Article

Effectiveness of Permeable Reactive Bio-Barriers for Bioremediation of an Organohalide-Polluted Aquifer by Natural-Occurring Microbial Community

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Abstract: In this study, a bioremediation approach was evaluated for the decontamination of an aquifer affected by the release of organohalides by an industrial landfill. After preliminary physico-chemical and microbiological characterization of the landfill groundwater, the stimulation of natural organohalide respiration by the addition of a reducing substrate (i.e., molasse) was tested both at microcosm and at field scales, by the placement of an anaerobic permeable reactive bio-barrier. Illumina sequencing of cDNA 16S rRNA gene revealed that organohalide-respiring bacteria of genera *Geobacter*, *Sulfurospirillum*, *Dehalococcoides*, *Clostridium* and *Shewanella* were present within the aquifer microbial community, along with fermentative *Firmicutes* and *Parvarchaeota*. Microcosm experiments confirmed the presence of an active natural attenuation, which was boosted by the addition of the reducing substrate. Field tests showed that the bio-barrier decreased the concentration of chloroethenes at a rate of 23.74 kg d^{-1}. Monitoring of organohalide respiration biomarkers by qPCR and Illumina sequencing revealed that native microbial populations were involved in the dechlorination process, although their specific role still needs to be clarified. The accumulation of lower-chloroethenes suggested the need of future improvement of the present approach by supporting bacterial vinyl-chloride oxidation, to achieve a complete degradation of chloroethenes.

Keywords: microbial bioremediation; organohalide respiration; permeable reactive bio-barrier; microcosms; reductive dehalogenases; *Chloroflexi*

1. Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) are widely used in industrial activities as solvents for waxes, resins, fats, rubbers, oils and in metal degreasing. They can also be found in different household products as paint and dry-cleaning products. Because of their intensive use, they are among the prevalent contaminant compounds all over the world. Their presence in the environment is mainly due to their inadequate disposal [1,2]. PCE is toxic for humans, but the metabolites resulting from its degradation, which are TCE, 1,1- and 1,2(*cis-trans*)-dichloroethene (DCE) and vinyl chloride (VC), are even more toxic [3,4]. In particular, VC and TCE are carcinogenic, and they have been included in group 1 by the International Agency for Research on Cancer (IARC) [5]. PCE and TCE form a dense non-aqueous phase liquid (D-NAPL) with higher density with respect to water. Penetrating through permeable groundwater aquifers, D-NAPL causes the formation of contamination plumes.

Chloroethenes (CE) can be degraded under both anaerobic (organohalide respiration, OHR) and aerobic (metabolic and co-metabolic oxidation) conditions. High chlorinated ethenes are easily dechlorinated under anaerobic conditions with the consequent accumulation of DCE and VC in the distal part of contaminated plumes. Conversely, these two compounds are oxidized more efficiently under aerobic conditions [6].

Only few bacterial genera are known to perform anaerobic OHR (i.e., organohalide-respiring bacteria, OHRB), including the obligate organohalide-reducers *Dehalococcoides* and *Dehalogenimonas* (phylum *Chloroflexi*) *Geobacter*, sulfate-reducing bacteria as *Desulfomonile* and *Desulfuromonas* (class Deltaproteobacteria), *Desulfitobacterium* and *Dehalobacter* (phylum Firmicutes) and *Sulfurospirillum* (phylum *Campylobacterota*, formerly *Epsilonproteobacteria*) [7–9]. For complete biodegradation of CE, different bacterial consortia are involved in different steps of the degradation pathways [10]. The genes involved in OHR are reductive dehalogenase-homologous genes (*rdh*). Among these, tetrachloroethene reductive dehalogenase (*pceA*) is involved in the reduction of PCE to TCE [11], trichloroethene reductive dehalogenase (*tceA*) catalyzes TCE reduction to DCE or of DCE to VC [12], vinyl chloride reductase (*bvcA*) reduces DCE to VC and VC to ethene [13] and vinyl chloride reductase (*vcrA*) catalyzes the dechlorination of VC to ethene [14].

During OHR, CE are used as electron acceptors and each chlorine atom is replaced by one hydrogen atom, which derives from the electron donor H_2 [15]. Reducing power and carbon sources are produced during the fermentation of organic compounds which are converted to H_2, CO_2 and small organic acids by fermentative microorganisms (i.e., *Parvarchaeota*) [16] in anaerobic environment such as groundwater. Lactic and butyric acid deriving from the fermentation of organic compounds can also be used as electron donors by OHRB [17,18]. In the anaerobic section of contaminated aquifers, although OHRB are favored by a low concentration of hydrogen, they compete with methanogens, acetogens and sulfate-reducing bacteria for the use of hydrogen [19–21].

In order to remediate the sites affected by CE contamination, bioremediation has been demonstrated to be an appealing approach in terms of both costs and environmental impact compared to physicochemical treatments [22]. In order to improve OHR, organic compounds (small organic acids) are added to contaminated aquifers to promote microbial fermentations, which in turn increase the reducing power available to OHRB.

To ameliorate environmental and economic sustainability of bioremediation actions, fermented biomass deriving from wastes of agri-food industry (such as molasses from sugar beet) are evaluated as possible substrates to produce reducing power to OHRB in anaerobic aquifers, via the use of permeable reactive bio-barrier technology. Moreover, this in situ technology avoids the production of contaminated landfilled byproducts. Permeable reactive bio-barrier technology is considered cheaper than permeable reactive barrier, and pump and treat methods. According to Battelle [23], the respective final costs for 1000 L of treated groundwater were: 0.3 $ for permeable reactive bio-barrier, 1.1 $ for permeable reactive barrier and 2.06 $ for pump and treat method.

Although OHR is very well studied at the laboratory scale, in field analyses aimed at demonstrating the role of OHRB in reductive dehalogenation and the effect of bio-stimulation interventions at contaminated sites are poorly investigated [24].

In this study, an aquifer affected by CE contamination due to the leaching of a landfill hosting petrochemical wastes was considered. In order to determine the feasibility of a bioremediation intervention consisting of the injection of molasse into permeable reactive bio-barriers, laboratory based-microcosms and in situ OHR responsiveness were assessed. To evaluate the effectiveness of the treatment, the microbial community inhabiting the aquifer was analyzed using environmental genomics over a two-year bio-stimulation.

2. Materials and Methods

2.1. Site Description and Pilot Scale Experiment

The study area (17 ha) is located in Italy, next to a former industrial waste disposal site (16 hectares with a total waste mass of 1,700,000 tons). The area is surrounded by a brackish

lagoon to the South-Eastern side, and it lies 3 m below the sea level. A drainage channel, bordering the study area to the North-Western side, drains waters from agricultural fields.

The groundwater beneath the landfill is affected by multiple contaminations (chlorinated compounds, petroleum hydrocarbons and BTEX, arsenic and heavy metals) released by the break of a clay lent underneath the industrial landfill. During the past years, a contaminated groundwater plume has been released at -10 m below ground level, flowing from the South-Eastern to the North-Western side of the area. Since 1995, the aquifer was secured by an hydraulic barrier functioning with pump and treat technology.

Due to high operation costs, low efficiency and sustainability of the approach, the feasibility of an in situ bioremediation technology based on microbial OHR process was evaluated at laboratory and at pilot field scales. Pilot plant (Figure 1) was constituted by a permeable reactive bio-barrier for addition of a reducing substrate directly into the aquifer. According to the flow direction of the aquifer, the contaminated plume runs from piezometers Pz22 and Pz25 (located at the landfill), through piezometers Pz13 and Pz16 upstream the permeable reactive bio-barrier, and through Pz10 and Pz3 downstream the permeable reactive bio-barrier, respectively.
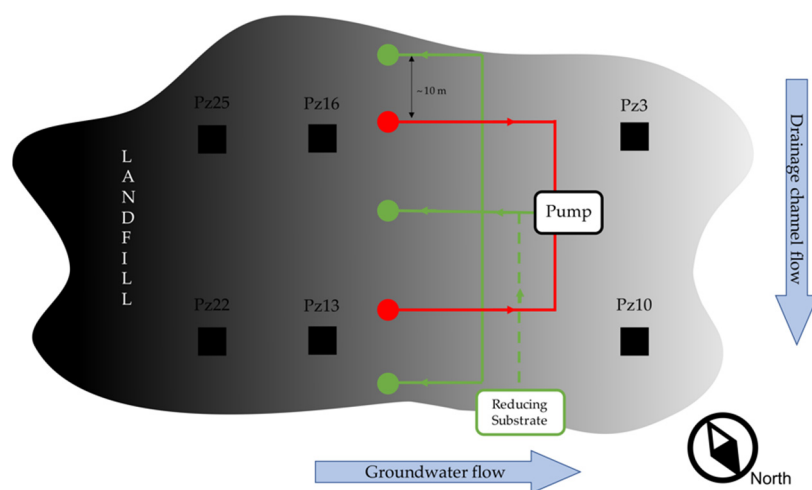

Figure 1. Position of the monitoring piezometers near the permeable reactive bio-barrier. Red lines indicate the extraction wells, whereas green lines indicate the injection wells.

The reducing substrate was an engineered molasse derived from fermentation of vegetable (i.e., *Saccharum Officinarum* L., *Gramineae*, *Beta vulgaris* L. and *Chenopodiaceae*) waste and it was added at a concentration of 1.8 mL L^{-1}. It is a viscous liquid (density 1300 kg m^{-3} and dynamic viscosity of 1500 mPa s, pH 5), with total COD of 494.5 g L^{-1}, nitrogen content of 3.8 g L^{-1} and total residue of 77 wt.% including sodium, potassium and magnesium.

In the present study, groundwater samples were collected over a 20 month-time frame from two series of piezometers forming two different transects of the aquifer: Pz22-Pz13-Pz10 and Pz25-Pz16-Pz3 (Figure 1).

2.2. Microcosm Experiments

The OHR potential of the native groundwater microbial community and its stimulation by the addition of the reducing substrate were determined in microcosm experiments. Groundwater samples from piezometers Pz13, Pz16, Pz10 and Pz3 were placed in glass bottles (1 L) completely filled in order to avoid oxygenation. Bottles were brought to laboratory in cooler bags and stored in the dark at 4 °C until use.

Three conditions were considered: abiotic control, native groundwater (GW) and reducing substrate-supplemented groundwater (GW-RS). Serum bottles (100 mL) were added with 50 mL of groundwater sample, reducing substrate (1.8 mL L^{-1}, *v/v*) when appropriate, and resazurin (0.1%, *w/v*), under anaerobic chamber (N_2:CO_2:H_2, 85:10:5%,

v/v) (Forma Scientific, Marietta, USA). Abiotic controls were prepared with groundwater autoclaved 3 times with 72 h-intervals at 22 °C. Serum bottles were sealed with butyl rubber septa with aluminum crimps, under anaerobic cabinet. Microcosms were set up in triplicate and incubated at 20 °C in static conditions.

2.3. Chemical Methods

In microcosm experiments, CE were quantified by gas-chromatography mass spectrometry (GC-MS) after 6 and 12 month-incubation. The analysis of volatiles compounds was conducted with GC 7890A gas chromatograph associated to 5975C mass spectrometer (Agilent, Palo Alto, CA, USA).

Water samples (1 mL) were placed in GC headspace 10 mL vial (Agilent Technologies, Santa Clara, CA, USA), which was rapidly sealed with a magnetic screw cap equipped with silicon/polytetrafluoroethylene septa (PTFE). Headspace (HS) volatile compounds were collected using HS syringe. Injection volume 1500 μL with HS syringe installed on CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA). Chromatography was performed on a Porabond Q (25 m × 0.32 mm × 5 μm) column (Agilent Technologies, Santa Clara, CA, USA) with helium as carrier gas at a constant flow of 3.0 mL min^{-1} with a split ratio of 3:1 and a split flow of 7.5 mL. GC interface, MS source and quad temperatures were 260 °C, 230 °C and 150 °C, respectively. Oven temperature conditions were 42 °C for 2 min, then 10 °C min^{-1} ramp until 260 °C and held at 260 °C for 7 min. Mass spectra were recorded in scan mode in the 10 to 200 mass-to-charge ratio range by a 5975B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) at an ionization energy of 70 eV and a scanning speed of 7 scans s^{-1}. Chromatograms and spectra were recorded and processed using the Enhanced ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Ethenes relative concentration (%) was calculated referring the chromatographic area of the specific compound to the total chromatographic area obtained for all the compounds detected in sample. All the analyses were performed in triplicate.

At the pilot-scale field side, CE were quantified by GC-MS every four months over a 20 month-time frame in piezometers upstream (Pz13 and Pz16) and downstream (Pz10, and Pz3) the permeable reactive bio-barrier. Groundwater was sampled following equilibrium based static headspace preparation (EPA5021A 2014 method). The analyses were outsourced (AGROLAB Group, Altavilla Vicentina, Italy) and performed in triplicate by using GC-MS method for volatile organic compounds (EPA8260D 2018).

2.4. Nucleic Acid Extraction from Groundwater Samples

For nucleic acid extraction, groundwater was sampled (20–60 L) every four months over a two-year time, from two piezometers within the landfill (Pz22 and Pz25) and from the piezometers upstream (Pz13 and Pz16) and downstream (Pz10 and Pz3) the permeable reactive bio-barrier. The samples were collected into sodium hypochlorite-washed polyethylene containers, brought to laboratory in cooler bags and stored in the dark at 4 °C until use.

From each water sample, the biomass was filtered onto mixed cellulose/ester filters (MediaKap™ ME2M-050-18S ∅ 0.2 μm) (Cole-Parmer, Vernon Hills, IL, USA) using a peristaltic pump apparatus (Masterflex L/S Economy Variable-Speed Drive, 20 to 600 rpm with Masterflex L/S Easy-Load Head for High-Performance Tubing, PSF/CRS) (Cole-Parmer, Vernon Hills, IL, USA). Filters were stored at −20 °C until processing. The biomass was removed from the filters by phosphate-buffered saline (PBS) solution (0.1 M, pH 7.2) washings and subsequent centrifugation (10,000 rpm for 5 min at 10 °C). From all sampling points, triplicate samples were obtained and all downstream analyses were conducted in triplicate.

DNA was isolated with DNA PowerSoil® Isolation kit (Qiagen, Hilden, Germany) from all samples, and quantified with Spectrophotometer Power Wase XS2 (BioTEK Instruments, US). RNA was extracted using RNA Power Soil® Total RNA Isolation kit (Qiagen, Hilden, Germany) from groundwater sampled from landfill Pz22. Residual genomic DNA

was removed with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) (1 U µg^{-1} of RNA) according to the following protocol: 30 min-incubation at 37 °C, EDTA 0.5 M addition (4 µL) followed by 10 min-incubation at 65 °C. RNA was reverse-transcribed with iScript^{TM} cDNA Synthesis Kit (BIO-RAD) according to the manufacturer's protocol. Agarose gel electrophoresis and full-length 16S rRNA genes PCR amplification of non-retro-transcribed RNA (protocol in SM) were used to determine the purity of RNA extractions.

2.5. Real Time Quantitative PCR

16S rRNA gene copy number of Bacteria, Archaea, *Geobacteraceae* (*Geo*), and *Dehalococcoides* (*Dhc*), of trichloroethylene reductase gene (*tceA*) and vinyl chloride reductase gene (*vcrA*) were quantified by real time quantitative PCR (qPCR). The primer sets used for each target gene are described in Table S1. The thermal protocol for bacterial 16S rRNA gene was applied according to Fierer et al. (2005) [25]. The thermal protocol for archaeal 16S rRNA gene was: initial denaturation for 15 min at 95 °C, 40 cycles of 1 min at 95 °C, 30 s at 60 °C and 1 min at 72 °C. Melting curve was set from 60 °C to 95 °C with an increment of 1.6 °C s^{-1} for 5 s. The thermal protocol for *Dhc*, *tceA* and *vcrA* genes was: initial denaturation for 15 min at 95 °C, 40 cycles of 1 min at 95 °C, 40 s at 58 °C and 40 s at 72 °C. Melting curve was set from 58 °C to 95 °C with an increment of 1.6 °C s^{-1} for 1 min. Each reaction mixture contained 1× of Titan HotTaq Probe qPCR Mix (Bioatlas Science of Life, Estonia), 0.25 µM of forward and reverse primers, 10 ng of DNA and PCR-grade water (AppliChem, Darmstadt, Germany) to a final volume of 20 µL. Standard curves were set up through amplification of plasmids carrying the insert of each target gene (Table S2).

2.6. Illumina MiSeq Sequencing of 16S rRNA Genes

Illumina MiSeq sequencing of the V3-V4 region of 16S cDNA (Bacteria and Archaea) was used to characterize the active microbial community present within the landfill (Pz22) and in one piezometer (Pz16) at the beginning of the bioremediation intervention and after 20 months. Sequencing was performed at the Research Resources Center (RRC, University of Illinois, Chicago-USA) using the primer pairs CS1341F/CS2806R and CS1ARC344F/CS2ARC806R for Bacteria and Archaea, respectively, as described previously [26].

Demultiplexed and trim sequences were processed with QIIME2 [27]. Forward and reverse reads were merged with vsearch [28] and sequences were denoised with DADA2 [29]. Representative Amplicon Sequence Variants (ASVs) were selected by setting a 100% sequence identity [30] and aligned to the GreenGenes database version (gg128 version) (http://greengenes.lbl.gov, accessed on 16 June 2020) for taxonomy assignment.

Bacteria genera putatively directly or indirectly involved in OHR were retrieved according to Adrian and Löffler 2016 [22]. Fermentative bacterial and archaeal taxa were inferred according to the literature [31–34].

The sequences obtained in this study were deposited in GeneBank within the PRJNA744480 Bioproject (https://www.ncbi.nlm.nih.gov/sra/PRJNA744480, accessed on on 15 May 2021) and in the Dataverse repository (https://dataverse.unimi.it/dataverse/INAIL-ID52, accessed on on 15 May 2021).

2.7. Statistical Analyses

All statistical analyses were performed using base and accessory packages of the R program v. 4.0.3 [35]. One way analysis of variance (ANOVA) [36] and Tukey's b test were applied to determine significant differences ($p \leq 0.05$) in: 1) the chemical parameters measured in the different treatments and incubation times in the CE degradation microcosms, and 2) the markers genes quantified with qPCR among different piezometers and over time. Pearson correlation coefficient ($p \leq 0.05$) was calculated to determine any significant correlation between the quantification of OHR gene targets and the physicochemical parameters.

To highlight the phyla retrieved with Illumina sequencing that were significantly different in the two sampling points, *t*-test ($p \leq 0.05$) was used. To further investigate genera that are significantly different over time, differential abundance was calculated through quantile-adjusted conditional maximum likelihood (qCML) with EdgeR package [37,38]. Common dispersion and tagwise of sequences were determined [39], and pairwise comparisons were performed at $p \leq 0.05$ [40].

3. Results

3.1. Landfill Groundwater: Chemical Characterization and OHR Biomarkers

The aquifer was a reducing environment, with neutral pH and oxygen concentrations below 1.5 mg L^{-1} in Pz22 and below 1 mg L^{-1} in Pz25, suggesting that the system was nearly anoxic.

All CE were present in the landfill (Pz22 and Pz25) with concentrations of 3–6 orders of magnitude higher than European law limits (Directive 2000/60/EC) (Table 1). In particular, VC concentrations were 72,000.00 µg L^{-1} and 6200 µg L^{-1} in Pz22 and Pz25, respectively, with a law limit of 0.5 µg L^{-1}.

Table 1. Chemical parameters and OHR biomarkers in landfill piezometers at the beginning of the pilot scale experiment.

Parameter/Target	Unit	Law Limits Directive 2000/60/EC	Pz22	St. Dv.	Pz25	St. Dv.
PCE	µg L^{-1}	1.1	7300.00	802.1	2860.00	622.3
TCE	µg L^{-1}	1.5	43,000.00	15,143.8	7300.00	1850.2
1,1 DCE	µg L^{-1}	0.05	22,800.00	16,607.9	1370.00	640.9
1,2 DCE	µg L^{-1}	60	17,000.00	6455.0	5640.00	1683.6
VC	µg L^{-1}	0.5	72,000.00	62,649.8	6200.00	4346.6
ethene	µg L^{-1}	-	19,800.00	282.8	980.00	195.2
Eh *	mV	-	−126.00		−85.00	
pH		-	7.4		7.5	
DO **	mg L^{-1}	-	1.20		0.9	
Eub	log(gene copies L^{-1})	-	9.42 ± 0.08		9.79 ± 0.12	
Arc	log(gene copies L^{-1})	-	5.70 ± 0.06		7.27 ± 0.15	
Geo	log(gene copies L^{-1})	-	3.34 ± 0.21		3.89 ± 0.05	
Dhc	log(gene copies L^{-1})	-	6.42 ± 0.02		5.17 ± 0.11	
tcrA	log(gene copies L^{-1})	-	7.80 ± 0.10		7.05 ± 0.14	
vcrA	log(gene copies L^{-1})	-	6.77 ± 0.05		6.01 ± 0.04	

* Eh = Redox potential. ** DO = dissolved oxygen.

OHR biomarkers (*Dehalococcoides* 16S rRNA genes, *tceA* and *vcrA*) were present in the landfill in the range of 10^5 and 10^7 gene copies L^{-1} at DNA level, and of 10^3 and 10^6 transcript copies L^{-1} (at RNA level). *Geobacteraceae* 16S rRNA genes were present in the order of 10^3 gene copies L^{-1}.

3.2. Active Microbial Community of Landfill Groundwater

Illumina MiSeq 16S rRNA cDNA sequencing was performed on groundwater sampled from piezometer Pz22, in order to detect metabolically active Bacteria and Archaea communities and to verify the presence of microbes involved in OHR.

The active bacterial community was dominated by *Proteobacteria* and *Firmicutes* (49.14% and 39.85%, respectively) (Figure 2), with all bacterial genera with a relative abundance above 1% belonging to these two phyla. Within the *Firmicutes*, *Fusibacter* was dominant with a relative abundance of 31.66%, followed by *Sedimentibacter* and *Coprococcus* (1.15% and 4.81%, respectively). Within the *Proteobacteria*, *Xanthomonadaceae* and *Caulobacteraceae* were the most abundant (8.23 and 7.96%, respectively), followed by *Mycoplana* and *Thermomonas* at 2%, and *Alcaligenaceae* and *Phyllobacteriaceae* at 1.5%. *Actinobacteria*, *Bacteroidetes* and *Chloroflexi* were present with lower relative abundances (3.91%, 2.48% and 2.69%, re-

spectively). Inferred functionality analyses showed that active OHR bacteria in the landfill were dominated by *Shewanella* that accounted for the 11.39% of the total Bacteria, while other OHRB were below 1% (Figure 2).
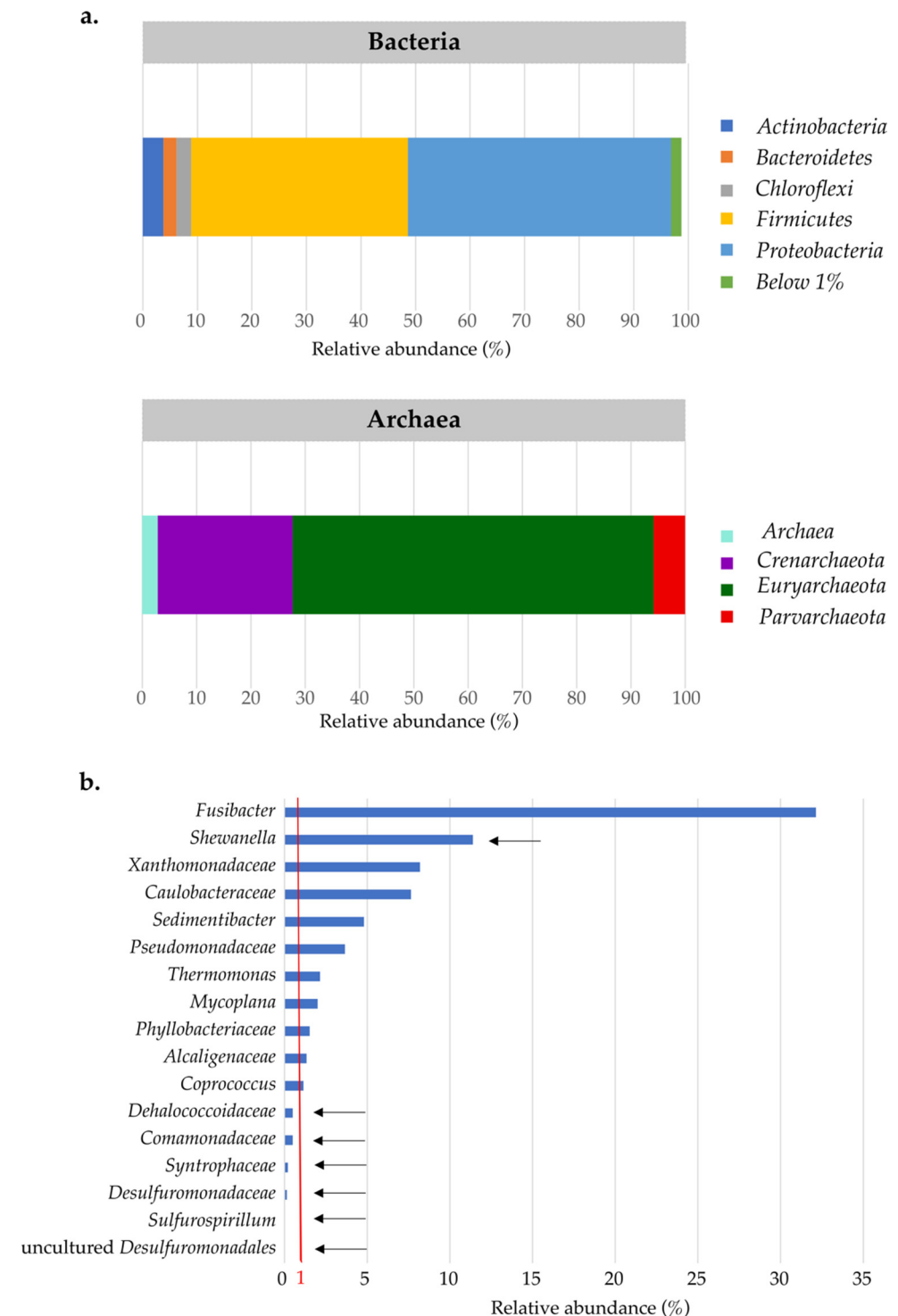

Figure 2. Composition of the active microbial community resident in groundwater underneath the landfill sampled at Pz22, as determined by RNA-based Illumina sequencing of 16S rRNA of Archaea and Bacteria at phylum level (**a**). Active bacterial genera present in Pz22 either with a relative abundance above 1% or that are inferred as OHRB according to the literature (**b**); arrows indicate OHRB genera or family.

Euryarchaeota was the main component of the active archaeal community (66%) (Figure 2), with 60.14% belonging to the *Methanomicrobia* class and 3.35% to the *Methanobacteria*. *Crenarchaeota* and *Parvarchaeota* accounted for the 24.1% and the 5.77% of the total.

3.3. Determination of Natural OHR in the Aquifer by Microcosm Incubations

Anaerobic microcosms were set up with groundwaters sampled from different piezometers in order to determine the ability of indigenous microbial communities to perform reductive dehalogenation of CE, gaining information on natural attenuation processes at the site. Moreover, the effect of the addition of a reducing substrate was tested, with the aim to determine the possibility of accelerating the bioremediation process in the aquifer.

GC-MS analyses at time zero showed that in Pz16 and Pz3 microcosms the concentration of highly CE (PCE and TCE) was significantly higher than in Pz13 and Pz10, which, conversely, displayed higher concentrations of low CE (DCE and VC) (Figure 3, $p < 0.05$ in Figure S1 in supplementary material). In accordance with the initial field monitoring, the distribution of different chlorinated solvents at the site was not homogeneous, evidencing that groundwaters from Pz16 and Pz3 derived from the proximity of the contamination source, whereas those in Pz13 and Pz10 were at the plume fringe.
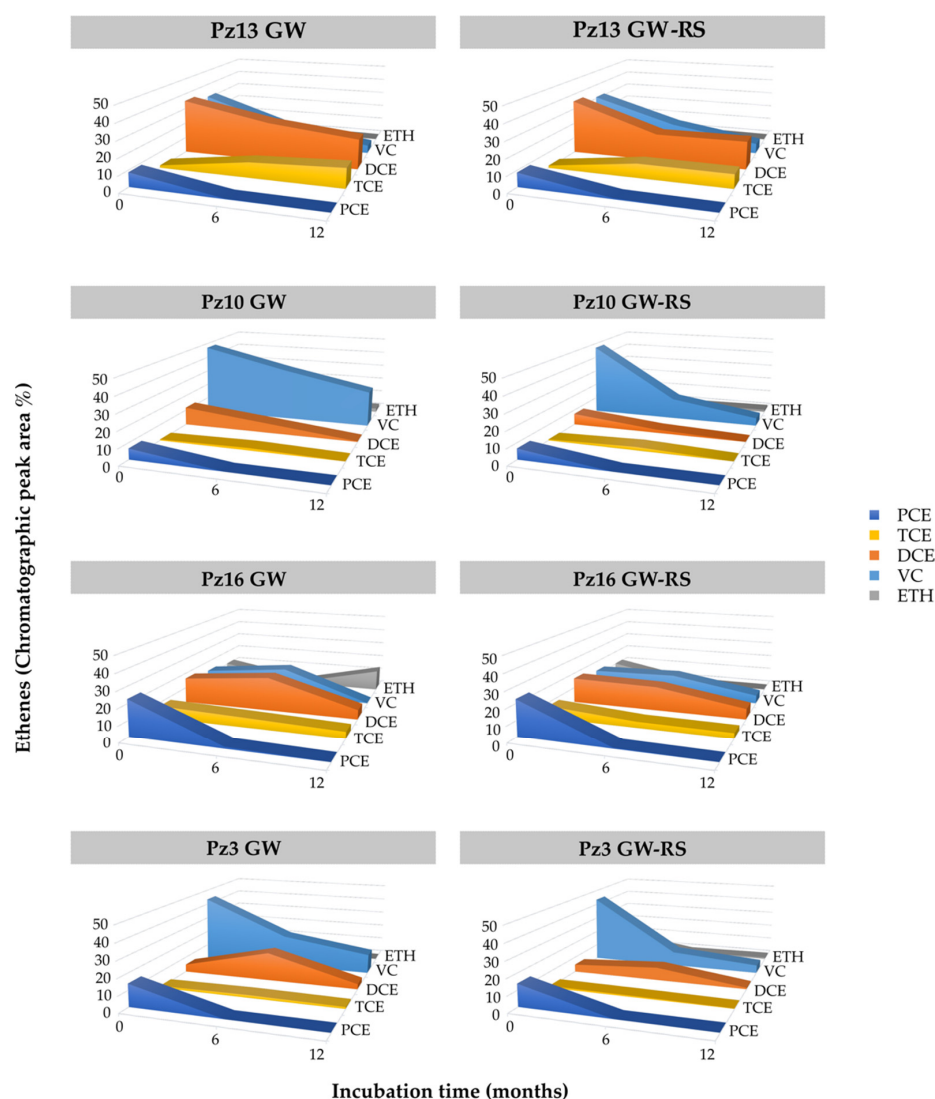

Figure 3. Relative abundance of CE in the microcosms with groundwater sampled from Pz13, Pz10, Pz16 and Pz3 incubated for 6 and 12 months without and with reducing substrate (GW and GW-RS, respectively).

Incubations with groundwaters sampled from transect Pz13-Pz10 started with a lower concentration of PCE and TCE, but higher concentration of DCE and VC, if compared with transect Pz16-Pz3. After 6 months of incubation, PCE was completely degraded in all microcosms, including the most contaminated sample Pz16 (Figure 3). Over time, all piezometers showed a different pattern of degradation. In fact, while in Pz16 TCE slightly decreased with the concomitant formation of DCE, in Pz13 TCE accumulated without further dechlorination to DCE. In Pz16 and Pz3 TCE was degraded completely and DCE and VC were degraded more efficiently than in Pz13-Pz10. The best performing incubation was observed in Pz16, where VC was completely degraded to ethene. These data point out that OHR was present and active in the native microbial communities in all sampling points, although not homogeneously distributed at the site.

The addition of molasse as reducing substrate had different effects on OHR in the different microcosms. In Pz16, it increased the degradation of TCE, DCE and VC after 6 months incubation, although an accumulation of 5% VC was observed after 12 months. In Pz13, the addition of the substrate promoted the degradation of TCE (12.45% and 8.8% without and with substrate addition, respectively) and to a lesser extent that of DCE (19% to 17.9% without and with substrate addition, respectively). VC degradation in Pz13 microcosm was not affected by substrate addition. In Pz10 and Pz3 microcosms, the addition of the substrate increased the degradation of TCE, DCE and VC already after 6 months incubation. After 12 months, substrate amendment significantly increased VC degradation in Pz10 and Pz3 augmented microcosm (23% and 12.4% vs. 5.12% and 5.17%, without and with substrate, respectively) (Figure 3, $p < 0.05$ in Figure S2 in supplementary material).

In sterilized microcosms, a negligible decrease of all CE was observed (data not shown).

3.4. CE Field Monitoring in Pilot-Scale Experiment

In light of the characterization of indigenous microbial community and of the results obtained with microcosm experiments, a permeable reactive bio-barrier was installed one year after, and groundwater was enriched with the reducing substrate. Over the pilot-scale experiment, all groundwaters showed highly reduced conditions, with negative E_h values ranging from -68 in Pz16 to -309 in Pz3 (Figure S2). Dissolved oxygen decreased to values below 1 mg L^{-1} in all sampling points and pH ranged between 6.5 to 7.5.

At the time zero, PCE, TCE and 1,1- and 1,2-DCE concentrations were higher in piezometers Pz16 and Pz13 upstream the permeable reactive bio-barrier with respect to the corresponding downstream piezometers Pz3 and Pz10 (Figure S3).

Over the 20 month-field monitoring, CE degradation/accumulation by means of ratio between final and initial concentration of each compound was different in the different areas of the aquifer (Figure 4). PCE and TCE were degraded in all piezometers except Pz13, whereas DCE was always degraded, although to a different extent.
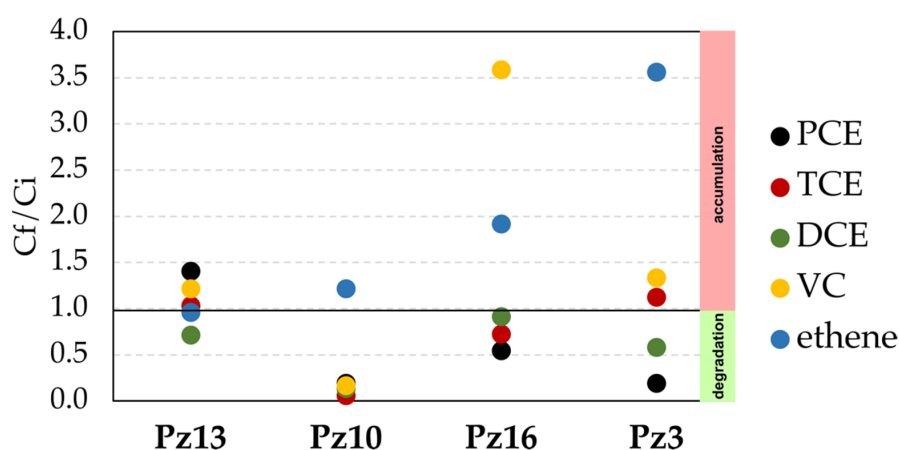

Figure 4. In situ CE degradation by means of ratio between final and initial concentration of CE in the groundwater sampled from piezometers Pz13, Pz10, Pz16 and Pz3.

The dynamics of VC showed completely different patterns in the different piezometers. In fact, while only in Pz10 a complete VC degradation could be observed, in all other piezometers this compound was accumulated, with the higher accumulation observed in Pz16. Ethene degradation was never observed. Quite the opposite, ethene concentration increased in all downstream piezometers (Pz10 and Pz3) and in Pz16.

The degradation rate of total CE from the beginning to the end of the experiment was of 23.74 kg day^{-1}.

3.5. Field Monitoring of Microbial Populations Involved in OHR

At time zero in all the four piezometers, total bacterial and archaeal 16S rRNA genes ranged from 10^8 to 10^{11} and from 10^2 to 10^7 gene copies L^{-1}, respectively (Figure S3). Members of the family *Geobacteraceae* and *Dehalococcoides* accounted for 10^2 to 10^6 copies of 16S rRNA genes L^{-1}. Genes involved in the reductive dehalogenation of TCE and DCE (*tceA*) and of VC (*vcrA*) varied between 10^4 to 10^7 copies L^{-1}, with *tceA* being more abundant than *vcrA* (Figure 5).
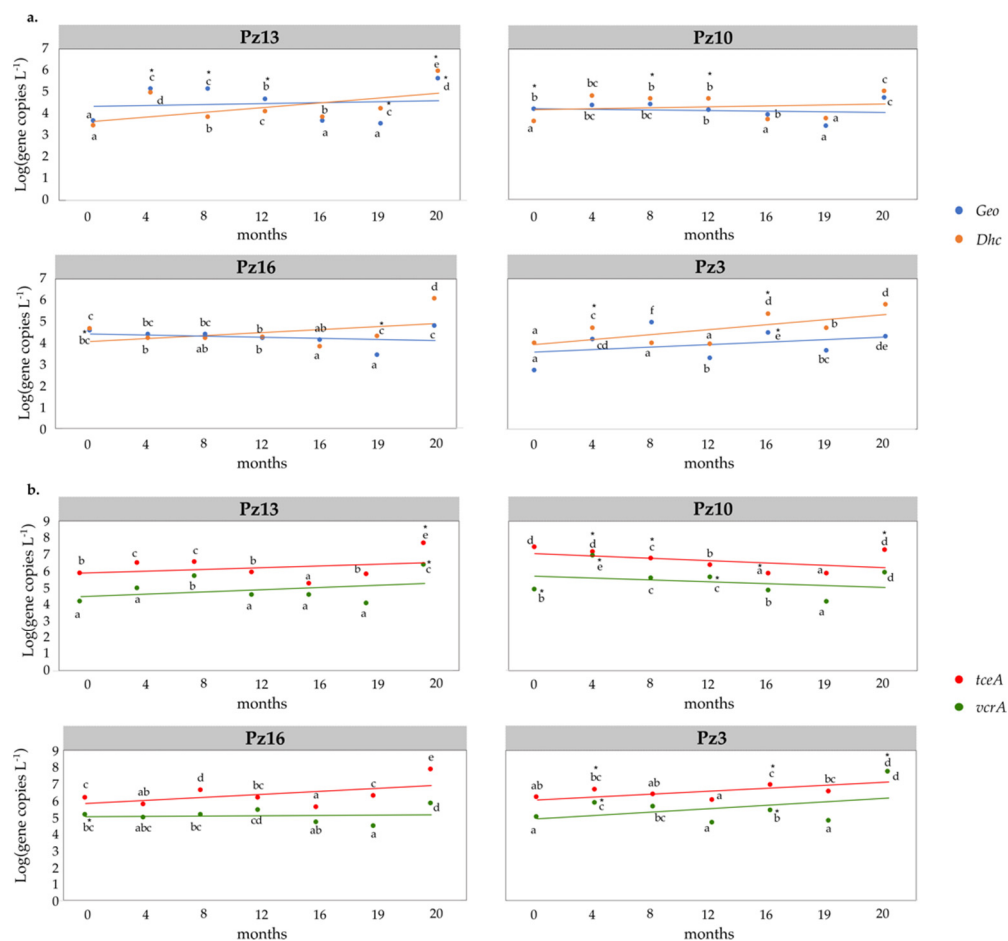

Figure 5. Gene copy abundance of phylogenetic gene markers for *Geobacteraceae* (*Geo*) and *Dehalococcoides* (*Dhc*) (**a**) and functional gene markers (*tceA* and *vcrA*) (**b**) in Pz13, Pz10, Pz16 and Pz3 during time. * indicates significant difference between piezometers of transect upstream and downstream injection wells of anaerobic bio-barrier. Lowercase letters indicate significant or not significant difference between different time (Tukey's test, $p \leq 0.05$).

After a 20 month-treatment, Bacteria increased significantly only in Pz13 while, Archaea increased significantly of one-two order of magnitude in all piezometers. *Geobacteraceae* and *Dehalococcoides* increased mostly in the downstream injection wells (Pz10 and Pz3). In both transects, *tceA* and *vcrA* increased from 10^5 to 10^6 gene copies L^{-1} in the

piezometers downstream the permeable reactive bio-barrier (Pz10 and Pz3). The difference of OHR marker genes between upstream and downstream piezometers was higher in transect Pz13-Pz10, compared to transect Pz16-Pz3 (Figure 5).

Pearson correlation indicated statistically significant positive and negative correlations between OHR biomarkers, CE concentrations and other physical-chemical parameters (Figure 6). Archaeal 16S rRNA gene copies showed significant negative correlations with the concentration of all CE except VC, while they were positively correlated with the pH. *Geobacteraceae* was significantly positively correlated with ethene concentration and *vcrA*. *Dehalococcoides* was significantly positively correlated with *vcrA* and *tceA*. The number of *vcrA* gene copies was significantly negatively correlated with TCE and DO.
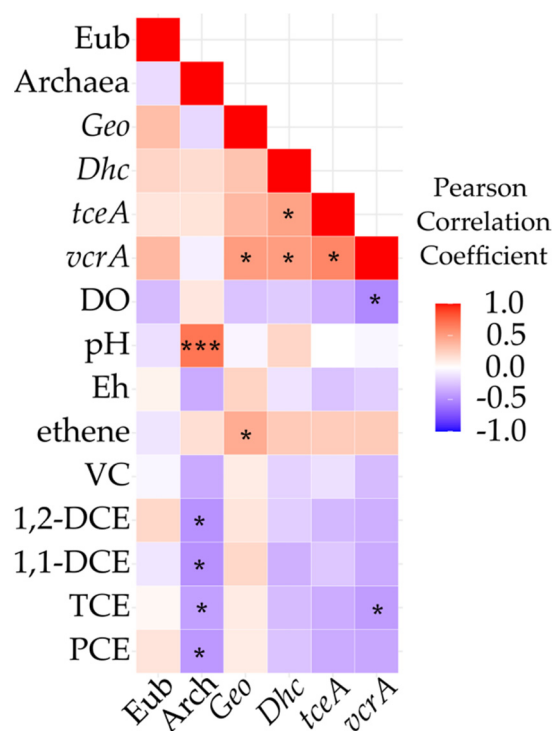

Figure 6. Pearson correlation indices among OHR biomarkers and physical-chemical parameters. Red shades indicate positive correlations and blue shades negative correlations. Asterisks indicates statistically significant correlations (* $p \leq 0.05$ and *** $p \leq 0.001$).

3.6. Effect of Substrate Addition on the Microbial Community of the Aquifer

The effect of reducing substrate addition on the microbial community, was analyzed in the groundwater sampled at Pz16 at the beginning and after 20 months of treatment.

At the beginning of the treatment, the bacterial community was dominated by *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Figure 7a). Over time, *Proteobacteria*, *Chloroflexi* and *Spirochaetes* significantly decreased with the concomitant increase of *Actinobacteria*, *Firmicutes* and *Tenericutes* ($p \leq 0.05$).

At the beginning, the archaeal community mostly included uncharacterized taxa (Figure 7b). After the treatment, the phylum *Parvarchaeota* significantly increased ($p \leq 0.05$), together with members of the phylum *Crenarchaeota*. *Euryarchaeota* accounted for approximately 1% of the total archaeal community at the beginning of treatment, but they were not detected after 20 months.

Differential abundance analysis revealed that the most contributors to the significant increase of *Firmicutes* were uncharacterized *Mollicutes* taxa, *Dehalobacterium* and *Desulfosporosinus* (Figure 8). On the other hand, *Proteiniclasticum*, *Dehalogenimonas*, *Porphyromonadaceae* family and *Arcobacter* significantly decreased over time.
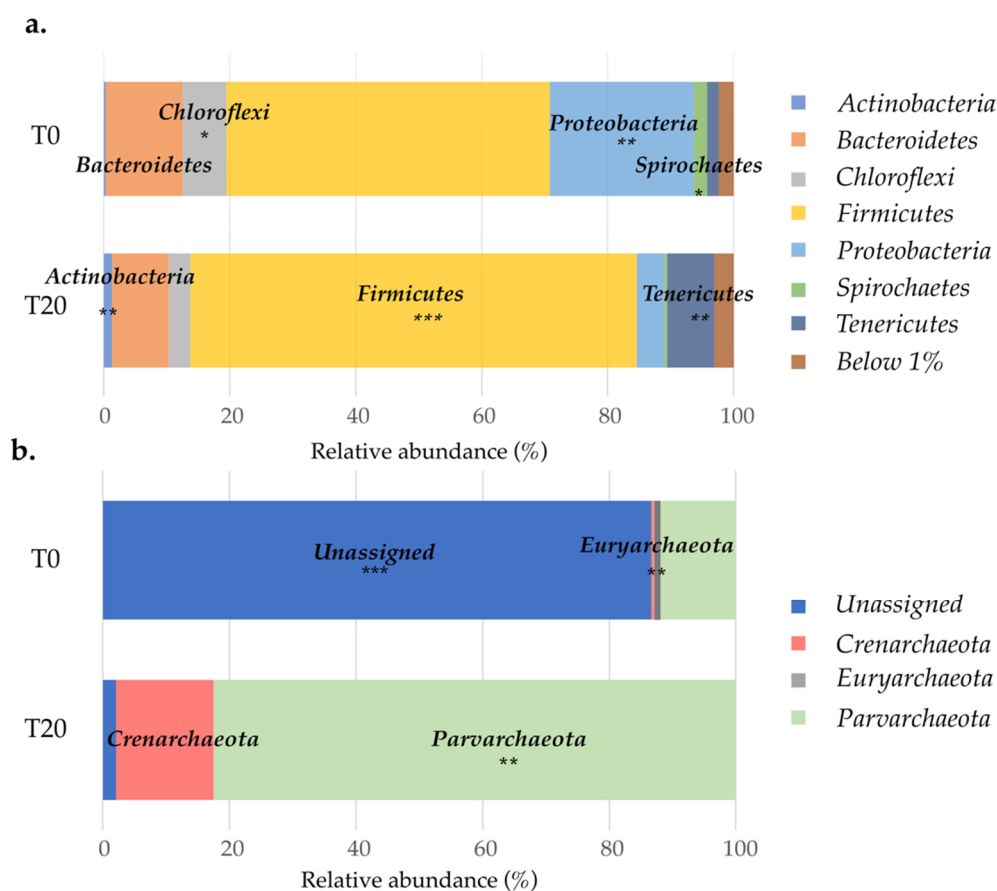
Figure 7. Composition of microbial community present in the anaerobic permeable reactive bio-barrier at the beginning (T0) and after 20 months (T20) of treatment, as determined by DNA-based Illumina sequencing of 16S rRNA of Bacteria (**a**) and Archaea (**b**) at phylum level. Asterisk indicates significant variation of relative abundance during the considered time frame (* = $p \leq 0.05$, ** = $p \leq 0.005$ and *** = $p \leq 0.0005$).

Most of the increase observed for the archaeal phylum *Parvarchaeota* over time was imputable to members of the uncharacterized order YLA114 that significantly increased from 0.03% to 79.74%. Members of the genus *Candidatus* Nitrososphaera significantly contributed to the increase of the phylum *Crenarchaeota* (*Miscellaneous Crenarchaeota Group* (MCG), from 0.43% to 7.22%).

Within the bacterial library, the following OHRB families and genera were detected: *Sulfurospirillum*, *Clostridium*, *Dehalococcoides*, *Desulfuromonadaceae*, *Syntrophaceae*, *Comamonadaceae*, *Geobacter* and *Shewanella* (Figure 8). *Shewanella*, *Clostridium* and *Geobacter* showed a relative abundance above 1%. With the exception of *Sulfurospirillum* and *Clostridium*, the other OHRB decrease after the treatment, although this variation was not statistically significant.

Differential abundance indicated that the treatment did not change the abundance of OHRB, but significantly affected a number of microorganisms indirectly involved in OHR (named 'OHR auxiliary bacteria') and fermentative microorganisms (Figure 8).
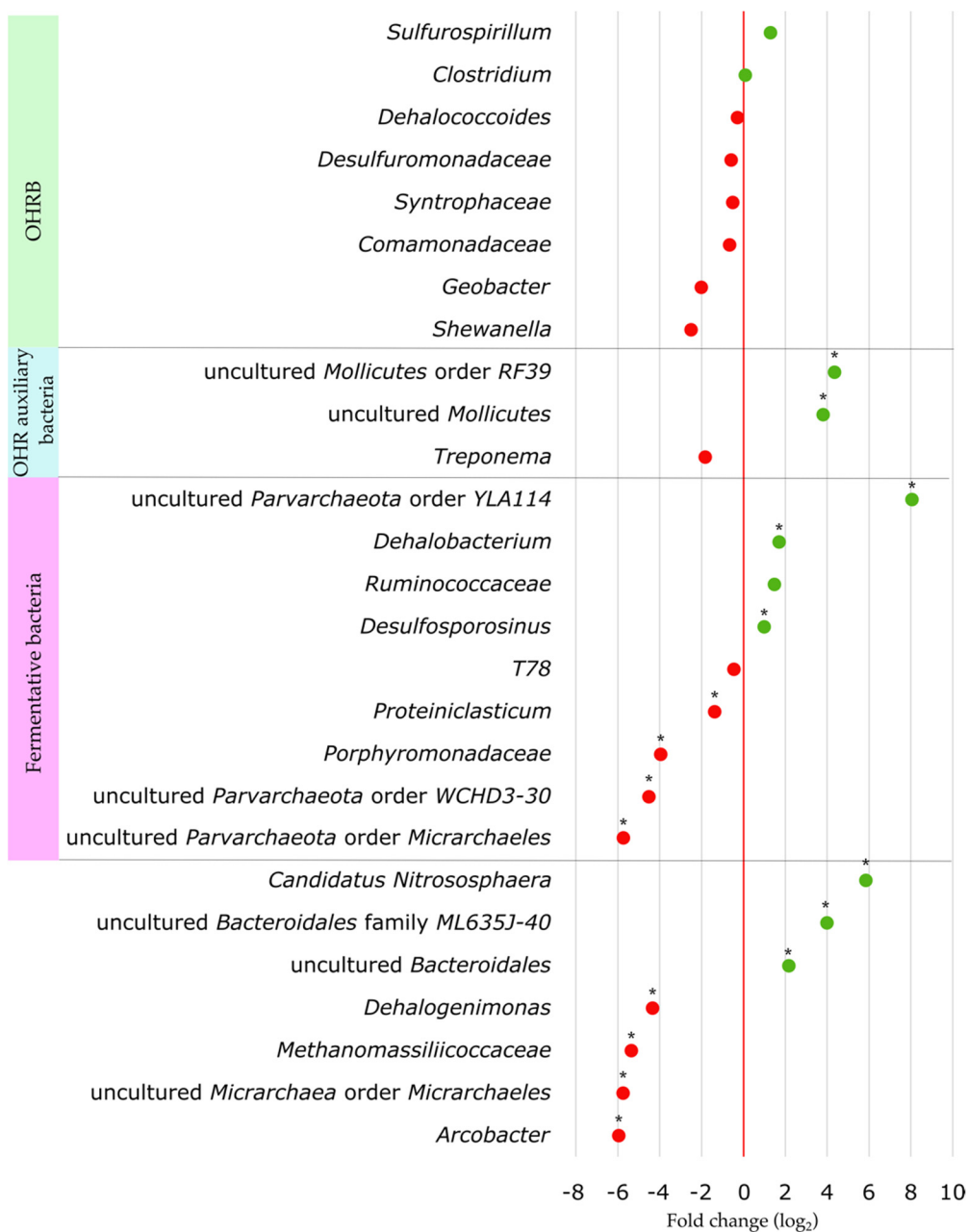
Figure 8. Differential abundance analysis performed on bacterial and archaeal taxa at genus level ($p \leq 0.05$) over 20 months of treatments. Relative abundance of OHRB, bacteria that have an auxiliary role in OHR activity and the fermentative bacteria involved in reducing power production. * indicates significant variation of relative abundance during the considered time frame ($p \leq 0.05$).

4. Discussion

4.1. Site Characterization

The present site is characterized by the proximity to a marine lagoon, within a petrochemical harbor. Brackish environments are recognized to be source of naturally-produced organohalides by marine eukaryotic and prokaryotic cells and by industrial pollution [41]. The pristine presence of organohalogens in marine environments may have selected for the development of different microbial dehalogenation metabolisms [42], such as OHR.

In the landfill analyzed in the present study, the concentration of CE was far exceeding the European law limits (2000/60/EC), and the pH and the redox potential were close to the optimal values for OHR activity. At pilot-scale level, these conditions were optimal

near the anaerobic permeable reactive bio-barrier. Due to the natural irregularity of the contamination plume, CE were unevenly distributed within the site at the beginning of the treatment, and along the anaerobic permeable reactive bio-barrier over the pilot scale experiment. Specifically, piezometer Pz13 was characterized by concentrations of CE higher than other piezometers, due to a non-linear hydrologic flow course from the landfill. This should be carefully considered when designing bioremediations actions, as evidenced in previous studies [43].

The phylogenetic and functional OHR biomarkers retrieved in the landfill and in the contamination plume at the beginning of the treatment were 4 orders of magnitude higher compared to similar biomarkers retrieved in a TCE-contaminated site analyzed by Lee and colleagues [44]. Similarly, to our study, Coubert and colleagues [45] determined that in highly contaminated aquifer reductive dehalogenases such as *tceA* and *vcrA* were expressed in the order of 10^5–10^7 mRNA copy L^{-1}, evidencing the suitability of natural microbial populations to perform reductive dehalogenation. In these conditions, OHR can be further incremented by a biostimulation approach [46].

At the beginning of the treatment, in the landfill and in the contamination plume, *Firmicutes* and *Proteobacteria* were the main members of the bacterial community. While in the landfill the archaeal community was characterized mainly by methanogenic microorganisms (*Euryarchaeota*, in particular *Methanomicrobia* genus) and anaerobic aromatic compounds degraders (*Crenarchaeota*), in the contamination plume *Parvarchaeota* was the most abundant archaeal phylum, together with a high number of uncharacterized taxa. These patterns were similar to the ones found in analogous untreated groundwaters [47,48], but also to contaminated wastewaters and urban rivers [49,50]. The high number of unassigned Archaea can be due to the presence of uncharacterized slow-growing bacteria, which are hard to investigate, and possibly promoted by harsh environment conditions in the contaminated aquifer [51]. Members of the Miscellaneous Crenarchaeota Group (MCG), retrieved at the site, are uncultivated Archaea present in different environments [52]. They can use a wide range of substrates as carbon source, suggesting an important role in the biogeochemical carbon cycling [53]. Furthermore, genes involved in the anaerobic degradation of aromatic compounds [54] and putative methane-metabolizing genes [55] were identified in their genomes.

Amplicon sequence variants of OHRB active at the landfill site comprised those of *Shewanella* and *Sulfurospirillum* genera, and of *Dehalococcoidaceae*, *Comamonadaceae*, *Synthrophaceae*, *Desulfomonadaceae* families and *Desulfomonadales* order. According to Maphosa et al. (2010) [56] and similarly to what observed in other terrestrial and sediment environments [57], the most abundant OHRB detected in the present study (i.e., *Geobacter*, *Sulfurospirillum*, *Desulfitobacterium* and *Shewanella*) are those characterized by versatile metabolic capabilities, rather than obligate OHRB (such as *Dehalococcoides* and *Dehalobacter*). In accordance with the presence of obligate and versatile OHRB, the quantification of functional OHR biomarker *tceA* was higher than that of *Dehalococcoides* genus. The presence of several OHRB populations at the site suggests that these microbial communities were primed by the presence of synthetic halides since decades, in addition to the possible presence of natural organohalides for a longer time [42], in the proximity of a marine environment.

Although facultative OHRB are usually characterized by a lower number of reductive dehalogenases [58], the retrieval of organohalide reductase transcripts in the analyzed groundwater confirmed the hypothesis that versatile OHRB were active at the site.

Among all OHRB, *Shewanella* [59] was the most abundant in the landfill and in the contamination plume. Members of the genus *Shewanella* are often found in brackish water environments, where they usually play a pivotal role in the biogeochemical cycle of several organic and inorganic compounds [60,61]. *Shewanella* spp. are known to couple the dissimilatory reduction of ferric iron (Fe(III)) to the oxidation of a wide range of organic contaminants, including organohalides, via the Fenton reaction [62]. These microorganisms are usually very versatile in terms of growth substrates. As an example, a marine

Shewanella sediminis isolate was found to be able to reductively dechlorinate PCE, with low dehalogenation kinetics, implying that the synthetic organohalide might not be its physiological substrate [59]. In future microcosm experiments, the role of *Shewanella* spp. in the OHR process in the present site will be investigated more in detail.

Other microorganisms retrieved in the landfill and in the contamination plume might have an indirect role in OHR, as in the case of several members of the phylum *Proteobacteria*. In fact, within the families *Xanthomonadaceae*, *Caulobacteraceae*, *Phyllobacteriaceae* and *Alcaligenaceae*, several species are aerobic or anaerobic polycyclic aromatic hydrocarbons degraders [63–66] or associated to OHRB, as in the case of *Thermomonas* [67]. *Sedimentibacter* and *Coprococcus* were found to release organic acids in soil amended with crop residues [68], suggesting a possible contribution to increase the reducing power to favor OHR. In previous studies, *Fusibacter* and *Mollicutes* (*Firmicutes* phylum) were detected in microcosms amended with PCE and TCE enrichment cultures, respectively [69,70]. Several members of the phyla *Bacteroidetes* and *Firmicutes* (*Acidaminobacteraceae*) were retrieved in hydrocarbon-degrading communities, although nothing is known about their specific ecological niche [71–74]. The role of the above-mention microbial taxa in CE degradation was never clarified, indicating that much work still needs to be conducted to elucidate many aspects of OHR.

4.2. Organohalide Respiration Affected by Reducing Substrate

In microcosms experiments, natural attenuation of CE was evidenced, and it was already present in the analyzed groundwater without reducing substrate addition, confirming that OHRB retrieved by Illumina 16S rRNA sequencing were active at the site. The presence of a natural attenuation was not always detected in contaminated site analyzed in previous studies [21]. Dechlorination rate of higher CE was, however, enhanced by the addition of reducing substrate [75] as sugar-based substrates, lactate, butyrate and hydrogen [21,76,77]. The present data suggested that, although in the presence of native microorganisms able to conduct natural attenuation processes, the addition of reducing substrate is a feasible strategy to accelerate and improve OHR.

In the microcosm experiments, higher CE were efficiently degraded, while DCE and VC were more recalcitrant. Although in the microcosm experiment an actual degradation of higher CE was observed, the same compounds appeared not to be degraded in situ after the treatment at pilot-scale level. This might be due to a constant replenishment of contaminants by the landfill, a situation that differed with other contaminated sites [78].

Both the microcosm incubations and the pilot-scale treatment by permeable reactive bio-barrier showed either a non-complete degradation or even an accumulation of VC.

VC accumulation in contaminated groundwaters was reported in several works both at microcosm and at field scale with or without the addition of reducing substrates [78–83]. The proportion of CE degradation appears to be dependent on the distance from the source of contamination. In fact, the higher the distance from the source of contamination, the lower the concentration of undegraded CE and the higher the accumulation of VC [81].

As stated above, VC degradation under anaerobic conditions showed thermodynamic limitations [84,85], which might be the reason for the observed accumulation. Since the treatment analyzed in the present study might accelerate OHR and, thus, VC accumulation, the production of VC under these conditions should be carefully monitored. These outcomes highlight the importance to find solutions for enhancing VC biodegradation, thus preventing its accumulation in the contamination plume. Kao and colleagues [76] showed that the increase of VC concentrations after the reduction of higher CE could be resolved by the injection of oxygen in the microcosm. Previous studies showed that in contaminated aquifers sequential anaerobic-aerobic biodegradation interventions can improve the efficiency of bioremediation [86–88]. Given the structure of the study site, a similar bioremediation set-up could be devised.

4.3. Impact of the Biostimulation Treatment on the Aquifer Microbial Community

In contamination plume, the addition of reducing substrate caused a fluctuation of total bacteria in the permeable reactive bio-barrier and it affected microbial species structure, while Archaea showed a significant increase in all piezometers.

Phylogenetic and functional gene targets did not follow a linear increase, but rather a fluctuation. This trend was present in another contaminated groundwater, under biostimulation with injection of vegetal oil [24]. Fluctuation of microbial populations might reflect both the fluctuation of contaminant release by the landfill as well as natural variations in groundwater flow.

In the present study, the abundance of the functional markers *tceA* and *vcrA* was higher than the phylogenetic markers (*Geobacter* and *Dehalococcoides*). Although this difference between phylogenetic and functional gene copies was not always noticed in CE contaminated aquifers [89], a similar phenomenon was observed by van der Zaan and colleagues [90]. This supports the hypothesis that in the studied aquifer different versatile OHRB might carry orthologous reductases. Pearson correlation evidenced that among the monitored OHRB, *Geobacteraceae* and *Dehalococcoides* were positively correlated with functional genes *tceA* and *vcrA*. *vcrA* gene copies number was negatively correlated with oxygen concentration in accordance with Liang et al. [91], confirming that this reductase is present in obligate anaerobic microorganisms that conduct OHR of VC [6]. At the contaminant plume, relative abundance of known OHRB was below 5% before and after 20 month-addition of reducing substrate. Nevertheless, chemical monitoring showed that the decrease of higher CE was enhanced, presuming the presence of uncharacterized OHRB. Using 454 pyrosequencing, Dugat-Bony and colleagues [92] observed that the abundance of known OHRB was never higher than 2%, even under biostimulation with lactate injection.

A number of bacterial genera retrieved in the aquifer showed a possible correlation with OHR activity. OHR was never described for *Desulfosporosinus*. However, different strains of *Desulfitobacterium* belonging to the same family (i.e., *Peptococcaceae*) are known to carry out OHR [9], and many OHRB are sulfur-reducing bacteria. In addition, *Desulfosporosinus* showed adaptation to high PCE concentration [93]. In this perspective, the analyzed aquifer might host new *Desulfosporosinus* strains involved in OHR processes. *Dehalobacterium* were found in areas contaminated by halogenated pollutants and its ability to ferment DCA was verified in vivo [94]. *Mollicutes* were found in TCE enrichment culture, but their role was not determined [70]. Future investigations with specific probes will be dedicated to ascertaining the role of these genera in OHR.

As already observed in previous studies [95,96], Archaea showed a negative correlation with the pH value, being favored by acidic conditions. The abundance of Archaea was negatively correlated with CE concentrations, likely excluding a possible involvement of these microorganisms in OHR. Moreover, such negative correlation might indicate a toxic effect of CE on archaeal populations. It is possible to assume that the increase of CE enhanced OHRB activity embezzling reducing substrate to Archaea. In the anaerobic permeable reactive bio-barrier, methanogenic *Euryarchaeota* decreased over time. Indeed, after 20 months, this class of Archaea decreased significantly. Among Archaea, members of the phylum *Parvarchaeota* increased after the addition of the reducing substrate. These microorganisms are included in the superphylum DPANN [97], and a previous metagenomic study suggested the ability to perform acetaldehyde fermentation [16]. Members of the Order YLA114 (*Parvarchaeota*), which significantly increased over time, are not well characterized but they were found in crude oil reservoir, suggesting a role in hydrocarbon degradation [98]. Together these data confirm previous observations and evidence that the addition of molasses as reducing substrates fuel the anaerobic degradation of organic compounds, thus enhancing reducing power in the system.

Syntrophic interactions between OHR, fermentative bacteria and Archaea are crucial for effective bioremediation of halogenated compounds, due to the production of H_2, acetate and micronutrients for OHR [99]. In situ addition of the reducing substrate affected the microbial communities living within the contamination plume in the proximity of the

permeable reactive bio-barrier. In fact, molasse addition increased the relative abundance of fermentative microorganisms. Bacterial fermentation was likely driven by fermentative members of the phylum *Firmicutes*, as previously observed in other CE-contaminated sites [100–102]. Members of the genera *Desulfosporosinus*, able to ferment lactate [31], and *Dehalobacter* significantly contributed to this increase.

It was shown that OHRB need specific physical-chemical conditions for their reductive activity [103]. When the environmental conditions are prohibitive for OHRB, their presence in the community is made possible by the activity of other microorganisms. *Actinobacteria* and *Proteobacteria* can protect strictly anaerobic OHRB as *Dehalococcoides* from O_2 and the consequences of oxidative stress through hypothetical different mechanisms such as the expression of superoxide dismutase (SOD) and the ruberythrin/rubredoxin scavenging system [101–103]. Cobalamin (B12), a corrinoid, is very important for OHR enzymes (Rdh) activity because it is a cofactor of these enzymes, but some OHRB are not able to produce it themselves [104]. *Spirochaetes* (*Treponema*) and *Sedimentibacter*, which are known to produce corrinoid cofactors [100,105], were retrieved in the studied aquifer and might have a protective role towards OHRB.

The 20-month treatment increased members of different taxa (*Tenericutes, Comamonadaceae, Nitrososphaera, Desulfosporosinus* and *Arcobacter*) previously retrieved in hydrocarbon-contaminated environments [83,106–112]. Since the aquifer was affected by the presence of a high quantity of hydrocarbons, not only CE, it is difficult to determine the role of all the microbial species which significantly varied over the course of the treatment.

5. Conclusions

This study demonstrated that the addition of a reducing substrate efficiently promoted microbial OHR in a heavily contaminated site, both at microcosm and at field scale. The activity of native OHRB was stimulated by the presence of several bacterial and archaeal fermentative microbial populations that contributed to reach the optimal conditions for OHR. Moreover, the decrease of CE observed in the aquifer could only be explained by the presence of still unexplored OHRB, which need further investigations. Given the observed accumulation of VC, a bioremediation set up based on coupling OHR to aerobic VC degradation should be envisaged at this specific studied site, in order to achieve a complete degradation of CE. Economic considerations on sustainability of this type of biostimulation actions will be compared with other remediation interventions.

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