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## Article

# Bioremediation of a Polluted Groundwater: Microbial Community Comparison of Treated and Untreated Aquifer through Next Generation Sequencing

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**Abstract:** Bioremediation is an active process for the detoxification of polluted ambient media employing the metabolism of microbes, while natural attenuation relies on physical, chemical and biological processes occurring without human intervention. A shallow aquifer (A0) was treated using a bioremediation approach through the amendment of whey to detoxify the most abundant contaminants: 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA), perchloroethene (PCE) and trichloroethene (TCE). A deeper aquifer (A1), showing lower concentration of the contaminants, was left untreated. In A0, a concomitant decrease of more chlorinated molecules 1,1,2,2-TeCA, PCE and TCE and an increase of less halogenated molecules such as trichloroethane (1,1,2-TCA), cis-dichloroethene (cis-DCE) and vinyl chloride (VC) were observed, suggesting that a reductive dechlorination took place. In contrast, the aquifer A1 did not show a significant decrease of contaminants during this period. A metagenomic approach (shotgun and 16S rRNA gene) was then used to investigate the microbial population of the two aquifers. A massive presence of the dehalogenator *Dehalococcoides mccartyi* (*D. mccartyi*) and a spectrum of different *Geobacter* species were detected in A0, after the treatment. The metagenome assembly of shotgun (SG) data further indicated a significant presence of methanogenic archaea, most likely from class *Methanomassiliicoccales*, at a level comparable to that of *D. mccartyi*. Instead, A1 was characterized by the species *Burkholderia*, *Curvibacter* and *Flavobacterium*. These results indicate that the autochthonous microbial consortia reflected the geochemistry of the two aquifers, with a dominant population thriving in an anoxic and nutrient rich environment implicated in reductive dehalogenation in A0 and a more diverse population, not able to decompose the pollutants, in A1.

**Keywords:** chlorinated solvents; polluted groundwater; bioremediation; microbial community; metagenomic analysis



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## 1. Introduction

Diffuse presence of organohalide compounds is primarily due to their release in the environment by anthropogenic activities such as paper production [1], metallic surfaces degreasing, dry cleaning [2,3], synthesis of plastic polymers such as polyvinyl chloride [4], etc. In particular, chlorinated ethenes and ethanes such as 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA), trichloroethane (1,1,2-TCA), perchloroethene (PCE), trichloroethene (TCE), and vinyl chloride (VC) are produced on large scale synthetically for industrial use or/and are generated as byproducts such as cis-dichloroethene (cis-DCE) trans-dichloroethene

(trans-DCE), 1,1,2-TCA and VC from partial degradation of more halogenated compounds. These widespread groundwater pollutants represent a serious threat to human health and the environment and being recalcitrant, they often persist in the subsurface for extended periods [5,6].

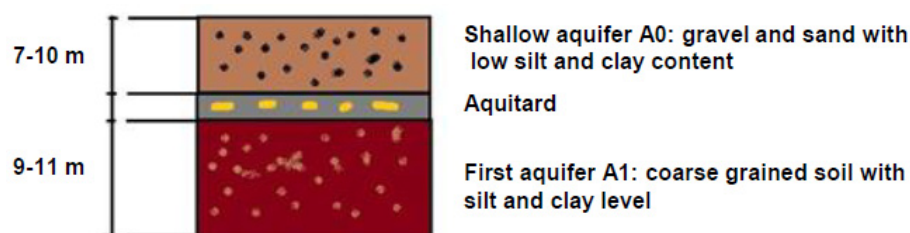
Since halogenated organic compounds are also both produced and degraded by microorganisms inhabiting the environment, microbial activity is pivotal for the cycling of such molecules [7]. From a metabolic point of view, microbial degradation of chlorinated ethenes can be performed by the following pathways [8]: anaerobic reductive dechlorination, anaerobic oxidation, aerobic/anaerobic cometabolism (fortuitous oxidation not leading to carbon for cell component or energy production) and aerobic assimilation (with VC and cis-DCE used as growth substrates). Anaerobic reductive dechlorination is an effective and well-studied pathway for the degradation of highly chlorinated ethenes and ethanes such as PCE, TCE and 1,1,2,2-TeCA, representing oxidized compounds employed by bacteria as electron acceptors in anoxic condition [9]. This process is carried on by microorganisms which need molecular hydrogen or organic carbon (organic acids) as carbon sources/electron donors in order to dechlorinate the contaminants [10–13]. For this reason, it is the metabolism that most likely happens during a bioremediation process involving the amendment of organic carbon to the polluted matrix. Different strains of *Dehalococcoides mccartyi* (*D. mccartyi*) are the only known microorganisms able to fully reductively dehalogenate PCE to ethene, but many other bacteria are known to be active in the partial dehalogenation process such as genera *Dehalobacter* [14,15], *Dehalospirillum* [16], *Dehalogenimonas* [17,18], *Desulfitobacterium* and *Desulfuromonas* [19]. *Sulfurospirillum* [20] and *Geobacter* have been found to be efficient in reducing PCE and TCE to cis-DCE [12,21] and appear to play a crucial role in this step. In general, organohalide respiring bacteria relies on other members of the environmental microbial community for the supply of fermentation products like hydrogen, acetate or formate. Some methanogens, such as the archaea *Methanosarcina*, have been described to dechlorinate PCE and TCE [22,23], and sulfate-reducing and iron-reducing microorganisms, have also been implicated in the process [24]. Besides anaerobic dehalogenation, also aerobic oxidation pathways have a role in the degradation of less halogenated organocompounds such as cis-DCE and VC in subsurface environments where oxygen is present. Members of *Polaromonas* (cis-DCE assimilating bacteria), *Burkholderia*, methanotrophs/methylotrophs and ammonia oxidizing microorganisms (involved in cometabolic dehalogenation) are of particular importance in these environments [25]. These naturally occurring bacteria can contribute to the process of natural attenuation which, without human intervention, generally leads to a slow and often inefficient partial removal of the contaminants.

Normally, bioremediation of sites polluted by toxic substances is achieved by the synergic metabolism of members of the autochthonous microbial community [26]. The first purpose of this study was to investigate in depth the composition of the complete microbial communities inhabiting two chlorinated organo-compound tainted aquifers, one treated with a bioremediation approach and the other left untreated and potentially hosting microbes capable of natural attenuation. The treatment of chlorinated solvents polluted aquifers with organic substances such as whey [27–30] is indeed a common technique to obtain environmental detoxification. The second purpose was to monitor the contaminant concentrations after ten months from the end of the treatment to verify if complete degradation was achieved. A genomic DNA shotgun (SG) and a 16S rRNA gene V3-V4 amplicon metagenomic analysis have been performed together with an investigation of the chemical composition of water. A comparison of the results obtained at the two overlaid aquifers has provided an insight of the microbial population of the two aquifers, while from the chemical analysis we witnessed a success when the treatment was in place and a bounce back of contaminant concentration after ten months from the end of the treatment.

## 2. Materials and Methods

### 2.1. Site Characterization

The site geology is constituted by alluvial and fluvio-glacial sediments, locally characterized by the presence of two aquifers (shallow aquifer–A0 and first aquifer–A1), which are separated by a thin clay layer (aquitard) at approximately 7–10 m of depth (Figure 1). Available data do not show any evidence of interconnection between the aquifers A0 and A1 in the contaminated site.



**Figure 1.** Hydrostratigraphy of sites. Schematic vertical representation of the hydrostratigraphy of the polluted site. Shallow aquifer (A0), first aquifer (A1).

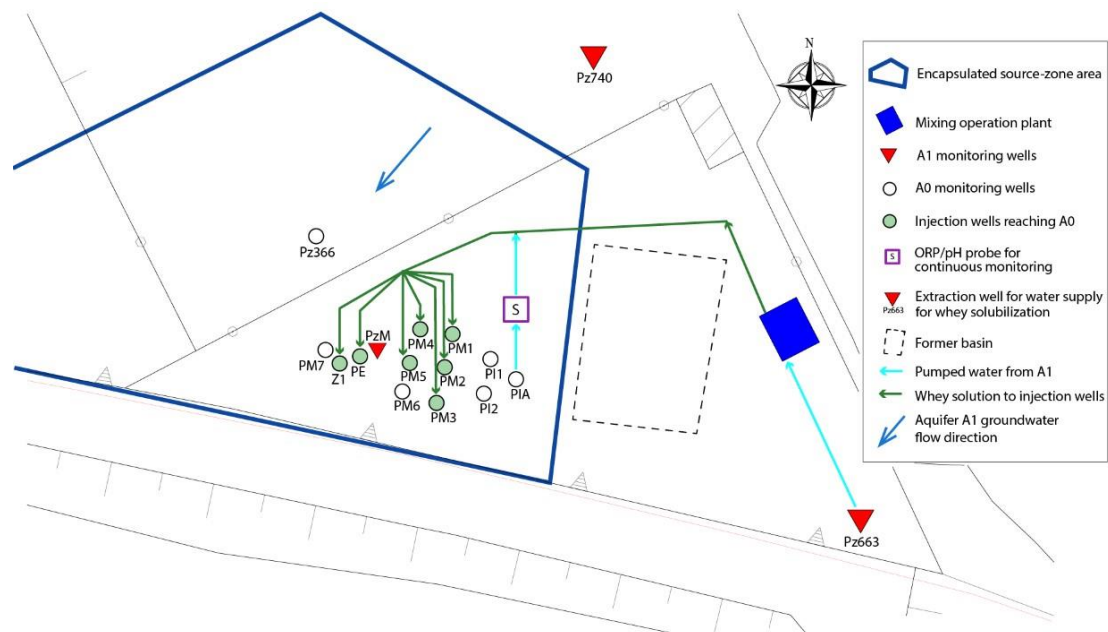
The industrial activity that was present on site has produced chlorinated solvents during an operational time of at least 50 years. The main source of contamination, detected in A0, was encapsulated by slurry walls embedded in the aquitard. Additionally, a hydraulic barrier with wells in both aquifers was installed downstream of the hotspot in order to capture the plume.

### 2.2. In Situ Field Test: Bioremediation Implementation

In situ bioremediation was performed on A0 through the injection of a concentrated whey solution. A total of 2000 kg of powder whey was dissolved in water in a tank (concentration of about 20 kg/100 L water) and gradually delivered into the A0 aquifer at a final concentration of 22 g/L through an automated system from the 21 February 2017 until June 2017. The whey solution was injected daily in piezometers PM1-PM6, PE and Z1 reaching A0 as illustrated in Figure 2. Monitoring was done through Pz366 and Pz663. The dose of substrate was calculated to maintain strong reducing conditions, to address the target contaminants and the alternative electron acceptors, such as iron, manganese, nitrates and sulfates, and to support reductive dechlorination in the aquifer [31].

### 2.3. Sample Collection and Total Community DNA Extraction

Groundwater was collected with a Grundfos (Milan, Italy) pump (MS3) from Pz366 (aquifer A0) and Pz663 (aquifer A1) after 5 min purge (Figure 2). The sampling for the microbial community analysis was performed in February 2017, right before the beginning of the treatment and 12 July 2017, after more than four months of whey injection. The pump was rinsed each time with clean water before sampling and the collected water was stored, at 4 °C, in plastic tanks (10 L) for about 5 days before filtration. Immediate filtration was indeed not possible for logistic reasons, but as described by Hinlo et al. [32], for short-term storage, refrigeration (3–5 days) is a feasible compromise enabling the detection of environmental low-density DNA. No genetic material was harnessed from the samples collected in February since the water presented high turbidity (probably due to silt) preventing filtration. For the groundwater collected in July, sample 366 was turbid due to the presence of iron and manganese (Table S1) whose oxides caused filter clogging and inefficient DNA extraction. To overcome this issue, sterile 200 mM EDTA was added directly to the sample at a final concentration of 20 mM with subsequent filtering through 5 µm and 0.22 µm mesh filters. Instead, sample 663 was limpid and clear and was filtered on 5 µm and 0.22 µm mesh filters without EDTA amendment. For each sample, 4 filters were prepared, each providing filtration of 300 mL groundwater for a total volume of 1.2 L.



**Figure 2.** A top-down view of the study site. Shown are the piezometers involved in the monitoring (Pz 366 and Pz 663) and the injection (PM1-PM6, PE and Z1). Water from piezometer Pz663 (A1) was pumped into a mixing operational plant (blue square) for preparing the whey solution which was then injected in piezometers reaching A0 (PM1-PM6, PE and Z1). A basin containing water and represented by the dashed line was present in the area but not used in the process. An impermeable barrier (blue line) isolates the shallow A0 aquifer in the test-site area with respect to the groundwater flow. The direction of A1 was from northeast to southwest.

Total genomic material was then extracted from the 0.22  $\mu\text{m}$  mesh filters using Dneasy Power Water (Qiagen, Germantown, MD, USA) and cleaned afterwards employing a sodium acetate–ethanol protocol. Briefly, a 4  $^{\circ}\text{C}$  cold 3 M sodium acetate solution was added to each sample at 1/10 of the sample’s volume and mixed. A 2.5 $\times$  volume of cold 100% ethanol was then added, and the sample was incubated at  $-20^{\circ}\text{C}$  for 1 h. The sample was then centrifuged for 15 min at 14,000 rpm at 4  $^{\circ}\text{C}$  and the supernatant was discarded. 250  $\mu\text{L}$  of cold 70% ethanol was then added to the pellet, the mix was centrifuged for 5 min at 14,000 rpm at 4  $^{\circ}\text{C}$ , and the supernatant was again discarded. The tubes with the pellet were left open at 37  $^{\circ}\text{C}$  for 10 min in order to remove of the remaining ethanol, and the DNA was finally resuspended in 50  $\mu\text{L}$  EB buffer (Qiagen, Germantown, MD, USA). The genomic material extracted from the 4 different filters was pulled together and quantified using Nanodrop (Thermo Scientific, Waltham, MA, USA) and Qubit (Invitrogen, Waltham, MA, USA) using dsDNA High Sensitivity assay measuring fluorescence at 485/530 nm showing a DNA concentration of 26.6 ng/ $\mu\text{L}$  for 663 and 77.6 ng/ $\mu\text{L}$  for 366.

#### 2.4. Sample Collection and Chemical Analysis

Sampling collection for the chemical analysis was performed starting on 2 February 2017 (before the beginning of the treatment which took place on the 21st of the same month) until May 2018. Chemical analysis was done immediately by Eurolab Italia (Nichelino, TO, Italy) for halogenated ethenes, ethanes and volatile fatty acids, and by Chelab S.r.l. (Merieux Nutrisciences Chicago, IL, USA) for ethenes. The analytical method used for the organohalogenated solvents in groundwater was the UNI EN ISO 15680:2005. Each sample was collected in vials, filled at the time of sampling and left untouched until the time of analysis. The vials were then inserted in a fully automatic sample treatment system for the addition of deuterated and surrogate internal standards, followed by the desorption of the compounds of interest by helium purging. The compounds present in the gaseous flow were trapped at sub-ambient temperature and subsequently injected into a capillary



column gas chromatography system; after separation, they were quantified in a mass selection detector (GC/MS). The calibration was checked before each sequence and every twenty samples.

The presence of short chain organic acids (fatty acids) in the water was determined applying the EPA methods 5030 C 2003 + 8260 D 2017. Samples were placed in glass containers and inserted into the autosampler serving the analytical system. The system is capable of injecting the required aliquots into the injection valve of the ion chromatograph comprising a suppressed conductivity detector (HPLC/IC); the detector collects and quantifies the ionic compounds after separation from the inorganic anions present. The calibration was carried out before each analytical sequence and checked every 10 samples. For the ethene analysis, the method used was the EPA protocol RSKSOP-175 2004. Briefly, the water samples were collected in the field without headspace, in a serum bottle and capped using a Teflon faced septum. A headspace was made by displacing 10% of the water with high purity helium. The bottle was shaken for 5 min, and a headspace sample was injected onto a gas chromatographic column where the gaseous components were separated and detected by a flame ionization detector.

### 2.5. Next Generation Sequencing

16S rRNA amplicons and total community genomic DNA were sequenced at Cemet GmbH (Tübingen, Germany). 16S rRNA gene V3-V4 amplicons were generated from 10 ng of DNA using forward primer S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and reverse primer S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') as described by Klindworth et al., 2012 [33]. Library generation was performed according to the recommendations provided by Illumina (San Diego, CA, USA). Amplicons were sequenced as 2 × 250 bp read pairs on a MiSeq instrument using MiSeq Reagent Kit v2. Approximately 60,000 read pairs were generated per 16S rRNA gene sample. SG samples were generated from 0.1 ng genomic DNA starting material following Illumina's Nextera XT protocol and sequenced as 2 × 100 bp paired reads on a NovaSeq 6000 instrument. Approximately 20 million read pairs were generated per SG sample.

Demultiplexing for both 16S rRNA gene and SG samples was performed with Illumina's bcl2fastq (v2.19), followed by trimming of adapter sequences with Skewer v0.2.2 [34]. Data quality was evaluated using FASTQC (v0.10.1, Wellcome Trust Sanger Institute, Hinxton, UK).

### 2.6. Sequence Read Processing, Classification of Metagenomic Profiles and Further Analysis

Following the removal of primers, raw 16S rRNA gene FASTQ files were filtered, as read pairs, with Trimmomatic v0.38 [35] applying parameters "MINLEN:200 AVEQUAL:30" and then combined into V3-V4 amplicon sequences using FLASH v.1.2.11 [36]. Taxonomic classification was performed using the SINTAX algorithm available in USEARCH [37] with the GTDB [38] bacterial and archaea SSU database (release 202: [https://data.gtdb.ecogenomic.org/releases/release202/202.0/genomic\\_files\\_all/ssu\\_all\\_r202.tar.gz](https://data.gtdb.ecogenomic.org/releases/release202/202.0/genomic_files_all/ssu_all_r202.tar.gz), accessed on 10 May 2021).

Raw SG FASTQ files were filtered with Trimmomatic v0.38 as read pairs applying parameters "MINLEN:90 AVEQUAL:30". Taxonomic classification was performed with KRAKEN2 [39] using the GTDB representative genomes available in release 202 ([https://data.gtdb.ecogenomic.org/releases/release202/202.0/genomic\\_files\\_reps/gtdb\\_genomes\\_reps\\_r202.tar.gz](https://data.gtdb.ecogenomic.org/releases/release202/202.0/genomic_files_reps/gtdb_genomes_reps_r202.tar.gz), accessed on 10 May 2021). The assembly of read pairs into contigs was performed with MEGAHIT [40] using SG data from both samples. No specific parameter was used when running the MEGAHIT program. A summary of the assembled MEGAHIT contigs is available in Table S2. Details of the METABAT2 results (grouping of assembled contigs into bins) such as N50 value and total contig length within each bin are available in Table S3. Contig binning was performed with METABAT2 [41] using bam files obtained with bowtie2 [42] by separately aligning read pairs from each sample to the MEGAHIT contigs. Taxonomic classification of METABAT2 bins was performed with

GTDBTk [43] using the GTDB release 202 database and completeness and contamination of metagenomes (bins) was estimated using CHECKM [44]. The detection of viral/phage genomes was done using VirSorter2 [45]. N50 values of metagenomes (bins) were calculated after sorting the contigs within each bin by decreasing length and determining the contig length, after which at least 50% of the overall bin length was covered. Relative abundance of metagenomes/bins was determined from the METABAT2 depth values for each contig. The relative abundance of a bin was determined by summing the product between the length and depth for each contig and dividing by the sum of the length of all contigs. Detection of reductive dehalogenase genes in the metagenomes was performed with DIAMOND [46] comparing the CHECKM predicted protein coding regions against the bacterial subset of the NCBI nr database. Coverage of 16S rRNA gene V3-V4 primer pairs was determined using the TestPrime tool available with the ARB-SILVA database (<https://www.arb-silva.de/browser/ssu-132/silva-ref-nr/testprime/>, accessed on 10 March 2021). A circular tree display of SG genus data and METABAT2 bins was performed with iTOL (<https://itol.embl.de/tree>, accessed on 25 July 2021).

### 3. Results

#### 3.1. Contaminant Concentrations

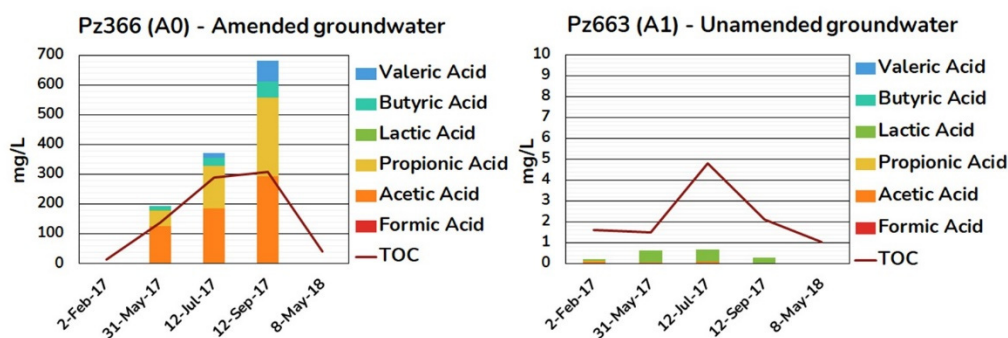
Chemical data, collected from the two studied aquifers A0 and A1 prior to treatment (Figures 1 and 2), showed that the initial concentration of pollutants in A0 was about 10 times higher than in A1. In A0, after the treatment has started (21 February) most of the initially present chlorinated ethanes and ethenes such as 1,1,2,2-TeCA (1400 µg/L), PCE (96 µg/L) and TCE (520 µg/L) decreased in concentration (down to 140, 3.7 and 19 µg/L, respectively), while less chlorinated transformation products, characteristic of reductive dehalogenation raised in concentration until September 2017 such as 1,1,2-TCA (from 8.8 to 31 µg/L), cis-DCE (from 10 to 610 µg/L), trans-DCE (from 3.5 to 480 µg/L) and VC (from 1.1 to 120 µg/L) (Table 1). Ten months after the end of the treatment (May 2018), we witnessed a bounce back in concentration of the initial contaminant 1,1,2,2-TeCA with respect to September 2017 (from 140 to 1860 µg/L), while TCE and PCE seem to remain constant (from 19 to 25.2 µg/L and from 3.7 to 6.4 µg/L). In the untreated aquifer A1, concentration of 1,1,2,2-TeCA, TCE and PCE remained relatively constant (from 70, 54 and 4.6 to 36, 78 and 5.2 µg/L, respectively) and transformation products like cis-DCE and trans-DCE VC and 1,1,2 TCA occurred only in low concentration up to 2.9, 1.2, 0.43 and 0.84 µg/L, respectively, in September 2017 (Table 1). This was probably due to a small connection between the two aquifers in proximity to the piezometers allowing some migration from A0 to A1. A bioremediation strategy on an area affected by a very similar composition in contaminants is reported by Aulenta et al. [30], in which they describe concentrations of initial 1,1,2,2-TeCA as high as 40 and 90 mg/L and TCE ranging from about 2 to 6 mg/L. Dechlorination intermediates like 1,1,2-TCA and cis-DCE were detected at concentrations higher than 6 and 5 mg/L. In a similar work, Rodriguez and colleagues [47] describe a decrease in concentration of TCE from 8400 to 400 µg/L while cis-DCE increased from 1600 to 3730 µg/L and presence of TCE and cis-DCE as high as 6000 and 4000 µg/L, respectively, was witnessed by Němeček et al. [29] during a thermally enhanced in situ bioremediation.

In A0, ethene was also detected (in September 2017) at a concentration of 68 µg/L, suggesting that a complete dechlorination of the initial PCE, TCE and 1,1,2,2-TeCA was taking place. 410 and 4500 µg/L of methane in September 2017 and May 2018 was also measured. In more detail, in A0, the presence of short chain fatty acids such as valeric, butyric, propionic and acetic is a result of the whey's metabolism by the autochthonous microbial community (Figure 3). In May 2018, the same short fatty acids were not detected. As previously explained, whey is a commonly employed organic substrate used for chlorinated ethanes and ethenes detoxification of polluted groundwater [27–29,48] since it is able to provide electron donors, reduced carbon, reduced redox condition and enhance the dissolution of those pollutants in the groundwater [30,49]. At the same time, fermentation

of such substrate brings to the formation of short chain fatty acids such as acetic, propionic, butyric, valeric.

**Table 1.** The pollutants and byproducts of reductive halogenation. Concentration ( $\mu\text{g/L}$ ) of pollutants and byproducts of reductive halogenation observed at the treated (A0) and the untreated (A1) aquifers. In the aquifer A0, a whey treatment was performed starting from the 21 February until June 2017. Aquifer A1 was left untreated. 1,1,2,2-TeCA: 1,1,2,2-Tetrachloroethane; TCE: Trichloroethene; PCE: Tetrachloroethene; cis-DCE: cis-1,2-Dichloroethene; trans-DCE: trans-1,2-Dichloroethene; TCA: 1,1,2-Trichloroethane; VC: Vinyl Chloride; ETH: Ethene; METH: Methane; n.d.: not determined.

Date	Pz366 (A0)					Pz663 (A1)				
	2 February 2017	31 May 2017	12 July 2017	12 September 2017	8 May 2018	20 February 2017	31 May 2017	12 July 2017	12 September 2017	8 May 2018
1,1,2,2 TeCA	1400	780	240	140	1860	70	37	84	53	36
TCE	520	110	17	19	25.2	54	96	110	23	78
PCE	96	4.2	6.5	3.7	6.4	4.6	4.9	11	6.0	5.2
cis-DCE	10	450	520	610	142	0.22	1.1	15	2.9	3.11
trans-DCE	3.5	240	410	480	330	0	<2.0	0.66	1.2	0.196
1,1,2 TCA	8.8	120	41	31	71	0	<2.0	1.1	0.84	0.25
VC	1.1	120	220	120	176	<0.025	<2.5	0.28	0.43	0.103
ETH	n.d.	n.d.	n.d.	68	<5	n.d.	n.d.	n.d.	<7.4	<5
METH	n.d.	n.d.	n.d.	410	4500	n.d.	n.d.	n.d.	<3.8	<5



**Figure 3.** Short chain fatty acid byproducts. Appearance of short chain fatty acid byproducts at the treated 366 (A0) aquifer. In the untreated 663 (A1) aquifer only traces of short chain fatty acids like lactic acid and acetic acid are present. Values reported for February are prior to the start of treatment which took place on 21 February. Values for May 2018 are ten months after the end of the treatment. TOC = Total organic carbon.

### 3.2. Microbial Community Composition

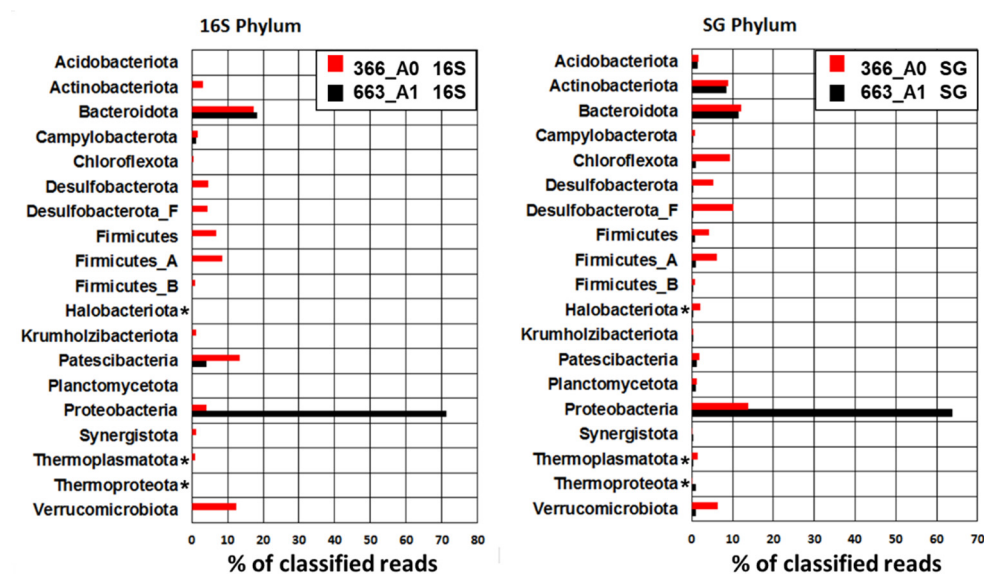
Following filtering of raw read data with Trimmomatic and 16S rRNA gene V3-V4 read pair merging (Table 2), the remaining 16S rRNA gene and SG read data was subjected to taxonomic classification using the GTDB taxonomy [38], with the USEARCH SINTAX algorithm (16S rRNA gene amplicons; [37]) or KRAKEN2 (SG data; [39]). While nearly all the 16S rRNA gene data was classified at the phylum level, classification of the SG data was very limited reaching only values around 10% (Table 2).



**Table 2.** The extent of taxonomic classification by USEARCH (16S) and KRAKEN2 (SG). Numbers indicate % of taxonomically classified read pairs (SG) or merged read pairs (16S). QC: quality control filter.\* Indicates merged amplicon.

Sample	Number of Reads	Number after QC	Phylum %	Class %	Order %	Family %	Genus %	Species %
663 (A1)16S	62,551	41,569 *	95.8	94.8	89.7	81.1	49.1	8.3
366 (A0) 16S	63,906	42,128 *	83.3	69.1	61.1	44.2	28.0	15.5
663 (A1) SG	20,825,527	14,757,073	52.46	51.69	49.71	47.86	39.20	23.19
366 (A0) SG	18,240,703	11,635,396	38.27	37.74	36.35	34.44	26.72	16.14

A significant difference in the bacterial community composition between aquifer A1 and A0 was evident already at the phylum level (Figure 4). While both 16S rRNA gene and SG data indicate an overall similar taxonomic profile, in the 16S rRNA gene V3-V4 data *Chloroflexota* were practically absent and a significant amount of *Patescibacteria* was present (Figure 4). A more detailed analysis using the SILVA [50,51] TestPrime tool revealed that only about 39% of *Chloroflexota* are covered by the V3-V4 primers used and that their abundance is likely to be underestimated by the 16S amplicons (Figure 4). For *Patescibacteria*, an emerging superphylum [52], absence of genome sequence information is the most likely cause for the being of lower relative abundance in the KRAKEN2 analysis. Other differences, for example the low amount of *Actinobacteria* for the 16S amplicons, most likely reflect intrinsic limitations of the two approaches—extensive known 16S sequence information, but also possibly incomplete primer coverage for 16S and the still relatively scarce information on genome sequence used to taxonomically classify SG data. The latter is also evident from the low percentage of SG reads actually assigned to a taxonomy (only  $\approx$  40–50%, Table S4), much lower than the near complete assignment for the 16S amplicons ( $\approx$  84–96%, Tables S5 and S6).



**Figure 4.** A 16S rRNA gene analysis at the phylum level. Community composition at the phylum level based on the 16S V3-V4 amplicon or the shotgun (SG) data. Only taxonomies representing more than 1% of classified reads are shown. Archaea are indicated by an asterisk (\*).

At lower taxonomic levels, the SG data for the aquifer A0 showed a strikingly different microbiome indicating a significant presence of the order *Dehalococcoidales* (Figure 5) that comprises the genus *Dehalococcoides* (Figure 6) and the species *D. mccartyi*, the only known

anaerobic dehalogenating bacteria capable of completely dechlorinate PCE to ethene [53]. A complete list of the KRAKEN2 classification is available in Table S7.

Given that classification methods such as KRAKEN2 rely on individual read pairs do not account for overall genome length and do not attempt to assemble the data into longer contigs or even metagenomes, the SG data was also examined applying a metagenomic binning approach. Using MEGAHIT [40] on all read pairs from samples A0 and A1, assembled contigs were obtained and grouped into metagenomes (bins) with bowtie2 [42] and METABAT2 [41].

The MEGABAT2 bins thus obtained were then taxonomically classified with GT-DBtk [43] (Table S8), checked for completeness and contamination with CheckM [44] (Table S9) and also examined with VIRSORTER2 [45] to detect the possible presence of phage genomes (Table S10). This allowed us to obtain an alternative view of the microbiomes present in each sample including an estimate for the relative abundance for each bin from the median depth values (coverage) for each bin from MEGABAT2 (Figure 7; Table S3).

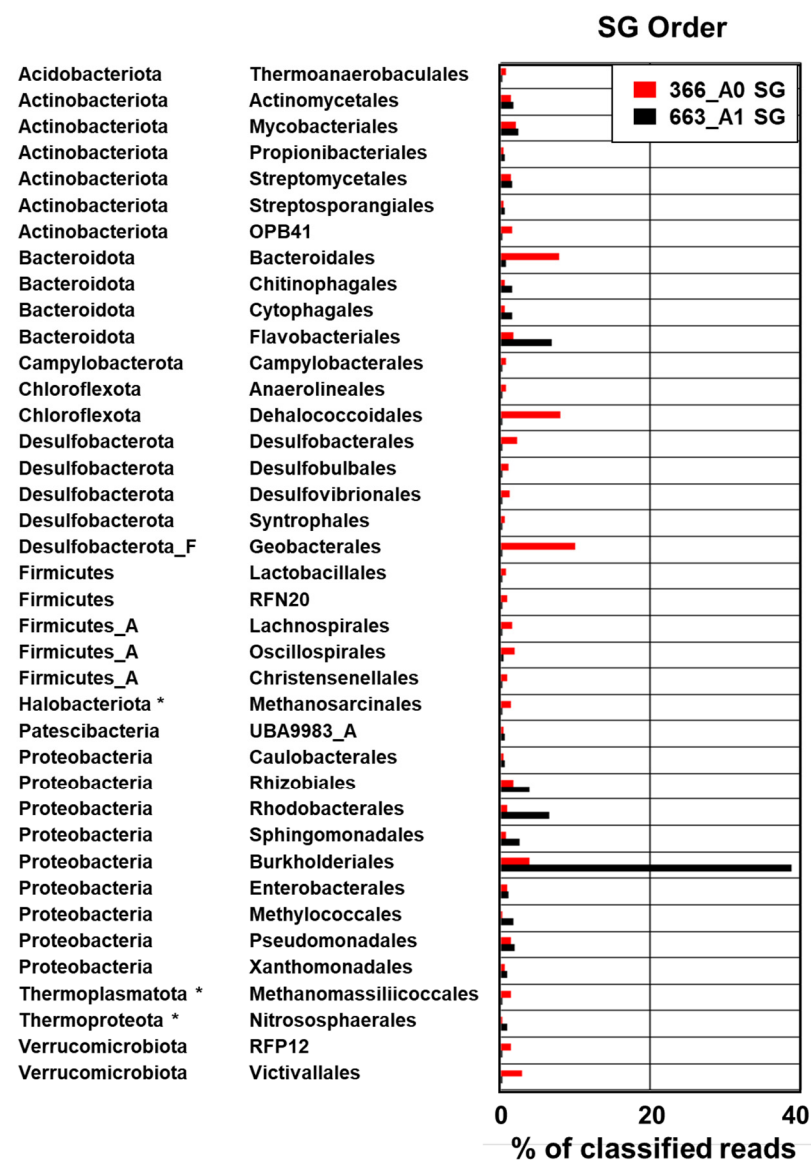
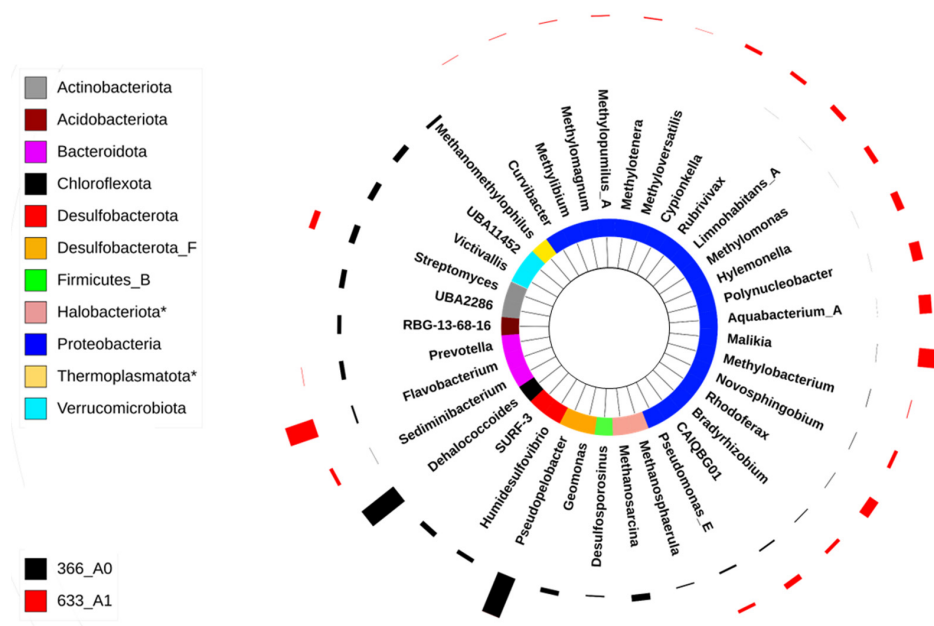
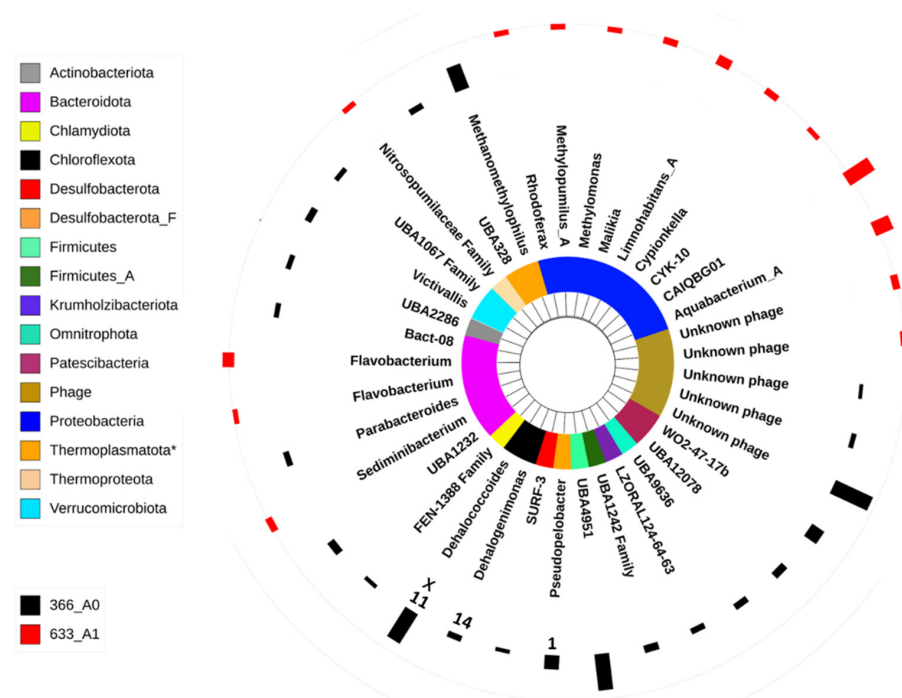


Figure 5. Shotgun (SG) data at the order level. Microbial community composition at the order level based on a KRAKEN2 analysis of the SG data. Only taxonomies representing more than 1% of classified reads are shown. Archaea are indicated by an asterisk (\*).



**Figure 6.** Shotgun (SG) data at the genus level. Microbial community composition at the genus level based on a KRAKEN2 analysis of the SG data. Values shown for each genus as a barplot (black 366\_A0, red 663\_A1) correspond to the percentage of taxonomically classified SG read pairs with maximal a value of 12.5% for *Pseudopelobacter*. Only genera representing more than 1% of classified SG reads are reported (sample A0\_top, sample A1\_bottom), plus a selected subset of genera. Archaea are indicated by an asterisk (\*).



**Figure 7.** A microbial community composition at the genus level according to the GTDBTK classification. METABAT2 relative abundance estimates (median METABAT2 depth value across all contigs in the bin) are shown as black (366\_A0) or red bars (633\_A1). with a maximal relative abundance value of 89.5 for a phage in sample A0. Phyla also present in Figure 7 are colored identical. Presence of reductive dehalogenase (Rdase) genes is indicated by numbers and the presence of a tetrachloroethene reductive dehalogenase (TceA) in *Dehalococcoides* by a 'x'. Archaea are indicated by an asterisk (\*).

An analysis of the relative abundances of each bin (assembled metagenome) revealed an essentially exclusive presence in either sample, with small overlap (Figure 6). This is consistent with the fact that the two aquifers are separated by a low permeability clay layer. Taxonomic classification of the bins with GTDBtk confirmed the presence in A0 of a *Dehalococcoides* species closely related to *D. mccartyi\_B* (99.26% sequence identity) as the dominant, most abundant bacterial species, closely followed by an archaea of genus *Methanomethylophilus*, an *Euryarchaeota* belonging to the class *Methanomassiliicoccales* (Figure 7) [54].

Interestingly, methanogenic archaea, which are also strictly anaerobes, have been reported to co-exist in *D. mccartyi* containing communities in a number of cases and supply essential co-factors and nutrients [24,55,56]. The presence of phages was also confirmed with an unknown phage representing the most abundant metagenome in sample A0 (Figure 7). Although no additional metagenomes could be classified at the species level in sample A0, a number of noteworthy genera were detected: *Dehalogenimonas* represents another *Chloroflexota* able to reductively dehalogenate chlorinated ethanes and ethenes [57]. Other noteworthy metagenomes are related to genus *Pseudopelobacter*, potential iron reducers [58] (Figure 7). *Dehalococcoides*, *Dehalogenimonas* and *Pseudopelobacter* are the only three genera present in Figure 7 and described in the literature as dehalogenator. Indeed, as reported below, the data here also evidences the presence in their genomes of the enzyme reductive dehalogenases (Rdases) which is pivotal in chlorinated solvents reductive degradation. The complete microbial community composition as classified by the metagenomic binning approach is available at different rank levels in Table S3.

The untreated sample A1 is instead characterized by a microbiome dominated by *Bacteroidota*, genus *Flavobacterium* (species closely related to *Flavobacterium* sp003634825 and *Flavobacterium glycinis*) and *Proteobacteria* (Figure 7), with genera CAIQBG01 (of family *Rhodocyclaceae*) and *Malikia* (species related to *Malikia granosa*), many of which are known for their capacity to degrade aromatic substances [59]. For sample A0, the presence of phages is also evident in the untreated sample A1, judging from the small size (around 300 kB) for two of the metagenomes (Figure 7, Table S3).

In a final step, the predicted protein coding sequences for all metagenome bins were confronted with the bacterial protein sub-division of the NCBI nr database. Rdases are a group of enzymes responsible for biological dehalogenation in organohalide respiring bacteria. Those are absent in sample A1 but could be identified in three metagenomes from sample A0: 11 in the metagenome closely related to *D. mccartyi\_B*, including tetrachloroethene reductive dehalogenase (TceA), 14 in the metagenome belonging to genus *Dehalogenimonas*, also known to reductively dehalogenate chlorinated alkanes [60] and alkenes [18] and 1 additional dehalogenase in the *Pseudopelobacter* metagenome (Figure 7). Sample A1 contains a large number of *Proteobacteria* metagenomes, a phylum that appears to be absent in the treated sample A0. Since *Dehalococcoides* is known to rely on external support of cobalamin for the growth, this essential factor is probably provided by the *Thermoplasmatota* (genera *Methanomethylophilus* and UBA328) and the *Firmicutes* genus UBA4951, all of which are known to be able to synthesize cobalamin [61,62].

## 4. Discussion

### 4.1. Hydrology Data

In northern Italy, a region with a long industrial history, subsoil pollution prevalently composed by 1,1,2,2-TeCA (1400 µg/L), TCE (520 µg/L) and PCE (96 µg/L) was subject to a bioremediation treatment. The underground site examined in this study is characterized by the presence of two overlaid aquifers, shallow (A0) and deep (A1), separated by a thin clay layer present all over the site with an average thickness of about 2 m. The direction of flow through the clay layer is determined by the head difference of the two aquifers. In A0, the hydraulic heads are about 2–3 m higher than the heads present in A1, giving rise to a vertical gradient with a downward direction. Even if clay is assumed to be impermeable (hydraulic conductivities lower than 1E-8 m/s), a thickness of 2 m can be passed in about

10 years, especially considering that in some portion of the site the clay layer might be less than 2 m or even be extremely thin (less than 0.5 m) or even absent. Furthermore, some of the site boreholes might not be perfectly sealed to avoid aquifer interchange, thus representing a minor migration path from the upper to the lower aquifer. This could be the reason why chlorinated byproducts and fatty acids were also found in minimum amounts in the deep aquifer A1.

#### 4.2. Chemical Data: Contaminants and Fatty Acids

The chemical data indicated that, prior to bioremediation treatment, a scarcely effective microbial community was unable to degrade the chlorinated contaminants (1,1,2,2-TeCA, PCE and TCE) that were dominant in the shallow aquifer A0. After three months, following whey injection, the contaminant PCE, 1,1,2,2-TeCA and TCE concentrations decreased with a concomitant appearance of less chlorinated compounds such as 1,1,2-TCA, cis-DCE, trans-DCE and VC (Table 1). This was indicative of an anaerobic dechlorinating metabolism taking place and operating toward a full detoxification since in September 2017 ethene was also detected. Methane was also present in September 2017 and May 2018, indicating strong reducing condition due to the metabolism of archaea methanogens (Table 1, Figures 4–7). Ten months after the end of the amendment injections, an increase of 1,1,2,2-TeCA concentration was detected meaning that the treatment was interrupted too early and a full aquifer decontamination was not achieved. The contaminant that was still embedded in the clay layer, which is not permeable to the nutrients and separates the two aquifers, was probably dissolving back to the groundwater. Indeed, being 1,1,2,2-TeCA, a chlorinated solvent with a specific gravity greater than water frequently present in aquifers, it forms a dense non-aqueous phase liquid (DNAPL) which sinks in low permeability clay layers spreading laterally [63]. Over time, this contamination both dissolves sparingly in water moving downgradient (forming a plume) and concentrates through diffusion in the clay zone [64]. Those low permeable areas, not affected by the bioremediation process, become reservoirs releasing contamination to the surrounding water and increasing the lifetime of the pollution through back-diffusion [65]. This explains why the pollutant's concentration may bounce back once nutrient's injection stops. TCE and PCE, which were also initial contaminants with a specific gravity greater than water, appear to have a similar concentration in September 2017 and May 2018. Ethene was absent in May 2018 probably because fully consumed by microbial metabolism while cis-DCE, trans-DCE and VC were still detected as well as 1,1,2-TCA meaning dechlorination of PCE, TCE and 1,1,2,2-TeCA was still slowly taking place, or such byproducts were not consumed. At the same time, short chain fatty acids such as acetic, propionic, butyric and valeric were found when the treatment was in place indicating an active microbial metabolism supported by the degradation of the whey organic carbon and pivotal for the pollutants' co-metabolism. They were not detectable ten months after the end of the injection, attesting a scarcity of nutrients and energy for microbial metabolism. As the carbon source derived from the whey's catabolism is depleted, also the short chain fatty acids decrease. Predicting and managing a successful in situ bioremediation project for chlorinated solvent impacted groundwater brought to a complete detoxification of the contaminants is extremely complex. This is due to the nature of the DNAPL, for which complete dissolution in water from the low permeability layers cannot be foreseen with current technologies [66].

Instead, in the deeper and untreated aquifer A1, no significant decrease in the concentration of the same contaminants was evident, and the temporary appearance of small amounts of less chlorinated molecules such as cis-DCE, 1,1,2-TCA and VC could be explained by the fact that some of the site boreholes might not be perfectly sealed, allowing some aquifer interchange and a transfer of degradation products between the two aquifers.



### 4.3. Microbial Composition

Metagenomic analysis focused on 16S rRNA amplicons or SG genomic fragments represent a powerful approach for elucidating the microbial population present in practically all existing habitats. Amplicons generated from the 16S rRNA gene variable regions such as V3-V4 can be compared with a vast number, nowadays more than several millions, of known 16S rRNA gene sequences. In the present study, divergent results have been obtained with the two methods (16S rRNA gene or SG), a situation known in the literature [67,68], with the 16S rRNA gene results missing out nearly completely *Chloroflexota*. One possible explanation for this discrepancy is the relatively low coverage (only  $\approx 39\%$ ) of phylum *Chloroflexota* by the primer pair used for generation of the V3-V4 amplicon, for S-D-Bact-0341-b-S-17 and rev S-D-Bact-0785-a-A-21, despite providing a generally broad overall coverage ( $\approx 87\%$ ) for bacteria [33,69]. Whole branches of taxa such as *Dehalococcoidales*, or even whole phyla such as archaea, can be missed. Alternative primer designs have been proposed [70,71]; however, use of multiple primer pairs can also introduce experimental bias during PCR amplification. In contrast, shotgun sequencing in principle avoids these limitations because every genomic DNA that is isolated from the sample is present and sequenced. The taxonomic classification of SG data requires large databases with complete genome sequences, but today only a relatively small fraction of the estimated bacteria and archaea universe is still covered. Importantly, the recent introduction of GTDB [38] has provided a more consistent and enlarged taxonomy system, allowing both 16S rRNA gene and SG data to be analysed within the same taxonomy framework. Using the most recent release of GTDB (RS202), a relatively large portion of reads could be classified, at least at the higher taxonomic ranks. The remaining unassigned reads most likely represented yet unexplored “dark bacterial matter” and possibly also indicate the presence of phages. The most complete picture of the microbial composition present in the two samples was obtained by a metagenomic binning approach aimed at assembling and classifying metagenomes. As anticipated by the 16S rRNA gene and SG results, the two samples demonstrate a non-overlapping metagenome composition. In the whey treated sample A0, a species closely related to *D. mccartyi* was identified as the dominant feature of the bacterial population. Interestingly, a series of other genera that have been reported to co-exist with *D. mccartyi* in dehalogenating consortia were also detected. The appearance of these other genera may be consistent with the dependence of *D. mccartyi* on an external cobalamin source [72], supplied most likely by Archaea, in particular the genus *Methanomethylophilus*, which is present with a relative abundance similar to *D. mccartyi*. Methanogenic archaea (e.g., *Methanosarcinales*), known to co-exist with *D. mccartyi* and often supplying cobalamin as essential co-factor or electron donor and carbon sources such as acetate and  $H_2$  [73,74], and able also to dechlorinate PCE and TCE [22,23], were detected in relatively small amounts during the taxonomic classification.

Other than the well-known and highly abundant organohalide-respiring *D. mccartyi*, additional genera identified in the SG sample A0 have been implicated in dechlorinating processes. Amongst them, the *Euryarchaeota*, genus *Methanosarcina* is a PCE and TCE dehalogenator [22,23] while the genus *Methanosphaerula* has been recently described as a member of an anaerobic dechlorinating enrichment [75]. Species belonging to genus *Geomonas* (*Geobacter*) are often present in environments tainted with chlorinated ethenes and play a pivotal role in anaerobically dechlorination of PCE to cis-DCE [12,21,55], as does *Sulfurospirillum* [20]. Similarly, spore-forming sulfate reducing bacteria belonging to *Desulfosporosinus*, previously known as *Desulfotomaculum*, is a genus reported to dehalogenate PCE to TCE, 1,1-DCE and VC [76].

Results for the untreated sample A1 showed instead a completely different and much more diverse composition of genera inhabiting the oligotrophic groundwater environment, some of which could be potentially involved in natural attenuation of chlorinated ethenes via cometabolic aerobic oxidation, for example the cosmopolitan *Polynucleobacter*, which is ubiquitous in lentic freshwater habitats [77]. Other examples are the order *Burkholderiales* and genus *Pseudomonas*, known to possess both halogenating and dehalogenating

enzymes [7] and able to co-metabolically degrade TCE employing mono and dioxygenase enzymes [25,78,79].

*Curvibacter* is a genus inhabiting well water [80] but is also present in a stable microbial consortium able to degrade chlorinated ethenes in a simulated groundwater column [81]. The obligatory aerobe genus *Flavobacterium* is normally present in soil and freshwater, but it is also found in groundwater contaminated by chlorinated solvents undergoing natural attenuation [82]. Another example is the genus *Bradyrhizobium*, soil bacteria possessing halogenating and dehalogenating enzymes [7] and capable of oxidative dechlorination of the insecticide methoxychlor [83]. Among the less abundant genera, several methanotrophic and methylotrophic bacteria (*Methylomagnus*, *Methylibium*, *Methylobacterium*, *Methylomonas*, *Methyloversatilis*) express the enzyme methane monooxygenase (MMO) capable of degrading chlorinated compounds. MMO can generate the unstable TCE-epoxide from TCE, which then breaks down into not chlorinated compounds, carbon dioxide and chloride salts [84–86].

Overall, microbial composition and chemical analysis evidenced that in the treated shallow aquifer A0, an anaerobic microbial community was active in the dechlorination of the initial pollutants while in the first aquifer A1 a more diverse natural population was not efficient in degrading the contaminants in a short period of time but might be implicated in a slower natural attenuation.

Finally, the metagenomic binning approach has also provided evidence for a significant portion of phages within the sample microbiomes, with an unknown phage representing the most abundant metagenome in the treated sample A0. Attempts to further classify the (putative) phage metagenomes failed due to a relatively low sequence homology to known phage encoded proteins.

In summary, using a panel of metagenomic methods a first approximate quantitative and qualitative picture of the microbial community present within a whey-treated groundwater sample has been obtained. Although genome databases are constantly expanding, they still lack sufficient “cover”, in particular at the genus and species taxonomic level, necessary to obtain a more complete and detailed taxonomic resolution. In addition, we have found that phages can also be present in relatively high abundance. For their pivotal roles in biogeochemistry and microbial ecology, they should be, then, always included in a metagenomic analysis.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w14162456/s1>, Table S1: Chemical analysis Pz 366; Table S2: Details MEGAHIT; Table S3: Summary METABAT2; Table S4: SG Phylum classification; Table S5: 16S Phylum classification; Table S6: 16S Species classification; Table S7: SG Species classification; Table S8: GTDBTk classification; Table S9: CheckM results; Table S10: Visorter2 results.

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