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BIOLOGICAL REMOVAL OF 1,2-DICHLOROETHANE FROM A REAL POLLUTED GROUNDWATER BY USING ENRICHED BACTERIAL CONSORTIA UNDER AEROBIC AND ANAEROBIC CONDITIONS

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Palermo, 2022 November 21

To the Editor of
International Biodeterioration & Biodegradation

Dear Editor,

please find an original research full length Article titled: **Biological removal of 1,2-dichloroethane from a real polluted groundwater by using enriched bacterial consortia under aerobic and anaerobic conditions**

by

Federica De Marines, Ilenia Cruciata, Gaetano Di Bella, Daniele Di Trapani, Maria Gabriella Giustra, Laura Scirè Calabrisotto, Pietro Greco Lucchina, Paola Quatrini, Gaspare Viviani.

The research topic concerns the bioremediation of groundwater contaminated by chlorinated solvents. The aim of the work was to gain insights about the feasibility of 1,2-dichloroethane (1,2-DCA) removal through an anaerobic biological process in a laboratory-scale permeable reactive barrier (PRB). The PRB effect is enhanced by the use of an ecofriendly biopolymer, poly-β-hydroxybutyrate (PHB), as electron donor for reductive dechlorination reactions; the anaerobic column experimental system is run in parallel with an aerobic column system.

Chlorinated solvents contamination is a global environmental problem regarding soil and groundwater and the interest in the development of new *in situ* bioremediation and nature-based technologies is growing.

The novelty of our study relies in the use of a real contaminated groundwater, collected from a Site of National Interest located in Sicily, still unexplored from a microbiological point of view. The column systems were inoculated with dehalogenating consortia obtained by enrichment cultures on 1,2-DCA from the same groundwater. Our results provide, for the first time, useful preliminary indications towards the evaluation of 1,2-DCA biodegradation pathways, both under anaerobic and aerobic conditions, when treating real contaminated groundwater. We combined an engineering approach, based on the monitoring of 1,2-DCA biodegradation in experimental column plants under aerobic or anaerobic conditions, with a microbiological one, based on 16S RNA gene-based metagenomics. For both conditions, we suggest the microbial taxa responsible of biodegradation processes.

For all the afomentioned reasons we consider our submission appropriate for the scope of the journal, and we hope the same for you.

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Kind regards
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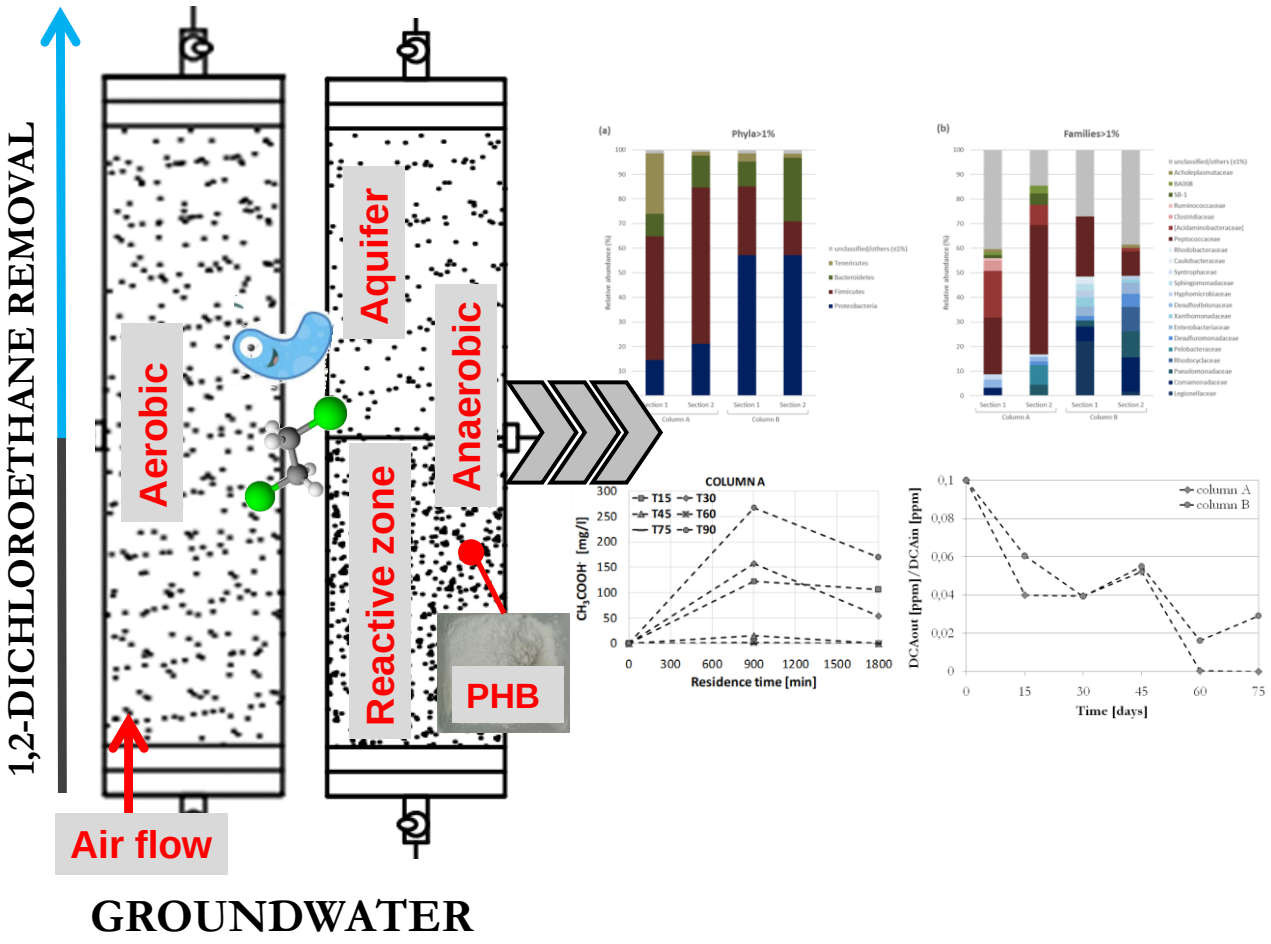
Highlights

- Complete anaerobic 1,2-DCA degradation sustained by ecofriendly biopolymer.
- PHB acts as a slow-releasing carbon source and acetate accumulation is detected.
- Metagenomic analysis reveals the presence of dehalogenating bacteria.
- Feasability of a sustainable remediation technique by the use of PHB.
- Establishment of aerobic (co)-metabolism, probably carried out by *Pseudomonas* spp.

Graphical Abstract



BIOLOGICAL REMOVAL OF 1,2-DICHLOROETHANE FROM A REAL POLLUTED GROUNDWATER BY USING ENRICHED BACTERIAL CONSORTIA UNDER AEROBIC AND ANAEROBIC CONDITIONS

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Abstract

Permeable reactive barriers (PRBs) based on biological reductive dechlorination processes represent a promising technology for the remediation of groundwater contaminated by chlorinated solvents. The aim of this work was to gain insights about the feasibility of chlorinated solvents removal through an anaerobic biological process in a laboratory-scale PRB, based on the use of poly-β-hydroxybutyrate (PHB) as electron donor, and comparing the results with those achieved under aerobic conditions. The experimental plant consisted of two Plexiglas cylindrical columns filled with silica sand and fed with 1,2-dichloroethane (1,2-DCA) contaminated groundwater: in column A the simulated PRB contained powder PHB under anaerobic conditions, in column B an inlet air flow rate ensured aerobic conditions. Both columns were inoculated with dechlorinating bacterial consortia obtained from the contaminated groundwater. Results from column A proved that PHB can be fermented and used as a slow-releasing carbon source for sustaining reductive dechlorination, as revealed by acetate production and complete 1,2-DCA removal. The microbial community detected in column A at the end of the experimental period was mainly enriched in sulfate reducing bacteria that could act as both dechlorinating and fermenting agents. Column B showed a slight lower 1,2-DCA removal efficiency likely related to the establishment of aerobic (co)-metabolic processes, probably carried out by *Pseudomonas* spp.

Keywords: Biological reductive dechlorination; Aerobic (co)-metabolic process; Chlorinated solvents; Poly-β-hydroxybutyrate; Permeable reactive barriers.

Abbreviations:
1,1,1-TCA, 1,1,1-trichloroethane; 1,2-DCA, 1,2-dichloroethane; BRD, biological reductive dechlorination; DNAPL, dense non-aqueous phase liquid; DO, dissolved oxygen; EC, electric conductivity; OHRB, organohalide respiring bacteria; ORP, oxidation reduction potential; OTU, operational taxonomic unit; P&T, Pump & Treat; PCE, tetrachloroethylene; PHB, poly-β-hydroxybutyrate; PRB, permeable reactive barrier; SNI, Site of National Interest; SRB, sulfate reducing bacteria; TCE, trichloroethylene; VFA, volatile fatty acid; ZVI, zero-valent iron.

1. Introduction

Chlorinated solvents are organic compounds belonging to the class of chlorinated aliphatic hydrocarbons and they are a subgroup of organ-halogen compounds. These compounds have been widely used in various industrial sectors, as degreasers of mechanical and electronic parts, dry cleaning of metals and clothes, as solvents and raw materials in the chemical-pharmaceutical industry. The major issues related to these compounds are represented by high toxicity and carcinogenicity; furthermore, they are poorly soluble and degradable, and very often, because of improper handling and disposal methods, they can be released into the environment. For this reason they can be found in the environmental matrices, promoting in particular aquifer contamination (APAT IRSA-CNR, 2003; Bradley, 2003; Anam et al., 2019; Aulenta et al., 2019). To date, in fact, several cases of soils and groundwater contamination by chlorinated solvents have been reported in technical literature (Majone et al., 2015; Pierro et al., 2016, 2017; Aulenta et al., 2019).
Generally, chlorinated solvents discovered in the environment belong to the class of chlorinated methanes, ethanes and ethenes, such as 1,2-dichloroethane (1,2-DCA), 1,1,1-trichloroethane (1,1,1-TCA), carbon tetrachloride, methylene chloride, chloroform, tetrachloroethylene (PCE) and trichloroethylene (TCE). Most of them have some peculiar chemical-physical characteristics that describe their behavior in soils and groundwater, including a high vapor pressure, a greater density than water and a very limited water solubility (Ajo-Franklin et al., 2006). Consequently, most of the chlorinated compounds, if released in high quantities, are prone to form a dense non-aqueous phase liquids (DNAPLs). Moreover, the high density favors their rapid percolation through the unsaturated soil, reaching the aquifer, where these compounds settle down to the impermeable bottom and are then dispersed along the water flow direction (Sleep and Sykes, 1989; Cohen and Mercer, 1993; Pierro et al., 2016).
Conventionally applied remediation processes, such as Pump & Treat (P&T), are ineffective to solve DNAPLs contamination; these treatments, in fact, rarely allow reaching the low standard limits imposed by Regulations for water reinjection into groundwater; therefore, the final destiny is often the discharge into surface water bodies. Moreover, this treatment is based on contaminant phase transfer, regardless its effective degradation (Starr and Cherry, 1994; Beretta, 2000; Majone et al., 2009).

This led to the interest in the development of new in situ remediation technologies, including permeable reactive barriers (PRBs), based on physical-chemical and biological processes (Majone et al., 2015; Fan et al., 2017; Cameselle and Gouveia, 2018; Li et al., 2019). This technology presents potential advantages over the P&T in terms of lower management costs, related to lower energy demand, absence of external discharges to be treated and disposed as well as less site disturbance issues. Furthermore, this technology promotes the contaminant degradation, rather than its phase transfer.
Concerning PRB based on physical-chemical processes, zero-valent iron (ZVI), activated carbon and natural and synthetic zeolites, have been widely applied (Accardi et al., 2012; Baric et al., 2012; Piscitello et al., 2020; Rossi et al., 2021). ZVI is the most frequently employed reactive media, especially for the treatment of chlorinated hydrocarbon plumes, referring in particular to chlorinated ethenes and ethanes (Odziemkowski et al., 1998; Furukawa et al., 2002). It has been demonstrated that ZVI can effectively degrade these pollutants to nontoxic compounds, such as ethene and ethane, through a series of dechlorination reactions (Baric et al., 2012). However, PRB-ZVI technology is affected by some operational issues, the main one likely being the long-term performance decrease due to loss of ZVI reactivity and/or porosity reduction (Song and Carraway, 2005; Henderson and Demond, 2007). Indeed, the alkaline pH (8–9) and the presence of ferrous and ferric ions within the barrier, due to ZVI corrosion, can lead to the precipitation of minerals, which depends on groundwater chemical composition, which can form a passive layer over the iron surface. This aspect might hamper the electron transfer process,

thus reducing the iron reactivity (Odziemkowski et al., 1998; Furukawa et al., 2002). Additionally, iron precipitation could cause pore occlusion, resulting in a greater flow velocity, thus reducing the treatment effectiveness due to the decrease of the residence time within the PRB (Morrison and Bartlett, 2009; Baric et al., 2012). Moreover, some compounds, e.g., chloromethane, dichloromethane and 1,2-dichloroethane, do not undergo chemical reduction in the presence of ZVI (Song and Carraway, 2005; Baric et al., 2012). Among them, 1,2-DCA, a carcinogenic compound, is of particular concern because it is one of the most widespread groundwater contaminants (De Wildeman et al., 2003).

It has been demonstrated that most of chlorinated solvents can be effectively degraded by biological processes carried out by bacteria. The biodegradation of chlorinated compounds can occur through an oxidation (aerobic) or reductive (anaerobic) reaction and it can follow a direct or cometabolic pathway (Frascari et al., 2015). In the oxidative direct pathway, the microorganism consumes the chlorinated compound, which acts as an electron donor and growth substrate; conversely, in cometabolism, an oxygenase enzyme, synthetized by the microorganism for the uptake of a growth substrate, fortuitously catalyzes the oxidation of the chlorinated compound. Generally, low-chlorinated compounds are degraded through a oxidative direct pathway; in contrast, polychlorinated compounds can be effectively degraded via biological reductive dechlorination (Furukawa et al., 2002), provided that specific microorganisms and available electron donors are present (which is usually the limiting factor in natural reductive biodegradation processes) (Baric et al., 2012, 2014).
The biological reductive dechlorination (BRD) is a natural process where indigenous microorganisms present in the groundwater ecosystem through an anaerobic multi-step reaction convert highly chlorinated parent compounds to less chlorinated daughter products and finally to non-toxic end products, like as ethene and ethane (Xiao et al., 2020; Lin et al., 2021; Amanat et al., 2022). In this anaerobic respiratory process, called dehalorespiration, organohalide respiring bacteria (OHRB) utilize the chlorinated compounds as terminal electron acceptors (Hug et al., 2013). BRD is an electron consuming process and it is often limited by the lack of suitable electron donors that results in an incomplete process and leads to the accumulation of undesired by-products (Li et al., 2021; Yan et al., 2021; Amanat et al., 2022). Therefore, it is possible to enhance and sustain BRD through the addition of fermentable substrates by supplying the microorganisms with sources of electron donors (e.g., hydrogen, butyrate, lactate).

To date, poly-β-hydroxybutyrate (PHB) derived both from pure and mixed microbial culture has proven to be effective as a slow-release electron donor for the reductive dechlorination process (Aulenta et al., 2008; Pierro et al., 2017; Amanat et al., 2020, 2021, 2022) and various studies (Baric et al., 2012, 2014; Matturro et al., 2018; Amanat et al., 2021, 2022) confirmed the feasibility to use it as a reactive medium in a PRB. Heretofore, the most common way to produce PHB is to use pure bacterial cultures and selected substrates; however, this involves high costs and makes the production process economically unsustainable (Ivanov et al., 2014; Villano et al., 2014). For these reasons, in recent years, more sustainable processes based on the use of mixed microbial cultures (MMC) (Valentino et al., 2019; Amanat et al., 2021) and low-cost feedstocks (such as wastewaters) (Morgan-Sagastume, 2016; Kourmentza et al., 2017; Corsino et al., 2022) have been extensively studied. This represents an economically and environmentally sustainable solution, according to the principles of circular bio-economy. To date, there are few experimental studies in which PHB has been used as a fermentable substrate to support reductive dechlorination (Aulenta et al., 2005a , 2005b; Baric et al., 2012, 2014; Amanat et al., 2020, 2022). Moreover, these studies have been conducted on a microcosm or laboratory-scale and using synthetic waters contaminated with chlorinated compounds (PCE, TCE, 1,2-DCA). This aspect, if on one hand simplified the understanding of the mechanisms occurring within the studied

system, on the other hand, did not allow to appreciate the possible issues related to the treatment of a real contaminated water in which there are many other compounds that can cause interferences.
In general, the chlorinated compounds investigated in the aforementioned studies are prone to be refractory to aerobic biodegradation, excepting the lower chlorinated compounds; for this reason, the experiments have been carried out ensuring anaerobic conditions within the system.

In this context, the aim of the present study was to evaluate the feasibility of bioremediation of a real groundwater contaminated by chlorinated solvents through a biological process in a permeable reactive barrier, filled with PHB to be used as slow-release electron donors to sustain the BRD process. PHB fermentation, in fact, is able to support the long-term growth of microbial consortia including dechlorinating bacteria. In order to fulfil the aim of the study, an experimental bench-scale apparatus was realized, consisting in two parallel Plexiglas cylindrical columns (column A and column B) filled with silica sand. Column A was additioned with powder PHB and run under anaerobic conditions, while column B was filled only with silica sand and operated under aerobic conditions. Both columns were fed with a real contaminated groundwater for the entire experimental duration. At the beginning of the experiment the columns were inoculated with two different enrichment cultures obtained from the same contaminated groundwater under anaerobic PHB conditions and under aerobic conditions.

The novelty of this study relies in the use of a real groundwater contaminated by chlorinated solvents, mainly 1,2-DCA, collected from a Site of National Interest (SNI) located in the Sicilian territory; the results from this study can provide useful preliminary indications towards the evaluation of 1,2-DCA biodegradation pathways, both anaerobic and aerobic, when treating real contaminated groundwater.

2. Materials and Methods

The experimental campaign had an overall duration of 180 days. In particular, during the first 90 days, enrichment cultures were set up from the real chlorinated solvents contaminated groundwater in order to favor the autochthonous dechlorinating bacterial population. A dechlorinating bacterial consortium was enriched in presence of PHB powder, as electron donor for the reductive dechlorination process, under anaerobic conditions. In parallel, an enrichment culture on 1,2-DCA was carried out from the same groundwater under aerobic conditions, in order to biostimulate oxidative processes. During the subsequent 90 days, a pilot study was carried out on a laboratory-scale plant fed with the real contaminated groundwater inoculated with the dechlorinating bacterial consortia mentioned above.

2.1. Contaminated groundwater characterization

The real groundwater was sampled from a Sicilian SNI, characterized by significant industrial activities in the last decades and where soil and groundwater have been documented as chronically contaminated by organic and inorganic pollutants. The groundwater sample was stored at 4 °C in a container with no headspace and hermetically sealed, in order to limit, as much as possible, the exposure to atmospheric oxygen, maintaining anaerobic conditions and limiting the contaminants volatilization. The groundwater was mainly contaminated by 1,2-DCA and was characterized by high values of electrical conductivity as well as chlorides and total dissolved solids. Among the pollutant compounds found in the real groundwater, 1,2-DCA, a carcinogenic compound, is of high concern since it is one of the most widespread groundwater contaminants (Marzorati et al., 2006). In Table 1, the main characteristics of the real groundwater used in this study and the analytical methods adopted in their determination are shown.

Table 1. Chemical-physical characteristics of groundwater and analytical methods for their determination.

PARAMETER	ANALYTICAL METHOD	UNIT OF MEASURE	CSC D.LGS. 152/06	MEASURED CONCENTRATION
REDOX POTENTIAL	ASTM D1498-14 (III)	mV	-	-450,00
ELECTRIC CONDUCIBILITY	APAT CNR IRSA 2030 Man 29 2003	µS cm^{-1}	-	9.400,00
pH	APAT CNR IRSA 2060 Man 29 2003	pH	-	7,30
TOTAL DISSOLVED SOLIDS	SM 2540 C 2017	mg l^{-1}	-	7.000,00
CHLORIDE	EPA 9056A 2007	mg l^{-1} (as Cl^-)	-	1.800,00
NITRATES	EPA 9056A 2007 (PRI)	mg l^{-1} (as NO_3)	-	< 0,88
SULPHATES	EPA 9056A 2007	mg l^{-1} (as SO_4)	250	1.900,00
TOTAL CYANIDE	EPA 9014A 2014 T	µg l^{-1} (as CN)	-	< 5
METHYL-ter-BUTYLETHER	EPA 8260D 2018	µg l^{-1}	-	< 7,6
AMMONIUM	APAT CNR IRSA 4030 A2 Man 29 2003	mg l^{-1} (as NH_4)	-	2,00
HEXAVALENT CHROMIUM	APAT CNR IRSA 3150 C Man 29 2003	µg l^{-1} (as Cr)	5,00	< 0,84
TOTAL CHROMIUM	EPA 6020B 2014	µg l^{-1} (as Cr)	50,00	< 0,92
IRON	EPA 6020B 2014	µg l^{-1} (as Fe)	200,00	25.000,00
MANGANESE	EPA 6020B 2014	µg l^{-1} (as Mn)	50,00	1.700,00
CHLOROMETHANE	EPA 8260D 2018	µg l^{-1}	1,50	< 7,5
CHLOROFORM	EPA 8260D 2018	µg l^{-1}	0,15	< 1,3
VINYL CHLORIDE	EPA 8260D 2018	µg l^{-1}	0,50	520,00
1,2-DCA	EPA 8260D 2019	µg l^{-1}	3,00	20.000,00
1,1-DCE	EPA 8260D 2020	µg l^{-1}	0,05	18,00
TCE	EPA 8260D 2021	µg l^{-1}	1,50	< 7
PCE	EPA 8260D 2022	µg l^{-1}	1,10	< 6,9

2.2. Set up of enrichment cultures in microcosm

Enrichment cultures in microcosm were set up using the real groundwater sample from the contaminated site in order to enrich the autochthonous 1,2-DCA dechlorinating bacterial community. Anaerobic enrichment cultures were set up with groundwater and culture medium (Marzorati et al., 2006) (50% vol/vol) and biostimulated with poly-3-hydroxybutirrate (PHB) powder 3.4 mM to a final volume of 12 ml into 20 ml screw cap vials in an anaerobic box under an atmosphere of filter-sterilized N_2. The anaerobic cultures were incubated statically in the dark at room temperature. In parallel, aerobic enrichment cultures were set up with groundwater and culture medium (Coleman et al., 2002) and were amended with 1,2-DCA to a final concentration of 100 ppm and a mixture of volatile hydrocarbons (1% methane, 1% ethane, 0.01% C4-C6) in a final volume of 12 ml into 20 ml screw cap vials. The aerobic cultures were incubated in the dark at room temperature on a rotary shaker. In both anaerobic and aerobic conditions, abiotic controls were set up using filter-sterilized groundwater. All microcosms were prepared in triplicate. At the end of four established incubation times (0, 15, 30 and 45 days), a set of microcosms of each condition was analyzed by gas-chromatography as described in *2.4.* section. After 45 days of incubation, the obtained anaerobic and aerobic dechlorinating cultures were further enriched by transferring an inoculum (10% vol/vol) into fresh culture medium for 45 more days. These consortia were used to inoculate the columns as described below.

2.3. Continuous-flow experimental apparatus

Two parallel Plexiglas columns (length: 44 cm, inner diameter: 2.8 cm), namely column A and column B, were realized and continuously fed throughout the entire experimental laboratory-scale study (90 days). In particular, column A was conceived to simulate a first PRB portion (from 0 to 22 cm) and a down-gradient aquifer (from 22 to 44 cm); the PRB portion was filled with a mixture of silica sand and PHB at 5% on a weight basis; in contrast, the aquifer portion was filled with silica sand only. Column B was filled with silica sand only and at the flow inlet it was equipped with an aeration system in order to promote the aerobic metabolism.

Powder PHB was obtained from Sigma-Aldrich (CAS 29435-48-1, purity 99.9%). A 2 cm sand and coarse gravel layer was placed at the bottom of each column to homogenize the distribution of the water flow. Each column was equipped with a sampling port placed at the middle of the column; in addition, another sampling point has been realized at the outlet of the columns.

Before running the experiments, both columns were inoculated with the two enriched microbial communities previously obtained from the chlorinated solvents contaminated groundwater. In column A the dechlorinating consortium enriched on PHB under anoxic conditions was added with the specific aim of creating a biological reactive zone, where inoculated microorganisms could use the PHB fermentation products as electron donors. In column B, the dechlorinating consortium enriched on 1,2-DCA in presence of O_2 was inoculated with the aim of promoting an aerobic (co)-metabolism. In each column, 70 ml of groundwater were inoculated with 10% (vol/vol) bacterial culture at OD_{600} = 0.1 and left in batch conditions for one week, before starting the continuous groundwater feeding.

Finally, columns were covered and kept in the dark in order to simulate the same condition of a real aquifer, and also avoiding algae formation. Before the start-up of the experimental activity, column A was flushed with nitrogen gas to eliminate oxygen. Both columns were fed upward with the same real groundwater contaminated by 1,2-DCA at a concentration close to 20 mg l^{-1}. While running experiments, groundwater was continuously pumped into the columns by two peristaltic pumps at a flow rate of 0.1 ml min^{-1}, which corresponded to 30 hours of residence time or 1.3 cm h^{-1} of pore water velocity. The feed water was stored in sealed bags with a capacity of about 2 l, which were periodically refilled (autonomy of about 14 days). For column A, the bags were previously flushed with nitrogen gas to

eliminate oxygen ensuring anaerobic conditions. Furthermore, before each bags refill, groundwater was enriched in nutrients (urea - CH_4N_2O: 7.5 ml in 1 liter of water and potassium dihydrogen phosphate - K_2HPO_4: 8.6 ml in 1 liter of water) in order to avoid limitations for microbial growth. Nutrients were dosed according to Baric et al. (2014); in order to remove any residual dissolved oxygen in the feeding water of column A, a deoxygenation procedure, with sodium sulphite and cobalt chloride, was performed in accordance with Capodici et al. (2019).

Figure 1 shows a schematic layout (a) and a panoramic view (b) of the laboratory-scale columns.

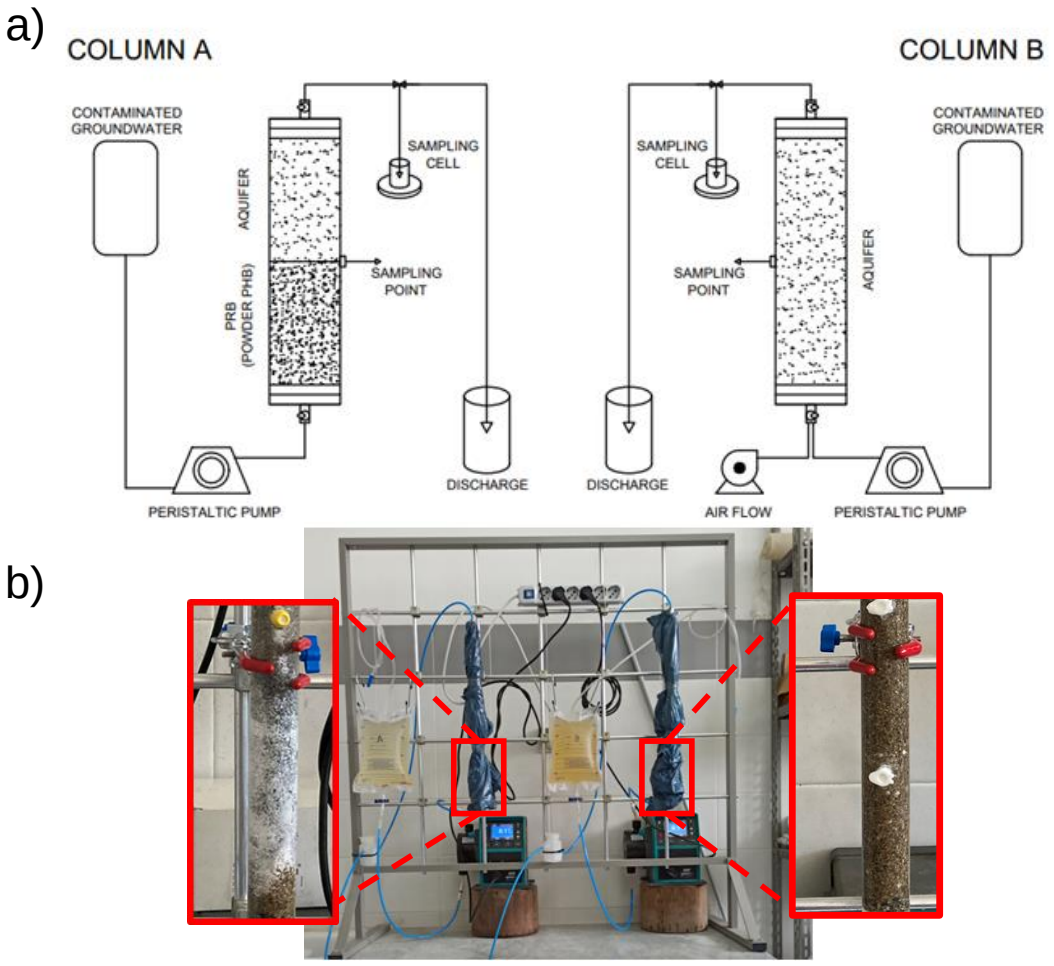


Figure 1. Schematic (a) and panoramic overview (b) of the laboratory-scale columns.

2.4. Analytical methods

During the experimental activity, the main operational parameters as dissolved oxygen (DO), redox potential (ORP), electric conductivity (EC) and pH have been regularly measured in a sampling cell, located at the outlet of both systems, by means of dedicated probes connected to a multimeter (WTW 3340) to monitor plant behavior.

Liquid samples were periodically collected (every 14 days) at the inlet, at the middle (corresponding to a retention time of 900 min) and at the outlet of each column (corresponding to a retention time of 1800 min). In column A, where the middle point of sampling corresponded with the outlet of PRB, the aim was to monitor respectively the features of the influent water, the PHB fermentation in terms of acetate

production, and the BRD progress in terms of 1,2-DCA biodegradation. In column B, the aim was to monitor the performance of the aerobic PRB in terms of 1,2-DCA oxidative biodegradation.
In detail, 1,2-DCA concentration in enrichment cultures and in columns was determined by headspace-gas chromatography/mass spectrometry using the *7000C GC/MS Triple Quad GC/MS System*, equipped with the column *Agilent 19091S-433UI*; helium was used as carrier gas, oven temperature was set at 240 °C and injection temperature was 270 °C. In column A, Volatile Fatty Acids (VFAs) were analyzed by ion chromatography (*DIONEX ICS-1100*) according to *APAT IRSA CNR 4020*.

2.5. Characterization of microbial communities by Illumina Miseq16S rRNA gene sequencing

The microbial community of the two portions (section 1 and 2 respectively, in column A corresponding to PRB and aquifer) of both columns was characterized using high throughput 16S rRNA gene amplicon sequencing. At the end of the experimental period, samples of sand were recovered from both sections of columns A and B. Metagenomic DNA was extracted from 0.5 g of each sample using the Fast DNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following manufacturer's instructions. Purity and concentration of extracted DNA were assessed using a Nano DROP ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). As reported in Corsino et al. (2022), genomic DNA obtained from each sample was used as template to amplify bacterial and archaeal V3-V4 hypervariable region of the 16S rRNA gene; the amplicons sequencing was carried out using the Illumina-MiSeq® platform; reads filtering and denoising were performed using Qiime2 tools version 2019.4 and taxonomy was assigned using trained Operational Taxonomic Units (OTUs) at 99% from Green Genes database version 13–8.

3. Results and discussion

3.1. Enrichment cultures in microcosm

Gas-chromatographic analysis conducted on anaerobic and aerobic enrichment cultures after 45 days incubation during the preliminary experimental study revealed a removal of 1,2-DCA of 90% in the PHB biostimulated anaerobic microcosms, and of 100% in the aerobic microcosms; a decrease of 1,2-DCA concentration was also observed in anaerobic/aerobic abiotic control microcosms, but to a lesser extent (30% and 70%, respectively) (Figure 2), thus confirming that a dechlorinating community was enriched in both conditions in microcosm. The obtained dechlorinating consortia were further enriched and used to inoculate the two columns of the continuous-flow experimental apparatus.

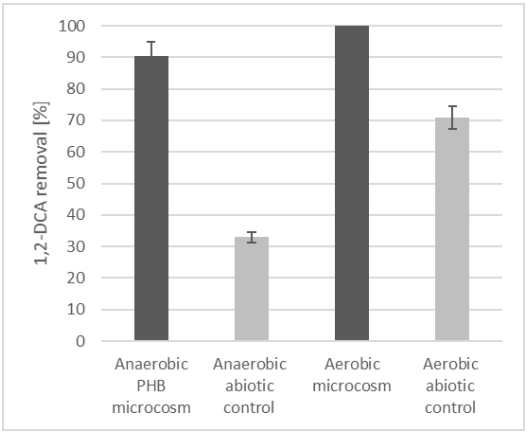


Figure 2. 1,2-DCA removal in PHB anaerobic microcosm, in aerobic microcosm and in their relative abiotic controls after 45 days of incubation.

3.2. Performance of continuous-flow experiments

3.2.1. Parameter monitoring

As expected, the two columns showed a different behavior throughout experiments. Figure 3 shows the parameters variation during the experimental campaign with continuous-flow into the columns.

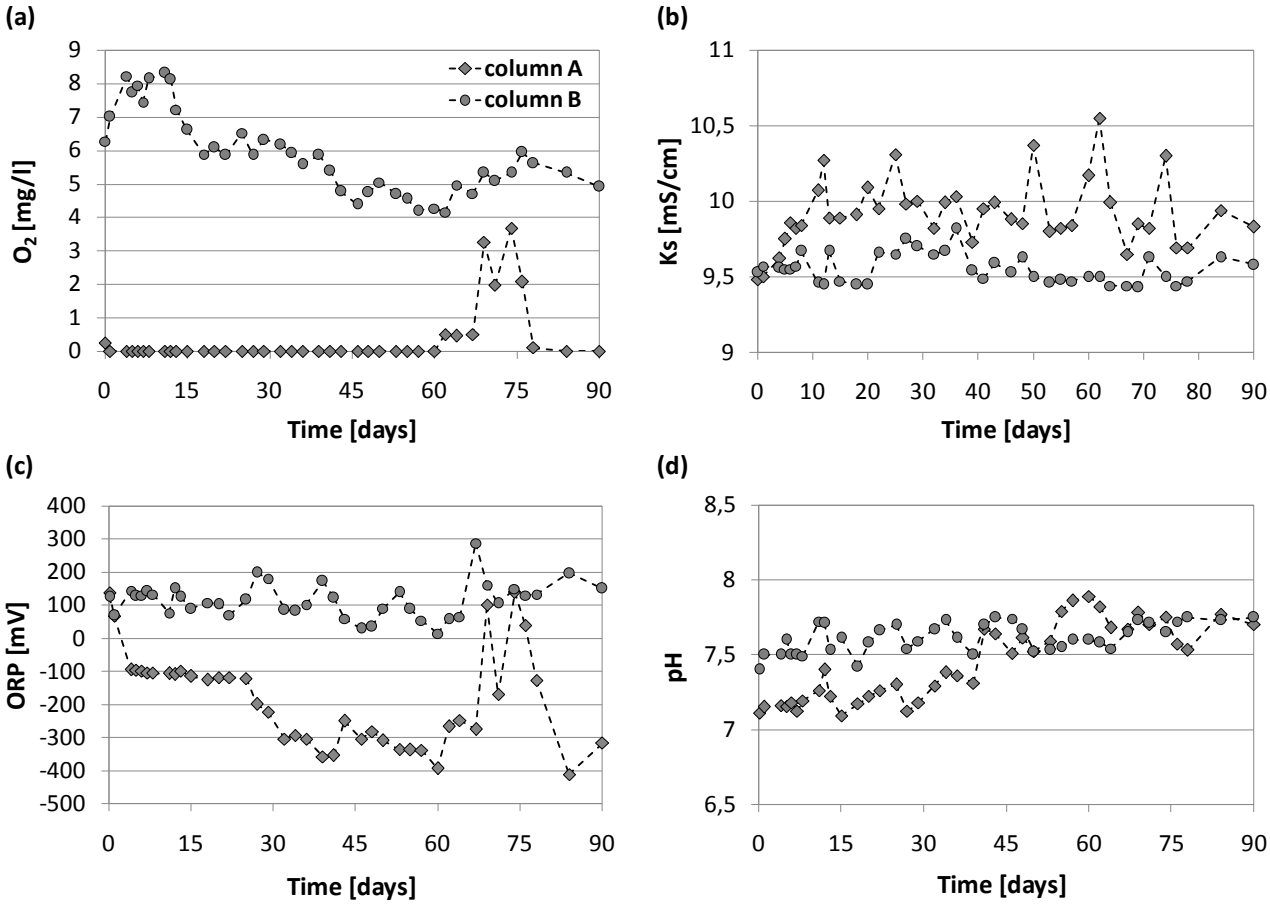


Figure 3. DO concentration (a), electric conductivity (b), redox potential (c), and pH (d) over time for column A and B.

Figure 3a reports the trend profile of DO concentration throughout experiments in the two columns. In particular, the DO concentration for column A was always equal to zero confirming the establishment of suitable conditions for the anaerobic pathways in the column, excepting a short time period (from day 60 to day 78) when a sudden increase of the oxygen concentration was observed. This result could be likely due to an improper water-bag storage. In contrast, in column B, the DO concentrations at the outlet of the system were significantly higher, thus promoting the suitable conditions for the aerobic (co)-metabolic pathway. Interestingly, a decreasing trend during experiments was observed, probably due to biotic oxygen consumption. These behaviors were confirmed by the ORP values measured at the outlet of both columns throughout experiments (Figure 3c). Indeed, in column A, the ORP always showed negative values, excepting the short period corresponding to the increase of oxygen concentration; these negative values confirmed the establishment of anaerobic conditions within column A. Conversely, the ORP values in column B were always strongly positive, thus indicating aerobic conditions, at least at the outlet of the system. Electric conductivity remained almost constant over time in both columns, with higher values observed in column A compared to column B (Figure 3b). The pH had a different behavior (Figure 3d); in both columns, it showed an increasing trend and it was higher in column A. This growing trend could be due to the acetate consumption process by microorganisms inside column A, thus causing an increase in alkalinity. This trend could be explained by a balance between two opposite phenomena occurring simultaneously: on one hand, PHB hydrolysis from which acetate is produced, which determines a pH decrease, while on the other hand, acetate consumption by microorganisms, which leads to a pH increase. It is possible that, from a kinetic point of view, consumption overwhelmed production thus determining a pH increasing trend.

3.2.2. PHB fermentation and acetate production in column A

Analysis of acetate concentration in column A showed that, under anaerobic condition, bacterial fermentation of PHB with production of acetate was enhanced (Figure 4). The registered trend of acetate concentration suggests an high rate of microbial activity inside the column A stimulated by the high specific surface area of the powder PHB that can explain the rapid biopolymer degradation (Baric et al. 2012).

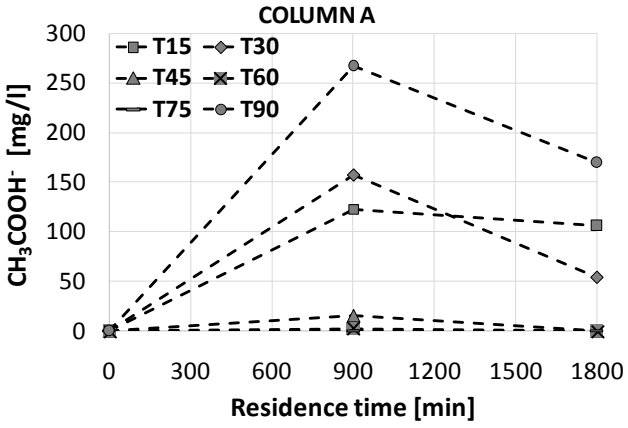


Figure 4. Acetate concentration trend over time along the column A at day 15, 30, 45, 60, 75 and 90.

Along column A, the highest values of acetate were observed at the end of the PRB, rather than at the exit of the column. This is probably due because PHB hydrolysis took place in the reactive portion of the column, resulting in the production of acetate, while in the aquifer this was consumed by

microorganisms and presumably used as growth substrate. However, the amount of acids deriving from the PHB was sufficient to sustain an efficient and prolonged dechlorination activity in the column, as better discussed below. Furthermore, it was observed that during the first 15 days the acetate production was higher than the consumption rate, denoting a greater fermentation activity compared to acetate consumption; consequently, no significant increase in pH was observed at this stage (Figure 5). Subsequently, from 30th to 60th day, the acetate consumption kinetics increased resulting in pH increase, as discussed previously. In fact, CH_3COOH^- concentration at day 45 decreased significantly in section 1 and was close to zero in section 2; this behavior was observed also at day 60, with acetate concentration close to zero in both sections. Thereafter, up to day 75, the acetate concentration remained close to zero in both sections but in this case for a zeroing of the fermentation kinetics which caused a reduction in the pH growth trend; this could be due to the inhibition of fermentation process caused by the unfavorable aerobic conditions established in that period inside the column. Finally, at experimental day 90, after optimal conditions for biopolymer fermentation were recovered within the system, a significant increase of acetate production was observed, reaching the maximum concentration of the entire experimental campaign, close to 267 mg l^{-1}.

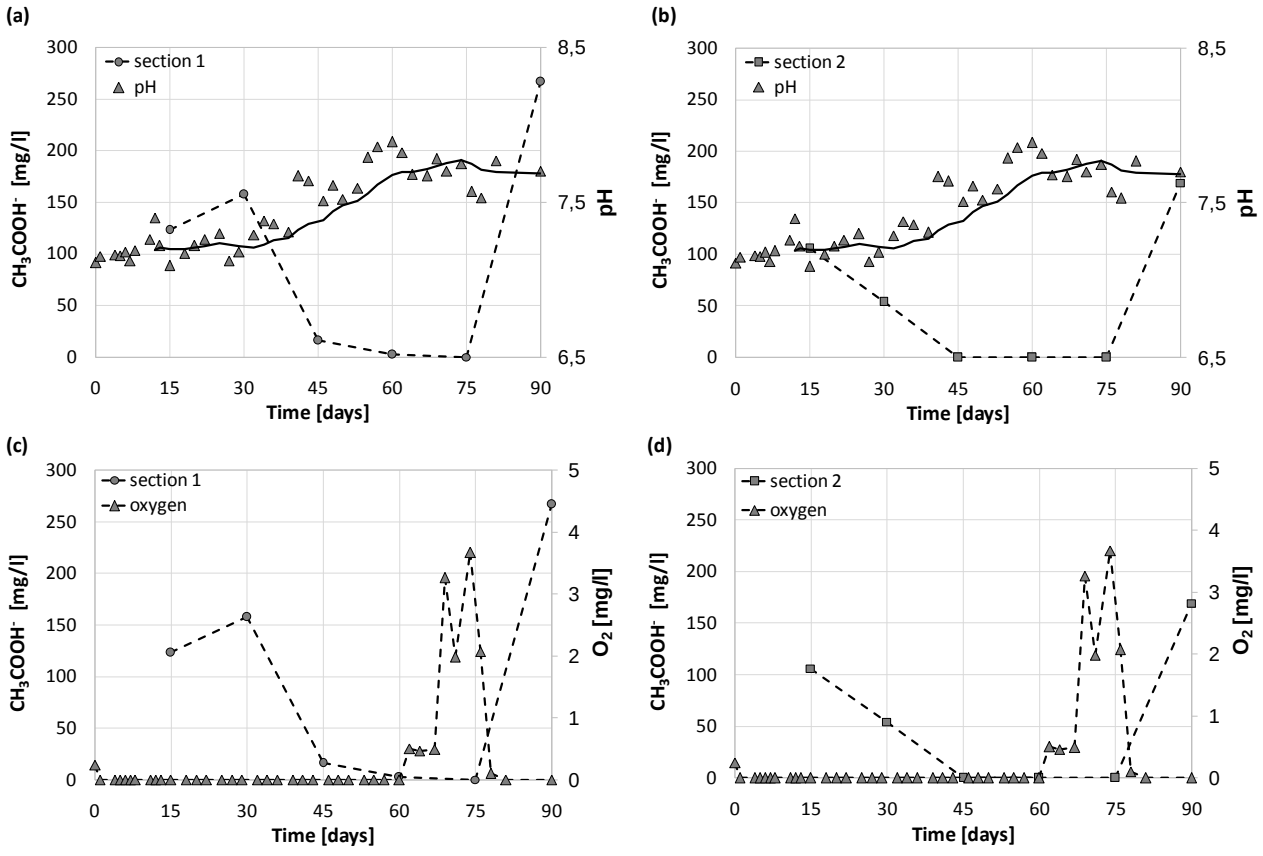


Figure 5. Correlation between pH and acetate in section 1 (a) and 2 (b) and DO and acetate in section 1 (c) and 2 (d) in column A.

3.2.3. 1,2-DCA concentration

Referring to column A, the 1,2-DCA was degraded to a concentration close to the standard limit imposed by Legislative Decree No 152/06 (1,2-DCA = 6 µg l^{-1}; CSC - contamination threshold concentration = 3 µg l^{-1}) on the 60th experimental day, while it was below the instrument detection limit after 75 days of operation. From the observed results, it was confirmed the effectiveness of the anaerobic pathway for

1,2-DCA degradation. On the contrary, in column B, the degradation rate was lower and the 1,2-DCA concentration was significantly higher than the CSC (1,2-DCA = 324 µg l^{-1}). 1,2-DCA degradation in column B was related to the establishment of an aerobic pathway, as it was confirmed by the dissolved oxygen and redox potential values achieved during experiments. However, the aerobic conditions did not allow a complete 1,2-DCA removal. Aerobic degradation of 1,2-DCA has been observed in previous studies (McCarty and Semprini, 1994), in which it has been shown that 1,2-DCA can be degraded both by direct oxidation and by a cometabolic process. Figure 6 shows the 1,2-DCA concentration trend along the two columns.

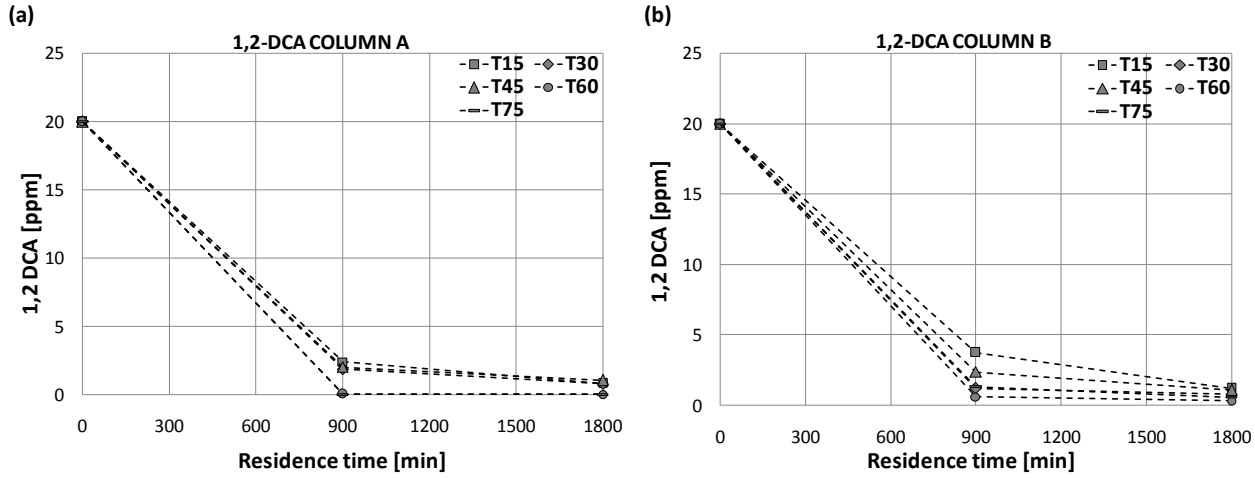


Figure 6. 1,2-DCA concentration trend along column A (a) and B (b).

Figure 7 shows the normalized trend of the effluent 1,2-DCA, for the two columns during the experimental period. In both columns very low values of the normalized 1,2-DCA were found, with a general decreasing trend, confirming a high 1,2-DCA degradation, with removal efficiencies always higher than 90%. In particular, in column A, after 75 experimental days, an almost complete removal of the 1,2-DCA was obtained, with an efficiency therefore close to 100%. The maximum removal efficiency observed in column B, on the other hand, was approximately 98%. From the achieved results, the suggestion is that for the operational conditions under study, the anaerobic pathway enhanced by PHB was more effective compared to the aerobic one.

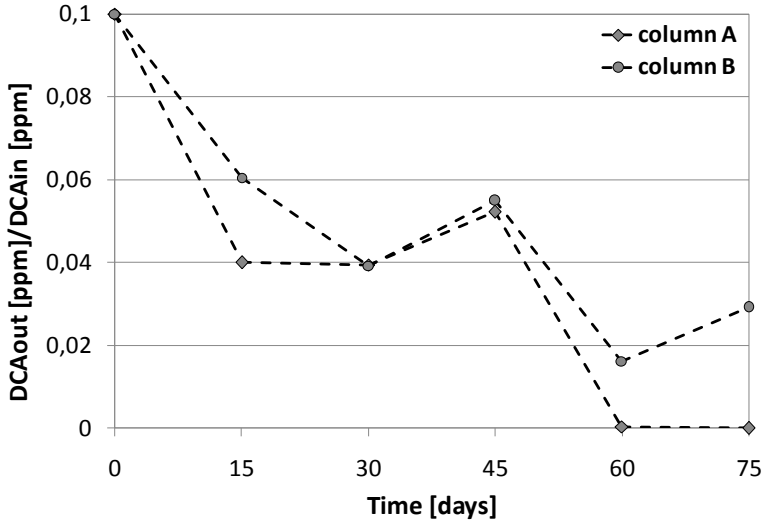


Figure 7. Normalized 1,2-DCA concentration trend over time for column A and B.

3.3. Microbial consortia in the columns

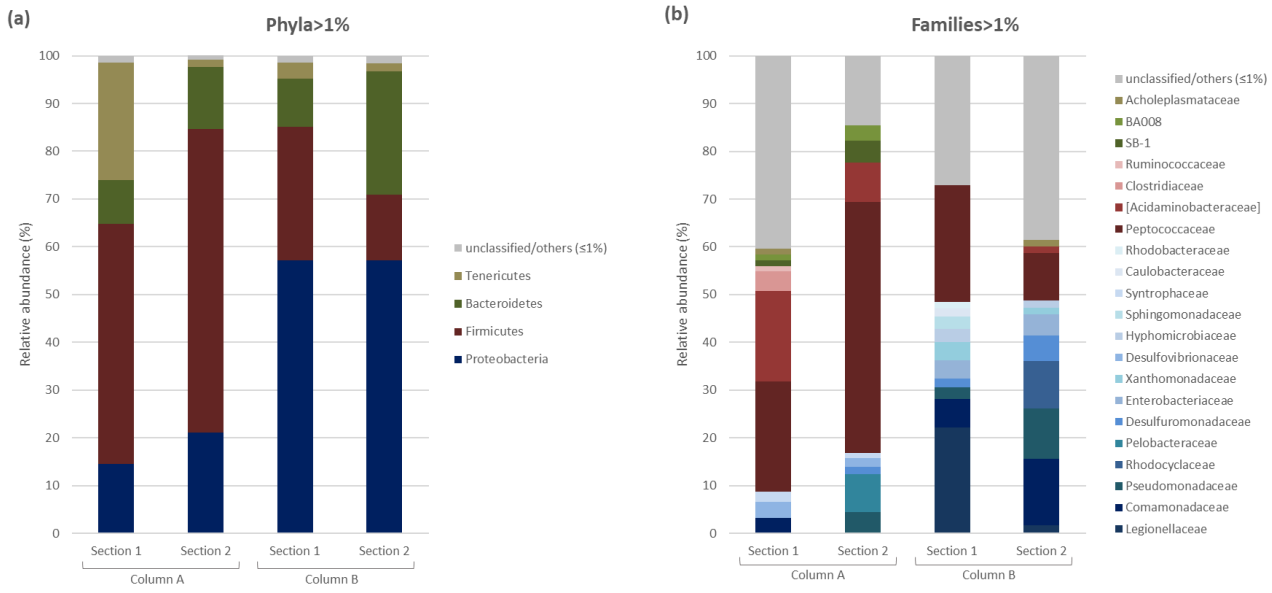
Microbial biomass of the columns was estimated as total double strand extracted DNA (dsDNA). Columns A and B had a similar biomass content that was higher in section 1 in respect to section 2 for both columns (Table 2). Illumina MiSeq 16S rRNA gene sequencing was performed on the metagenomic DNA extracted from the silica sand samples from columns A and B in order to detect and identify the bacterial communities and to verify the presence of microbes involved in BRD and direct or cometabolic oxidation of chlorinated solvents.

The summary of Illumina sequencing output is reported in Table 2. Clustering of sequences at 99% similarity, led to a similar number of operational taxonomic units (OTUs) in the two sections from column A (143 for section 1 and 131 for section 2), and a slightly higher number of OTUs in column B (Table 2). The clusters were taxonomically identified using Green Genes database version 13–8.

The histograms (Figure 8) show the composition of the two microbial consortia at the taxonomic level of phylum (Figure 8a), family (Figure 8b) and genus (Figure 8c). Taxonomic groups with relative abundance greater than 1% were taken into consideration.

Table 2. Microbial biomass of columns and summary of 16S Illumina sequencing output

Column	Section	Microbial biomass (dsDNA) (μg g^{-1}column content)	non-chimeric reads (n)	Bacterial diversity observed_OTUs (99% id.)
A	1	2.3	31640	143
A	2	1.6	29555	131
B	1	2.0	42446	214
B	2	1.8	32309	203



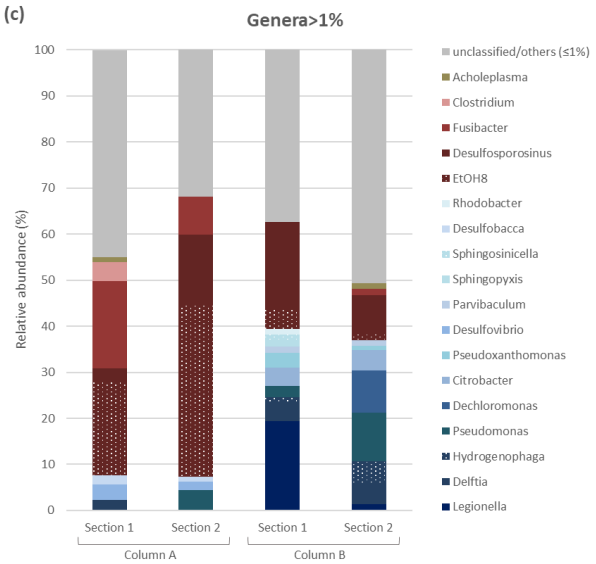


Figure 8. Relative abundances (%) of bacterial phyla (a), families (b) and genera (c) in section 1 and section 2 of columns A and B. Only taxa with relative abundance >1% in at least one sample are represented.

The microbial communities of columns A and B showed quite similar profiles at phylum level, although the relative abundances of the identified taxa varied between the two columns and between sections within each column. The most represented phyla in both columns were Firmicutes and Proteobacteria, followed by Bacteroidetes and Tenericutes. Column A was dominated by Firmicutes (50% and 64%, in section 1 and 2 respectively) followed by Tenericutes (25% and 1%). Conversely, column B was dominated by Proteobacteria (57% in both sections) followed by Firmicutes (28% and 14%).

At family level, column A was enriched in Peptococcaceae and Acidaminobacteraceae (Firmicutes) and to a lesser extent in Pelobacteraceae and Desulfovibrionaceae (Proteobacteria). Members of these families are almost exclusively obligate anaerobes confirming that anaerobic conditions were established throughout column A (Figure 3). Peptococcaceae, that include well known dehalogenating genera (Richardson, 2013), were represented in column A mainly by *Desulfosporosinus*, while Acidaminobacteraceae were represented by the genus *Fusibacter*. Both genera are sulfate and thiosulfate reducers but they have also a fermentative metabolism (Hippe and Stackebrandt, 2015; Ravot et al., 2015). Sulfate reducing bacteria (SRB) are known to be involved in the bioremediation of aromatic and chlorinated hydrocarbons (Dutta et al., 2022) and *Desulfosporosinus* in particular includes PCE-degrading species (Robertson et al., 2001), but the role of *Fusibacter* and *Desulfosporosinus* in dehalogenation of 1,2-DCA is not confirmed. *Fusibacter*, that was the most abundant identified genus in section 1 (19%), was detected also as the most abundant active genus resident in an organohalide polluted groundwater underneath a landfill stimulated with molasses (Bertolini et al., 2021). This genus, in column A could have a role as OHRB and also as fermenting agent responsible for acetate production from the degradation of PHB (Ravot et al., 1999).

The bacterial community of section 2 was enriched in *Desulfosporosinus* (15%) suggesting that this bacterium takes advantage of the presence of acetate, presumably released in section 1. Beyond Peptococcaceae, members of Pseudomonadaceae, Desulfovibrionaceae, Desulfuromonadaceae and Syntrophaceae, although detected in the column at lower abundance, include dehalogenating species (Dolinová et al., 2017; Türkowsky et al., 2018) which could be involved in the biodegradation of 1,2-DCA.

Surprisingly the most well-known OHRB genera within Peptococcaceae (i.e. *Dehalobacter* and *Desulfitobacterium*) were not detected but it is worth to note that a large part of the reads could not be assigned at genus level.

In column B, Proteobacteria were more than 57% and mainly made up of families Legionellaceae and Comamonadaceae in section 1 and Comamonadaceae, Pseudomonadaceae, Rhodocyclaceae and Desulfuromonadaceae in section 2. Firmicutes were mainly made up of Peptococcaceae (including the unidentified phylotype EtOH8) in both sections and included also Acidaminobacteraceae in section 2. The presence of aerobic families such as Legionellaceae and Comamonadaceae was expected. The Legionellaceae are aerobic Gram-negative bacilli that are found mainly in aqueous environments (Edelstein and Lück, 2015); they were probably present in the groundwater used to feed the columns and enriched in column B taking advantage of oxygen.

Beyond aerobic Legionellaceae and Comamonadaceae, column B hosts also facultative and anaerobic bacteria (i.e. Enterobacteriaceae and Peptococcaceae, respectively) suggesting that micro oxygen depletion zones can be formed inside the column. As above discussed, oxygen concentration in column B showed a slight decrease during the experiments from 8 to almost 5 mg l^{-1} thus suggesting oxygen consumption during microbial growth (Figure 3). Anaerobic Peptococcaceae were abundant in both sections of column B (although less abundant in section 2) and were mainly represented by SRB *Desulfosporosinus*, already described for column A. In addition, column B hosted also other families including known degraders of chlorinated solvents such as Comamonadaceae (6% in section 1 and 14% in section 2), Desulfuromonadaceae (1.8% and 5.4%) and Pseudomonadaceae (2.5% and 10.6%) (Dolinová et al., 2017; Bertolini et al., 2021). Within Pseudomonadaceae, members of the genus *Pseudomonas*, highly represented in column B (2.5% and 10.5%, in section 1 and 2 respectively), are known hydrocarbon degraders that are able to carry out cometabolic oxidation of chlorinated solvents (Alvarez-Cohen and Speitel, 2001). Within Comamonadaceae, the genus *Delftia* (4.2% and 4.5%) includes members that have been reported in anoxic-aerobic environments, where they could degrade halogenated aromatic compounds (Zhang et al., 2019).

4. Conclusions

From the results obtained for column A, it appeared that the powder PHB was effectively hydrolyzed and used as a substrate by autochtonous microorganisms that converted it into acetate, which accumulated up to concentrations of 267 mg l^{-1} after 90 days. The fast fermentation was determined not only by the favorable conditions established within the system, but also by the high specific surface of the biopolymer itself. This shows that poly-β-hydroxybutyrate in powder form can be effectively used to support a long-term reductive dechlorination process as it ensures long-term electron donor release, allowing the almost complete removal of the chlorinated compound. It was also observed that in the presence of PHB fermentation products the consortium was able to completely degrade 1,2-DCA. The microbial consortia established in column A and B derived from a 1,2-DCA dehalogenating mixed microbial communities obtained from a chlorinated solvents contaminated groundwater after enrichment in the presence of 1,2-DCA, with the addition of PHB under anaerobic conditions (column A) or under aerobic conditions (column B). The columns were fed with the same groundwater used for the enrichment but the composition of the two final communities was different as expected. This difference could only depend on PHB presence as well as the physical-chemical conditions of the two columns, referring in particular to dissolved oxygen concentrations. Anaerobic conditions in the presence of powder PHB favored the enrichment of fermentative/sulfate reducing bacteria in column A. The degradation of powder PHB was evidenced by acetate formation and also by microbial biomass proliferation. Alternate anoxic/aerobic conditions in column B enriched the consortium with a less

abundant and more diverse microbial community, composed of aerobic chemoorganotrophs and fermentative/sulfate reducing bacteria. Although the most frequently detected 1,2-DCA dehalogenating genera (such as *Dehalococcoides*, *Dehalogenimonas*, *Dehalobacter* and *Desulfitobacterium*) were not found, authors suggest 1,2-DCA was removed by members of the two consortia, mainly by reductive dehalogenation in column A enhanced by fermentation products from powder PHB degradation, and aerobic (co)-metabolism in column B.

In general, the experimental activity results confirm that biological processes can be effectively applied for the remediation of 1,2-DCA contaminated aquifers. Furthermore, this study confirmed the possibility to use a completely biodegradable polymer to stimulate biological reductive dechlorination, thus promoting the application of an environmentally sustainable remediation technique, with acetate production strongly affected by PHB form, significantly influencing the effectiveness of BRD process.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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