

Article

1,2-DCA Natural Attenuation Evaluation in Groundwater: Insight by Dual Isotope $^{13}\text{C}/^{37}\text{Cl}$ and Molecular Analysis Approach

Giovanna Carpani ¹, Massimo Marchesi ^{2,*}, Iliaria Pietrini ¹, Luca Alberti ², Luciano Massimo Zaninetta ³, Orfan Shouakar-Stash ⁴ and Francesca de Ferra ¹

¹ Decarbonization and Environmental R&D, Eni S.p.A., 20097 S. Donato Milanese, Italy; giovanna.carpani@eni.com (G.C.); ilaria.pietrini@eni.com (I.P.); fdeferra@gmail.com (F.d.F.)

² Department of Civil and Environmental Engineering, Politecnico di Milano, 20133 Milano, Italy; luca.alberti@polimi.it

³ Eni Rewind S.p.A., 20097 S. Donato Milanese, Italy; luciano.zaninetta@enirewind.com

⁴ Isotope Tracer Technologies Inc., Waterloo, ON N2V 1Z5, Canada; orfan@it2isotopes.com

* Correspondence: massimo.marchesi@polimi.it

Abstract: Natural attenuation (NA) processes represent a valuable option in groundwater remediation. At a heavily 1,2-dichloroethane (1,2-DCA) contaminated site, Compound-Specific Isotope Analysis (CSIA) in combination with Biological Molecular Tools (BMTs) were implemented as a rigorous characterization approach to evaluate the occurrence of Natural Attenuation in the proximity of the source area. By the use of microcosm experiments, the potential for natural and enhanced biodegradation under anaerobic conditions was documented, following the dichloroelimination pathway. Enrichment factors of -9.1% and -11.3% were obtained for ^{13}C while *Geobacter* spp. and reductive dehalogenase genes (*rdhs*) were identified as main site-specific biomarkers. At pilot scale, enrichments of 13.5% and 6.3% for $\delta^{13}\text{C}$ and $\delta^{37}\text{Cl}$, respectively, high levels of reductive dehalogenase (*rdh* group VI) along with the dominance of *Geobacter* spp. indicated the occurrence of significant dichloroelimination processes in groundwater under anaerobic conditions. By using the site-specific enrichment factors, degradation extents over approximately 70–80% were estimated, highlighting the relevant potential of NA in 1,2-DCA degradation in the vicinity of the source area at the site. The proposed fine-tuned protocol, including CSIA and BMTs, is proven to be effective as a groundwater remediation strategy, properly assessing and monitoring NA at site scale.

Keywords: 1,2-dichloroethane (1,2-DCA); Compound-Specific Isotope Analysis (CSIA); Biological Molecular Tools (BMTs); natural attenuation; groundwater; enhanced bioremediation; microcosm experiments



Citation: Carpani, G.; Marchesi, M.; Pietrini, I.; Alberti, L.; Zaninetta, L.M.; Shouakar-Stash, O.; de Ferra, F. 1,2-DCA Natural Attenuation Evaluation in Groundwater: Insight by Dual Isotope $^{13}\text{C}/^{37}\text{Cl}$ and Molecular Analysis Approach. *Water* **2021**, *13*, 728. <https://doi.org/10.3390/w13050728>

Academic Editor: Domenico Cicchella

Received: 16 December 2020

Accepted: 23 February 2021

Published: 7 March 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Chlorinated hydrocarbons are among the most frequent groundwater contaminants in many industrialized countries, as they are used as chemical intermediates, refrigerants, solvents, cleaners etc. [1]. In particular, 1,2-dichloroethane (1,2-DCA), listed among the priority pollutants with a toxicity comparable to that of tetrachloroethane (PCE) and trichloroethane (TCE) is widely used as a precursor in the manufacture of vinyl chloride (VC) and, in the past, it was added as a scavenger to leaded gasoline [1]. It has been demonstrated that spills or leakages result in accumulation and persistence of this contaminant in the environment [2]. 1,2-DCA has also been classified as a probable human carcinogen by the U.S. Environmental Protection Agency. This classification and the tendency to collect indicate both an environmental and human health risk due to acute and long-term effects [1].

For remediation of sources and extended plumes of 1,2-DCA contamination Pump and Treat (P&T) systems are generally implemented, although they are usually expensive

and not very effective in reducing the contamination, unless used on a long term basis [3]. On the contrary, attenuation processes, either natural attenuation (NA) and/or enhanced natural attenuation (ENA), are less expensive and are particularly low carbon-footprint remediation systems which can play a major role in achieving remediation, where applicable [4]. With regards to biodegradation processes, the main actors of the natural attenuation (NA) of organochlorides such as 1,2-DCA are microorganisms that can dehalogenate or oxidize/mineralize these toxic molecules because of specific enzymatic systems. In anaerobic conditions, frequently detected in aquifers and sediments, *Dehalobacter* spp., *Dehalococcoides* spp. (that is, *D. mccarty* strain 195) and *Desulfitobacterium* spp. (that is, *D. dichloroeliminans* strain DCA1) are primarily responsible for the conversion of 1,2-DCA, mainly using reductive dehalogenases (RDs), a class of enzymes which are capable of eliminating chlorine from molecules [5–7]. During reductive dehalogenation, vinyl chloride (VC) is formed as an intermediate of the dehydrodechlorination step due to the cleavage of one atom of chlorine using H as an electron donor and 1,2-DCA as a terminal electron acceptor. This step can be followed, in specific conditions, by a hydrogenolysis to ethane. Another possibility is dichloroelimination, where two chlorine atoms of 1,2-DCA are removed, converting the contaminant into ethene without production of toxic chlorinated intermediates (such as VC, which is even more toxic than the precursor). Therefore, the latter presents the favored process for the remediation of polluted groundwater. In this respect, several authors described 1,2-DCA specific RD gene clusters in the metagenome of a 1,2-DCA dehalorespiring enrichment culture from a contaminated aquifer [8,9]. Aerobic biodegradation of 1,2-DCA is also feasible, occurring via enzymatic oxidation or hydrolytic dehalogenation reactions. *Xanthomonas autotrophicus* GJ10 and *Ancylobacter aquaticus* AD20 for example are known for aerobic 1,2-DCA degradation through a hydrolytic dehalogenation pattern, whereas *Pseudomonas* strain DCA1 is able to degrade 1,2-DCA by means of a monooxygenase enzyme [10]. The presence of the specific catabolic gene (that is, molecular biomarker) and of the main bacterial strain (that is, taxonomic biomarker) involved in the biodegradative process can be assessed by the use of quantitative PCR (qPCR) analysis. Moreover, the characterization of the autochthonous bacterial community can be determined by the sequencing of the gene 16S rRNA through next generation sequencing (NGS) systems [11].

At present, valuable assessment of biodegradation processes can be reached by a multidisciplinary approach that combines the two Biological Molecular Tools (BMTs) cited above, with the Compound Specific Isotopic Analysis (CSIA) approach [12]. The latter is a promising methodology that permits assessing the extent, and often the type, of degradation involved in processes of NA of contaminants (that is, hydrocarbons and chlorinated solvents) in groundwater. During biodegradation, molecules containing lighter isotopes (that is, ^{12}C , ^1H , ^{35}Cl) are favored by the microorganisms, inducing a relative enrichment of molecules containing the heavier isotope (that is, ^{13}C , ^2H , ^{37}Cl) in the remaining contamination [13–17]. Such isotope fractionation (expressed by the use of enrichment factors, ϵ) varies based on the reaction mechanisms, and various enrichment factors ϵ of 1,2-DCA have been described for different degradation pathways. With regards to ^{13}C , a wide ϵ range, from -3‰ to -32‰ was demonstrated for aerobic microbial degradation [10,18], while enrichment factors concerning strictly anaerobic environments from approximately -7‰ to -33‰ were reported [19–21]. Recently, ^{37}Cl and ^2H fractionations were also investigated [22] and the use of a multiple isotope approach has been successfully applied in laboratory and field investigations, highlighting the degradation mechanism in the field. Palau et al. [23] revealed the existence of dehalogenation processes within 1,2-DCA plumes at two field sites by the use of ^{13}C and ^{37}Cl .

The aim of this study is to characterize the natural attenuation processes at a contaminated site impacted by 1,2-DCA, particularly in the vicinity of the source area where the highest concentrations were detected and where a pilot scale test for enhanced bioremediation was to be implemented. At laboratory scale, microcosm experiments were carried out in order to test the potential for 1,2-DCA biodegradation, to estimate the associated $\delta^{13}\text{C}$ fractionation, and to investigate several biological markers. At field scale, microbiological

(that is, Illumina NGS sequencing) and molecular analysis (that is, quantification of the reductive dehalogenase gene (*rdh*)) were used in combination with the $\delta^{13}\text{C}$ and $\delta^{37}\text{Cl}$ isotope approach for a detailed characterization of the biodegradation processes, including potential pathways and degradation extent estimation.

2. Materials and Methods

Microcosm experiments were carried out in order to test the potential for biodegradation at the site and to estimate the related site-specific carbon enrichment factor. At field scale, groundwater samples were collected for 1,2-DCA concentration, isotope analysis including ^{13}C -CSIA and ^{37}Cl -CSIA, and for additional molecular and microbiological analysis.

2.1. Site Description

The contaminated site is located at an industrialized area heavily contaminated with chlorinated solvents in northern Italy. The pilot area, about 200 m², represents a historical contaminant source for 1,2-DCA linked to a storage tank (Figure 1). From ground level to 3–4 m of depth b.g.l. (below ground level), the lithology is characterized by fill material and a horizon of silty fine sand (or silty-sand), often showing unsaturated conditions and occasionally recharged by infiltration waters. The subsequent layer consists mainly of clays, extending to approximately 9 m b.g.l. From this depth, a silty fine sand (or silty-sand) horizon starts, of a variable thickness of approximately 2 m, hosting the upper confined aquifer. The impermeable base of the aquifer is made up of gray clays locally peaty reaching down to 13–15 m b.g.l., where a layer of medium and coarse sands, locally silty, hosts the lower confined aquifer. This lower confined aquifer reaches on average to a depth of 40 m b.g.l. The Plant site overview and specific monitoring wells are shown in Figure 1.

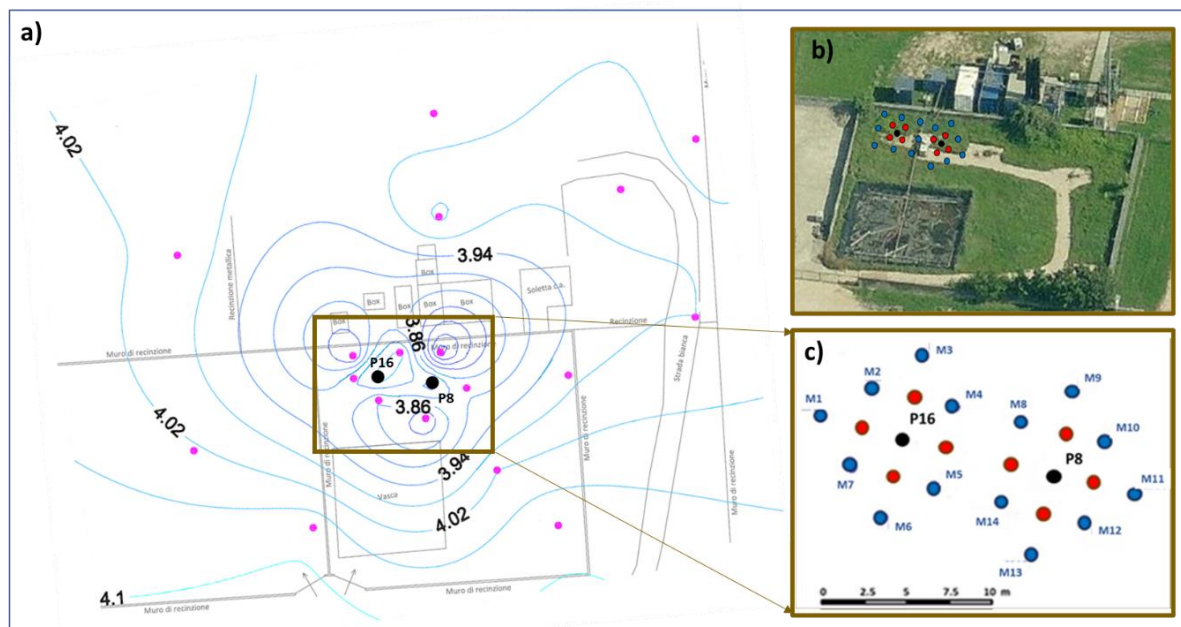


Figure 1. (a,b) Plan view of the site, with detail (c) of the pilot area including the monitoring well distribution: the monitoring wells P8 and P16 are in black while the monitoring wells for the detailed pilot area characterization of are in blue (red points indicate other wells not used for the purpose of this study); blue lines (a) are piezometric lines of the upper confined aquifer.

1,2-DCA has been found in high concentration, on the order of thousands mg/L, in the upper confined aquifer, with concentration decreasing over time, at least for P16, close to the source area, as shown by monitoring data from 2004 to 2015 (Figure 2a), when a pilot experiment for enhanced bioremediation was implemented in the area. Groundwater

velocity within the upper confined aquifer is very low as the gradient is flat (below 0.5‰) and hydraulic conductivity ranges between 8×10^{-6} and 3×10^{-5} m/s.

Despite the high concentrations, previous studies [8,24,25] showed an intrinsic potential of degradation at the site, also indicated by a significant decrease in concentration over time for example for P8 (Figure 2a). To investigate the role of natural attenuation at the site, 14 monitoring wells, screened only on the upper confined aquifer were installed in the vicinity of P8 and P16 (Figure 1c).

The site is characterized by reducing conditions, with dissolved oxygen < 0.2 mg/L and low redox potential (that is, averaging values of $-200/300$ mV) in the entire pilot area, indicating a mainly anaerobic environment.

Groundwater samples for site characterization were collected from the 14 new monitoring wells, as well as from P8 and P16 in June 2015 for 1,2-DCA concentration quantification and isotope analysis, including ^{13}C -CSIA and ^{37}Cl -CSIA. On selected monitoring wells (M2, M4, M6, M10 and M13) additional groundwater samples were collected for molecular and microbiological analysis. For P16, molecular and microbiological analysis were also carried out during a monitoring period from 2013 to 2015.

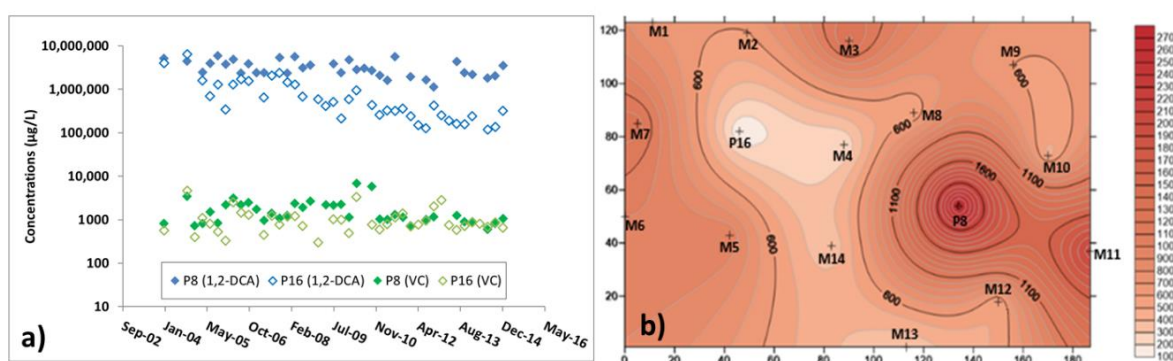


Figure 2. 1,2-dichloroethane (1,2-DCA) and vinyl chloride (VC) concentrations during the monitoring period from 2004 to 2013 for P8 and P16 (a). The absence of VC formation confirms a dihaloelimination mechanism; map distribution of 1,2-DCA concentration (mg/L) during the sampling campaign in 2015 (b).

2.2. Microcosm Experiments

A series of microcosms were prepared in order to test the biodegradation potential for natural attenuation (indicated as NA) and for further enhanced biodegradation applications (enhanced natural attenuation, indicated as ENA) and to estimate the site-specific ^{13}C enrichment factor.

Laboratory microcosms (that is, anaerobic conditions) were performed on fresh (NA) and enrichment cultures (ENA, 39th refresh culture), prepared from water samples collected from P16, where the highest decrease in 1,2-DCA concentration over time was observed. After being withdrawn during the periodic monitoring sampling in November 2012, cultures were set up in duplicate in 50 mL serum bottles for several enrichment cycles. The groundwater was degassed under a N_2 flow to eliminate all the contaminants originally present, then 0.5 mL of BTZ 100× (content of medium: ammonium chloride NH_4Cl , potassium dihydrogen phosphate KH_2PO_4 , magnesium chloride hexahydrate $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, calcium chloride dihydrate $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, and the buffer HEPES 50 mM), 1 mM cysteine, 400 µL of a 1000× vitamin solution (content of solution: 100 mg of Vitamin B12, 80 mg of p-aminobenzoic acid, 20 mg of D(+)-biotin, 200 mg of Nicotinic acid, 100 mg of Calcium pantothenate, 300 mg of Pyridoxine hydrochloride, and 200 mg of Thiamine-HCl $\times 2 \text{H}_2\text{O}$ in 1 L of distilled water), and 200 µL of a 200× trace elements solution (content of solution: 12.800 g of Nitrilotriacetic acid-NTA, 1.350 g of $\text{FeCl}_3 \times 6 \text{H}_2\text{O}$, 0.100 g $\text{MnCl}_2 \times 4 \text{H}_2\text{O}$, 0.024 g of $\text{CoCl}_2 \times 6 \text{H}_2\text{O}$, 0.100 g of ZnCl_2 , 0.025 g of $\text{CuCl}_2 \times 2 \text{H}_2\text{O}$, 0.010 g of H_3BO_3 , 0.024 g of $\text{Na}_2\text{MoO}_4 \times 2 \text{H}_2\text{O}$, 1.000 g of NaCl, 0.120 g of $\text{NiCl}_2 \times 6 \text{H}_2\text{O}$, and 0.026 g of $\text{Na}_2\text{SeO}_3 \times 5 \text{H}_2\text{O}$ in 1 L of distilled water) were added; lactate 1 mM was

provided as an electron donor while 1,2-DCA was added. After checking for complete 1,2-DCA consumption, subsequent dilutions were carried out on a weekly basis, again using fresh BTZ 1× medium at a 1:2 or 1:10 dilution rate, depending on the growth and activity of the culture.

All the microcosms were prepared similarly for the enriching phase, with a proportion of 1:2 of mineral medium BTZ 1× as supplementary salt solution and sample water purged with sterile oxygen-free nitrogen, in a total volume of 50 mL. All the microcosms were amended with 1 mM cysteine, 400 µL of a 1000× vitamin solution, and 200 µL of a 200× trace elements solution. Then, soda 10 N was added to all the microcosms to reach a pH of 7.0, 1 mM of lactate was added as an electron donor, and DCA was added to reach a desired initial concentration of 100 ppm. All microcosms were immediately sealed after the last addition with Teflon-faced septa and aluminum crimp seals and incubated in the dark at 26 °C. The abiotic control microcosms were prepared by incubating parallel serum bottles containing all the amendments with an additional 1% (*w/v*) of a 10% stock solution of sodium azide (NaN₃) as a bacteriostatic agent.

Both types of microcosms (biotic and control) were sacrificed at regular time intervals (0, 8, 16, 24, 32, 40, 44, 48, 52, 56, and 64 h) until complete consumption of 1,2-DCA. Separate aliquots for the quantification of 1,2-DCA concentration and for the ¹³C-CSIA analyses were collected.

2.3. 1,2-DCA Concentration, Isotope Analysis and Enrichment Factor Estimation

The concentrations of 1,2-DCA and the presence of ethene were determined by head-space gas chromatography on a 6890N Agilent gas chromatograph equipped with a FID and a DB5MS column (Agilent Tech, length 60 m × ID 0.25 mm × film thickness 0.25 µm, Cernusco sul Naviglio, Italy). During the analysis program, split mode with a ratio of 1:10 was used with an initial GC temperature hold of 35 °C for 5 min, followed by an increase to 120 °C at a rate of 10 °C/min and a final hold for 4 min. The 1,2-DCA limit of detection was about 1–2 µg/L. A five-point calibration curve was prepared prior to sample analyses using a standard solution of 1,2-DCA.

For ¹³C-CSIA, a gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) system was used (TRACE-Ultra/delta V plus, ThermoFisher Scientific, Rodano, Italy) combined with a headspace solid-phase micro extraction method (CAR/PDMS 75 µm, Supelco). The GC, equipped with a DB 624 column (Restek, Cernusco sul Naviglio, Italy) was configured in split mode (10:1) and the oven program was set initially to 40 °C for 2 min, heated to 220 °C at a rate of 10 °C/min, with a final hold of 2 min. Internal standards were used every 6 injections in order to obtain VDPB reference values, while samples were analyzed in duplicates; the overall precision of the method was 0.3‰. The analyses were performed at the Geo-Lab isotopic facility, ENI, Milan, Italy.

For ³⁷Cl-CSIA, a method by gas chromatography continuous flow isotope ratio mass spectrometry (GC-IRMS) using a 6890 (Agilent) coupled to a MAT 253 (ThermoFisher Scientific) was adopted, again with a solid-phase micro extraction (SPME) method and a similar GC set up and conditions as used for ¹³C-CSIA. Internal standards were used every 6 injections in order to obtain VSMOC reference values, while samples were analyzed in duplicates; the overall precision of the method was 0.2 ‰. The method is described in [23] and the analyses were performed at the Isotope Tracer Technologies Inc. laboratories, Waterloo, ON, Canada.

Carbon and chlorine isotope ratios are expressed using the δ-notation (Equation (1)),

$$\delta^h E_{sample} = \frac{R(^hE/^lE)_{sample}}{R(^hE/^lE)_{standard}} - 1 \quad (1)$$

where *R* is the isotope ratio of heavy (^{*h*}*E*) and light (^{*l*}*E*) isotopes of an element *E* (¹³C/¹²C and ³⁷Cl/³⁵Cl), and δ values were reported in per mil (‰). The relationship between

isotope fractionation and the extent of 1,2-DCA transformation in laboratory experiments was evaluated by a modified form of the Rayleigh distillation (Equation (2)):

$$\ln \frac{R_t}{R_0} = \ln \left(\frac{\delta^h E_t + 1}{\delta^h E_o + 1} \right) \cong \varepsilon_{bulk} \cdot \ln f \quad (2)$$

where f is the remaining fraction and ε_{bulk} the enrichment factor.

2.4. Characterization of Microbial Communities and Quantification of the Catabolic Gene of *Geobacter-spp.*

2.4.1. Microbial Population Analysis. DNA Extraction and Illumina-MiSeq® Sequencing

500 mL of the groundwater collected from the selected monitoring wells were filtered employing a vacuum filtering kit (that is, flask, Buchner funnel and Orange Scientific 0.45 µm paper filter). Genomic DNA was extracted from the half part of the filter using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's protocol.

PCR amplifications of the extracted genomic DNA were performed in two independent 50 µL reactions containing 25 µL of Green Taq® G2 Master Mix 2× (Promega, Madison, WI, USA), 5 µL of each primer (10 µM), 12.5 µL of nuclease-free water, and 2.5 µL of DNA. The amplification of the V5-V6 hypervariable region of the 16S rRNA gene (fragment length 244 bp) employed primers 783F and 1027R with a barcode, a specific 6-bp sequence that allows the differentiation of samples, at the 5' position. Cycling conditions for the amplifications were 94 °C for 5 min, 29 cycles of 94 °C for 50 s, 47 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 5 min. Subsequently, the amplification products were separated by electrophoresis on EtBr stained 1% agarose gel and the specific bands were excised and purified using Wizard SV Gel and a PCR Clean-UP System (Promega, Madison, WI, USA) kit following the manufacturer's protocol. Finally, the concentration (ng/µL) of the purified DNA was determined using a Qubit® 2.0 Fluorimeter. Once all the libraries were prepared, the sequencing was carried out at Parco Tecnologico Padano (Lodi, Italy) using the Illumina-MiSeq® platform. The sequences of the amplified fragment (subdivided into samples in different FastQ format text files) were subsequently processed using a pipeline based on Usearch software [26], the Ribosomal Database Project (RDP) classifier [27], and an "in-house" Python script developed to describe the structure of the bacterial community.

Since the sequenced fragment has a length of 290 bp, the sequences of the two 250 bp reads were superimposed to obtain a single sequence of the entire fragment. This operation was performed using the fastsh_mergepairs tool of Usearch that produces the reverse-complement sequence of one of the two reads and aligns it to the other read under the perfect alignment condition. Reads that did not perfectly match were discarded. The fastq_mergepairs tool of Usearch was also used to remove low quality score sequences (number of uncorrected bases > 0.5) according to the information reported in the FASTQ file. Moreover, the sequences were converted into FASTA format. Then, the Usearch cluster_otus command was used to combine the sequences obtained from all samples in a single file and representative sequences for each OTU were defined (cluster). Singletons (that is, sequences that occur only once in the entire dataset) were removed and the representative sequences were defined with a 97% similarity cut-off.

The representative sequences of OTUs were classified by the Bayesian Ribosomal Database Project (RDP) classifier.

The abundance of each OTU within a microbial community was estimated by aligning sequences of each sample with representative ones and assigning them to the most similar OTU sequence. The processing was carried out using the usearch_global tool of Usearch. Finally, a script (count_illumina.py) described in [28] was used to convert the output generated by the usearch_global command into a tab-delimited text file where each OTU was related to its respective abundance in each sample.

2.4.2. Quantitative Polymerase Chain Reaction (qPCR) for Dehalogenase (*rdh*) Gene Determination

The presence and the quantification of the catabolic gene *rdh* in the genomic DNA extracted from the sampled groundwater and from the enrichment cultures of well P16 was estimated by quantitative PCR (qPCR)-amplification with primers 552F (5'-ATGACCAATGAA ATAGCTAATGA-3') and 625R (5'- TGTTGGCCTTGTTGTCGCACACT-3'). The qPCR was performed in a total mix volume of 30 μ L, composed of 15 μ L of FluoCycleII Sybr reaction mix (Euroclone, Pero, Italy), 0.3 μ L (1 μ M) of each primer, 11.4 μ L of nuclease-free water, and 3 μ L of genomic DNA. Cycling conditions for the amplifications were 95 $^{\circ}$ C for 4 min, followed by 40 cycles of 95 $^{\circ}$ C for 15 s, 52 $^{\circ}$ C for 30 s, and 56 $^{\circ}$ C for 45 s, with acquisition of the fluorescence at the end of each 72 $^{\circ}$ C elongation step.

The relative concentration of *Geobacter* spp. was further assessed by quantitative gene amplification with specific primers designed on the rRNA *Geobacter* 16S sequence (sequence GeoQ1F: 5'-CCTCAGTTGCCATCATTAAGT-3'; sequence GeoQ2R: 5'-GGTATCGCAACTC TTTGTAC-3') of the genomic DNA extracted from the enrichment cultures of well P16. This qPCR was performed in a total mix volume of 30 μ L, composed of 15 μ L of FluoCycleII Sybr reaction mix (Euroclone, Pero, Italy), 0.3 μ L (1 μ M) of each primer, 10.4 μ L of nuclease-free water, and 4 μ L of genomic DNA. Cycling conditions for these amplifications were 95 $^{\circ}$ C for 5 min, followed by 40 cycles of 95 $^{\circ}$ C for 20 s, 56 $^{\circ}$ C for 20 s, and 60 $^{\circ}$ C for 45 s, with acquisition of the fluorescence at the end of each 60 $^{\circ}$ C elongation step.

3. Results and Discussion

3.1. 1,2-DCA $\delta^{13}\text{C}$ Fractionation in Microcosms

The periodical evaluation of 1,2-DCA concentration by GC-FID analyses showed almost complete consumption of the contaminant within 64 h for all the stimulated microcosms ENA (Figure 3, top). Complete 1,2-DCA consumption was also observed for the microcosms without amendment (natural conditions, NA), although it required a longer experimental time of about 15 days (Figure 3, bottom). These trends confirmed the high potential of biodegradation of the native consortium in the aquifer under natural conditions and particularly after lactate amendment (in accordance with [8,24]). Abiotic controls also showed a slight decrease in 1,2-DCA concentrations for NA microcosms only throughout the incubation period (15% maximum over 15 days, data not shown), most likely due to volatilization processes; such decrease in concentration due to abiotic process was taken into consideration during estimation of the kinetic parameters and enrichment factors. The calculated first-order constant rate values (k) of 1,2-DCA degradation kinetics and half-life ($t_{1/2}$) values are 0.039 h^{-1} and 18 h, respectively, for the stimulated experiments and 0.011 h^{-1} and 66 h for those under natural conditions.

The metabolic pathway followed during 1,2-DCA degradation shows that the consumption of this contaminant can be associated to the direct formation of ethene, which, in the case of the amended experiments, reached its maximum concentration after several hours of incubation time; after reaching the peak, the ethene concentration started to decrease again (data not shown). Such a decrease appears to be the result of biodegradation. It is reasonable to suppose that, given the low residual concentration of 1,2-DCA, the bacterial consortium began to consume ethene. In many cases, 1,2-DCA dehalogenating bacteria are known to transform 1,2-DCA into vinyl chloride (VC) first, and to ethene in a second stage. In the present case, the absence of VC formation leads to the hypothesis that the degradation pathway involves a dihaloelimination mechanism, which favors the removal of the two halogen atoms at the same time, leading to the direct formation of the non-toxic compound.

With regards to the $\delta^{13}\text{C}$ results, data from abiotic controls did not present any fractionation over time (data not shown), confirming the absence of any degradation processes over the incubation period. For the stimulated and natural conditions experiments, the consistent decrease in 1,2-DCA concentration was accompanied by a significant $\delta^{13}\text{C}$ enrichment, with values up to +9.0‰ and +6.1‰, respectively (Figure 3).

Average enrichment factors of $-11.3 \pm 0.3\%$ and $-9.1 \pm 0.3\%$ with good linear correlation were obtained ($R^2 > 0.95$) (Figure 3) respectively for the stimulated (ENA) and natural condition experiments (NA). Compared to the wide range for 1,2-DCA biodegradation enrichment factors, such a slight difference suggests similar degradation pathways either for the enriched or fresh cultures respectively during the stimulated (ENA) and natural condition (NA) experiments. Most likely the key microorganisms responsible for the contaminant degradation have not changed significantly during the enrichment cycles, other than being present in higher concentration and hence resulting in faster degradation rates.

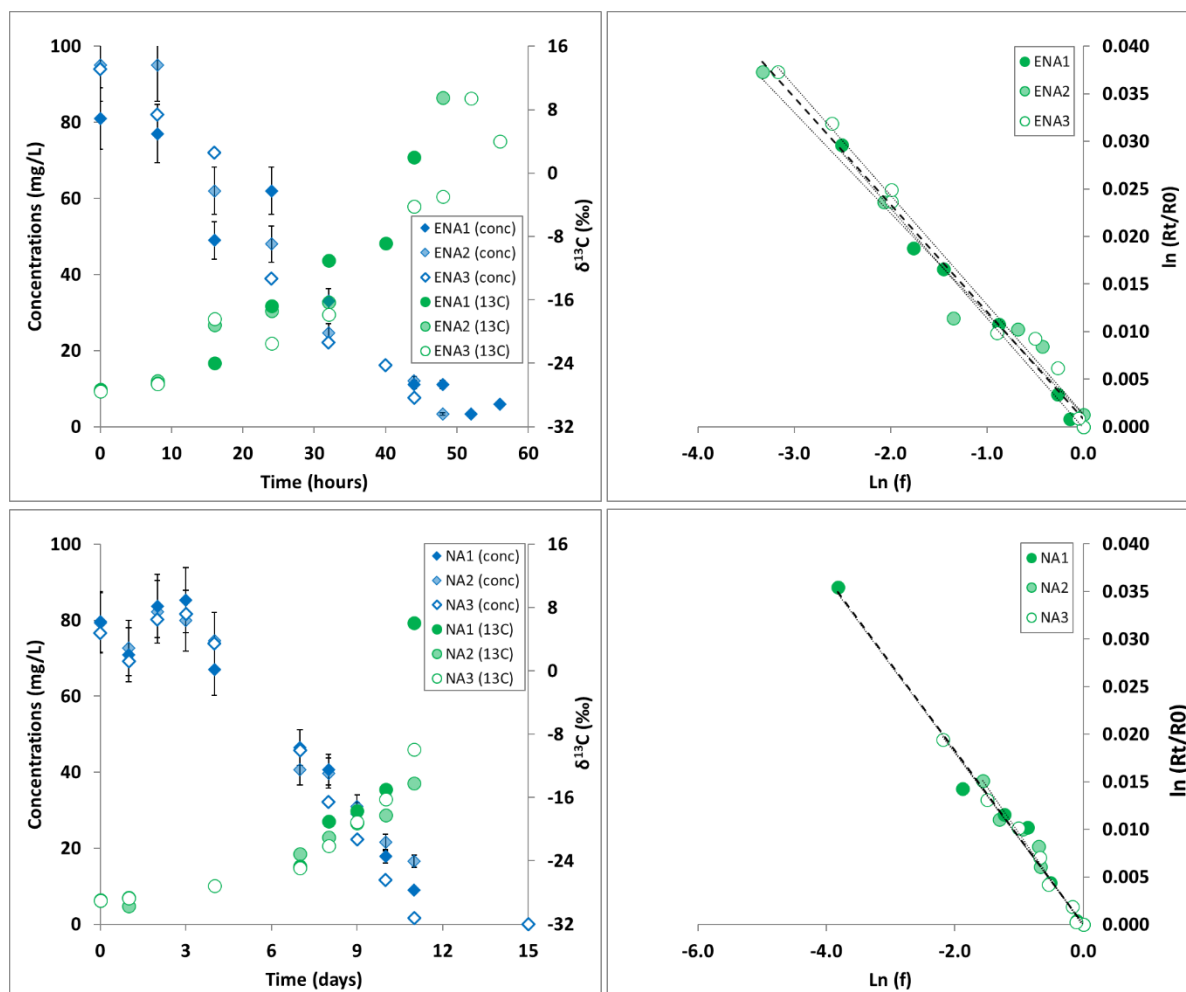


Figure 3. Microcosm experiments results: 1,2-DCA concentrations and $\delta^{13}\text{C}$ results over time for enhanced natural attenuation (ENA) microcosm experiments; numbers 1–3 indicate experiment number) and natural attenuation (NA) (microcosm experiments; numbers 1–3 indicate experiment number) on the left; $\text{Ln}(f)$ and $\text{Ln}(R_t/R_0)$ for enrichment factor estimation (on the right).

Results from the microcosm experiments fall within the large range of enrichment factors documented in literature and associated to 1,2-DCA biodegradation, from approximately -3% to -33% [10,19–21,23]. While significantly higher than those related to an oxidation pathway (ranging from -3% to -5% [10]), the estimated enrichment factors were lower than those reported for dihaloelimination pathways in the presence of *Dehalogenimonas*-containing culture and *Dehalococcoides* spp. (enrichment factors from -26% to -33% , [21,23]). Hirschorn et al. [20] documented enrichment factors similar to those obtained in the present study in anaerobic enrichment cultures originating from contaminated sites, with values from $-7.3 \pm 0.2\%$ to $-16.7 \pm 0.5\%$; lactate stimulation was applied and the dihaloelimination reaction mechanism was identified as the predominant pathway

responsible for biodegradation. Additionally, high ethene concentrations were measured similarly to those measured in the present study. These authors hypothesize that different enzymes or enzymatic reaction pathways (stepwise versus concerted dihaloelimination) may control isotopic fractionation during 1,2-DCA dihaloelimination. The enrichment factors were significantly higher than those related to an oxidation pathway characterized by the activity of monooxygenase and ranging from -3% to -5% [10].

The enrichment factors calculated in the present study can be used to characterize the 1,2-DCA isotopic signatures associated to dechlorination due to natural attenuation and for future biostimulation actions planned at the site (enhanced attenuation).

3.2. Characterization of Microbial Communities and Quantification of the Catabolic Gene and of *Geobacter* spp. from Microcosm Experiments

With the aim of a complete protocol for monitoring the attenuation processes in the pilot field trial, the presence of the catabolic gene *rdh* was determined in the genomic DNA extracted from the enriched cultures of the well P16 (Figure 4). During the experiments, the values of the number of copies of the gene increased from an order of 10^3 in natural condition to an order of 10^6 in the enriched cultures. Interestingly, in the enriched cultures the distribution pattern of this gene is consistent with the presence at similar levels of the *Geobacter* spp. (that is, number of copies of the specie increase from an order of 10^3 in the first refresh to an order of 10^5 – 10^6 in the last two refreshed cultures). The presence and quantity of other dehalogenating strains that could lead to a similar pathway (that is, *Dehalococcoides* spp. and *Dehalogenimonas* spp.) were also investigated, showing concentrations under the threshold value associated to real activity (that is, 10^3).

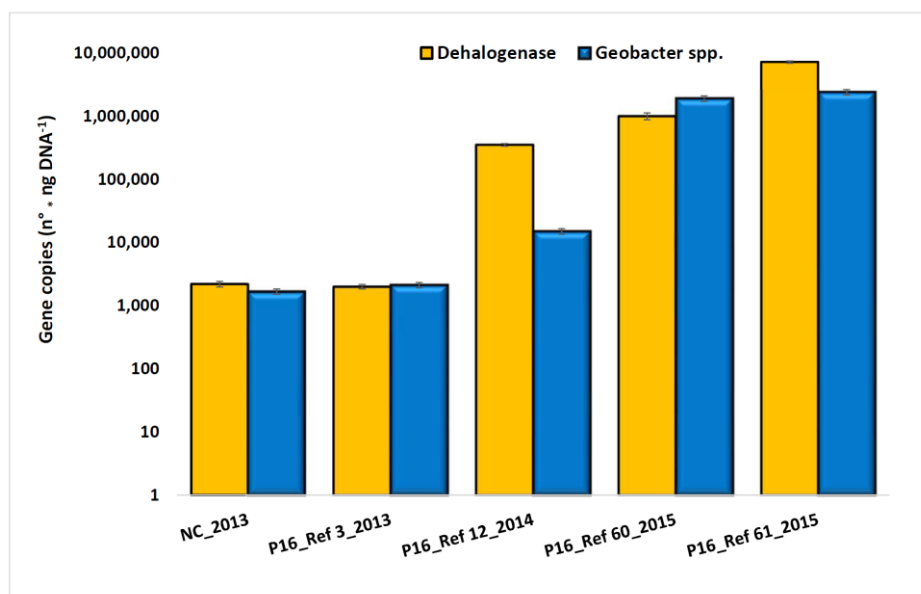


Figure 4. Quantitative PCR (qPCR) experiments results: determination of the reductive dehalogenase gene (*rdh*) and *Geobacter* spp. 16SrRNA copy number in subsequent enrichment cultures. NC is the non-enriched culture (that is, no refresh) microcosm at the beginning.

In addition to this evidence, the following metagenomic sequencing indicates a physical linkage of the reductive dehalogenase gene to the *Geobacter* chromosome (see Figure 5a,b). These results, also reported in the international application patent (WO2018/137798 A1), represent a deeper interpretation of the first results described in [29].

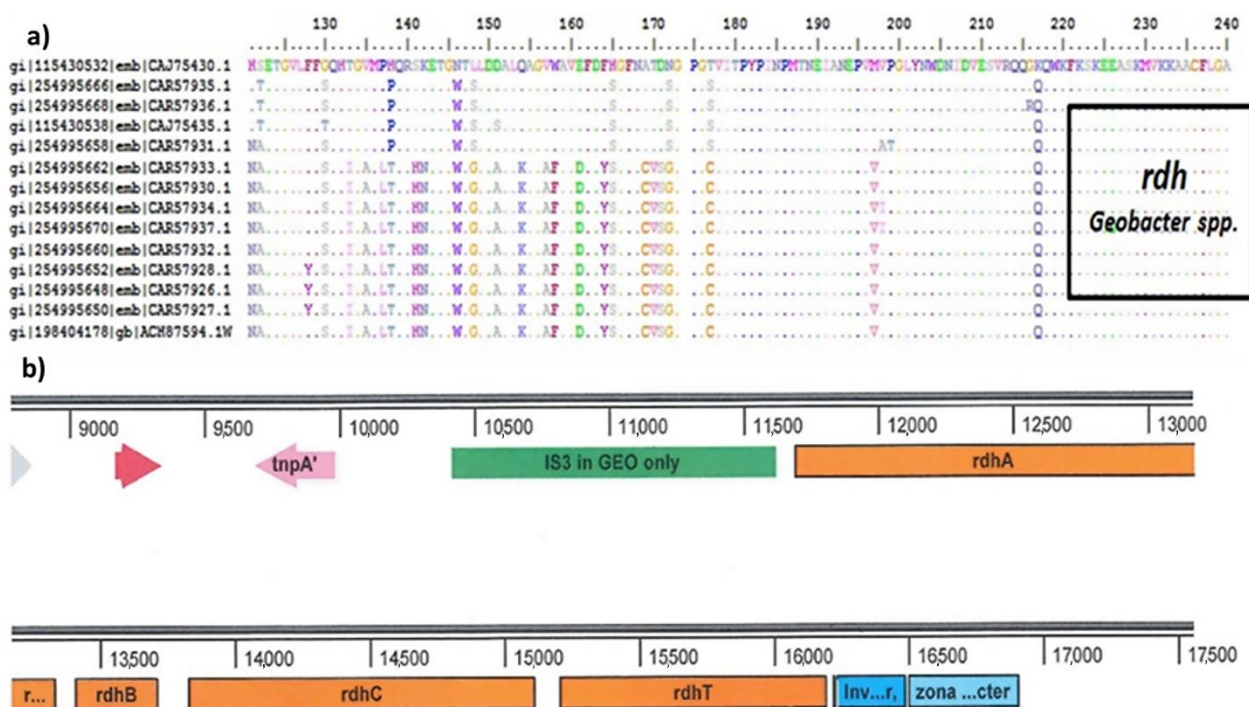


Figure 5. (a) Sequence of the area surrounding the *rdh* gene in an enrichment culture of *Geobacter* spp. and (b) characterization of the reductive dehalogenase operon.

3.3. Microbiological and Isotopic Results from the Pilot Area

1,2-DCA concentration varied significantly in the pilot area between the monitoring points, for example, decreasing from 4300 mg/L to 111 mg/L within 8 m distance. Furthermore, concentrations do not follow a clear spatial trend or delineate or distribute along a plume direction (Figure 3). Concentrations are most likely affected not only by degradation processes but also by other processes, such as diffusion, due to the proximity to the source area and due to the low permeability materials characterizing the aquifer.

Nevertheless, significant enrichment of ^{13}C has been measured. ^{13}C values vary from -25.8‰ to -12.3‰ , toward more enriched values at lower 1,2-DCA concentrations (Figure 6). The only exceptions are represented by M13, with a trend toward enriched ^{13}C values but with increased concentrations and M7, with a relatively higher ^{13}C enrichment compared to other monitoring points. These differing trends could be related to the presence of a dissolution process from the nearby source and/or attenuation by non-degradative process(es), related for example to 1,2-DCA transport along the aquifer. It also cannot be excluded that different biodegradation processes are insisting in the vicinity, as indicated by a completely different biological and molecular characterization at least for M13 (Figure 7).

Hence P8, which showed almost no reduction in 1,2-DCA concentrations between 2004 to 2015 (Figure 2a), presents the most depleted ^{13}C value of -25.8‰ . On the contrary P16, where the 1,2-DCA concentrations historically showed a decrease over time (Figure 2a), an enriched $\delta^{13}\text{C}$ value of -20.6‰ suggests the existence of a biodegradation process in containing and reducing 1,2-DCA concentrations at the site.

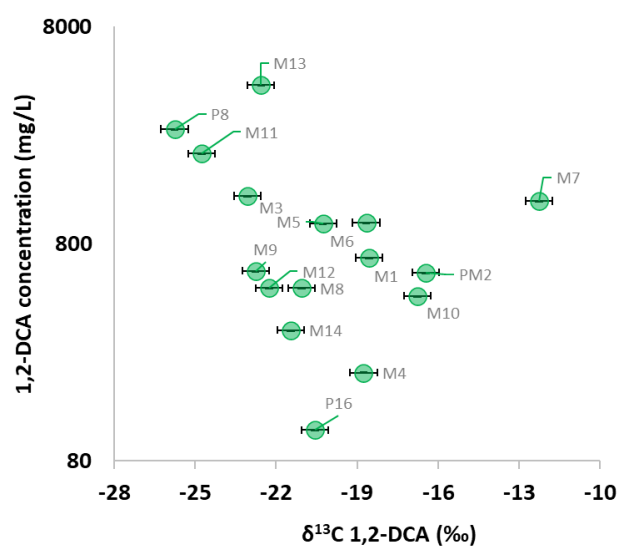


Figure 6. $\delta^{13}\text{C}$ and concentrations data from the pilot area.

While most of the literature on CSIA applications to chlorinated solvents addresses characterization issues related to groundwater contaminant plumes, few studies have investigated CSIA application within source areas. On a pilot scale experiment, Morrill et al. [30] demonstrated that continuous dissolution processes in the vicinity of a PCE source prevent the use of ^{13}C for biodegradation estimation. On the contrary, the present study shows larger $\delta^{13}\text{C}$ enrichment for 1,2-DCA within several meters from the suspected source, hence demonstrating that $\delta^{13}\text{C}$ data can still be possibly used to estimate biodegradation processes in the case of 1,2-DCA in the vicinity of a source area: this possibility probably derives from the high degradation rates for 1,2-DCA compared to PCE.

By using the site-specific enrichment factors of -9.1‰ and -11.3‰ obtained by the microcosm experiments, degradation extents over approximately 70 to 80% were estimated, highlighting the importance of natural attenuation processes in reducing 1,2-DCA concentrations in the vicinity of the source.

With regards to the microbiological results, the study on the bacterial population determined by Illumina sequencing confirms the presence of an anaerobic aquifer environment. In fact, the species distribution of the native consortia showed that *Geobacter* spp. (an iron and manganese reducing bacterium) is the prevalent species at the site, with a relative abundance ranging from 20% (well M13) to 58% (wells M2 and M10) of the total rRNA 16S gene (Table 1 and Figure 7). Furthermore, a sulphate-reducing species (that is, relative abundance of *Desulfosporosinus* spp. equal to 12% in well M2 and 8% in well M4) and other anaerobic bacteria such as *Clostridium* spp. (relative abundance of 10% in well M2 and 5% in well M4), are consistently present. As reported in different studies [31–33], all bacteria belonging to these groups can play a role in both hydrocarbons and chlorinated solvents degradation. On the other hand, in this environment methanogens do not appear to have a significant role and the detection of low levels of *Dehalogenimonas* spp. (group included in the cluster “Other genus < 2%”), suggesting a possible minor role of this organohalide-respirer at the site.

Conversely, the prevalence of *Malikia* spp. in well M13 (relative abundance of 66%), that belongs to the aerobic family *Comamonadaceae*, could be considered anomalous in this predominantly anaerobic environment. However, the close co-existence of anaerobes and aerobes has been shown in similar heterogeneous aquifer environments and it has been demonstrated to occur in liquid culture by Aburto et al. [34].

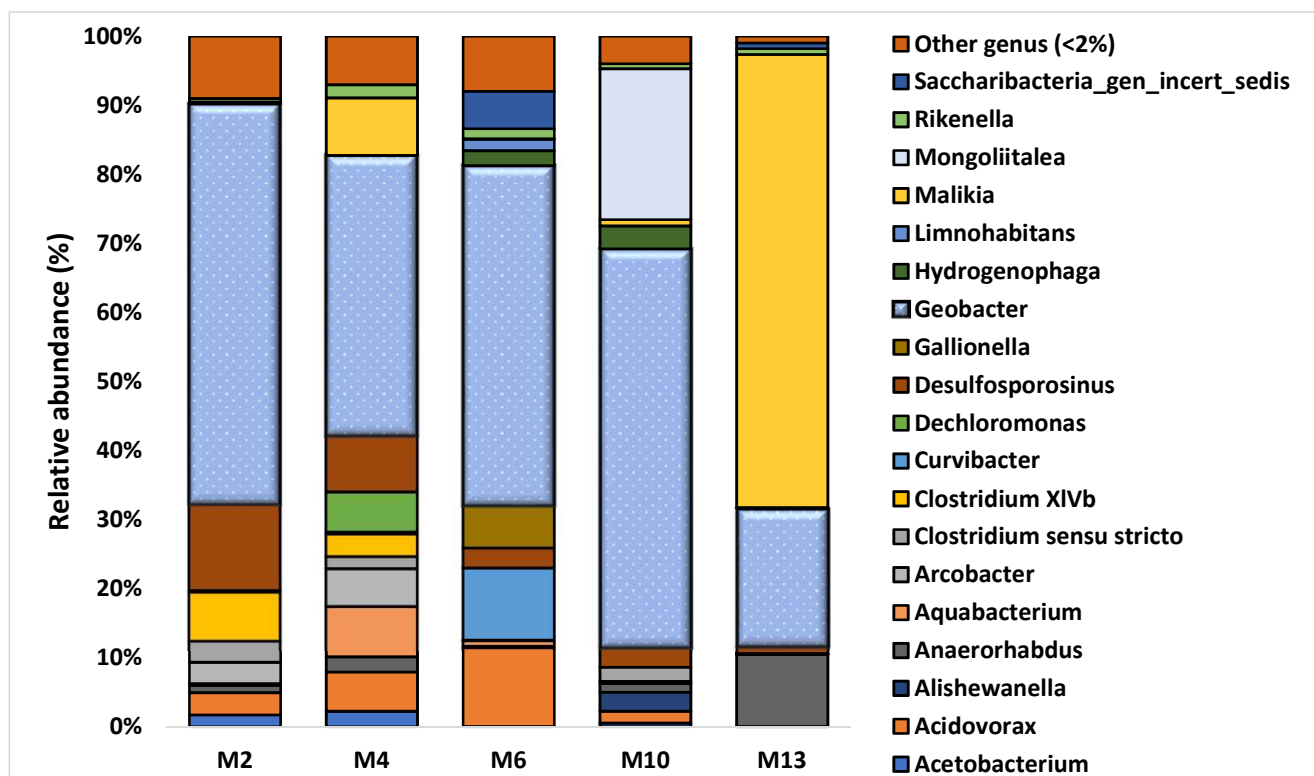


Figure 7. Illumina sequencing results: composition and relative abundance of the species identified in five representative wells in the pilot test area. *Geobacter* spp. (light blue with white points) is the strain mainly represented.

To confirm the findings obtained in the microcosms, the presence of both the dehalogenase catabolic gene *rdh* and the rRNA gene indicating the presence of *Geobacter* spp. were determined in the genomic DNA extracted from groundwater collected in 2015 (June) in the selected area (M2, M4, M6, M10 and M13). Both markers were detected at the site with the gene *rdh* concentration (gene associated to the dihaloelimination pathway) present at values ranging from 10^5 to 10^6 of gene copy number (Table 1). These values are similar to those assessed in the last enriched cultures set up with samples originating from well P16 (Figure 4). Moreover, the values of the *rdh* gene copy number in the groundwater sampled from the area closely matched the level of 1,2-DCA, with the highest value of the copies of the gene in monitoring well M6, which also shows the highest concentration of 1,2-DCA equal to 1000 ppm (Table 1). On the other hand, the relative abundance of *Geobacter* spp. is almost the same in all the native microbial communities of the different wells, with the only exception for sample M13, where the lowest *Geobacter* rRNA gene copy number value was recorded.

Table 1. Comparison between 1,2-DCA concentration values (ppm), *rdh* gene copy number and relative abundance (% of the total species) of *Geobacter* spp. in different monitoring wells of the pilot test area.

	M2	M4	M6	M10	M13
1,2-DCA concentration (ppm)	349	204	1000	460	255
<i>rdh</i> gene copies (n° * ng DNA-1)	5.63×10^5	6.81×10^5	1.42×10^6	1.07×10^6	1.78×10^5
Relative abundance of <i>Geobacter</i> spp. (%)	58	41	49	58	20

The results obtained separately through molecular and isotopic analyses show good agreement, confirming the combination of the proposed techniques as an excellent monitoring method in cases of application of bioremediation as remediation treatment.

Robust natural attenuation was demonstrated by the presence and activity of biological markers such as dehalogenases (novel *rdh* genes and their RNA expression), species-specific markers (detected and quantified by qPCR and metagenomics) and 1,2-DCA isotopic analysis in cultures and in situ. Data from laboratory cultures, from well water, and from stable consortia defined the reactions occurring and optimal amendments to accelerate them.

Site characterization by molecular techniques revealed that *Geobacter* spp. appears as the primary species responsible for this degradation process. This result was combined with the amount of RD genes (*rdhs*) determined by quantitative PCR (qPCR), to evaluate the possible contribution of NA at the site. Furthermore, in order to proceed with a pilot test of enhanced natural attenuation (ENA), an integrative monitoring protocol including isotopic (that is, $\delta^{13}\text{C}$), microbiological (that is, Illumina sequencing) and molecular analysis (that is, quantification of gene *rdh*) was adopted.

3.4. Additional Insights from the Dual ^{13}C and ^{37}Cl Isotopic Approach at the Pilot Area

With regards to ^{37}Cl , values from +2.0‰ to +8.6‰ were measured. A trend similar to that of ^{13}C is observed, with ^{37}Cl moving toward more enriched values at lower 1,2-DCA concentrations (data not shown), confirming the importance of biodegradation processes at the site. By plotting ^{13}C over ^{37}Cl data, results correlate quite consistently to a linear trend (Figure 8). Such behavior indicates possibly a major, or multiple releases over time of 1,2-DCA (but characterized by similar ^{13}C and ^{37}Cl values) followed by biodegradation. Most likely, in the case of different sources, scattered $^{13}\text{C}/^{37}\text{Cl}$ values would have been found; such constraint, among several other conditions, is very important for estimating biodegradation extents at contaminated sites via application of the Rayleigh equation.

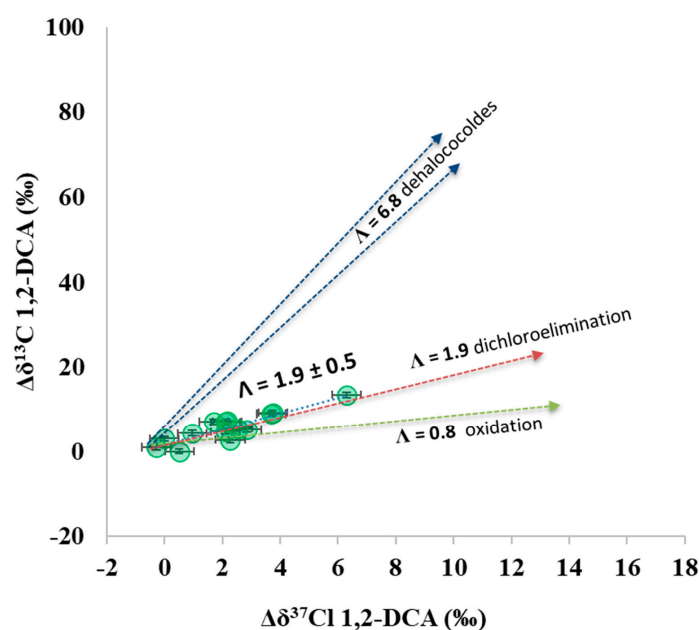


Figure 8. Dual isotope approach reporting the variation of $\delta^{13}\text{C}$ and $\delta^{37}\text{Cl}$ for the pilot area; Λ values for oxidation, dichloroelimination and *Dehalococcoides* sp. are the values reported in Palau et al., 2017a.

By considering the dual-isotope approach using $\delta^{37}\text{Cl}$ and $\delta^{13}\text{C}$, a linear correlation with a Λ of 1.9 ± 0.5 was obtained, although with a low R^2 (<0.90), (results from M13 and the nearby M12 were not considered because of the different microbiological results). One of the reasons for such low correlation is that the measured fractionations still remain restrained, compared to potentially larger fractionations, for example for carbon there were large enrichment factors of -9.1% and -11.3% estimated from the microcosm experiments. The obtained Λ of 1.9 ± 0.5 is very similar to the Λ of 2.1 ± 0.1 documented by

other authors also at field site conditions [23]. By comparing the obtained Λ to measured Λ for specific pathways some considerations can still be made. The obtained Λ is significantly different from the Λ indicated in the case of biodegradation processes by *Dehalococcoides* spp. containing microcosms, characterized by higher Λ of 6.8 ± 0.2 , confirming that *Dehalococcoides* spp. activity may not be relevant at the site. A difference also with oxidation processes in the case of *Pseudomonas* spp. is suggested, although because of the restrained fractionation such evidence cannot be ruled out by isotopic evidence only. Oxidation containing *Pseudomonas* spp. microcosm experiments [23] showed a Λ of 0.78 ± 0.03 , smaller than the Λ of 1.9 ± 0.5 measured in this study. In accordance with this, biological and molecular analysis did not exhibit a significant presence of *Dehalococcoides* spp., nor *Pseudomonas* spp., this latter is frequently detected along with other aerobic oxidative species in subsoil. On the contrary, Λ is similar to the Λ of 1.89 ± 0.02 available in literature for dihaloelimination by *Dehalogenimonas* spp. containing microcosms. The dihaloelimination pathway is confirmed by the absence of VC (or presence only in trace concentration) and also by the formation solely of ethene instead of VC during the microcosm biodegradation experiments. Still, no significant *Dehalogenimonas* spp. were revealed by the biological and molecular analyses; thus, other species must be responsible for 1,2-DCA dihaloelimination in the field. Since *Geobacter* spp. showed a large enrichment during the refresh microcosm experiments phase and was shown to be among the dominant species within the characterized wells and initial fresh microcosms, it is proposed that *Geobacter* spp. may be the main species responsible for 1,2-DCA biodegradation. Because analogues $\delta^{37}\text{Cl}$ and $\delta^{13}\text{C}$ fractionations (similar Λ) were observed, it is possible that *Geobacter* spp. dihaloelimination mechanisms could be similar to that proposed in [23] for *Dehalogenimonas* spp., namely a concerted mechanism with similar reduction mechanism of the halogenated substrate via halogen-cobalt bond formation in microbial reductive dehalogenases [35].

4. Conclusions

The potential for natural and enhanced attenuation was demonstrated by the use of anaerobic microcosm experiments, which showed almost complete degradation within 15 days and several hours by the use of native (fresh) and enriched cultures under natural (NA) and stimulated conditions (ENA) respectively. Degradation occurs by dichloroelimination hence no other chlorinated compounds (in particular VC, more toxic) are produced.

As part of an integrated monitoring protocol BMTs, techniques such as qPCR and NGS, as well as CSIA were developed from the laboratory experiments. *Geobacter* spp. as well as high levels of *rdh* were selected as indicative of biodegradation activity since they appear linked and responsible for the biodegradation pathway. Carbon stable isotope analysis confirms to be a proper tracer, with enrichment factors of -9.1% and -11.3% obtained for natural and enhanced conditions, respectively.

Significant enrichment on ^{13}C but also ^{37}Cl in combination with relative high presence of *Geobacter* spp. were documented in the field, at a detailed characterization level within the pilot area. The revealed decrease in concentration in the vicinity of the source area denoted efficient NA processes, hence reducing both 1,2-DCA concentrations and plume spreading at the natural conditions.

Dichloroelimination was confirmed to occur at the pilot scale by the combined use of ^{13}C and ^{37}Cl , moreover site specific ^{13}C enrichment factors led to estimate an extended degradation up to 70 to 80%.

The described coupled approach allows reducing any interpretative uncertainties. While CSIA itself can provide evidence for biodegradation processes and may be used for both qualitative and quantitative estimation, the interpretation is strengthened by the integration with BMTs, which are powerful tools to gain insights into the structure of the microbial community. By identifying the strain involved, BMTs offer the possibility of confirming the presence of microbial communities suitable for those natural attenuation processes assessed by the use of stable isotopes. Moreover, the functional biomarkers identification allow identifying the most likely degradation pathways occurring; such

information is crucial when using stable isotope data to evaluate natural attenuation processes as fractionations are intimately related to the site specific degradation pathways.

This paper highlights the relevance of the here proposed fine-tuned protocol in characterizing and estimating natural attenuation processes among groundwater remediation practices; moreover, by coupling BMTs and CSIA, lab and field results advocate the NA processes' role in reducing contaminant concentrations in the vicinities of source areas and not only along groundwater contaminants' plumes.

Author Contributions: G.C., F.d.F. and I.P. were involved both in the design of the study and in the processing of all the biological parts of the study, mainly focused on the set-up of the several microcosms including the chemical monitoring of the contaminant. L.A., M.M. and O.S.-S. developed the hydrogeological aspects and the isotopic part. All the above authors made fairly equal contributions to the writing of the paper. L.M.Z. was the referent for all the field activities and documentation. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Eni Rewind S.p.A. within the two projects "Novel Water treatment Processes"-Line "Chloro-organics biodegradation" and "Fingerprinting project".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All the data are represented in figures. No others databases area available.

Acknowledgments: Thanks also to Umberto Traldi, Thermo Fisher Scientific, and all the staff from the isotopic facility of ENI S.p.a., in particular Corrado Barbieri, for their kind help and assistance with regard to the isotope analysis.). Thanks also to Parco Tecnologico Padano (Lodi, Italy) that carried out the Illumina-MiSeq[®] sequencing.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the analyses and interpretation of data and in the writing of the manuscript.

References

1. United States Environmental Protection Agency. *Proposed Designation of 1,2-Dichloroethane (CASRN 107-06-2) as a High-Priority Substance for Risk Evaluation*; U.S. EPA.: Washington, DC, USA, 22 August 2019.
2. Falta, R.W.; Bulsara, N.; Henderson, J.K.; Mayer, R.A. Leaded-Gasoline Additives Still Contaminate Groundwater. *Environ. Sci. Technol.* **2005**, *39*, 378A–384A. [[CrossRef](#)]
3. Voudrias, E.A. Pump-and-Treat Remediation of Groundwater contaminated by Hazardous Waste: Can it really be achieved? *Global Nest J.* **2001**, *3*, 1–10.
4. Chen, Q.; Fan, G.; Na, W.; Liu, J.; Cui, J.; Li, H. Past, Present, and Future of Groundwater Remediation Research: A Scientific Analysis. *Int. J. Environ. Res. Public Health* **2019**, *16*, 3975. [[CrossRef](#)]
5. De Wildeman, S.; Diekert, G.; Van Langenhove, H.; Verstraete, W. Stereoselective Microbial Dehalorespiration with Vicinal Dichlorinated Alkanes. *Appl. Environ. Microbiol.* **2003**, *69*, 5643–5647. [[CrossRef](#)]
6. Duhamel, M.; Edwards, E.A. Microbial composition of chlorinated ethene-degrading cultures dominated by Dehalococoides. *FEMS Microbiol. Ecol.* **2006**, *58*, 538–549. [[CrossRef](#)] [[PubMed](#)]
7. Duhamel, M.; Edwards, E.A. Growth and yields of dechlorinators, acetogens, and methanogens during reductive dechlorination of chlorinated ethenes and dihaloelimination of 1,2-dichloroethane. *Environ. Sci. Technol.* **2007**, *41*, 2303–2310. [[CrossRef](#)]
8. Marzorati, M.; Balloi, A.; de Ferra, F.; Corallo, L.; Carpani, G.; Wittebolle, L.; Verstraete, W.; Daffonchio, D. Bacterial diversity and reductive dehalogenase redundancy in a 1,2-dichloroethane-degrading bacterial consortium enriched from a contaminated aquifer. *Microb. Cell Fact.* **2010**, *9*, 12. [[CrossRef](#)] [[PubMed](#)]
9. Grostern, A.; Edwards, E.A. Characterization of a Dehalobacter Coculture That Dechlorinates 1,2-Dichloroethane to Ethene and Identification of the Putative Reductive Dehalogenase Gene. *Appl. Environ. Microbiol.* **2009**, *75*, 2684–2693. [[CrossRef](#)]
10. Hirschorn, S.K.; Dinglasan, M.J.; Elsner, M.; Mancini, S.A.; Lacrampe-Couloume, G.; Edwards, E.A.; Lollar, B.S. Pathway dependent isotopic fractionation during aerobic biodegradation of 1,2-dichloroethane. *Environ. Sci. Technol.* **2004**, *38*, 4775–4781. [[CrossRef](#)] [[PubMed](#)]
11. Ansorge, W.J. Next-generation DNA sequencing techniques. *New Biotechnol.* **2009**, *25*, 195–203. [[CrossRef](#)] [[PubMed](#)]
12. Marchesi, M.; Alberti, L.; Shouakar-Stash, O.; Pietrini, I.; de Ferra, F.; Carpani, G.; Aravena, R.; Franzetti, A.; Stella, T. ³⁷Cl-compound specific isotope analysis and assessment of functional genes for monitoring monochlorobenzene (MCB) biodegradation under aerobic conditions. *Sci. Total Environ.* **2018**, *619–620*, 784–793. [[CrossRef](#)]

13. Hunkeler, D.; Aravena, R.; Butler, B.J. Monitoring Microbial Dechlorination of Tetrachloroethene (PCE) in Groundwater Using Compound-Specific Stable Carbon Isotope Ratios: Microcosm and Field Studies. *Environ. Sci. Technol.* **1999**, *33*, 2733–2738. [[CrossRef](#)]
14. Meckenstock, R.U.; Morasch, B.; Griebler, C.; Richnow, H.H. Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. *J. Contam. Hydrol.* **2004**, *75*, 215–255. [[CrossRef](#)] [[PubMed](#)]
15. Hunkeler, D.; Meckenstock, R.U.; Sherwood Lollar, B.; Schmidt, T.C.; Wilson, J.T. *CSIA EPA “A Guide for Assessing Bio-Degradation and Source Identification of Organic Groundwater Contaminants Using Compound Specific Isotope Analysis (CSIA)”*; EPA 600/R-08/148; U.S. Environmental Protection Agency: Washington, DC, USA, 2008.
16. Carter, J.F.; Barwick, V.J. *Good Practice Guide for Isotope Mass Spectrometry*; FIRMS: Bristol, UK, 2011; ISBN 978-0-948926-31-0.
17. Alberti, L.; Marchesi, M.; Trefiletti, P.; Aravena, R. Compound-Specific Isotope Analysis (CSIA) Application for Source Apportionment and Natural Attenuation Assessment of Chlorinated Benzenes. *Water* **2017**, *9*, 872. [[CrossRef](#)]
18. Hunkeler, D.; Aravena, R. Evidence of Substantial Carbon Isotope Fractionation among Substrate, Inorganic Carbon, and Biomass during Aerobic Mineralization of 1,2-Dichloroethane by *Xanthobacter autotrophicus*. *Appl. Environ. Microbiol.* **2000**, *66*, 4870–4876. [[CrossRef](#)] [[PubMed](#)]
19. Hunkeler, D.; Aravena, R.; Cox, E. Carbon Isotopes as a Tool to Evaluate the Origin and Fate of Vinyl Chloride: Laboratory Experiments and Modeling of Isotope Evolution. *Environmen. Sci. Technol.* **2002**, *36*, 3378–3384. [[CrossRef](#)] [[PubMed](#)]
20. Hirschorn, S.K.; Dinglasan-Panlilio, M.J.; Edwards, E.A.; Lacrampe-Couloume, G.; Lollar, B.S. Isotope analysis as a natural reaction probe to determine mechanisms of biodegradation of 1,2-dichloroethane. *Environ. Microbiol.* **2007**, *9*, 1651–1657. [[CrossRef](#)]
21. Schmidt, M.; Lege, S.; Nijenhuis, I. Comparison of 1,2-dichloroethane, dichloroethene and vinyl chloride carbon stable isotope fractionation during dechlorination by two Dehalococcoides strains. *Water Res.* **2014**, *52*, 146–154. [[CrossRef](#)]
22. Palau, J.; Shouakar-Stash, O.; Mortan, S.H.; Yu, R.; Rosell, M.; Marco-Urrea, E.; Freedman, D.L.; Aravena, R.; Soler, A.; Hunkeler, D. Hydrogen Isotope Fractionation during the Biodegradation of 1,2-Dichloroethane: Potential for Pathway Identification Using a Multi-element (C, Cl, and H) Isotope Approach. *Environ. Sci. Technol.* **2017**, *51*, 10526–10535. [[CrossRef](#)]
23. Palau, J.; Yu, R.; Mortan, S.H.; Shouakar-Stash, O.; Rosell, M.; Freedman, D.L.; Sbarbati, C.; Fiorenza, S.; Aravena, R.; Marco-Urrea, E.; et al. Distinct Dual C–Cl Isotope Fractionation Patterns during Anaerobic Biodegradation of 1,2-Dichloroethane: Potential To Characterize Microbial Degradation in the Field. *Environ. Sci. Technol.* **2017**, *51*, 2685–2694. [[CrossRef](#)]
24. Marzorati, M.; De Ferra, F.; Van Raemdonck, H.; Borin, S.; Alliffranchini, E.; Carpani, G.; Serbolisca, L.; Verstraete, W.; Boon, N.; Daffonchio, D. A Novel Reductive Dehalogenase, Identified in a Contaminated Groundwater Enrichment Culture and in *Desulfitobacterium dichloroeliminans* Strain DCA1, Is Linked to Dehalogenation of 1,2-Dichloroethane. *Appl. Environ. Microbiol.* **2007**, *73*, 2990–2999. [[CrossRef](#)]
25. Marzorati, M.; Borin, S.; Brusetti, L.; Daffonchio, D.; Marsilli, C.; Carpani, G.; De Ferra, F. Response of 1,2-dichloroethane-adapted microbial communities to ex-situ biostimulation of polluted groundwater. *Biogeochemistry* **2005**, *17*, 41–56. [[CrossRef](#)]
26. Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **2010**, *26*, 2460–2461. [[CrossRef](#)]
27. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267. [[CrossRef](#)] [[PubMed](#)]
28. Espinoza-Tofalos, A.; Daghio, M.; Palma, E.; Aulenta, F.; Franzetti, A. Structure and Functions of Hydrocarbon-Degrading Microbial Communities in Bioelectrochemical Systems. *Water* **2020**, *12*, 343. [[CrossRef](#)]
29. Merlino, G.; Balloi, A.; Marzorati, M.; Mapelli, F.; Rizzi, A.; Lavazza, D.; de Ferra, F.; Carpani, G.; Daffonchio, D. Diverse Reductive Dehalogenases Are Associated with Clostridiales-Enriched Microcosms Dechlorinating 1,2-Dichloroethane. *BioMed Res. Int.* **2015**, *2015*, 242856. [[CrossRef](#)] [[PubMed](#)]
30. Morrill, P.L.; Sleep, B.E.; Seepersad, D.J.; McMaster, M.L.; Hood, E.D.; LeBron, C.; Major, D.W.; Edwards, E.A.; Sherwood Lollar, B. Variations in expression of carbon isotope fractionation of chlorinated ethenes during biologically enhanced PCE dissolution close to a source zone. *J. Contam. Hydrol.* **2009**, *110*, 60–71. [[CrossRef](#)]
31. Yuan, S.Y.; Chang, B.V. Anaerobic degradation of five polycyclic aromatic hydrocarbons from river sediment in Taiwan. *J. Environ. Sci. Health B* **2007**, *42*, 63–69. [[CrossRef](#)] [[PubMed](#)]
32. Foght, J. Anaerobic Biodegradation of Aromatic Hydrocarbons: Pathways and Prospects. *J. Mol. Microbiol. Biotechnol.* **2008**, *15*, 93–120. [[CrossRef](#)]
33. Xu, X.; Liu, W.; Tian, S.; Wang, W.; Qi, Q.; Jiang, P.; Gao, X.; Li, F.; Li, H.; Yu, H. Petroleum Hydrocarbon-Degrading Bacteria for the Remediation of Oil Pollution Under Aerobic Conditions: A Perspective Analysis. *Front. Microbiol.* **2018**, *9*, 2885. [[CrossRef](#)]
34. Aburto, A.; Fahy, A.; Coulon, F.; Lethbridge, G.; Timmis, K.N.; Ball, A.S.; McGenity, T. Mixed aerobic and anaerobic microbial communities in benzene-contaminated groundwater. *J. Appl. Microbiol.* **2009**, *106*, 317–328. [[CrossRef](#)] [[PubMed](#)]
35. Payne, K.A.P.; Quezada, C.P.; Fisher, K.; Dunstan, M.S.; Collins, F.A.; Sjuts, H.; Levy, C.; Hay, S.; Rigby, S.E.J.; Leys, D. Reductive dehalogenase structure suggests a mechanism for B12-dependent dehalogenation. *Nat. Cell Biol.* **2015**, *517*, 513–516. [[CrossRef](#)] [[PubMed](#)]