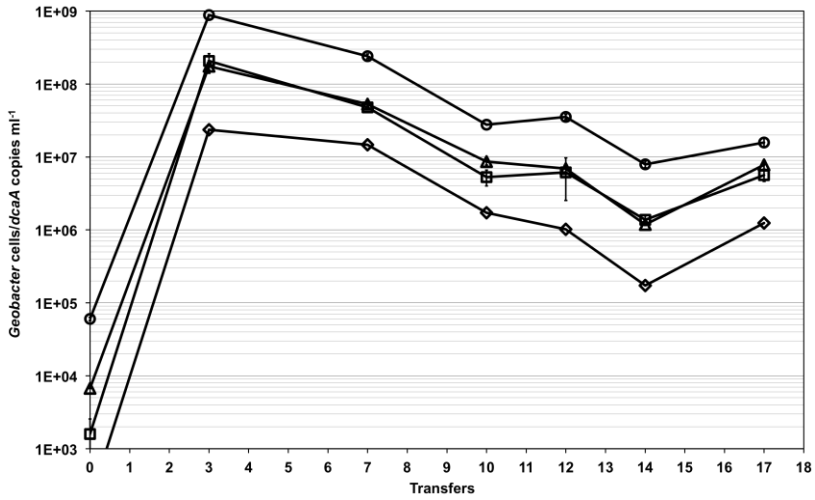

Figure 5 – Determination of the copy numbers of the dcaA gene (open squares) and of the cell numbers of total Bacteria (open circles), Geobacter (open triangles) and P. aeruginosa (open diamonds).

Assessment of the evolution of the dehalogenating consortium during active reductive dehalogenation

In order to clarify which bacteria was the main responsible for the degradation of 1,2-DCA, we exploited the potential of FCM. With the aim of this methodology we were able to characterize the two main populations in the dehalogenating consortia identified with the aim of the ILLUMINA *16S rRNA* libraries (Figure 6). Pure cultures of *Pseudomonas*, isolated and cultivated in aerobic conditions on R2A medium, were used as reference in order to tell apart the *Pseudomonas* population from the remaining part of the consortium strongly dominated by *Geobacter*. Panels C-H show the plots obtained enumerating the events in samples of the *Pseudomonas* pure culture (panels C, E and G) and in samples of the dehalogenating consortia (panels D, F and H). Panels C and D, show that the samples non-treated with Sybr Green gave a similar peak of auto-fluorescence (black curves). Similarly in panels E and F, which show the correlation between the side scatter (SSC-A), proxy of the morphology of the cells, and the auto-

fluorescence of the cell (FL1-A), do not allow to distinguish any difference among the populations of *Pseudomonas* and *Geobacter*. It can be speculated that due to the of their similar morphology and auto-fluorescence of the two species. However, after incubation with Sybr Green (panels G and H), in the mixed culture (panel H) is it possible to appreciate the presence of two distinct clusters of events, which correspond to two separate peak of fluorescence in panel D (red curves). Comparing the peaks in panels C and D and the clusters of events in panels G and H, it can be speculated that there is a correspondence between the peak of auto-fluorescence associated to the *Pseudomonas* pure culture in panel C and the correspondent peak in panel D. A similar correspondence can be found among panels G and H, where the cluster of events associated to *Pseudomonas* in the pure culture (panel G) in exactly in the same position as in the co-culture (panel H). Using the enumeration of events obtained correlating side scatter (SSC-A) with the fluorescent signal (FL1-A) generated after incubation with Sybr Green (as shown in panel H of Figure 6), we were able to distinguish the two main populations of the anerobic dehalogenating consortia. We thus performed this kind of analysis at different times during the dehalogenation process and we were able to correlate directly the growth of the populations inside the consortium with the depletion of 1,2-DCA. This confirms the metabolic nature of 1,2-DCA degradation process, which allow the dehalogeanting bacterium to obtain energy for growth. Since, as shown above, the strain of *P. aeruginosa* was found not to be able to use 1,2-DCA as terminal electron acceptor, and the qPCR assays demonstrated a tight correlation among the quantifications of the the *16S rRNA* copy numbers of *Geobacter* and the copy numbers of the *dcaA* gene, taken together these results suggest that the Uncultured *Geobacter* sp could be considered as the responsible for the degradation of 1,2-DCA.

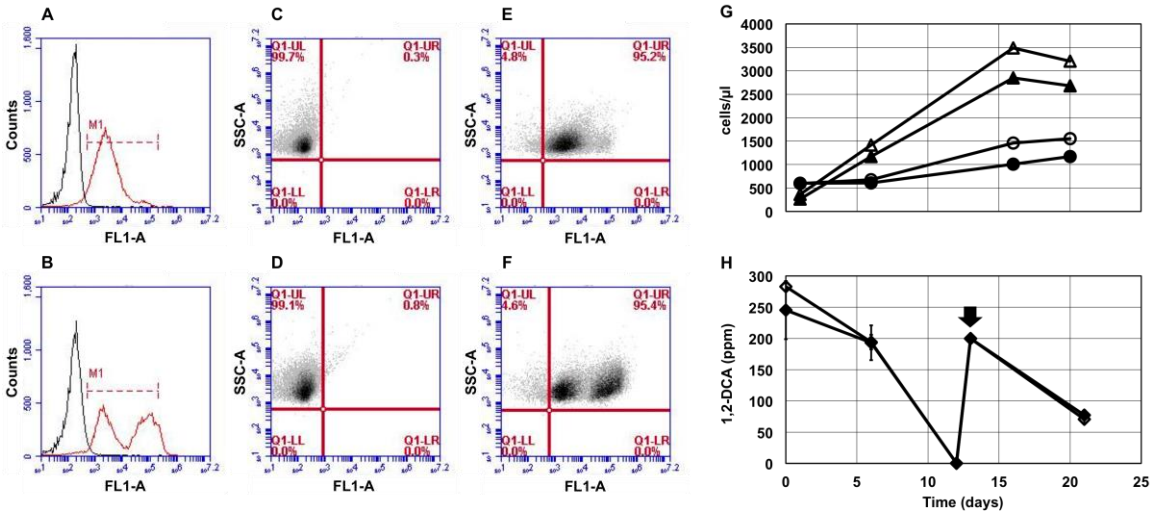

Figure 6 – Flow cytometric characterization of an enriched dehalogenating co-culture. A-F) Flow Cytometer plots showing for the reference pure culture of P. aeruginosa (A, C and E) and for the dehalogenating co-culture transfer t23-a (B, D and F) the event counts (A and B) and the clusters of events before (C and D) and after (E and F) the incubation with Sybr Green. G) Microbial counts of the Geobacter and Pseudomonas populations at different times during active degradation of 1,2-DCA. Open and filled triangles, respectively transfer culture t23-a and t23-b; open and filled circles, respectively t23-a and t23-b. H) Degradation kinetics of 1,2-DCA. Open and filled diamonds, respectively t23-a and t23-b. The black arrow indicates the respike of 1,2-DCA after depletion.

SUPPLEMENTARY MATERIALS

METHODS

Extraction of pyocyanin (PYO) produced by *P. aeruginosa*.

Procedure for the extraction of PYO form the liquid cultures of *P. aeruginosa* was adapted from Essar *et al.*, 1990). *P. aeruginosa* cultures were grown shaking in R2A medium for 48 h. Aliquots were then centrifuged at 12000 rpm for 15 minutes and then filter sterilized. Five volumes of supernatant were mixed with 3 volumes of chloroform and centrifuged 10 minutes at 10000 rpm. The organic phase was subsequently retrieved and acidified with HCl 0.2 M (1:2) then centrifuged again at 10 minutes at 10000 rpm. Absorbance at 520 nm of the aqueous red colored phase

was then measured with spectrophotometer and the value obtained multiplied by 17.072 to yield PYO concentration in µg/ml.

FIGURES

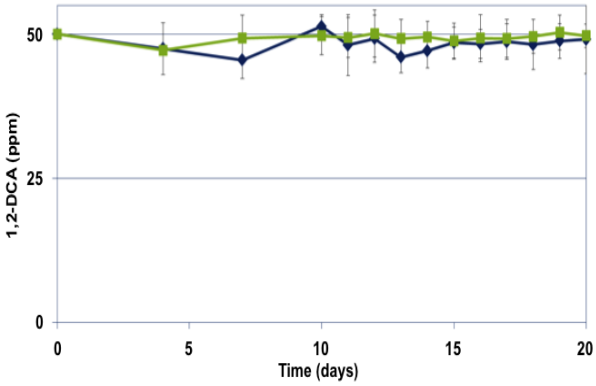

FIGURE 1S - DEGRADATION KINETICS OF 1,2-DCA OF ANAEROBIC AND AEROBIC MICROCOSMS INOCULATED WITH PURE CULTURES OF P. AERUGINOSA. FILLED SQUARES: ANAEROBIC MICROCOSMS; FILLED DIAMONDS: AEROBIC MICROCOSMS.

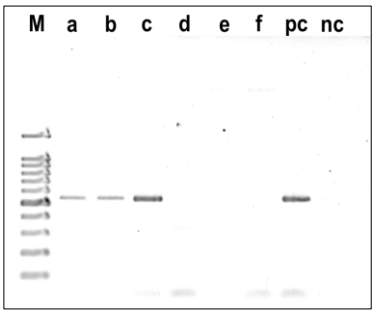

FIGURE 2S - PCR PRODUCTS OF THE AMPLIFICATION WITH PRIMER-SET DH3F/DH3R LOADED ON A 1.0% AGAROSE GEL. M: MASSRULER™ DNA LADDER MIX (THERMOFISHER SCIENTIFIC, USA); A-C: DNA ISOLATED FROM DEHALOGENATING MICROCOSMS OF THE SERIES 8AF;D-E: DNA ISOLATED FROM PURE COLONIES OF P. AERUGINOSA GROWN ON R2A AGAR; PC: POSITIVE CONTROL, THE EXPECTED ≈450 BP FRAGMENT OF THE DCAA GENE AMPLIFIED FROM THE DNA OF A BAND EXCISED FROM A DCAA-PCR-DGGE GEL, LIKE THE ONE SHOWN IN FIGURE 3A; NC: NEGATIVE CONTROL OF THE PCR

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CHAPTER 6

The role of secondary metabolites of *P. aeruginosa* in the dynamics of a 1,2-DCA dehalogenating co-culture

ABSTRACT

During studies on 1,2-DCA dehalogenating microbial enrichments, proceeding from polluted groundwater samples, a stable dechlorinating community was achieved, clearly co-dominated by two main genera: *Pseudomonas* and *Geobacter*. The role in the co-culture of the former was then investigated, as it was previously assessed its inability to degrade 1,2-DCA, whereas its presence seemed to be essential for maintaining of dechlorination capability throughout the subsequent transfers. Following stimulation with exogenous, putative electronic shuttles, namely pyocyanin, alone and together with rhamnolipids, previously described as responsible for mechanisms of electron shuttling, the dechlorination performances and the bacterial population dynamics were monitored. Pyocyanin then, was quantified in each microcosm, to verify its production at the given conditions. Its presence was confirmed in every experiment, suggesting that the real limiting factor for achieving an efficient electronic flow through the populations are rhamnolipids.

INTRODUCTION

Bioremediation approaches are considered as promising and effective means for the clean-up of environments contaminated by chlorinated compounds (Yu *et al.*, 2013; Wang *et al.*, 2015;Juwarkar *et al*. 2010). However, if on one side the potential for reductive dehalogenation of microbial communities have been extensively studied in the last decades (Freedman & Gossett, 1989; Distefano *et al*., 1992; Maymó-Gatell *et al*., 1999; Marzorati *et al.*, 2005; Grostern & Edwards, 2009; Dugat-Bony *et al.*, 2012; Vandermeeren *et al.*, 2015), the role and the importance of the interactions that can take place among microorganisms in a community and the influence that such interactions can have on the extent of the biodegradation have been poorly investigated so far (Duhamel & Edwards, 2007; Hug *et al.*, 2012; Perez-de-Mora *et al.*, 2014).

One typical interaction described is the conversion, by fermenting microorganisms, of the initial electron-donors supplied to H_2, recognized as a main intermediate used as electron-donor by different known dechlorintors, such as *Dehalococcoides* and others representatives (Yang & McCarty, 1998; He *et al*., 2002). Another possibility is for instance that the non-dehalogenating population assist the primary chlororespires providing directly essential nutrients (Yan *et a*l., 2013). Recently a growing body of research is showing that different other mechanisms can be involved in the interaction among different species, in particular favoring the transfer of electrons, which is also a key process in reductive dehalogenation. In particular, *Pseudomonas* secondary metabolites, pyocyanin (PYO) and rhamnolipids (RL) proved their involvement in promoting extracellular electron transfer, both between different microbial populations and towards poised electrodes in microbial fuel cell (MFC) experiments (Rabaey *et al*, 2005; Boon *et al*., 2008 ; Pham *et al*., 2008).

In our previous work, a stable 1,2-DCA dehalogenating consortium was enriched from the complex microbial community of a heavily 1,2-DCA polluted

groundwater. The microbial composition of the consortium, enriched throughout more than 30 consecutive transfers, was characterized with the aim of *16S rRNA* ILLUMINA libraries, showing a clear co-dominated by representatives of 2 genera: an uncultured *Geobacter* sp. and a *Pseudomonas aeruginosa* strain. Data obtained suggested an active role in the degradation of 1,2-DCA by the *Geobacter* strain, whereas the ability to degrade 1,2-DCA of the isolate of *P. aeruginosa* was tested with negative results (chapter 5)

The objective of the present study was to determine the role of the strain of *P. aerugionsa* in the co-culture. A possible interaction between the two strains and a possible beneficial effect of the non-dechlorinating *P.aeruginosa* on the performance and survival of the putative dechlorinating *Geobacter* strain was hypothesized due to the difficulty to separate the *Geobacter* sp. from *P. aeruginosa*, together with the positive correlation among *P.aeruginosa* presence into the culture and the stability of the dechlorinating activity through time and transfers. In particular the present work was aimed at elucidating the putative role of secondary metabolites from *P. aeruginosa* in promoting electron transfer between the two dominating species of the community as a possible drivers that enabled the enrichment of the co-culture over more than 30 transfers. The effects of *P. aeruginosa* secondary metabolites, namely pyocyanin (PYO) and rhamnolipids (RLs) on 1,2-DCA dechlorination rate and on the total population size of the enriched mixed culture were monitored through gas chromatography (GC) and flow cytometry (FCM). The proportions of the two populations was monitored as well, combining FCM with FISH (Flow-FISH) (Rigottier Gois *et al.*, 2005).

MATERIALS AND METHODS

Preparation of *P. aeruginosa* supernatant (PJ)

P. aeruginosa strain LGM 1242 was grown in aerobic conditions at 30°C, shaking for 48 h, in assembled R2A medium. 50 ml aliquots were retrieved and centrifuged at 13000 rpm for 20 minutes, filter-sterilized and flushed with N_2.

PYO synthesis by photolysis by Phenazine methosulfate, purification and quantification by spectrophotometry

A 1 mg/ml solution of Phenazine methosulfate (PMS) (Sigma-Aldrich, USA) was prepared in Tris HCl 100 mM and exposed during 4 hours to sunlight, rapidly turning from bright yellow to deep, dark blue. Solution was then added of 1 volume of chloroform and organic phase was recovered and acidified with HCl 0.2 M until turning to red. Aqueous phase was retrieved and basified with 0.4 M Borate-NaOH buffer pH 10 until turning back to blue. Again, 1 volume of chloroform was added and organic phase was dehydrated with anhydrous Na_2SO_4. Subsequently the solvent was evaporated with rotavapor to yield PYO powder. Success of the synthesis and purity of the extracted PYO were evaluated by H-NMR.
Concentration of PYO was determined in *P. aeruginosa* pure culture and in experimental MCs. The same extraction procedure described above was followed, with lesser volumes. The acidified, red aqueous phase was loaded in a spectrophotometer and absorbance was measured at 520 nm. The value obtained was then multiplied for 17.072, to yield the concentration of PYO in mg/L (Essar *et al.*, 2005).

Set-up of the microcosms

Vials were prepared with modified BTZ-3 minimal medium (43 mg/L NH_4Cl, 0.5 g/L KH_2PO_4, 0.2 g/L $MgCl_2 \cdot 6\ H_2O$, 0.01 g/L $CaCl_2 \cdot 2\ H_2O$ and 50 mM Hepes/NaOH pH7), and a 1:200 dilution of a trace elements solution (12.8 g/L

nitrilotriacetic acid, 1.35 g/L $FeCl_3 \cdot 6\ H_2O$, 0.1 g/L $MnCl_2 \cdot 4\ H_2O$, 0.024 g/L $CoCl_2 \cdot 6\ H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.1 g/L $ZnCl_2$, 0.025 g L^{-1} $CuCl_2 \cdot 2\ H_2O$, 0.01 g/L H_3BO_3, 0.024 g/L $NaMoO_4 \cdot 2\ H_2O$, 0.12 g/L $NiCl_2 \cdot 6\ H_2O$, and 0.026 g/L $Na_2SeO_3 \cdot 5\ H_2O$, 1 g/L NaCl). Eventually, resazurin was added to the solution at 1 mg/L. Before getting sealed and autoclaved, vials containing 42 ml of the medium were reduced 4 days in an anaerobic glove box Concept 400 (Ruskinn technology, UK) under atmosphere formed by 85% N_2, 10% H_2 and 5% CO_2. Moreover, all MCs were added, just prior to inoculum, with a mix of seven B vitamins (100 mg/L Vitamin B_{12}, 80 mg/L p-aminobenzoic Acid, 20 mg/L D(+)-biotin, 200 mg/L Nicotinic Acid, 100 mg/L Calcium Pantothenate, 300 mg/L Pyridoxine hydrochloride and 200 mg/L, Thiamine hydrochloride dehydrate; Seven Vitamin Solution, SVS), with 2 mM of Na Formate, 2 mM of Na Acetate, 4 µg/ml of Vancomycin hydrochloride hydrate (Sigma-Aldrich, USA), 1 mM of Dithiothreitol (DTT) (Sigma-Aldrich, USA), and 180 mg/L 1,2-DCA. All reagents, but resazurin stock solution (0.025%), were fluxed with N_2, and stored in vials to minimize O_2 contaminations. Each triplicate series differed from the other just for a 1:25 fraction, replaced, respectively, with PJ for PJ series, with anoxic R2A medium for R2 series, with a solution of dH_2O and PYO (C_f in microcosms 1 mg/L), for PC series, and with a solution containing both PYO and RLs (final concentration in microcosms, 1 mg/L PYO and 1 mg/L RLs) for PR series, and dH_2O for the control NN series.

Evaluation of the degradation performance

Concentrations of 1,2-DCA and presence of ethylene and vinyl chloride (VC), were evaluated by headspace analysis of 500-µl samples retrieved from the microcosms with gas-tight precision syringe (Hamilton, USA) and injected in split-less mode in a 25 m long, 0.5 mm wide bore capillary VOC-2 column (Mega, Italy), mounted on a GC 86.10HT (Dani, Italy) equipped with FID. Thermal conditions were set as follow: Injector, 220°C; oven, 60°C; FID, 220°C. N_2 was used as carrier gas at 0.6

bar of pressure. Detection was made with double attenuation of peaks. Measures were taken at 2-3 days intervals.

FISH probes hybridization

1-ml samples were retrieved in 1.5-ml tubes from each culture and centrifuged at 13000 rpm for 5 minutes in order to collect the cells at the bottom of the tube. The pellet was subsequently suspended in 1 ml of 4% paraformaldehyde solution in PBS 1x pH 7.2 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) for cells fixation. Centrifugation and re-suspension was repeated in 0.5 ml of 1 mg/ml lysozyme solution in TE buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) in order increase the permeability of the fixed cells. This solution was incubated at 37°C for 10 minutes. After the incubation, lysozyme solution was washed away through another step of centrifugation-suspension in 0.5 ml of TE buffer. Washing buffer was discarded through centrifugation and the permeable cells were subjected to hybridization through suspension and incubation ON at 37°C, in the dark, in hybridization buffer (900 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.1% SDS, 30% formamide) containing 5 ng/µl of two probes (Table 1), each one specific for a region of the *16S rRNA* gene of *Geobacter* and *Pseudomonas*. The probe GEO3-A, specific for *Geobacter*, was labeled with fluorescein isothiocyanate (FITC), while the probe PSE449, specific for *Pseudomonas*, was labeled with cyanine 5 (Cy5). After ON incubation, the cells were centrifuged and subsequently re-suspended in washing buffer (64 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.1% SDS). The hybridization protocol was then completed with a final centrifugation and suspension of the hybridized cells in 1X PBS pH 7.2 (Rigottier Gois *et al.*, 2005).

Name	target	Sequence	Fluorophore	Excitation/emission λ (nm)	Reference
GEO3-A	Geobacter spp.	CGCAACACCTAGTACTCATC	FITC	490/525	Richter *et al.,* 2007
Pse449	Pseudomonas spp.	ACAGAATAAGCACCGGCTAAC	Cy5	649/670	Bergmark *et al* ., 2012

Table 1: Combinations of FISH probes and fluorophores used in this study

Flow cytometry (FCM) analysis

Total cell count

FCM analysis was carried over with a C6 Accuri (Becton Dickinson, USA) at a flow-rate of 14 µl/min, a core-size of 10 µm, with F-SC threshold set at 5000 and S-SC at 4000. All samples for total cell count were stained with 1X SYBR Green for 10 minutes at 37°C and fluorescence shifting cells were counted with filter FL-1. Samples for total cell count were retrieved from the cultures at 2-3 days intervals.

Flow-FISH

Flow-FISH analysis was carried over with the same instrument and the same conditions described above. Setup experiment was performed with the same sample treated in quadruplicate with FISH protocol as above. The first reaction used only PSE449 probe and fluorescence shift of Cy5 was detected through FL-4 filter; second reaction contained only GEO3-A probe and fluorescence shift of FITC was detected through FL-1 filter; third reaction was performed with both probes and fluorescence shifts were measured in parallel through both channels; fourth and last setup was performed with cells treated as above but incubated in hybridization buffer without probes to be subsequently marked with SYBR green for total count, to assess the yield of FISH experiment on total cell count (Rigottier Gois *et al.*, 2005). Only a preliminar analysis on R2 series was perform at the end of degradation and cells were marked with both probes to have an insight on the variation of the relative proportion of the main populations after stimulation with different putative electronic shuttles solutions.

RESULTS AND DISCUSSION

PYO synthesis from PMS

PMS *conversion* to PYO typically yields 60% of product as previously described by King and colleagues (1979). In our experiments, photolysis and extraction, starting from 40 mg of PMS produced tipically 18 mg of PYO, meaning a 45% yield. Presence of PYO was confirmed by H-NMR, which highlighted as well presence of some minor impurities.

Evaluation of the effects of PYO, R2A, RLs on degradation performances

A preliminary test on exhausted microcosms fed with 1mM Na-Formate and 1 mM Na-Acetate as the carbon- and electron-donors and 1,2-DCA as sole electron-acceptor, which apparently lost dechlorinating activity after about ten transfers was carried over adding to 4 microcosm replicates filter-sterilized, anoxic PJ. One out of 4 replicates successfully recovered the dechlorinating activity. This microcosm culture served as starter for a new series of transfer cultures named PJ (after the new amendant), and of course PJ was included in the recipe of the medium provided from then on. The dechlorinating kinetics of part of the PJ series are shown in Figure 1. The experiment set up to clarify the role of *P. aeruginosa* secondary metabolites, showed no enhancement of dechlorination in PYO (PC) amended replicates, compared to the unamended (NN) ones, whereas the latter showed a slightly better performance degrading all the available 1,2-DCA in 42 days, while one the PC replicates failed in achieving dechlorination. On the other hand, as expected, the R2A amended microcosms showed the best performance among all conditions tested, with nearly no lag phase before the onset of degradation, which took them less than 9 days to conclude. The presence of various putative electron donors in R2A medium (such as yeast extract, starch, and pyruvate) is very likely to be the cause of this improved performance. Surely, the most interesting degradation performance was given by the PYO and RLs amended

replicates (PR), which roughly equaled performance of PJ amended series, degrading all provided 1,2-DCA in about 9 days, and showing the same lag phase. These results, especially the latter, suggest that the combination of the two metabolites had some beneficial effect on the degradation and are consistent with the ones found by Pham *et al*. (2008).

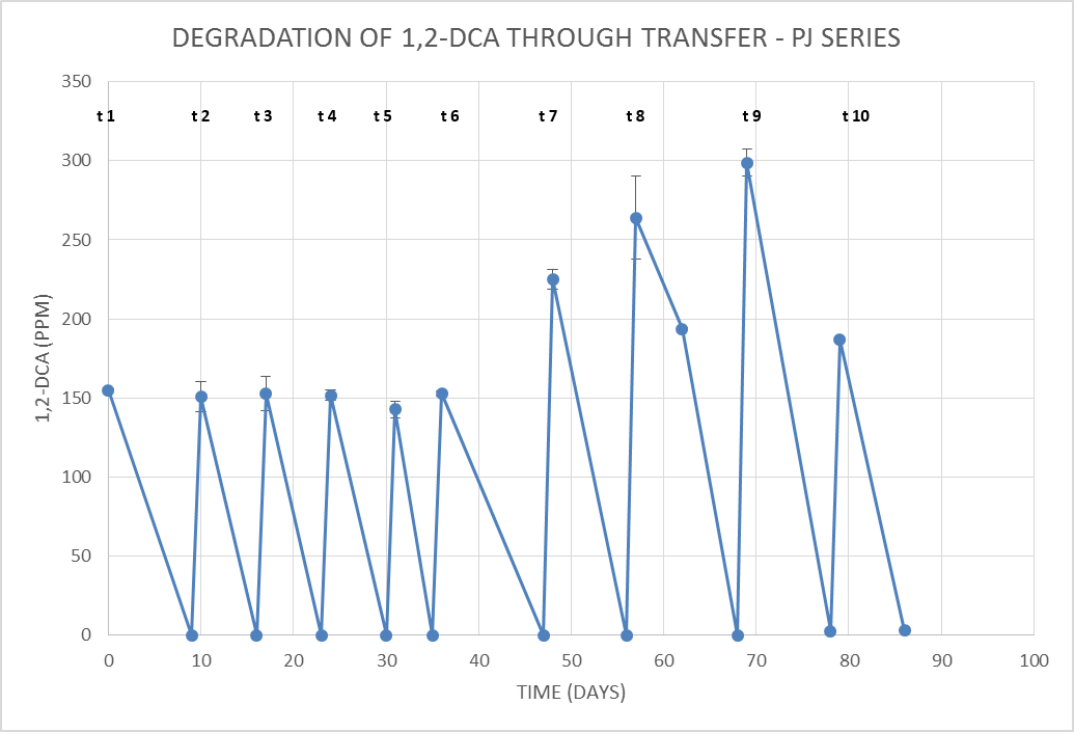

Figure 1: Dechlorinating kinetics for an extract of PJ series.

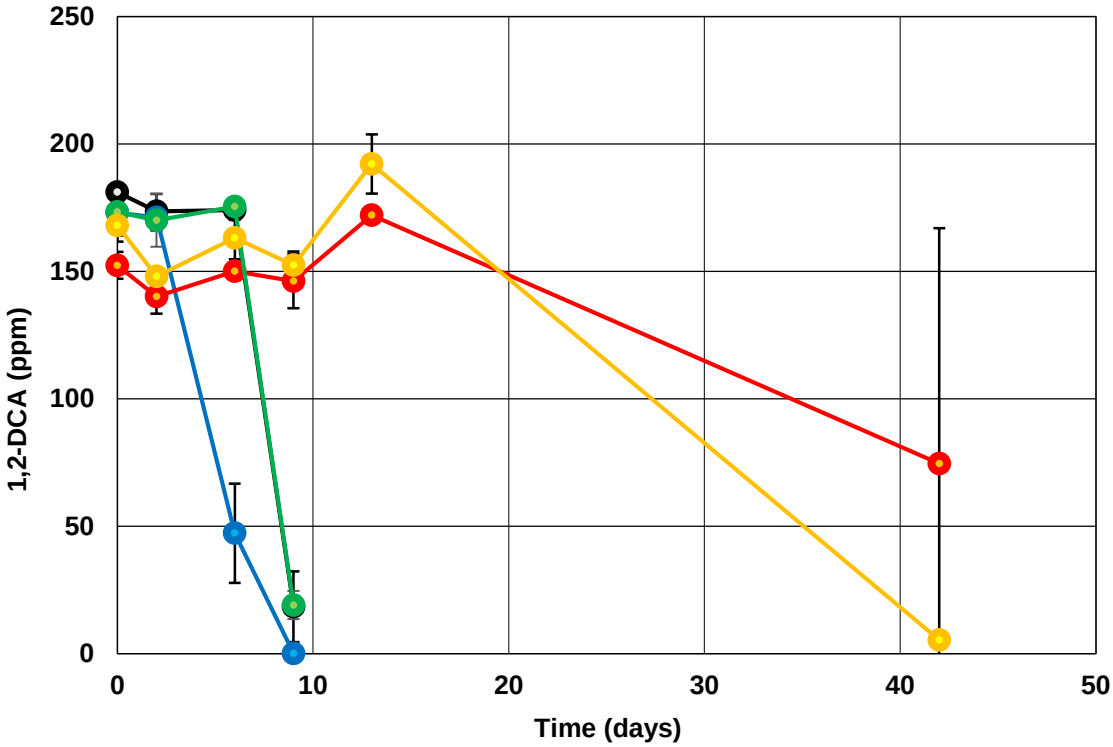

Figure 2: Degradation of1,2-DCA through time of the differently amended replicates

Total cell count

Total microbial population was monitored by FCM through degradation and all populations appeared to reach their peak concentration during between 5 and 8 days after inoculation, but while PC and NN replicates remained steadily between, respectively 3.80 x 10^5 cell/ml and 8.40 x 10^5 cell/ml, for PC MCs and 3.20 x 10^5 cell/ml and 8.81 x 10^5 cell/ml for NN MCs, during all the degradation course, R2 and PR series total cell concentration reached up to 3.62 x 10^6 cell/ml and 2.21 x 10^6 cell/ml, respectively, after 5 and 8 days, to decrease after 1,2-DCA depletion. Unlike these latter replicates, PJ cell concentration followed a more stable pattern, more similar to one of replicates PC and NN, with a peak at day 5 with 8.90 x 10^5 cell/ml.

Population proportion

Flow-FISH experiment could be carried over, as preliminary experiment, only on R2 cell aliquots retrieved at the end of 1,2-DCA degradation. Hybridization specificity and coverage were tested in quadruplicate with the hybridization procedure, differing only for the probe added. Replicate P exploited only PSE449 probe, replicate G exploited only probe GEO3-A, replicate PG used both and replicate B underwent all the process without probes, and was later stained with SYBR green to count total cells after hybridization procedure. In each of the experiments in which they were added, specific probes revealed an average proportion of *Pseudomonas* cells of 85.45% (std. dev. 1.06) and of *Geobacter* cells of 17.35% (std. dev. 1.34).

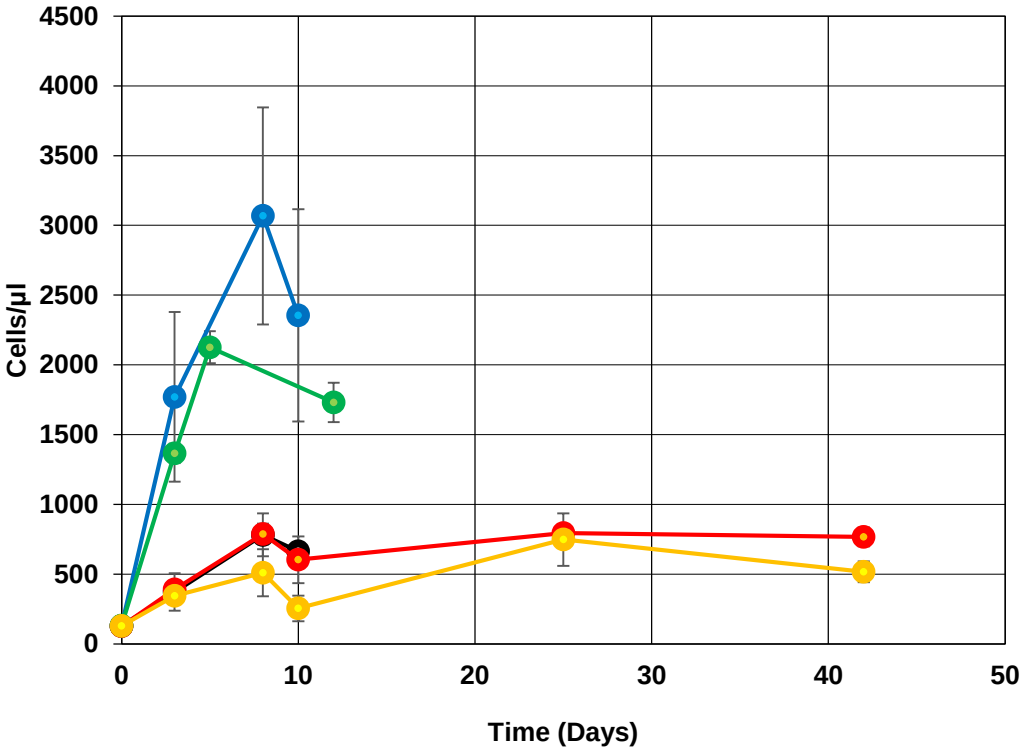

Figure 3 - Total cell count during degradation, measurement were taken until degradation was ongoing. Black, PJ; Blue, R2; Red, PC

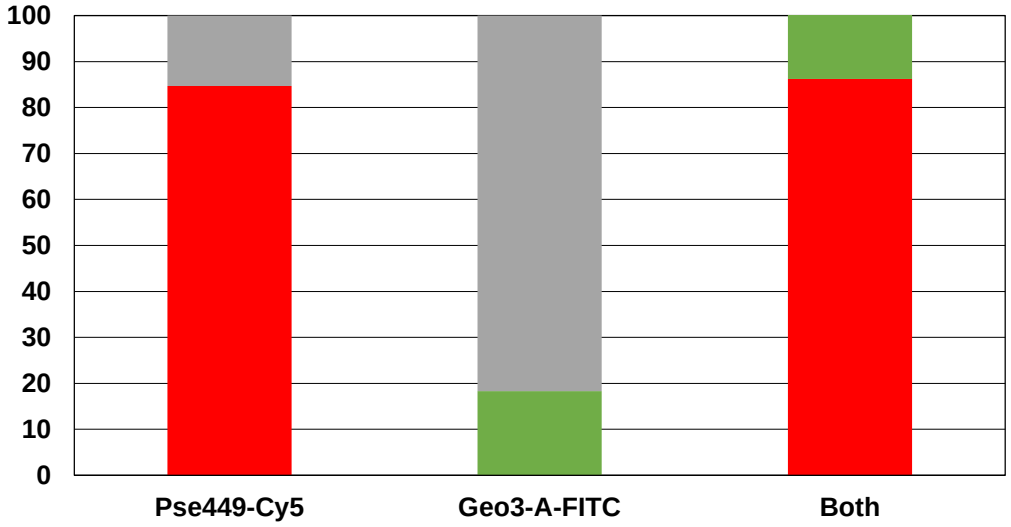

Figure 4 - Proportion of the populations of Pseudomonas and Geobacter determined through Flow-FISH on the cultures of the series R2. Red, percentage of Pseudomonas cells; green, percentage of Geobacter cells; grey, non labeled population.

Determination of PYO concentration after 1,2-DCA depletion

Aliquots of all cultures were collected after 1,2-DCA degradation and tested for PYO presence in the media, to assess if further PYO was produced during growth in dechlorinating conditions. PYO maximum average concentration was observed in PR replicates with 1.71 µg/ml and the lowest concentration detected in R2 MCs at 0.61 µg/ml. PYO concentration in PR was comparable to concentration detected in *P.aeruginosa* pure culture, grown in aerobiosis (PA). This allows us to say that PYO is produced in microcosms in the given conditions, in addition to the one provided to some experimental replicates as can be seen in Figur x.

Subtraction of hexogenous PYO stated by far the best production from PJ MC with 1.59 µg/ml, while PR production was only quantified at 0.76 µg/ml. PJ Hexogenous PYO concentration was below the limit of detection of our protocol and was obtained by dividing PA supernatant concentration by the dilution ratio applied (1:25).

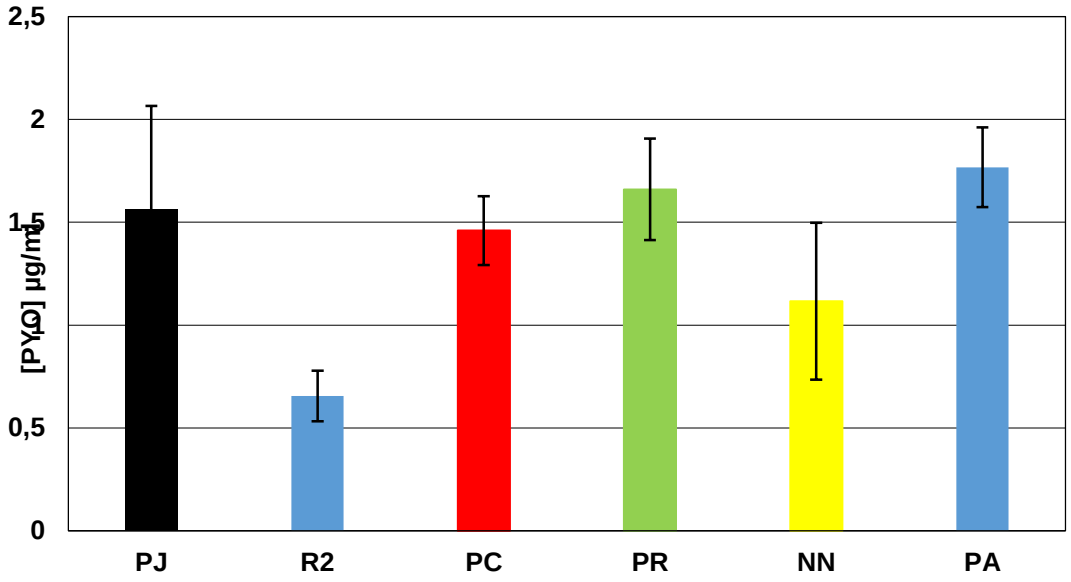

Figure 5: Concentrations of PYO measured after 1,2-DCA degradation7

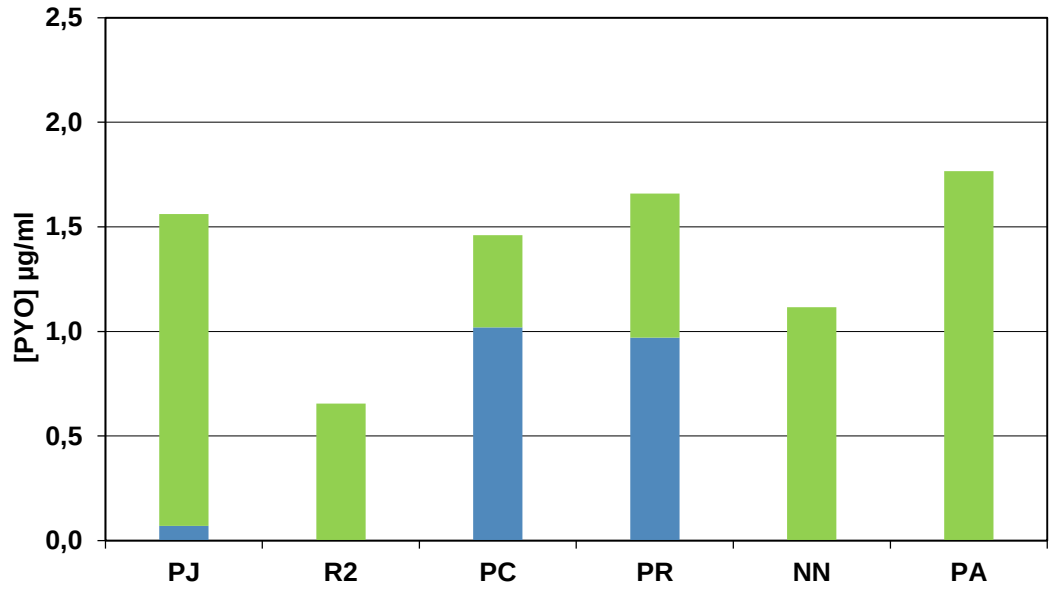

Figure 6: PYO concentration measured in each microcosm is compared to concentration of abiotic controls

DISCUSSION

We wanted to investigate *P. aeruginosa'*s role in the community, and the reasons behind its presence in the culture: two main issues, not necessarily excluding each other, seemed more likely to be the real causes. Since *P. aeruginosa* is mainly an aerobic bacterium it may have worked as a redox homeostasis agent whenever a small oxygen contamination leaked through the stoppers (e.g. when headspace samples for GC analysis were taken), but use of resazurin in the medium and of reducing agents, like dithiothreitol (DTT), hours prior to inoculum allowed to keep the redox condition of the cultures in control and minimize oxygen influence. A second, and indeed more fascinating hypothesis, was suggested by works on the role of pyocyanin (PYO) and rhamnolipids (RLs) secreted in *P. aeruginosa*'s supernatant on the electrochemical performance of a mixed culture (Pham *et al*., 2008). In this work, a *Pseudomonas* sp. strain CMR12a, known for its high PYO production (Perneel at al., 2007), was grown in coculture with a *Brevibacillu*s sp. strain PTH1 and yielded an increase in current production in an acetate fed microbial fuel cell (MFC), compared to the performance of CMR12a alone. In contrast, an experiment set up with the same conditions, but using a CMR12a mutant, lacking the gene *gacA* controlling secondary metabolisms, especially PYO and RLs production, failed in producing current. In the same way, PTH1 alone was unable to produce electricity, but both the former MFC proved able to produce current when supplemented with supernatant from CMR12a WT or when supplemented with synthetic PYO and RLs, respectively up to 10 and 1 mg/L, performance was significantly lower when RLs were omitted and no current production was observed when PYO was lacking. This set of experiments strongly suggested a key role of the secondary metabolites of *Pseudomonas* sp CMR12a in promoting extracellular electron transfer, even in strains normally incapable of performing such task. Moreover, PYO was previously found to increase current density towards poised electrodes and promote IET in MFC experiments (Rabaey

et al., 2005; Boon *et al*., 2008). On the other hand, our dechlorinating enrichment cultures consisted of a PYO producing *P. aeruginosa* strain and an Unc. *Geobacter* sp., whose most studied closely related species showed unparalleled ability to acquire and transfer electrons to, and from, a variety of chemical and biological agents, as well as MFC anodes and cathodes.

The mutual benefits for the two species that suggest this possibility as very likely are, from one side, the need of an electron sink for *P. aeruginosa* to perform anaerobic acetate, or formate oxidation without nitrites, or nitrates in the medium and, on the other hand, the intrinsic feature of *Geobacter* genus to scavenge electrons with extremely adaptable responses and to use them for their reductive metabolism, in this case 1,2-DCA conversion to ethane. Moreover, the positive reaction of our preliminary test with PJ, induced us to think that the relatively low concentrations of nutrients, together with the anaerobic conditions severely limited *P. aeruginosa* production of secondary metabolites, making them a limiting factor for efficient electron transfer. This was documented at least for RL production by *P. aeruginosa*, since even the best results for RL production under denitrifying conditions measured less than one third of the yield for aerobic conditions (Chayabutra *et al*., 2000). This is what apparently happened for the tested strain as well, since the aerobic grown bacterium makes the medium foamy, causing problems during N_2 flushing of supernatant, on the other hand, none of the MC ever appeared foamy.

For these reason, we hypothesized that the amendment of the medium with *Pseudomonas* metabolites in concentration comparable to the ones found in the cited experiments could remove such limits enhancing electron flow between bacterial populations and, in turn, dechlorination rate, as well as population growth. Maximum degradation rate were observed in MCs of series R2, PJ and RP, from the most performing to the less, with an average rate of, respectively, 19.2, 18.1 and 17.1 ppm/day. The remaining replicates achieved a very slower dechloriation rate, with an average of 3.9 ppm/day for NN MCs and 1.9 ppm/day for PC

replicates. From one side this data seem to exclude the role of PYO alone in the dechlorination reaction in MCs, and suggest a role from unmetabolized nutrient in the supernatant as booster of the dechlorination process. Nevertheless, the combined action of PYO and RLs suggests that RLs addiction to PYO amended MCs can boost dechlorination at levels comparable with the ones reached by PJ series. This is consistent with data from literature, that suggest a concurrent role of RLs in electron transfer process, maybe caused by its documented effect, causing the increase of Zeta potential of hydrophobic molecules (PYO, in this case), allowing them to disperse better in hydrophilic medium, on one side, and making them more accessible for cells, on the other. This could mean that PYO alone could not allow efficient electron transfer between the two populations, until poised at the right potential to work as electron shuttle, as by RLs action. On the other hand, RLs seem to be the real limiting factor in the system, since PYO is produced in the medium in all conditions, in a comparable range between all MCs.

For what concerns the performance of R2 MCs, it was somehow expected due to the relative richness of this medium compared to the BTZ-3 medium, in facts, some of its ingredients, like pyruvate and yeast extract, as well as glucose has been described as possible electron donors either for reductive dechlorination reaction, or for *Geobacter* metabolism, or both (Rotaru *et al*., 2011). Moreover, another R2A ingredient is riboflavin, which was escribed as a putative electron shuttle substance, as well (Von Canstein *et al*., 2008).

For what concerns cell population increase, this seemed very strong in R2 and RP MCs, consistent with the high dechlorination rate observed, in contrast PJ unexpectedly yielded a significantly lower population growth, in spite of the good dechlorinating performance. This could be explained in terms of different population proportion, since for R2 MCs, less of the 20% of the events measured could be classified as *Geobacter*, with the remaining falling under *Pseudomonas* group. In contrast, previous barcoding analysis made on the course of the enrichment of the PJ culture showed a ratio favorable to *Geobacter* (data not

shown) in the enrichment that served as inoculum from this experiment, and was prepared exactly as the PJ replicates described here above. Moreover, R2A contains fermentable substrates for *P. aeruginosa*, namely glucose and pyruvate, which can support the growth of *P. aeruginosa*, as described by Glasser *et al*. (2014), equilibrating its redox cycle both through pyruvate reduction to lactate and through PYO redox cycling, in our case probably completed by Unc. *Geobacter* sp. as electron sink through re-oxidation of PYO.

R2 MCs constantly presented lowest PYO concentration, in spite of the fast dechlorination rate; this is very likely to happen because of different parallel metabolisms taking place. In facts, the variety of putative electron donors present can draw electron flow away from the PYO route, making it less necessary for survival of both species. In all other MCs, concentrations varied around a narrower range, confirming production of PYO in all conditions and suggesting an important role of this substance in the economy of the microbial community, especially when no further electron donors were present, like, instead, was the case of R2 replicates that produced lower concentration of PYO.

In conclusion, further experiment are needed to definitely ascertain the role of secondary metabolites of *P. aeruginosa* in promoting electron flow through the two codominant species and, in turn facilitating reductive dechlorination of 1,2-DCA. In particular amendment with supernatant after growth of CMR12a_Reg mutant (Pham *et al*., 2008), or analog *Pseudomonas* strains deficient in secondary metabolisms which lead to production of both PYO and RLs could be the suited experiment to conclude this work.

SUPPLEMENTARY MATERIAL

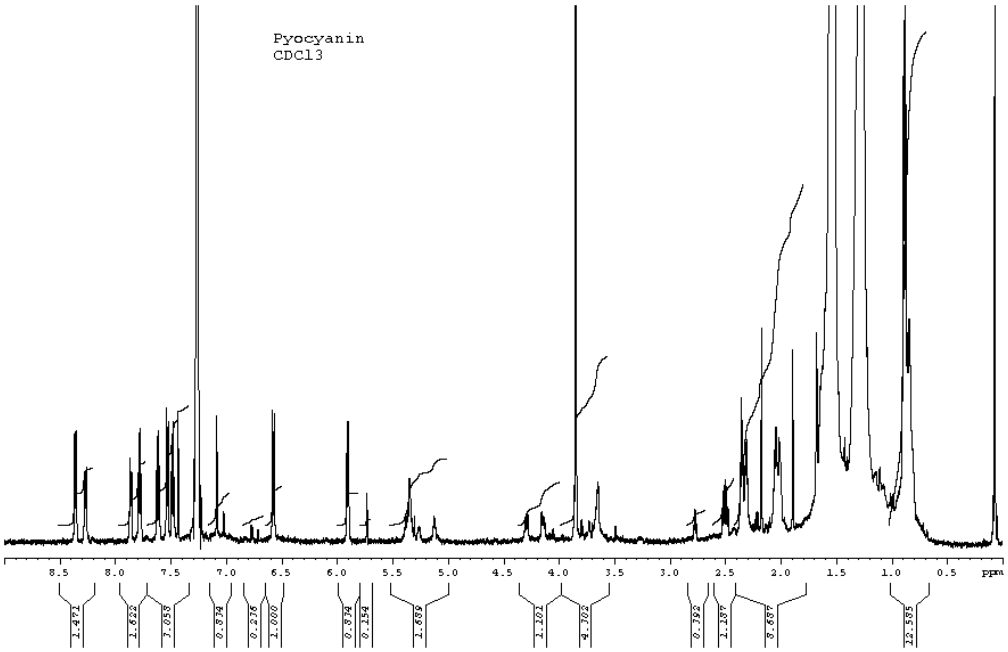

FIGURE 1 SM: H-[1]NMR ANALYSIS OF PYOCYANIN: ARROW INDICATES PEAKS CONSISTENT WITH SUDHAKAR *ET AL*. (2011).

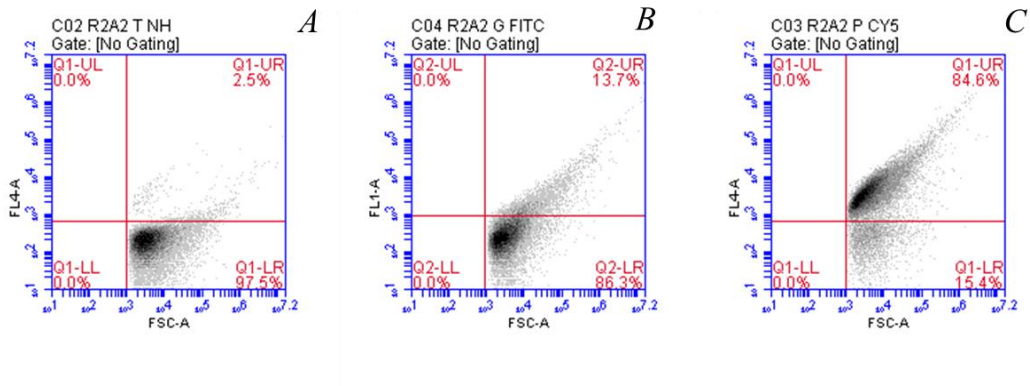

FIGURE 2 SM: FLOW FISH ANALYSIS EXAMPLE, PANEL A) UNMARKED; PANEL B) GEO3-A-FITC DYE; PANEL C) PSE449-CY5 DYE

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CHAPTER 7

General conclusions

In the first part of this work, response of microbial community proceeding from the lower layer of a 1,2-DCA contaminated aquifer to lactate biostimulation was investigated in terms of population diversity and relative abundance. Reductive Dehalogenases (RDs) specific for 1,2-DCA diversity was screened, as well and the recovered sequences were compared to the ones previously retrieved in the upper layer of the aquifer (Marzorati *el al.*, 2010). Clone libraries for community, before and after lactate treatment, ranked a Shannon index of 3.33 before and of 2.88 after lactate amendment, reflecting for both abundance and evenness of the species present. This suggests that the shift in the biodiversity of the bacterial community occurred following the biostimulation treatment. Contextually a small increase in community organization, from 48% to 58%, was registered showing potential for enrichment of dehalogenating community through consecutive transfers of the culture. Remarkably, none of the dominant population from before biostimulation conserved its status after treatment: *Geobacter* related OTUs, for example, disappeared after treatment, in contrast, before the treatment they belonged to the most highly represented genus (43%). The most abundant OTUs following biostimulation fell under 4 main groups, namely *Parabacteroides* (25.61%) *Acidaminobacter* Unc. (24.39%) *Clostridiales* sp. (15.85%) and *Malikia* (15.85%). Interestingly only among *Clostridiales*, known 1,2-DCA dechlorinators can be found, but none of the related species previously described was detected, suggesting the presence of a novel 1,2-DCA dechlorinator. On the other hand, phylogenetic analysis on the retrieved dehalogenases indicated the presence of a highly intra-similar group of RDs which shared 99% nucleotide identity with WL rdhA1 (Grostern & Edwards, 2009) from *Dehalobacter* sp. WL, which was previously recovered in the upper aquifer (Marzorati el al., 2007). This suggests the

redundancy of function, namely 1,2-DCA dechlorination, in the investigated environment, with potential sharing, through HGT mechanisms of similar RDs across species never previously described as possible dechlorinators.

In the second part of this work, we described a dechlorinating mixed culture of an Unc. *Geobacter* sp. which is a putative dechlorinating microorganism, the first in its genus that has been observed performing dichloroelimination of 1,2-DCA. This bacteria evaded all isolation attempts and several enrichment attempts failed when the dechlorinating bacteria was outcompeted by other faster growing poulations. For this reasons, the main object of this study can be classified as a fastidious anaerobe and every approach to study its metabolism was forcedly indirect and always needed to take into account response of 1,2-DCA adapted microorganisms, which did not participate in degradation of solvent, but used the organic acids added to MCs, for either fermenting metabolisms, like homoacetogenesis, or other unclear pathways. In the first enrichment trials, cysteine, which was previously added as reducing agent, appeared to cause important drawbacks, causing the production of abundant FeS precipitates, in absence of inorganic sulfur sources. The toxicity of sulfide ions for several bacteria has been described previously (Oleszkiewicz *et al*., 1989) and substitution of cysteine with DTT solved this issue. A very fine and patient regulation of the amendment mix composition, resulted in a stable simplified dechlorinating community, in which the favorite partner for the putative dechlorinator proved to be a *P. aeruginosa* strain, unable to perform dechlorination, but probably involved in exoelectrogenic activity, supported by its characteristic secondary metabolites.

An overall view of this project, highlights the extreme complexity underlying the mechanisms of interaction in the simplified dehalogenating co-culture we achieved. Environmental bacteria are by definition very adaptable, in contrast to the hyper-specialized endosymbionts and parasites. Thus their response to stimuli can be extremely variable and unpredictable. In addition the microgradients of temperature and of chemical composition can subsist even in small environments like

microcosms and can induce slightly different metabolic regulation, especially when a highly responsive microorganism, is involved. As described in the section dedicated to *Geobacter*, this genus stands out for a complex regulatory mechanism and is able to deploy an extremely sophisticated net of survival strategies thanks to its great versatility.

Working with anaerobic co-cultures is a very empirical task due to the complex responses sometimes highlighted in experiments (not shown in this work) that produced no consistent results, thus continuous precautions are required to avoid the onset of unexpected complications in data interpretation. Nevertheless, we managed to describe an enriched dechlorinating co-culture, in which we retrieved an Unc. *Geobacter* sp. that is a putative 1,2-DCA dechlorinator, the first in its genus. We managed to link the growth of *Geobacter* population to the growth of dehalogenase number into the culture by qPCR experiment, and we managed to show population growth during 1,2-DCA degradation by flow cytometry experiments.

We obtained an extremely simplified enriched culture, which proved able to conserve dechlorinating activity through several transfer and to recover after cryopreservation at -80°C, which could be a powerful bioaugmentation source for environments in which no dechlorinators are present. At the same time, we tried to elucidate the dynamics that exist between the two dominating species of the co-culture, investigating a way to boost Interspecies Electron Transfer (IET) and, in turn, dechlorinating activity. Eventually, we partially demonstrated the involvement of *P. aeruginosa* secondary metabolites as key intermediates to boost IET, pinpointing them as a possible driver that enabled he formation of this peculiar co-culture.

Exoelectrogenic bacteria are gaining more attention every day, and their possible beneficial applications are being thoroughly investigated, in order to obtain the knowledge needed to use them to operate fuel cells and wastewater treating implants, in order to exploit their, yet abundantly unexplored, full potential.

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APPENDICES

Publications

Articles

- Merlino, G., Balloi, A., Marzorati, M., Mapelli, F., Rizzi, A., Lavazza, D., de Ferra, F., Carpani, G., and Daffonchio, D. (2015). Diverse Reductive Dehalogenases Are Associated with Clostridiales-Enriched Microcosms Dechlorinating 1, 2-Dichloroethane. BioMed Research International vol. 2015, Article ID 242856, 11 pages.

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- Merlino, G., Balloi, A., Mapelli, F., Rizzi, A., Lavazza, D., de Ferra, F., Carpani, G., Borin, S., and Daffonchio, D. "Achievement of an enriched anaerobic 1,2-DCA dehalogenating simplified co-culture containing a novel putative 1,2-DCA degrader *Geobacter* sp. associated to previously identified 1,2-DCA-specific reductive dehalogenase". EBC VI – 6th European Bioremediation Conference. Chania (Greece), 28th June – 2nd July 2015.
- Merlino, G., Balloi, A., Mapelli, F., Rizzi, A., Lavazza, D., de Ferra, F., Carpani, G., Borin, S., and Daffonchio, D. "Unusual bacterial community assembly of a simplified consortium reductively dechlorinating 1,2-dichloroethane". BAGECO 13 – Bacterial Genetics and Ecology: the microbial continuity across changing ecosystems. Milan (Italy), 14th – 18th June 2015.
- Merlino, G., Barbato, M., Mapelli, F., Lavazza, D., Borin, S., Carpani, G., Vitale, E., de Ferra, F., and Daffonchio, D. "New putative 1,2-dichloroethane dehalorespiring bacteria". DehaloCon - A Conference on anaerobic biological dehalogenation. Jena (Germany), 23rd – 26th March 2014.
- Carpani, G., Vitale, E., Daffonchio, D., Merlino, G., Lavazza, D., and De Ferra F. "Enrichment of alternative rdhs in 1,2-DCA simplified dehalogenating consortia". DehaloCon - A Conference on anaerobic biological dehalogenation. Jena (Germany), 23rd – 26th March 2014.
- Merlino, G., Barbato, M., Mapelli, F., Lavazza, D., Borin, S., Carpani, G., Vitale, E., de Ferra, F., and Daffonchio, D. "Enrichment and identification of new putative dehalorespiring bacteria able to degrade 1,2-dichloroethane". MedRem - Microbial resource management for polluted marine environments and bioremediation. Hammamet (Tunisia), 16th – 18th January 2014.

Posters

- Merlino, G., Barbato, M., Mapelli, F., Lavazza, D., Borin, S., Carpani, G., Vitale, E., de Ferra, F. and Daffonchio, D. "Diversity and evolution of 1,2-DCA Reductive Dehalogenases in polluted marine and freshwater environments". MD2013 - Microbial Diversity: Microbial interactions in complex systems. Turin (Italy), 23rd – 25th October 2013.
- Borin, S., Merlino, G., Mapelli, F., Lavazza, D., Barbato, M., Carpani, G., de Ferra, F., Vitale, E. and Daffonchio, D. "Reductive dechlorinating microbiomes in contaminated seawater sediments and groundwaters". SAME 13 - Aquatic Microbial Ecology. Stresa (Italy), 8th – 13th September 2013.

Teaching experiences

- Teaching assistant at FACILIS summer school for grad student (Milan, July 2014)
- Teaching assistant for Microbial technologies laboratory course, held by Prof. S. Borin (Milan, November 2014)