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Research Article

Microbial Degradation of Chlorinated Solvents: A Microcosm Study and a Microbial Genetic Analysis to Remediate a Contaminated Area in Central Italy

Federica Matteucci[1], Annarosa Sprocati[2], Chiara Alisi[2], Salvatore Chiavarini[2], Claudia Ercole[1*], Maddalena Del Gallo[1]

[1]Department of Life, Health and Environmental Sciences, University of L'Aquila, Italy

[2]Technical Unit for Environmental Characterization, Prevention, and Recovery ENEA, Rome, Italy

[*]**Corresponding author:** Claudia Ercole, Dipartimento MeSVA Università degli Studi dell'Aquila Via Vetoio - 67100 Coppito - L'Aquila, Italy. Tel: +390862433283; Email: claudia.ercole@univaq.it

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Abstract

A combination of microcosm studies, Polymerase Chain Reaction (PCR) analysis and Denaturing Gradient Gel Electrophoresis (DGGE) analysis was performed to assess the indigenous reductive dechlorinating potential in a Tetrachloroethene (PCE) and other chlorinates solvents-contaminated aquifer in a Central Italy industrial area located in the coastal site of the Vibrate valley (Teramo).

The identification of the conditions to promote the bioremediation of the area, as well as establishing the identity and properties of the microorganisms involved in Reductive Dehalogenation (RD) were the main goals of this study. A previous microcosm study indicated the presence of an active native dechlorinating population in the subsurface, but probably inhibited by co-contaminants in the groundwater, or more likely by lack of nutritional factors. 19 microcosms were set up under strictly anaerobic conditions, added with different electron donors and some also with an anaerobic sludge inoculum and monitored for one year.

A combination of PCR and DGGE analysis was utilized to evaluate composition, dynamics and activity of dechlorinating bacteria, indigenous and not, at the different phases of RD. We concluded that in the contaminated site an enhanced anaerobic dechlorination of PCE and TCE may take place through the appropriate addition of electron donors, mineral medium and/or through bioaugmentation by dechlorinating cultures, even coming from an anaerobic sludge.

Keywords: Anaerobic Microcosms; Microbial Bioremediation; Organochlorine Solvents Contamination; PCR; DGGE

Introduction

Chlorinated Aliphatic Hydrocarbons (CAHs), such as Perchloroethene (PCE), Trichloroethene (TCE) are widespread groundwater pollutants. They are among the most common pollutants in industrial sites due to their extensive use in chemicals production, metal degreasing and dry cleaning. These compounds are persistent in the groundwater, toxic and sometimes carcinogenic; their presence in the environment poses important health hazards and has prompted investigations concerning their fate in the subsurface through a range of biotic and abiotic reactions [1].

PCE and TCE have density higher than water, so they can spread through mobilization of groundwater, but also percolates into the ground until trapped above finer grained layers [2]. They form DNAPLs (Dense Non-aqueous Phase Liquid) that sink through permeable groundwater aquifers until a non-permeable zone is reached [3-5]. CAHs tend to dissolve slowly into flowing water, serving as a long-term source of groundwater contamination difficult to remediate with conventional techniques [6,7].

Chlorinated solvents, once released into the environment from different industrial activities, are subject to both chemical-physical and, especially, microbial degradation. They often behave as electron acceptors due to their substituents' electronegativity, and are then reduced. Most abiotic transformations are slow, but they can still be significant within the time scales commonly associated with groundwater remediation. Biotic reactions are typically much faster, if appropriate conditions are present such as sufficient substrate, nutrients and suitable microbial population. In particular, the Reductive Dehalogenation (RD) occurs mostly in compounds with a high number of halogen substituents (i.e. Tetrachloroethene and Trichloroethene) which are totally unaffected by aerobic microorganisms [8].

Such dechlorination reactions can be a threat to living organisms if, under uncontrolled conditions, daughter compounds - more toxic than the parent compounds - are formed, such as the carcinogen vinyl chloride from PCE [1]. On the other hand, reductive dechlorination has also received considerable attention as a reliable and cost-effective strategy for the removal of chlorinated solvents from contaminated environments, because either less chlorinated daughter compounds formed are more biodegradable under aerobic conditions or non-chlorinated, harmless end-products are obtained [9].

Many cases of serious contamination of groundwater - that might hamper their use in industry, agriculture and private - are present in Italy. Among these, the critical case of a Central Italy valley located in the Teramo province, characterized by a significant contamination by chlorinated solvents, was analyzed in the present study. In a previous study [3], after a monitoring campaign, we assessed the extension of pollution, importing data into GIS (Geographic Information System) and we collected samples from wells and core drillings to set up microcosms, to evaluate the potential for in situ natural or enhanced bioremediation. This study indicated the presence of an active native dechlorinating population in the subsurface, but possibly inhibited by co-contaminants in the groundwater, or more likely by lack of nutritional factors.

Microcosm studies were utilized to determine whether the addition of donors could stimulate reductive dechlorination, whether dechlorination could proceed past the DCE isomers, to assess competitive processes and to help explain observed field results [10]. In the present work we combined PCR and DGGE to detect dechlorinating bacterial species including Dehalococcoides ethenogenes, to assess dechlorinating potential under enhanced conditions at the site. Although D. ethenogenes is not the only dechlorinating bacterium that may be operative at a site, it has been found, thus far, to be an important organism exhibiting complete dechlorination to ethene [10]. DGGE is a fingerprinting technique that may further elucidate the bacterial community structure and allow assessment of the changing composition of bacterial communities in contaminated aquifers subjected to natural attenuation or enhanced bioremediation [11,12]. The DGGE is one of the best molecular tools applied to microbial ecology [13-15]. This technique of genetic fingerprinting is commonly utilized to provide a profile that represents the structure and the diversity of a microbial community from a specific environment, with high versatility, reliability and reproducibility [15]. Furthermore, the result of the DGGE can be coupled with the statistical analysis and the calculation of biodiversity indices to compare the bacterial communities of different environmental samples. Aim of the present study was to examine if an enhanced anaerobic dechlorination of PCE and TCE may take place in the contaminated site through an appropriate addition of electron donors, mineral medium and/or through bio augmentation by dechlorinating cultures, and to detect the microbial community involved in the different phases of dechlorination.

Materials and Methods

Field Site

The sampling site is an industrial area of a Central Italy valley located in the province of Teramo, morphologically developed between 40 and 75 m a.s.l. and located on a hilly area formed by the terraced alluvial deposits of the river Vibrate. The main activities carried out in the area, present and past, are: the assembly of electronic circuits, sealing and testing of electronic circuits, soldering of electronic components, screen printing, packaging products, manufacturing chemical products for industrial use, textile fasteners, jeans sandblasting, production of leather bags and accessories, manufacture of iron and aluminum, storage truck, washing aggregates, production of burglar and fire alarm systems, leather wash, and wholesale trade in industrial machinery. The whole area is characterized by a significant contamination by chlorinated solvents, in particular Tetrachloroethene and Trichloroethene.

Aquifer Sample Collection

Three locations, in three sites of the area and near the most contaminated wells, were chosen for soil and groundwater sampling, to set up microcosm bottles. Soil samples derived from core drillings were taken from sections of saturated soil near the impermeable clay zone (where there is the accumulation of DNAPL and therefore the greatest dechlorinating activity). The soil sections were then placed in a glass jars (2 L) and filled to the brim with groundwater from piezometers adjacent to the core drilling locations, to immediately ensure anaerobic conditions. Drillings, located according to the accessibility of places and pushed up to the waterproof substrate, were performed with the method of drilling continuous core, with simple core barrel, dry and without drilling fluids. At the end of each maneuver sampling, we proceeded with the rods and core barrels cleaning by high-pressure cleaner, to remove any residue from the previous maneuver. The drilling was followed by temporary lining hole. To avoid contamination by lab

strains, all materials taken to the site from the lab were swabbed with ethanol before being shipping. Soil-groundwater samples were stored after collection, and then shipped overnight to the laboratory.

Experimental Design

Nineteen microcosms were set up in 250 mL serum bottles with a soil suspension (70 mL) from the contaminated site drillings and a liquid part (mineral medium or contaminated groundwater from the site), in different conditions: abiotic control, soil without amendments as a biotic control, soil with amendments (lactate, yeast extract and butyrate), and soil with anaerobic sludge (20 mL) from a wastewater treatment plant [16]. The set-up of the experiment is described in (Table 1).

Microcosm samples	Electron donor	Anaerobic sludge	MM
1	Abiotic control	no	yes
2	Biotic control	no	yes
3	Lactate 3mM	no	yes
4	Yeast extract 3mM	no	yes
5	Butyrate 3mM	no	yes
6	Lactate 3mM	yes	yes
7	Butyrate 3mM	yes	yes
Microcosm samples	**Electron donor**	**Anaerobic sludge**	**MM**
8	Biotic control	no	yes
9	Lactate 3mM	no	yes
10	Butyrate 3mM	no	yes
11	Yeast extract 3mM	no	yes
12	Lactate 3mM	yes	yes
13	Butyrate 3mM	yes	yes
Microcosm samples	**Electron donor**	**Anaerobic sludge**	**MM**
15	Biotic control	no	no
16	Yeast extract 3mM	no	no
17	Yeast extract 3mM	yes	no
18	Lactate 3mM	no	no
19	Butyrate 3mM	no	no
20	Butyrate 3mM	yes	no

Table 1: Simulated wastewater of different concentrations.

Each microcosm was added with electron donors and carbon source (lactate or butyrate 3mM), yeast extract 5 mg /L and an 86 mL of mineral medium (MM) containing: resazurin 0.1% w/v (redox indicator), NH_4Cl 0.5g/L, $MgCl_2*6H_2O$ 0.1 g/L, $CaCl_2*2H_2O$ 0.05 g/L, K_2HPO_4 0.4 g/L, 5 mL of 5% w/v Na_2S, 2 mL of 10% w/v $NaHCO_3$ and a metal solution containing NTA, $FeSO_4*H_20$, $MnSO_4*H_20$, $CoCl_2*6H_2O$, $ZnSO_4*7H_2O$, H_3BO_3, $NiCl_2$ and $Na_2MoO_4*2H_2O$ [17]. Microcosms with contaminated groundwater, instead of mineral medium, were set up to investigate the possibility of a natural attenuation in the area. Microcosms were sealed with Teflon-faced butyl rubber stoppers (Wheaton, Millville, NJ) and aluminum crimp caps [10]. Bottles and caps were previously autoclaved to reduce the emission of volatile organic compounds [17] and avoid microbiological contamination. Headspace was flushed with a 70% N_2- 30% CO_2 gas mixture, to ensure anaerobic conditions [18]. Microcosms were incubated statically in the dark at room temperature (18-22 °C) for one year and monitored every 15 days; the same scheme was used for the soil samples from S3 drilling, with MM and groundwater. Each condition was set-up in duplicate, to verify reproducibility [3].

Analytical Methods

For the determination of chlorinated compounds and ethene, gaseous samples (100 µL) were removed from the headspace of microcosms using gas-tight syringes (Hamilton) and analyzed with a gas chromatograph equipped with a capillary column and a mass spectrometer (GC-MS, Agilent series). Standards for PCE, TCE, cis-DCE, VC and ETH were prepared by adding a known amount of each compound to a serum bottle with the same headspace-to-liquid ratio as our microcosm bottles [18]. Concentrations of volatile compounds are expressed as molar fraction (%). It was assumed that DCE produced from more highly chlorinated ethenes was cis-DCE, which has a different calibration constant from trans-DCE due to a higher Henry's constant [19].

Molecular Study of Microcosms

DNA Extraction

Microcosms were monitored every 15 days. For the determination of chlorinated compounds and ethene, gaseous samples (100 µL) were removed from microcosm headspace of and analyzed by gas-chromatography [3]. For the biomolecular analysis, homogeneous samples (2 mL) were taken from microcosms every 15 days, and analyzed at six different times (from T0 to T5), corresponding to the different phases of the dechlorination, according to the gas-chromatograph results. At the end of the experiment, these samples were thawed and centrifuged for 10 minutes at 10,000 rpm to separate the solid from the liquid phase, to extract more efficiently DNA from the single solid phase. Bacterial genomic DNA was extracted from 0.5 g of solid phase with a bead beating technique using a "ZR Soil Microbe DNA

MiniPrep kit" (D6001, Zymo Research, CA, USA), according to the manufacturer's protocol. The DNA was then filtered to remove humic acids and polyphenols that could inhibit the PCR.

DGGE Analysis

DGGE was carried out using the DCode Universal Mutation Detection System (BioRad, California, USA). The analysis was performed in two series of microcosm in which the dechlorination has ended up to ethene formation and for all samples at different phases of dechlorination. 16S rDNA gene was amplified using the universal primer 9bfm 5'-GAGTTTGATYHTGGCTCAG-3'(Eubacteria, position 9-27 *E. coli*) and primer 1512r 5'-ACGGHTACCTTGTTACGACTT-3'(Eubacteria and Archaea, position 1492-1512 *E. coli*) [20]. PCR mixtures (50 µl) contained 5 ng of template DNA, 25 µl 2 x Master Mix (Bioline, London, UK) and 20 pmol of each primer. The PCR reaction conditions consisted of one cycle of 95˚C for 5 min; 30 cycles of 95˚C for 1 min, 56˚C for 1 min and 72°C for 1 min; and a final extension at 72˚C for 10 min. The products from this universal PCR were used as the template for a nested PCR, where the variable V3 region of 16S rDNA was enzymatically amplified with primers that conserve regions of the 16S rRNA genes [21]. The nucleotide sequences of the primers are as follows: primer 341f-GC, 5'- CGCCCGCCGC GCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGG GAGGCAGCAG-3'(corresponding to position 341-359 in *E. coli*, with an additional 40-nucleotide GC-rich sequence (GC clamp) at 5' end) and 518r, 5'-ATTACCGCGGCTGCTGG-3'(corresponding to position 518-534 in *E. coli*) [22].

All primers were synthesized by BioFab Research (Italy). PCR samples were resolved on 8% polyacrylamide gels in 1x TAE buffer with a denaturant gradients 45% to 65% (urea and formamide) in DCode apparatus (Bio-Rad, CA, USA) run at a constant voltage of 60 V and at temperature of 60˚C for 18 h [23]. After electrophoresis, the gel was stained with GelRed (Biotium, CA, USA) for 30 min, rinsed with distilled water and then visualized by UV GelDoc XR System (BioRad). The image was acquired and elaborated by 1-D Quantity One (BioRad) analysis software and the main bands were individually excised from the gel and incubated at 4˚C overnight in 30 µl sterile distilled water to elute the DNA. Aliquots of 1 µl from each band was amplified with the primers 341f (without GC-clamp) and 518r, purified with CL6B resin and sequenced. All fragments were purified and concentrated on Sepharose CL6B200 (Sigma, St. Louis, MO) following the manufacturer's instructions.

A phylogenetic analysis was performed using MEGA 5.1 [24] and the evolutionary history was inferred using the neighbor-joining method [25]. The sequencing was performed by the Genechron Laboratory (Ylichron Srl), with automated Sanger sequencing technology. The identification of the bands was obtained by inserting the sequences in the NCBI database using the online search program BLAST (Basic Local Alignment Search Tool - http://blast.ncbi.nlm.nih.gov/Blast.cgi) and identifying the species present in the database with the highest probability of similarity. DGGE gels images were processed to derive a set of ecological indices useful to the understanding of microbial dynamics within the process of reductive dechlorination, such as: Simpson's diversity index (1 -D); Simpson's evenness index (Ed); Range-weighted richness (Rr); Rate of change (Δt); Functional organization (Fo), according to Simpson [26] and [27].

Results and Discussion

Microcosm Study

In the abiotic controls and in the non-amended microcosms, dechlorination was not taking place and PCE was not degraded (results not shown). This indicates that chemical and abiotic degradation of organochlorine is no possible and is not even feasible a natural attenuation. All microcosms prepared only with Yeast Extract as an electron donor, even in the presence of mineral medium showed a low dechlorinating activity (results not shown). This is in contrast with the literature in which yeast extract appears to be an effective electron donor. Indeed, in most of the cases, hydrogen produced during organic compounds fermentation, is typically the direct electron donor for the reductive dechlorination [8]. A key point concerning the choice of fermentable organic substrates is the possible competition for hydrogen that can establish between dechlorinators and other H2 utilizing microorganisms such as methanogens [28,29]. Previous studies demonstrated that dechlorinators have the potential to outcompete other H2-utilizers when H2 is present at low concentration, due to dechlorinators' higher affinity for hydrogen [30,31]. Consequently, the utilization of substrates that are slowly fermented, such as butyrate or lactate, would result in a competitive advantage to dechlorinators over other H2-utilizer microorganisms [28] lactate-amended microcosms showed the shortest lag-phase (i.e., time prior to the onset of dechlorination) and the highest initial dechlorination rate; similar results were obtained by [32].

In lactate-amended microcosms (Figure 1A) a complete degradation of PCE and TCE with formation of ethene occurred. The dechlorinating activity began after a lag period of about 60 days, and led to a rapid accumulation of TCE, with the formation, by hydrogenolysis, of cis-DCE. After reaching a maximum concentration, DCE was transformed into VC, and later into ethene. At the end of incubation, the percentage of VC was 2%, while the percentage of ethene was 98%, and the process of reductive dehalogenation ended up. The rate of PCE dechlorination gradually increased with time and shifted toward more reduced end-products. In butyrate-amended microcosms (Figure 1B) dechlorinating activity began after a lag period of about 4 months and led to a gradual accumulation of TCE, with the subsequent and fast formation of DCE. The concentration of TCE remained

high for about 3 months, until both TCE that DCE were degraded to VC; with the decrease of VC concentration, slow formation of ethene occurred. At the end of the incubation, only part of VC was converted into ethylene, with consequent accumulation within the microcosm. In particular, the percentage of VC and ethylene was, respectively, 86% and 18%.

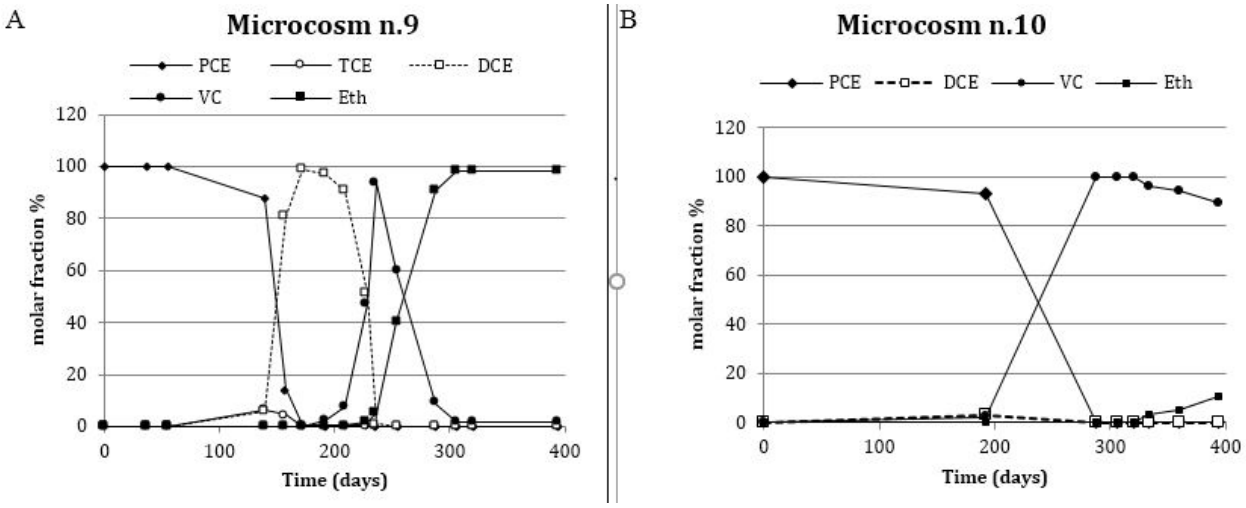

Figure 1: Time course of reductive dechlorination of PCE in microcosms 9 and 10 amended with lactate (A) and butyrate (B), and PCE, MM, yeast extract.

Microcosms bio augmented with the anaerobic sludge, dechlorinating Tetrachloroethene to cis-Dichloromethane, Vinyl Chloride and also Ethene more quickly than the amended ones. In lactate-amended microcosms (Figure 2A) dechlorinating activity began after a lag period of about 20 days, and led to a rapid accumulation of TCE and subsequently of cis-DCE. DCE was gradually transformed into VC; Ethene formation from VC was a slow process. At the end of the incubation all VC was converted to ethylene: the percentage of VC was 5% while the percentage of Ethene was of 95%. Butyrate-amended microcosms (Figure 2B) had a lag period shorter than the corresponded treatments without digester sludge inoculum, but a longer lag period than treatment added with lactate and digester inoculum.

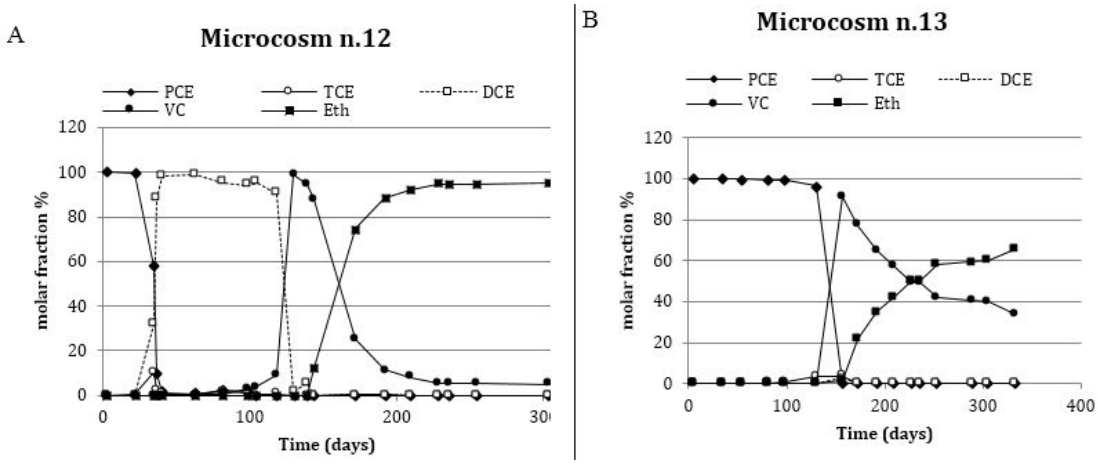

Figure 2: Time course of reductive dechlorination of PCE in microcosms 12 and 13 amended with lactate (A) and butyrate (B), and digester sludge, PCE, MM, yeast extract.

A limiting factor in the experiment was the conservation of microcosms at rest. In fact, all microcosms, which had stopped at the production of VC, put in a shaker, showed a rapid recovery of dechlorinating activity, with the completion of the final step of the Ethene formation by hydrogen lysis of VC (results not shown).

The results of microcosm studies have provided information on natural attenuation processes in the contaminated aquifer, and the capability of accelerating bioremediation processes, through the addition of amendments or microorganisms in the aquifer. The electron donors tested, lactic acid, butyric acid, and yeast extract were compared both in the presence and in the absence of anaerobic Mineral Medium. No microcosms prepared with groundwater only, showed any dechlorinating activity, even in the presence of lactate and butyrate.

This indicates that the addition of a mineral medium with metal compounds and nutrients is necessary for the activation of the indigenous microbial community. This experimental evidence underlines also the importance of the metal complexes in the process of reductive dechlorination. The microcosm experiments showed that: the natural attenuation cannot be applied because of the absence of dechlorinating activity detected in biotic control treatments and in those ones that reproduce the natural conditions present in groundwater.

The probable presence of native microorganisms capable of performing full dechlorination was confirmed by the microcosms that showed dechlorinating activity without digester sludge addition; the electron donors tested, except yeast extract, activated or increased the rate of dechlorination compared to biotic control, but the substrate that stimulated the greater this activity was the lactate. The addition of anaerobic digester sludge had as a main advantage, to determine a lower lag phase and the formation of products moved toward ethene. As reductive dechlorination occurred only in microcosms with the addition of MM, the limitations of the RD chloromethane at the site is possibly to be ascribed to inhibitory effects of co-contaminants in the groundwater, or more likely, to the absence or deficiency of nutritional factors.

DGGE Analysis

DGGE analysis was performed on samples from two microcosms in which complete dechlorination to ethylene was observed: the first (n. 12) with the anaerobic digester sludge inoculum, the second (n. 9) without the addition of sludge. The 42 genera isolated from DGGE gel of the two microcosms and sequenced, are clustered in the phylogenetic tree in (Figure 3). Microcosm n. 12, as described previously, was set up with lactate as electron donor, with the anaerobic mineral medium and the anaerobic digester sludge. Its DGGE profile shows a complex bacterial community with the major phyla of Firmicutes, such as *Staphylococcus sp*, *Streptococcus sp., Bacillus sp.,* the Proteobacteria, such as *Aeromonas sp.* (class Gammaproteobacteria) and *Geobacter sp.* (class DeltaProteobacteria), Chloroflexi, such as *Dehaloccocoides sp.* and uncultured Chloroflexi, and finally Actinobacteria, Bacteroidetes, Spirochetes and Synergistetes with the respective genres uncultured Actinobacterium, uncultured Bacteroidetes, uncultured *Synergistetes sp.* and uncultured *spirocheta sp*.

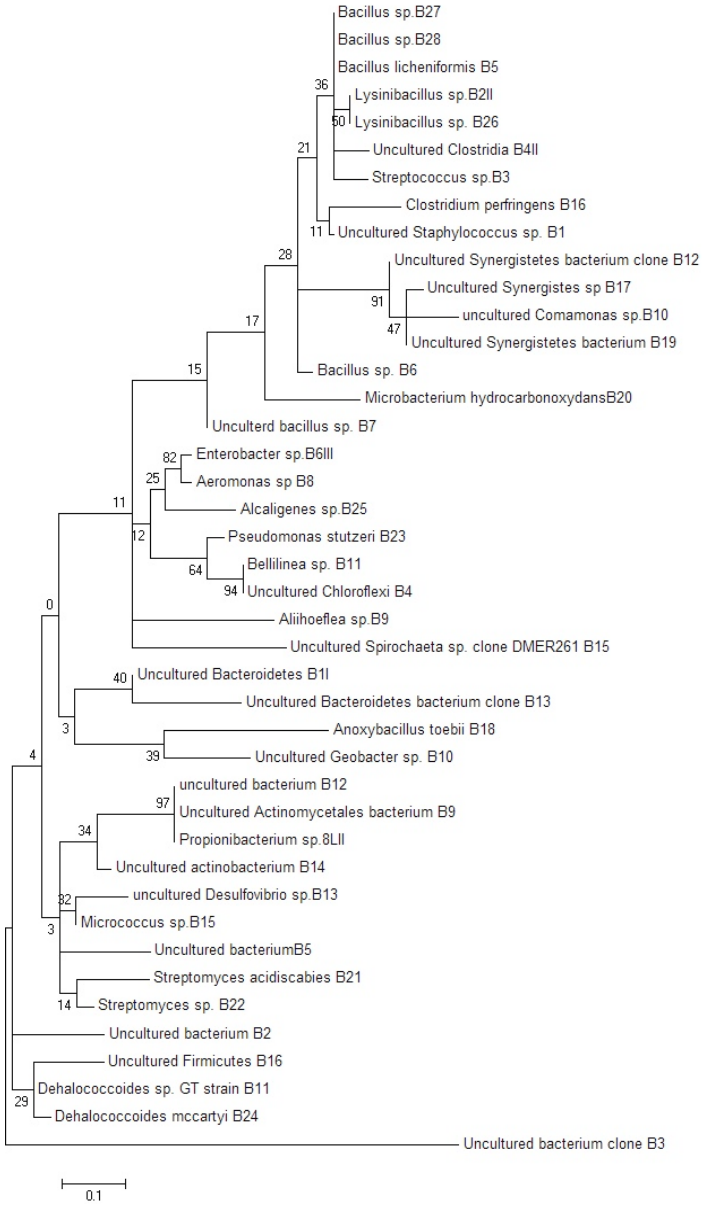

Figure 3: Phylogenetic tree based on partial 16S rRNA gene sequences (about 1300 bp) of the acrylamide gel bands of microcosms 9 and 12. The tree was constructed using UPGMA algorithm.

DE halogenating bacteria and dehalorespiring species belong to the three phyla most represented in the DGGE profile, i.e. Chloroflexi, Formicates and Proteobacteria [33]. It's important the presence of Dehalococcoides genus, in particular the strain GT. It is known that the genus "Dehalococcoides" was firstly described in 1997 with Dehalococcoides ethenogenes strain 195 as a strictly anaerobic bacterium capable of reductively dechlorinating PCE to VC and ethene [34,35], but more detailed investigations concluded that neither trans-DCE nor VC could serve as an electron acceptor

for growth of strain 195 and that utilization of these compounds is apparently metabolic. Some studies revealed extensive metabolic capability even among other Dehalococcoides populations [36,37] and different strains can utilize a wide range of halo organic compounds. In particular, strain GT can utilize halogenated compounds such as TCE, 1,1-DCE, cis-DCE e VC for [33].

A confirmation of this is that the band corresponding to Dehalococcoides GT was identified in the last two lanes, which correspond to the times T4 and T5, relative to the complete degradation of VC in Ethene (results not shown). Uncultured Chloroflexi was identified in the first two lanes corresponding to the early stages of reductive dehalogenation. Thus we hypothesized that Chloroflexi is probably involved in the degradation of PCE and TCE. Uncultured Chloroflexi is a group of relatively different dehalogenation bacteria and is considered as a phylogenetic sister cluster of Dehalococcoides within the phylum Chloroflexi. Bacteria from this cluster were found in many dehalogenations enriched cultures, coming from sites contaminated by chlorinated compounds, particularly from PCBs [38].

Geobacter is a genus of bacteria with the capability of reducing iron and other metals and, particularly, to dechlorinating PCE [39]. It belongs to the Proteobacteria phylum, and it was detected in the first phases of dechlorination. The other genera belonging to the Proteobacteria phylum, identified in the profile of the bacterial community of the microcosm 12, is Aeromonads, which may be associated with sewage or sewage sludge. In fact, microorganisms belonging to the genus Aeromonads, aerobic or facultative anaerobes, are identified in the routinely water research methodology as indicators of pollution and pathogens. Their detection in the waters seems to follow a seasonal pattern in relation to water temperature and also seems to have a positive correlation with the presence of dissolved chlorine [40]. The Bacteroidetes phylum is composed of three major classes of Gram-negative anaerobic bacteria, widely distributed in the environment, including soil, sediments and sea water. Microorganisms belonging to the Bacteroidetes class are associated with the activity of anaerobic digesters; in fact, they were all times, possibly corresponding to bacterial groups present in the sludge from the anaerobic digester use to inoculate the microcosm. The other bacterial groups belonging to Actinobacteria and Synergistetes phyla are related to the sludge degradation in anaerobic digesters.

The microcosm n. 9, as previously described, was set up with the lactate as electron donor, with the anaerobic mineral medium but without the anaerobic digester sludge. The sequenced strains belong to the major phyla of Firmicutes, such as *Clostridium sp*., *Bacillus sp., Lysinibacillus sp*., *Geobacillus sp*., *Anoxybacillus* sp., the Proteobacteria, such as *Enterobacter sp*., *Pseudomonas sp*. (Gammaproteobacteria class)*, Aliihoeflea sp*. (Alphaproteobacteria class), *Comamonas sp*. and *Alcaligenes sp*. (Betaproteobacteria class); the Actinobacteria, such as *Arthrobacter sp*., *Propionibacterium sp*., *Halomonas sp*., *Microbacterium sp*., *Streptomyces sp*.; Chloroflexi, such as *Dehaloccocoides sp*. and *Bellilinea sp*., and finally the less represented Bacteroidetes and Synergistetes. As mentioned above, the three phyla most represented in the DGGE profile of the microcosm n 9, ie. Chloroflexi, Firmicutes and Proteobacteria, are considered in literature mostly related to dehalogenation, while the Actinobacteria are also known for their degrading ability of the soil [33]. It's important the presence of Dehaloccoides genus, strain BAV1, in particular. BAV1 has the same physiological characteristics of strain GT (mentioned above). The halogenated compounds that this strain can utilize are 1,1-DCE, cis-DCE e VC [33]. A confirmation of this is that the band corresponding to strain BAV1 was identified in the lane corresponding to the time corresponding exactly to the degradation of VC in Ethene. It is interesting also the presence of Uncultured *Bellilinea sp.,* belonging to the Chloroflexi phylum and Anaerolineae class.

The class Anaerolineae has been recently proposed as asubclade in the phylum Chloroflexi [41]. Analyses based on sequences of 16S rRNA and the 16S rRNA gene have revealed that members of this class are widely distributed in many different ecosystems, such as the subsurface, sediments, hot springs and aerobic and anaerobic sludge [42,43], suggesting their ubiquity and functional significance in such environments. *Bellilinea sp* is Gram-negative bacterium which grows under strictly anaerobic conditions [44]. It is interesting to observe that the band corresponding to *Bellilinea sp.* is found in particular in the last two dechlorinating times, i.e. in times relative to the dechlorination of VC to ethene, which means that it could also be involved in reductive dechlorination. Microorganisms belonging to the phylum Firmicutes are very well represented with 9 strains. Interesting is the presence of the genus *Desulfuvibrio sp.,* a group of sulfate-reducing bacteria including a dehalogenating species [33] and of *Geobacillus sp.*

It is important to note the complexity of the microbial community found in the microcosm 9, although it was not enriched with the digester sludge, but there was only stimulation of bacterial activity with the addition of anaerobic mineral medium and electron donor: all the dechlorination was performed by native microorganisms. Moreover, except for Dehalococcoides, many bacteria commonly described in the literature for their involvement in the degradation of chlorinated ethenes, in particular in breathing of PCE and TCE, such as *Dehalobacter spp.* [45] *Desulfuromonas spp.* [46,47], and *Sulfospirillum spp.* [48] were not detected in any of the two profiles. They perform, however, an incomplete dechlorination leading usually to the accumulation of cis-DCE, or worse, of VC in groundwater. In both profiles, Dehalococcoides (with different strains) is present, but there are also other bacteria that might be related to reductive dehalogenation, considering their

environmental distribution, their characteristics, and timing (lane) in which they are identified. Examples of this type are *Bellilinea sp, Chloroflexi sp., Geobacter sp., Geobacillus sp., Desulfuvibrio sp.*

A microbial ecological community-analysis was performed on gels obtained from DGGE. The comparison of the values of biodiversity indices in the two different experimental conditions corresponding to the two DGGE gels over time allows to obtain information's on the structure, diversity and function of microbial communities in the environment and under the set conditions. The bacterial communities of the two microcosms 9 (without the addition of anaerobic sludge) and 12 (with the addition of anaerobic sludge) were characterized and changes over time (long-term) were quantified, applying the analysis WMA (Moving-Window Analysis) to determine the rate of change in the community and introducing the Pareto-Lorentz curves to assess possible changes in terms of uniformity (evenness) in the community, as described in Table 2 (A e B). Figure 4 shows the diversity indices derived from data processing.

A

Time	Rr	1-D	Ed
T_0	162	0,95	0,98
T_1	145	0,94	0,97
T_2	127	0,91	0,95
T_3	98	0,93	0,97
T_4	82	0,92	0,97
T_5	50	0,91	0,96
$\Delta t = 43,9 \pm 7,3$			

B

Time	Rr	1-D	Ed
T_0	55,2	0,93	0,98
T_1	122	0,94	0,98
T_2	101	0,94	0,98
T_3	60	0,93	0,98
T_4	81	0,92	0,97
T_5	164	0,95	0,98
$\Delta t = 55,3 \pm 17,3$			

Table 2: Evolution of the microbial community, assessed by biodiversity indices, during the process of reductive dehalogenation in microcosm 9 (A) and 12 (B).

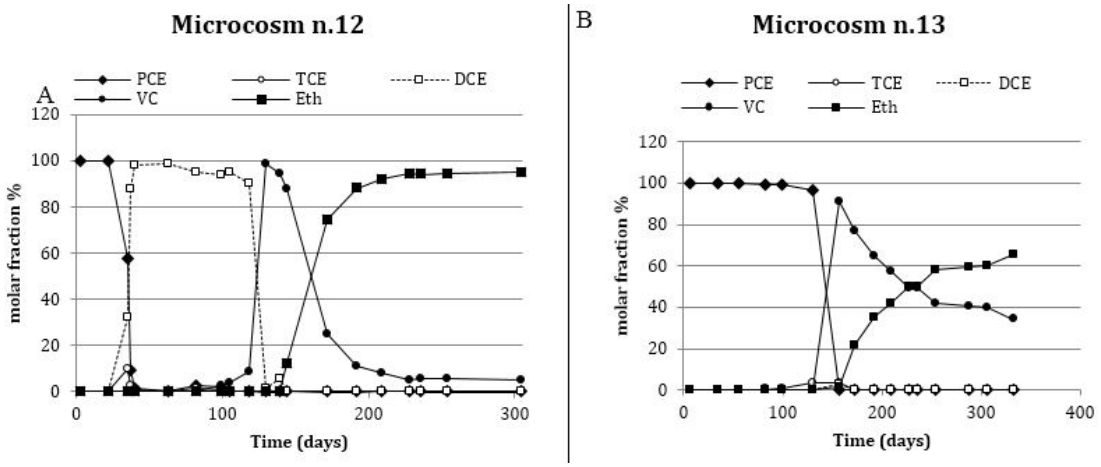

Figure 4: Change in biodiversity indices range-weighted richness (Rr) in relation to the composition of chlorinated solvents in the headspace of the microcosm 9 (left) and microcosm 12 (right).

These indices have so far been utilized to compare microbial communities from different environmental samples [27] but have not yet been applied to the study of communities in closed systems, as in the present case. The Rr trend in the microcosms 9 (without digester inoculum) detects a regular reduction from T0 to T5 (Figure 4A). The Rr value of 162 at T0 indicates a population consisting of a large number of species that is confirmed in the analysis Pareto-Lorentz (Figure 5); it shows, in terms of functional organization, a population less specialized in T0 (48%) compared to the later times, when the specialization increases and is maximum in the time T5 (61%), passing through T1 (56%) and T4 (55%).

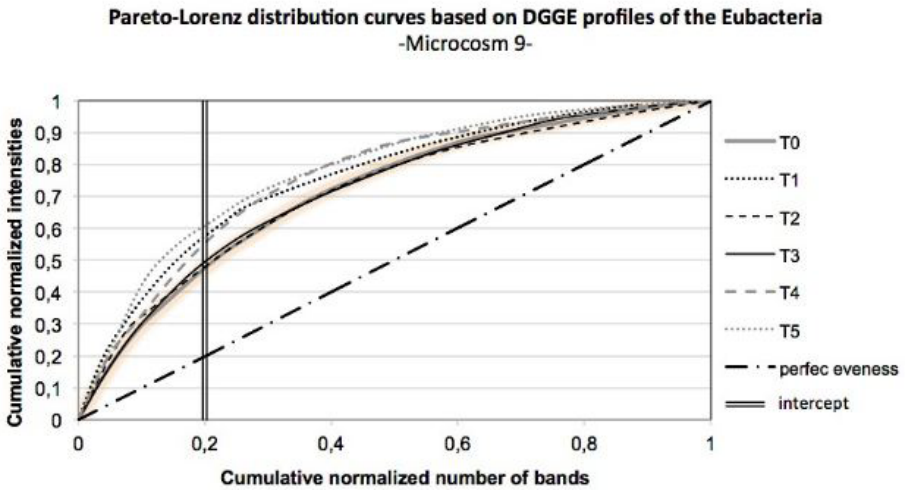

Figure 5: Pareto-Lorenz curve determined on the basis of the intensity of the eubacterial 16S fragments bands for the community of the microcosm 9.

It is interesting to note, at the T1 (PCE degradation to TCE), the discrepancy between functionality and richness data, since to high values of Rr corresponds also a discrete specialization, as well as a greater dynamism. The community detected within the microcosms 9 is characterized in general by high dynamism, with a Δt of 43.9 ± 7.3, then is characterized by the propensity of many species to become dominant in a short period of time depending on the conditions that gradually there. This condition ensures quick recovery from stress conditions (such as the release or production of a new chlorinated compound) and alternative routes to the use of the energy flow [49].

The situation of the microcosm 12, in general, can be considered as a bioaugmentation approach that theoretically corresponds to increasing the metabolic capacity of the microbiota considered and, therefore, the genetic diversity [50]. Conversely, at time T0 the initial Rr value of Eubacteria is 55,2, so much less than the value identified in correspondence of T0 in the microcosm 9. Unlike the latter, the trend of Rr in the microcosm 12 (with digester sludge) detects an irregular trend from T0 to T5 (Table 2B and Figure 4B), with a peak (Rr = 122) in correspondence of T1, which corresponds to the reduction of TCE to cis-DCE, and with very similar values with T2 of microcosm 9 that corresponds also to the reduction of cis-DCE (Rr = 127). The species richness tends to decrease until the production of ethene, when it tends to increase again getting maximum at T5 with the almost complete conversion of VC to ethene (Figure 4B).

This situation is partially confirmed by the Pareto-Lorentz analysis (Figure 6) that indicates a balanced community with an average functional organization (values ranging from 43% to 53%). The population is less specialized at T0 (43%), then increases and becomes maximum at the time T4 (53%), passing through T1 (52%) and T5 (49%). It is interesting to observe, in this case too, in correspondence of T1 (degradation of TCE to cis-DCE), the inconsistency between the data of functionality and richness, since a greater specialization and a greater dynamism corresponds to high values of Rr. The community detected within microcosm 12 is characterized in general by a greater dynamism than Microcosm 9 (Table 2B), with a Δt of 55.3 ± 17.3; one reason could be that the microcosm 12 reaches more quickly the complete degradation of PCE in ethylene.

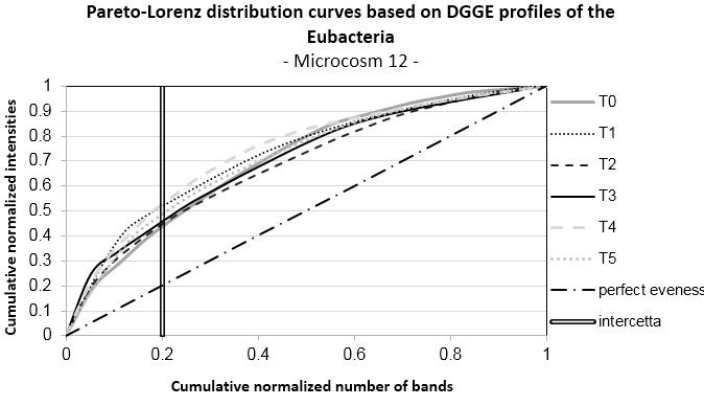

Figure 6: Pareto-Lorenz curve determined on the basis of the intensity of the eubacterial 16S fragments bands for the community of the microcosm 12.

Conclusions

Based on microcosm experiments we concluded that: i) the natural attenuation cannot be applied to the remediation of the area because of the absence of dechlorination observed in treatments that reproduced the natural conditions present in groundwater (biotic controls and microcosms prepared with only groundwater); ii) in the soil of the contaminated site, dechlorinating native populations

are present because all microcosms, amended with mineral medium and electron donors, showed dechlorinating activity; iii) dechlorinating activity of indigenous microorganisms is triggered by the presence of anaerobic mineral medium and its absence represents a limiting condition for their metabolic dechlorinating activity; iv) the addition of an anaerobic digester inoculum had the effect of significantly increasing the speed of degradation of chlorinated contaminants; v) all electron donors tested (lactate, butyrate), except yeast extract, stimulate the dechlorinating activity when compared to biotic control; vi) the electron donor that stimulates more dechlorinating activity is lactic acid, also in the presence of inoculum digester. Therefore, this demonstrates that the autochthonous microorganisms of the soil, if properly stimulated, may be able to perform all the stages of the reductive dehalogenation of chlorinated ethenes up to ethene.

The molecular and ecological analysis of microbial communities inside microcosms in which the reductive dechlorination led to the formation of ethene, showed the presence of a complex and varied community, with a high dynamism and functionality. Microorganisms such as Dehalococcoides - that, as we know, is the only organism that the literature indicate to be able to completely degrade PCE to Ethene - are present; but we found also less well-known anaerobic bacteria that are likely involved in crucial stages of the reaction of degradation of tetrachloroethene *(Bellilinea sp, Chloroflexi, Geobacter sp., Geobacillus sp., Desulfuvibrio sp.)*. The addition of anaerobic digester sludge, even from the point of view of molecular biology, did not led to significant increase in species richness and functionality of microbial populations but only in dynamism. In conclusion, we can assert that in the contaminated site of the Vibrate valley it is possible to implement an enhanced bioremediation, favoring the complete degradation of chlorinated contaminants through the addition of an anaerobic mineral medium and a suitable electron donor, lactic acid in particular.

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