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# Molecular characterization of microbial communities in a peat-rich aquifer system contaminated with chlorinated aliphatic compounds

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## Abstract

In an aquifer-aquitard system in the subsoil of the city of Ferrara (Emilia-Romagna region, northern Italy) highly contaminated with chlorinated aliphatic toxic organics such as trichloroethylene (TCE) and tetrachloroethylene (PCE), a strong microbial-dependent dechlorination activity takes place during migration of contaminants through shallow organic-rich layers with peat intercalations. The in situ microbial degradation of chlorinated ethenes, formerly inferred by the utilization of contaminant concentration profiles and Compound-Specific Isotope Analysis (CSIA), was here assessed using Illumina sequencing of V4 hypervariable region of 16S rRNA gene and by clone library analysis of dehalogenase metabolic genes. Taxon-specific investigation of the microbial communities catalyzing the chlorination process revealed the presence of not only dehalogenating genera such as *Dehalococcoides* and *Dehalobacter* but also of numerous other groups of non-dehalogenating bacteria and archaea thriving on diverse metabolisms such as hydrolysis and fermentation of complex organic matter, acidogenesis, acetogenesis, and methanogenesis, which can indirectly support the reductive dechlorination process. Besides, the diversity of genes encoding some reductive dehalogenases was also analyzed. Geochemical and 16S rRNA and RDH gene analyses, as a whole, provided insights into the microbial community complexity and the distribution of potential dechlorinators. Based on the data obtained, a possible network of metabolic interactions has been hypothesized to obtain an effective reductive dechlorination process.

**Keywords** Bioremediation · Reductive dechlorination · Chloroethylene · 16S rRNA · Microbial community

## Introduction

Chlorinated ethylene contaminants such as perchloroethylene (PCE) and trichloroethylene (TCE) are commonly found in groundwater due to their frequent industrial use since the beginning of the last century (Balderacchi et al. 2014; McCarty

2010). Dichloroethylene isomers (mainly 1,2-cis dichloroethylene—cDCE) and vinyl chloride (VC) may be found associated to PCE and TCE in groundwater as by-products of anaerobic/reductive microbial dechlorination (Bradley and Chapelle 2010). Since VC is a known human carcinogen (IARC 2008) and is also highly soluble and volatile (Mackay et al. 2006), its possible occurrence in ground-water is of primary concern. In the past few years, several studies have used the Compound-Specific Isotope Analysis (CSIA) in combination with molecular biological analysis to evaluate the rate of reductive dechlorination (Chiu et al. 2013; Damgaard et al. 2013; Kotik et al. 2013). In particular, analyses applying isotope fractionation together with molecular biology tools have been conducted to measure the dehalogenation efficiency in heterogeneous aquifers, where halogenated groundwater is mixed with open water bodies (Nijenhuis et al. 2007; Imfeld et al. 2011). In this respect, dechlorinating bacterial communities have been mostly investigated through quantitative Polymerase Chain Reaction (qPCR) (Damgaard et al. 2013; Courbet et al. 2011).

However, as primers targeting specific microbes were used, the information obtained on the composition of the entire bacterial community was limited because microbial dehalogenase activity depends on metabolic interactions with other microorganisms (Damgaard et al. 2013; Hunkeler et al. 2011; Clark et al. 2018; Hermon et al. 2019). Therefore, to acquire a full overview of the entire microbial communities involved in putative mutualistic relationships between specific bacterial and/or archaeal groups is necessary to clarify the microbiology behind the dechlorination process (Lee et al. 2015). To this end, some studies have been done in the past but using low-resolution microbial community analysis methods such as clone libraries and Terminal Restriction Fragment Length Polymorphism (TRFLP) of 16S rRNA amplicons (Macbeth et al. 2004; Rahm et al. 2006). Therefore, a series of open questions on the microbiology related to the dehalogenation processes in aquifers contaminated by toxic PCE and TCE remains.

Different molecular methodologies have characterized functional genes for the dehalogenation of organic solvents in anaerobic and aerobic environments. Several reductive dehalogenase (RDH) genes have been examined using both microarray-based methods and PCR-based protocols, and different sets of primers proved useful for the identification of numerous RDH genes from a variety of samples, allowing their detection in some genera of anaerobic bacteria such as *Dehalococcoides*, *Dehalobacter*, and *Desulfitobacterium* (Futagami et al. 2009; Regard et al. 2004). Alternatively, the aerobic degradation of halogenated organic solvents is performed through cometabolic transformation by aerobic bacteria containing oxygenase enzymes with broad substrate range which fortuitously degrade VC, cDCE, and 1,1,2,2-tetrachloroethane using phenol, toluene, ethene, methane, propane, or ammonia as the growth-supporting substrate (Mattes et al. 2010; Cappelletti et al. 2018).

The aquifers in the subsoil of the city of Ferrara (Emilia-Romagna region, northern Italy) are affected by chlorinated ethylene contamination at several field sites with concentrations up to 10 mg L<sup>-1</sup> for single contaminants (Gargini et al. 2011). The contamination originated from the industrial spill of solvents or improper disposal of industrial wastes between the 1960s and the 1970s (Nijenhuis et al. 2013). Whereas dechlorination products cDCE and VC generally occur in groundwater associated with primary compounds PCE and TCE, at some of the sites of Ferrara VC, the composition of the contaminant plumes downgradient to the source dominates, suggesting the presence of exceptionally high dechlorination activity. Filippini et al. (2016) observed that dechlorination of PCE and TCE takes place during contaminant migration through shallow organic-rich layers with peat intercalations. Such layers are ubiquitous in the subsoil of Ferrara, and they were proposed to act as a “reactor” stimulating the degradation of contaminants with the accumulation of VC.

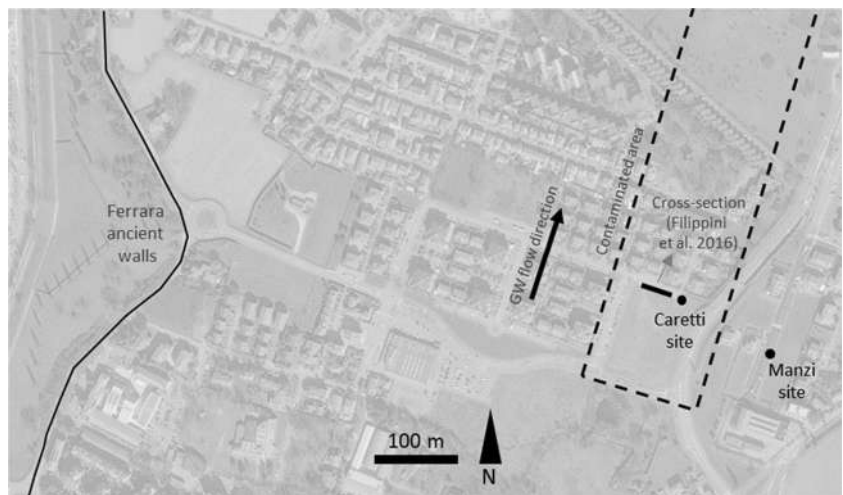
Here, Illumina sequencing of V4 hypervariable region of 16S rRNA gene in combination with clone library analysis of dehalogenase metabolic genes was applied to analyze the composition of the microbial community that drives the peculiar dehalogenation activity observed inside the organic-rich layers in the subsoil of Ferrara. The results were interpreted to show that the microbial communities catalyzing the chlorination process are composed not only of dehalogenating bacterial genera such as *Dehalococcoides* and *Dehalobacter* but also of other groups of non-dehalogenating bacteria and archaea involved in hydrolysis and fermentation of complex organic matter, acidogenesis, acetogenesis, and methanogenesis. Overall, both previous and present results allowed us to propose a working model in which syntrophic relationships within microbial communities are required to obtain an effective reductive dechlorination process.

## Materials and methods

### Description of sampling sites

Sediment samples were collected in June 2017 in a contaminated site in Ferrara, called “Caretti site” (hereinafter referred to as C), and in a second site 130 m southeastward, called “Manzi site” (hereinafter referred to as M) (Fig. 1). Filippini et al. (2016) thoroughly investigated the distribution of chloroethylene contaminants in the multilayered aquifer-aquitard system below the C site. Their research was focused along a cross section 60 m deep and 60 m wide adjacent to the local source of contamination (Fig. 1). The vertical distribution of contaminant concentrations was reconstructed in detail along with the easternmost of three vertical profiles (labeled MC4–5 in Fig. 2) which was the most contaminated one. Sediment samples were collected from cores and analyzed for total contaminant concentration. Estimated pore water concentrations assuming equilibrium phase partitioning (Filippini et al. 2020) showed peaks of dechlorination products cDCE and VC of 145 and 61 mg L<sup>-1</sup>, respectively, within an organic-rich sub-layer between 5.9 and 7.5 m below sea level (bsl) (Fig. 2). A groundwater sample collected along the same profile showed PCE, TCE, and cDCE concentrations of 0.4, 0.8, and 63.6 mg L<sup>-1</sup>, respectively, consistent with averaged pore water concentrations from sediments, whereas the VC was much higher in the groundwater sample (124.2 mg L<sup>-1</sup>), suggesting that sediment sample analysis led to an underestimation of the actual VC concentration (Filippini et al. 2016). Ethylene was also detected in groundwater in a concentration of 169.1 mg L<sup>-1</sup>, proving some complete dechlorination inside the organic-rich layer. A degree of dechlorination (DOD) of 0.8 was estimated for the groundwater sample, where a DOD equal to 0 or 1 indicates no dichlorination or complete dichlorination, respectively

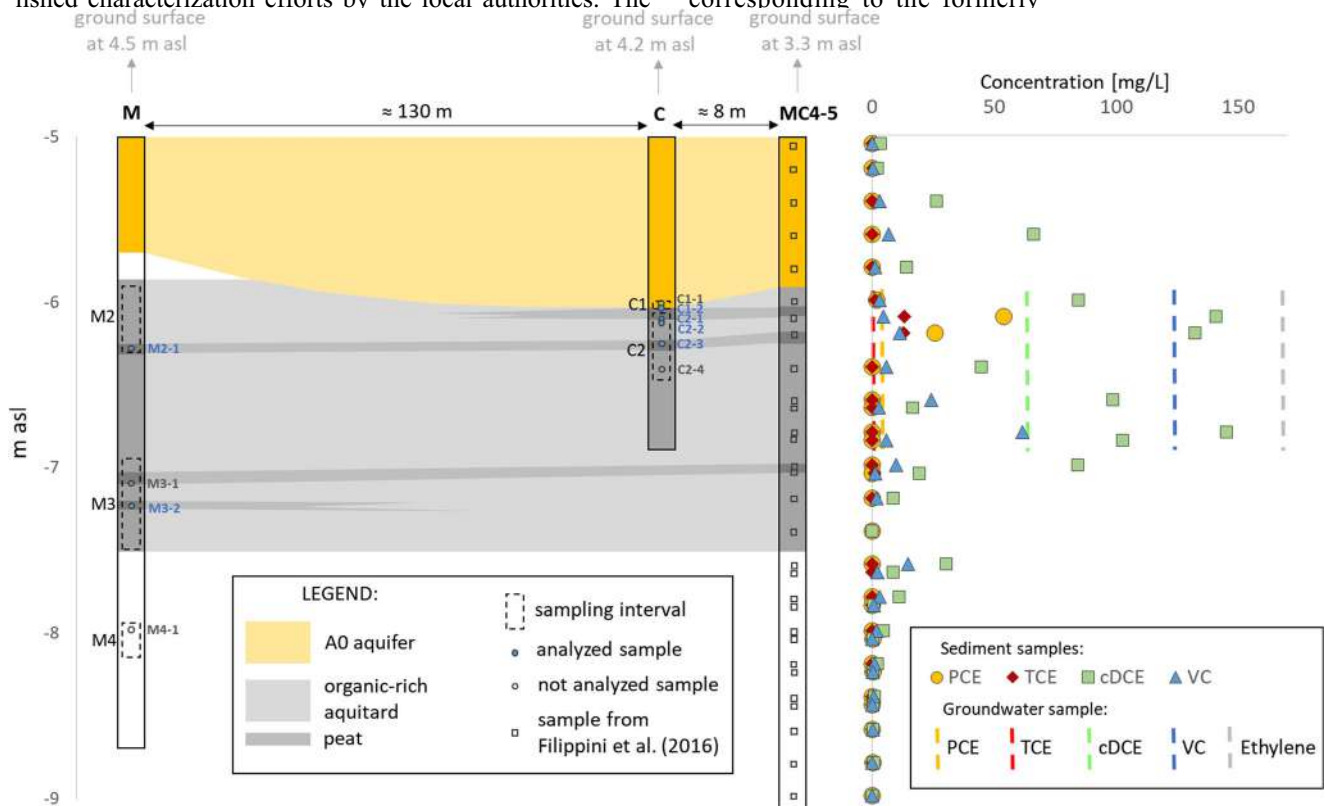
**Fig. 1** Location of the study area. The black arrow indicates the main direction of groundwater flow. The dashed rectangle delimits the contaminated area



(Damgaard et al. 2013). The CSIA was performed by Filippini et al. (2016) showing isotopic fractionation within the organic-rich sub-layer that is consistent with the occurrence of reductive dechlorination. The same authors observed Eh values down to  $-280$  mV, oxygen concentrations lower than  $0.4 \text{ mg L}^{-1}$ , and methane concentrations higher than  $30 \text{ mg L}^{-1}$  in the groundwater of the C site, corresponding to a methanogenic geochemical condition.

The M site is considered uncontaminated based on unpublished characterization efforts by the local authorities. The

samples from this site were used to make a comparison between contaminated and uncontaminated sediments of the same aquifer-aquitard system. The new sampling locations were carefully selected based on previous characterization efforts. At the C site, which is well characterized in terms of aquifer-aquitard geometries, lithologies, hydrogeochemistry, and 3D contaminant distribution (Nijenhuis et al. 2013; Filippini et al. 2015, 2016, 2020), the new sampling was performed a few meters apart from the most contaminated hot spot in the source area, corresponding to the formerly



**Fig. 2** Stratigraphic control points and sampling depths at the C and M sites. Profile MC4-5 from Filippini et al. (2016) is also depicted together with the vertical distribution of contaminant concentrations from sediment and water samples

investigated profile MC4–5. Such location was selected to ensure the collection of samples representative of strongly contaminated conditions since chloroethenes are known to assume complex distributions in the underground with abrupt changes in contaminant concentration over short distances (Parker et al. 2003). At site M, the new samples were collected close to an existing control point (piezometer) that allowed excluding the occurrence of chloroethene contamination in groundwater. Among existing piezometers at the M site, the selected one is the closest to the C site to maximize the correlability of geological layers between the two sites.

In both sites, the sediment samples were collected between 6 and 8 m bsl, i.e., the depth of the peat-rich layers showing the highest dechlorination activity at the C site. The depth of sampling was reached via percussion drilling. The technique allows advancing in the ground without the use of fluids that may disturb the sample. While advancing in the ground, a stainless-steel casing was put in place to avoid the collapse of the borehole or vertical cross-contamination. Cores 1.5 m long were recovered inside transparent plastic liners of 4 cm inner diameter. The use of transparent liners allowed for the selection of the sampling intervals of interest in the field by visual inspection, without the need of opening the liners. Three sampling intervals were selected at the M site corresponding to peat-rich layers (M2 to M4 in Fig. 2).

In contrast, two intervals were selected at the C site corresponding to a peat-rich layer and the bottom of a sandy aquifer layer overlying the peat (C1 and C2). The selected sampling intervals were separated from the rest of the liner using a hacksaw, plugged at both ends with plastic caps, and stored at  $-20\text{ }^{\circ}\text{C}$ . The remaining liners were opened in the field and used for stratigraphic description. The liner sections selected for sampling were opened in the lab. The cores were scratched at the surface to remove disturbances caused by drilling, and a total of 6 samples were collected for DNA extraction and subsequent PCR amplification (Table 1).

**Table 1** List and description of samples analyzed in this work

Sample ID	Site	Depth (bsl)	Matrix	$\mu\text{g}$ DNA per 1 g soil	Sequenced reads	ASVs	Shannon	Inverse Simpson's	Evenness
C1.2	Caretti	6.05 m	Peat	2.64	51,725	696	5.250	73.421	0.802
C2.1	Caretti	6.09 m	Interface between peat and organic rich aquitar	3.24	26,119	499	5.012	57.384	0.807
C2.2	Caretti	6.17 m	Organic rich aquitar	1.40	55,678	641	5.043	57.005	0.780
C2.3	Caretti	6.25 m	Peat	1.02	49,787	399	4.514	32.047	0.754
M2.1	Manzi	6.28 m	Peat	1.84	34,428	784	5.774	131.217	0.866
M3.2	Manzi	7.23 m	Interface between peat and organic rich aquitar	1.46	57,010	1075	5.741	86.631	0.822

## DNA extraction, 16S rRNA gene amplification, and Illumina sequencing

The sediment samples were extracted for their total DNA using the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's protocol with slight modifications as previously described (Cappelletti et al. 2016). The extracted DNA was used as a template for PCR amplification targeting the V4 hypervariable region of the 16S rRNA gene using the primer pair 515F (5'GTGCCAGCMGCCGCGGTAA3') and 806R (5'GGACTACHVGGGTWTCTAAT3') (Caporaso et al. 2010) modified with an Illumina adaptor sequence at the 5' end. PCR reactions were performed in a final volume of 50  $\mu\text{L}$  containing 10 ng of total DNA, primers 500 nM, 1 $\times$  Takara Ex Taq buffer with  $\text{MgCl}_2$ , dNTP mix 200  $\mu\text{M}$ , and Takara Ex Taq Polymerase 0.5 U. The thermocycling program included 1 cycle at  $95\text{ }^{\circ}\text{C}$  for 10 s, 30 cycles at  $95\text{ }^{\circ}\text{C}$  for 10 s,  $58\text{ }^{\circ}\text{C}$  for 30 s,  $72\text{ }^{\circ}\text{C}$  for 30 s, and a final extension at  $72\text{ }^{\circ}\text{C}$  for 2 min. Amplicons were submitted to the library preparation and Illumina MiSeq sequencing platform for indexing and pair-end sequencing (2  $\times$  250 bp; reagent kit, v2) at the sequencing service BMR Genomics SRL. The sequence analysis of 16S rRNA amplicons was performed by using QIIME2 version 2018.4 (Bolyen et al. 2019) and DADA2 package version 1.5.0 (Callahan et al. 2016). The demultiplexed and primer-clipped reads were trimmed based on quality and length. Trimmed sequences were dereplicated, denoised, and merged, and chimeras were removed. The taxonomic assignment of the resulting 16S rRNA gene amplicon sequence variants (ASVs) was performed by querying the ASVs against the SILVA SSU 132 reference database (Quast et al. 2013). The taxonomy nomenclature was further manually checked and modified according to the most recently published studies. Accordingly, *Bathyarchaeia* (former class of *Crenarchaeota*) and *Aminicentantia* (former class of *Acidobacteria*) were renamed as *Candidatus* (Ca.) *Bathyarchaeota* (Meng et al. 2014) and Ca. *Aminicentantes* (Rinke et al. 2013) phyla, respectively. One ASV belonging to C2.3 sample and affiliated to the *Escherichia/Shigella* genus

was removed from further analysis being recognized as possible laboratory contaminant. Metabolic pathways contributing to PCE and TCE degradation were predicted from ASVs through the PICRUSt2 pipeline. Functional profiling was performed only for the most abundant ASVs (relative abundance > 1.0%). To further improve the reliability of the functional prediction, we focused only on ASVs having a Nearest-Sequenced Taxon Index (NSTI) value below 0.15. The NSTI score indicates to which extent a microorganism in a sample is related to a set of reference genomes in the PICRUSt database.

The Illumina sequencing raw data were deposited in the Sequence Read Archive of NCBI under accession number PRJNA635257. The 64 most abundant ASVs were submitted to the NCBI GenBank database under accession numbers MW136710-MW136773.

### Clone library of dehalogenases metabolic genes

A set of primers selected to amplify from the total DNA different types of reductive dehalogenase homologous genes (*rdhA*) (Hug and Edwards 2013) was chosen accordingly to the most abundant microbial species retrieved from the 16S rRNA gene analysis. The list of *rdhA* primer used along with the expected product size, primer sequences, and melting temperature is summarized in Table S1. Around 10 ng of total DNA was added to a 50- $\mu$ L (final volume) PCR reaction mixture containing Takara Ex Taq buffer with  $MgCl_2$  (10 $\times$ ; Takara Bio Inc., Tokyo, Japan), primers 200 nM each, dNTP mix 200  $\mu$ M, Takara Ex Taq Polymerase 1.25 U. PCR amplification conditions were applied according to Hug et al. reference (Hug and Edwards 2013) with some minor changes as shown in Table S2. The *rdhA* PCR products were purified using the Qiagen PCR purification kit and eluted in 50  $\mu$ L of Qiagen elution buffer. Aliquots (30 ng) of purified PCR products were cloned into the TOPO-TA vector (Thermo Fisher Scientific) and transformed into chemically competent *Escherichia coli* DH5 $\alpha$  cells following the manufacturer's instructions. White colonies isolated on LB agar plates containing 100  $\mu$ g mL<sup>-1</sup> ampicillin, 50  $\mu$ g mL<sup>-1</sup> kana-mycin, and 40  $\mu$ g mL<sup>-1</sup> X-gal were screened through colony PCR with the vector primers T7 and T3 (Invitrogen). After confirmation through electrophoresis, clones with the supposed correct size of the insert were sequenced in both forward and reverse directions using T7 and T3 primers (Invitrogen) using the sequencing service provided by Eurofins Genomics. Chimeras were identified and removed with Uchime2 v11 (Edgar et al. 2011). The clone library sequences of dehalogenases were submitted to the NCBI GenBank database under accession numbers MT561367 and MT561376.

### Phylogenetic and statistical analyses

Phylogenetic trees were constructed using, separately, (i) the ASVs resulting from the Illumina sequencing of V4 region of the 16S rRNA gene and (ii) the amino acid sequences of the *rdhA* genes obtained from in silico translation of the representative clones obtained from the clone library analysis. For each sequence included in the tree, the most closely related sequences retrieved from the NCBI database (Best Blast Hits) were downloaded. MEGAX (Kumar et al. 2018) was used to construct phylogenetic trees based on ClustalW sequence alignment and neighbor-joining clustering method with 1000 non-parametric bootstrap replicates for both the 16S rRNA gene (model: Jukes-Cantor; rates among site: uniform rates; gap/missing data treatment: pairwise deletion) and the amino acidic sequence of the *rdhA* genes (model: Poisson model; rates among site: uniform rates; gap/missing data treatment: pairwise deletion).

Diversity indices, richness estimations, and principal coordinate analysis (PCoA) were performed based on of ASVs using CALYPSO online software (Zakrzewski et al. 2017) and Primer-E v7 (Clarke and Gorley 2015).

## Results

### Stratigraphy at the C and M sites

The stratigraphy of boreholes M and C shows metric alternation of sandy aquifer layers and silty-clayey aquitard layers locally enriched in peat and organic matter. Such configuration is consistent with previous observations in the region (Molinari et al. 2007). The present work focuses on the aquitard layer section enriched in organic matter, where Filippini et al. (2016) observed the dechlorination of chloroethylenes fostered by the organic-rich sediments. The aquitard section of interest is extended for a few meters below the bottom of the shallowest aquifer layer (regionally known as the "A0 aquifer"; Regione Emilia-Romagna and ENI-AGIP 1998) (Fig. 2). The bottom of the A0 aquifer is around 6 m bsl. Below the aquifer bottom, a fine-grained sub-layer enriched in organic matter was identified in M and C boreholes containing centimeter intercalations of peats. The peaty horizons observed inside the organic-rich sub-layer are expected to have a remarkable later extent (Bruno et al. 2019), and it was possible to correlate some of them between boreholes M and C and with the previous borehole MC4–5.

### Diversity of microbial communities

The sampling depth and sequencing coverage resulted in being sufficient to describe the microbial diversity in each of the six samples under analysis (Fig. S1). As a result of the



processing of the demultiplexed fastq files with DADA2 package, a total of 284,110 total reads were obtained and clustered in 2781 sequence variants.

The microbial communities of the four contaminated C sediment samples (C1.2, C2.1, C2.2, and C2.3) showed lower richness, evenness, and diversity as compared to the two samples collected from the uncontaminated M site (M2.1 and M3.2) (Table 1). Among the C site samples, Shannon and Inverse Simpson's indices showed that the microbial communities' diversity decreased accordingly with the increment of the sampling depth. Moreover, the lean-peat samples (C2.1 and C2.3) showed less microbial richness than those located in the rich organic fraction of the semi-permeable layer and the aquifer fraction (C1.2 and C2.2), collected just above and below the peat horizon of the contaminated area (Table 1, see Fig. 2).

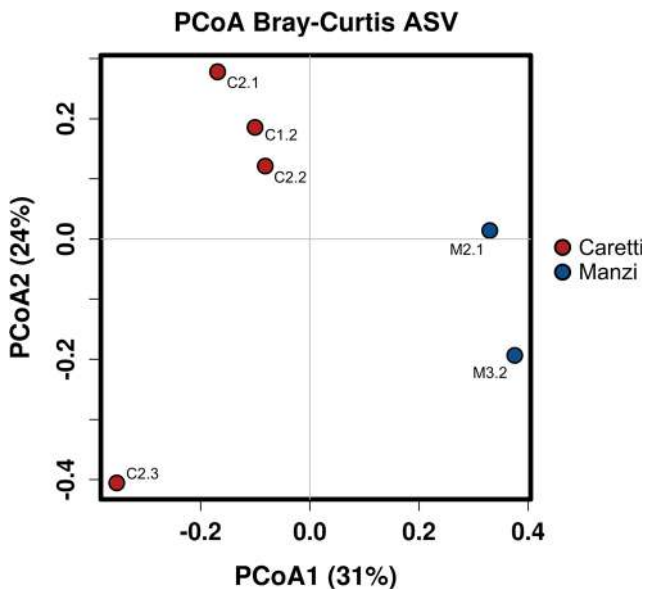
Principal coordinates analysis based on the taxonomy composition of the various sediments revealed that M samples grouped apart from C samples (Fig. 3). Among the four C samples, C1.2 and C2.1 shared higher taxonomic similarity. In contrast, the deepest sediment sample C2.3 was located separately from the other Caretti sediments on the plot (Fig. 3).

Bacteria were predominant in all analyzed samples representing at least 65% of each microbial community. Conversely, phylum-level taxonomic assignments of 16S rRNA gene sequences revealed that the Ca. Bathyarchaeota phylum was included in the top three microbial phyla of all six samples under analysis and represented the most abundant taxonomic group in the C samples C1.2 (24.5%), C2.1

(21.5%), and C2.2 (32.5%) (Fig. 4). These three samples were also abundant in *Chloroflexi* (7–17%) and *Elusimicrobia* (10–13%), whose relative abundances decreased accordingly to the sample deepness. Only in the C2.3 sample, *Firmicutes* represented the most abundant phylum (45.6%), followed by *Proteobacteria* (11.8%). Additional abundant phyla ranging between 5 and 10% of the total community in C sediments were *Spirochaetes*, Ca. Aminicenantes, and *Actinobacteria* (Fig. 4). The two samples M1.2 and M3.2, collected from the uncontaminated M site, were mainly constituted of *Chloroflexi* (35.3% and 23.9%, respectively), followed by Ca. Bathyarchaeota (12.7% and 19.6%, respectively). Other abundant phyla in the M sediments were *Elusimicrobia*, Ca. Aminicenantes, and *Spirochaetes* (Fig. 4).

The most abundant taxa were unclassified at low taxonomy levels. In particular, sequences affiliated to Ca. Aminicenantes and *Elusimicrobia* did not provide any more in-depth classification. *Coriobacteriia* represented the only defined class of the *Actinobacteria* phylum with the highest relative abundance of 2.5% in C1.2. Most of *Chloroflexi* classified up to the class level was *Anaerolineae* and *Dehalococcoidia*, the latter being more abundant in M site (Fig. 4). Some of the less abundant taxa were classified at lower levels and mainly identified in C2.3 sample, including members of the *Gracilibacteraceae* family and *Dehalobacter* genus (both belonging to *Firmicutes*) with relative abundances of 13% and 12%, respectively. Nevertheless, around 10% of *Firmicutes* in C2.3 remained unclassified at class level. *Deltaproteobacteria* and *Gammaproteobacteria* were the dominant classes of *Proteobacteria*, the first being more abundant in Manzi site (7% in M2.1 and 9% in M3.2) and the latter in Caretti site (1.5–8%). In Caretti, *Shewanella* and *Halomonas* represented the most abundant genera of *Gammaproteobacteria* showing the highest abundance in C2.3 (3.5% and 4.5%, respectively). Some bacterial members affiliated to the *Spirochaetes* phylum were classified up to the genus level, including *Spirochaeta* that represented 8% and 9% in C2.3 and M3.2, respectively (Fig. 4).

The dominance of Ca. Bathyarchaeota and *Elusimicrobia* observed in the phylum-level analysis among all samples was reflected in the ASV analysis (Table S3). Indeed, *Elusimicrobia*-affiliated ASV1 and ASV3 and Ca. Bathyarchaeota-affiliated ASV2 were among the dominant ASVs in C1.2, C2.1, and C2.2. The phylogenetic analysis of ASV1 and ASV3, along with other ASVs affiliated to the *Elusimicrobia* phylum, revealed sequence similarities with clones found in sediments and groundwater, and also present in enriched archaeal methanogenic communities detected in subsurface freshwaters (Fig. 5). In contrast, ASV2 and other abundant ASVs belonging to the archaeal Ca. Bathyarchaeota phylum showed their affiliation with sequences retrieved from the transitional zone between rich organic river estuary water and coastal seawater (Fig. 6).



**Fig. 3** Principal coordinates analysis (PCoA) based on ASV Bray-Curtis similarity of Caretti (red dots) and Manzi (blue dots) sediments. Dots closer together in the plots indicate samples with similar community composition. Variance explained in PCoA axis 1 = 31% and PCoA axis 2 = 24%

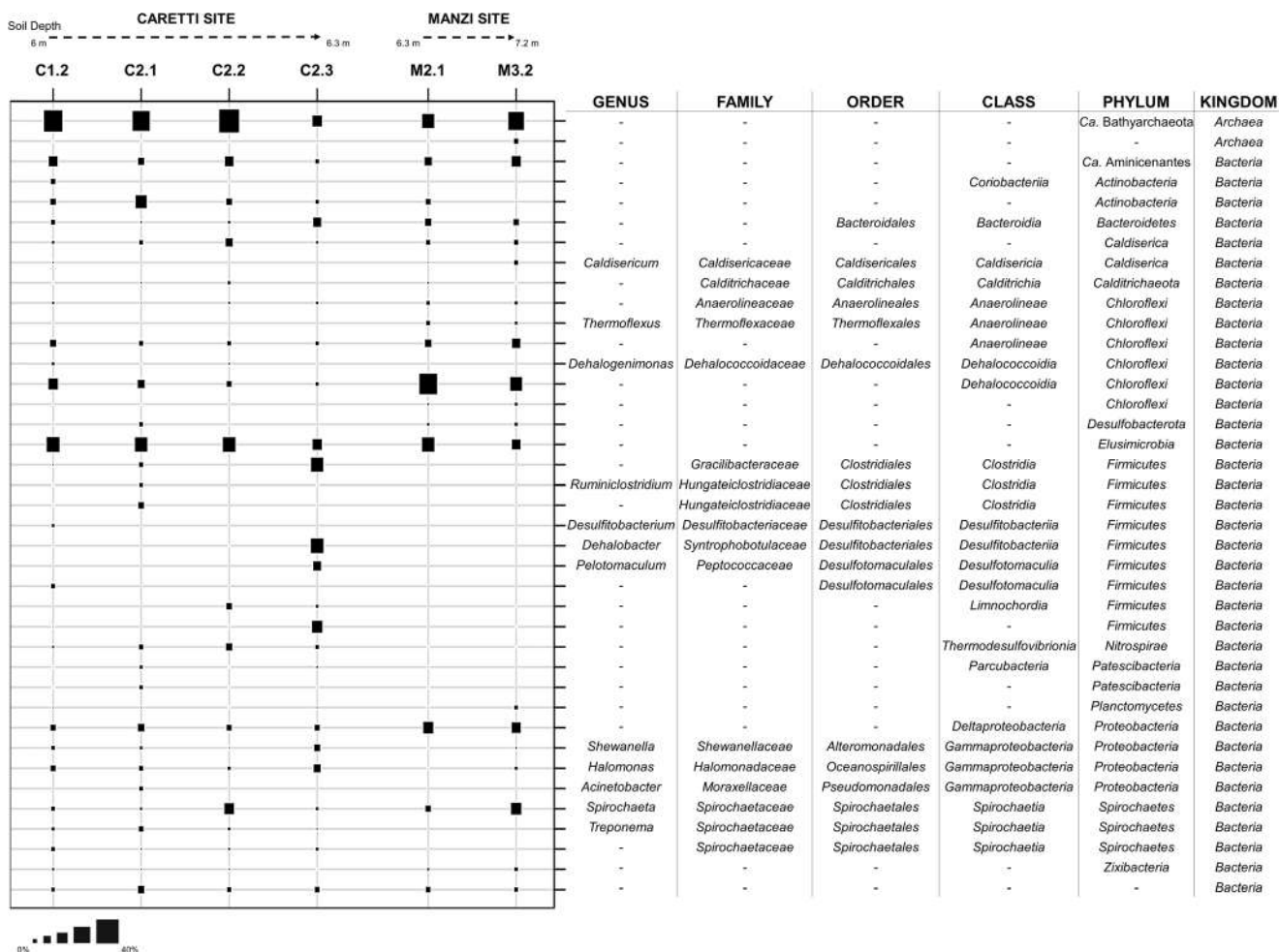


Fig. 4 Microbial community composition of samples collected from Caretti and Manzi sites. Microbial taxa with relative abundances > 1% in at least one sample are shown

These ASVs were also abundant in M sediments in which, however, ASV5 was dominant in M3.2 and affiliated to the *Spirochaetes* phylum (Fig. 5).

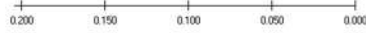
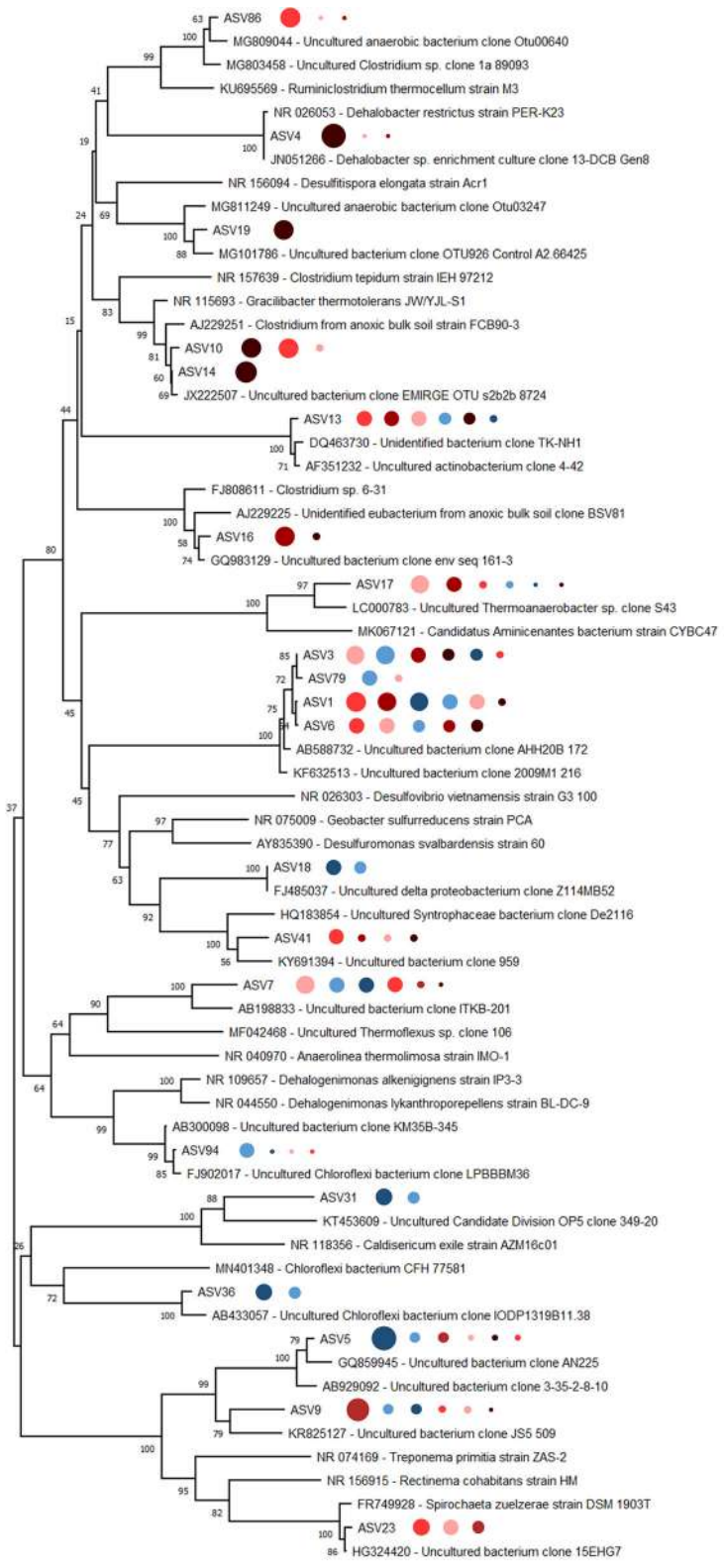
Interestingly, the abundance of *Chloroflexi* observed in the taxonomy analysis of sequenced reads did not find correspondence with the analysis of the most abundant ASVs (Table S3). This evidence indicates that *Chloroflexi* (in particular members of the *Dehalococcidia* class) were highly diversified in the M and C sediments and that the *Chloroflexi* species were evenly spread among many different ASVs which, therefore, were detectable at low abundance. In the shallower sediments of the C site (C1.2 and C2.1), the most abundant ASV of *Chloroflexi* was ASV7, which belonged to the *Anaerolineae* class and was phylogenetically affiliated to sequences recovered from subsurface sediments and groundwater contaminated by arsenic compounds (Fig. 5). In the Caretti deepest sediment C2.3, nine abundant ASVs were affiliated to the *Clostridia* class of *Firmicutes* and were almost totally absent in the other C and M sediments. Among these, the most abundant ASV (ASV4) was affiliated to the genus *Dehalobacter* and shared high nucleotide similarity with

sequences belonging to *Dehalobacter* spp. cultures that reductively dehalogenate different dichlorobenzene isomers (Fig. 5). Other two abundant ASVs (ASV10 and ASV14) clustered in the phylogenetic tree with clones belonging to the *Gracilibacteraceae* family and retrieved from subsurface aquifer sediments (Fig. 5).

### Functional inference of metabolic pathways involved in the synthesis of organic acids as reducing equivalents

We used PICRUST2 to predict the functional capacity of the most abundant ASVs contributing to the reductive dechlorination of PCE and TCE in C sediments. We focused on functions involved in the degradation of organic matter and the synthesis of reducing equivalents used by dehalogenating microbes in the reductive dechlorination. In this respect, for the most abundant ASVs in C samples with and NSTI < 0.15, functions were inferred from uncultured and environmental isolate-associated anaerobic niches such as hydrothermal vents, soil and aquatic sediments, and anaerobic digesters





*Clostridia*  
(Firmicutes)

*Actinobacteria*

*Limnochordia*  
(Firmicutes)

*Candidatus*  
*Aminicenantes*

*Elusimicrobia*

*Deltaproteobacteria*  
(Proteobacteria)

*Anaerolineae*  
(Chloroflexi)

*Dehalococcoidia*  
(Chloroflexi)

*Calditrichaeota*

*Dehalococcoidia*  
(Chloroflexi)

*Spirochaetia*  
(Spirochaetes)

- C1.2
- C2.1
- C2.2
- C2.3
- C2.3
- M2.1
- M3.2

Caretti  
Manzi

◀ **Fig. 5** Phylogenetic tree of the dominant bacterial ASVs retrieved in the sediments under analysis. Colored dots represent the samples where each ASV is present. The dot size indicates the abundance of each ASV in the microbial community composition

(Table S4). Pathways involved in the synthesis of reducing substrates were the acetate synthesis via CO<sub>2</sub> fixation (Wood-Ljungdahl pathway, WLP) and anaerobic digestion of carbohydrates, and CO<sub>2</sub> synthesis via formate dehydrogenase (Fig. 7). Notably, the functional capacity of Ca. Bathyarchaeota ASVs to synthesize acetate was associated with enzymes linking the carbonyl and methyl-branch of the WLP with the synthesis of acetate from Acetyl-CoA (Fig. 7). Likewise, acetate synthesis from CO<sub>2</sub> fixation via the WLP methyl-branch of acetogenic bacteria was predicted from a *Desulfitobacterium* ASV (Fig. 7). In contrast, phosphate acetyltransferase and acetate kinase, which are also involved in the synthesis of acetate, were inferred from ASVs affiliated to Ca. Aminicenantes, *Spirochaetales*, *Halomonas*, and *Caldithrix* (Fig. 7). Therefore, we found that among the most abundant ASVs in C samples, these were affiliated to microbes having the metabolic capacity of synthesizing acetate as reducing equivalent for the reductive dechlorination. These findings do not necessarily exclude the presence of taxa capable of producing other organic acids such as butyrate or lactate as products of fermentative processes.

## Reductive dehalogenases

Based on the results obtained from the sequencing of 16S rRNA, specific primers were used for the detection of the *vcrA*, *bvcA* and other *rdhA* genes (Table S2). VC-reductase genes *vcrA*-like were successfully amplified in samples C1.2 and C2.2 (Fig. 8), while the *bvcA* gene was not detected in any of the samples tested (Table S2). About the other reductive dehalogenases analyzed, only RDH22, i.e., the one containing the *crpA* cluster of the *Clostridiales* group, was found in sample C2.3. The finding of RDH22 in the only C2.3 sample agrees with the data obtained from the sequencing of the 16S rRNA, indicating an increase in the abundance of members of the *Clostridiales* order in that sample (Fig. 8).

## Discussion

Among the different environmental remediation procedures, the use of indigenous microbial communities to bio-remediate chlorinated ethylene aquifers is gaining increasing interest (Adrian and Löffler 2016). However, previous attempts to increase the reductive dechlorination process have been conducted in the absence of a thorough knowledge of the fundamental biogeochemical cycles governing the activity of key organohalide-respiring bacteria. To fulfill this flaw, in the

present work, we investigated the structure and composition of the whole microbial community present in an aquifer located in the subsoil of the city of Ferrara (northern Italy) contaminated with chlorinated ethylene compounds, along with the presence of different RDH genes.

The microbial diversity indices and estimated microbial population richness showed a partial reduction of both parameters in samples collected from the contaminated area C compared to those gathered from the non-contaminated area M (Table 1). This observation is not unusual as the influence of chlorinated solvents on the composition and diversity of microbial communities in enrichment procedures has previously been described (Atashgahi et al. 2017; Matturro et al. 2018). Furthermore, the microbial diversity was seen to decrease in parallel with the deepness of the different C sediments, and this phenomenon is likely due either to a higher concentration of VC at greater depths or a strong influence of depth on microbial diversity under anoxic conditions. Notably, the lean-peat samples (C2.1 and C2.3) showed a lower microbial richness of both the rich organic aquitard layer and the aquifer layer (C1.2 and C2.2). In recent years, several studies have examined the composition of peat microbial communities across different climate zones (Pankratov et al. 2008; Mishra et al. 2014). A common finding was that microbial communities in the peat-ecosystem were composed of a few dominant taxa reflecting the specific features of the peat (Andersen et al. 2013; Gilbert and Mitchell 2006) and this observation might possibly explain the reduction of microbial diversity in peat samples C2.1 and C2.3.

The Illumina sequencing analysis of 16S rRNA gene revealed the presence of a complex microbial community that synergistically supports the activity of reductive dehalogenation. Although the *Bacteria* domain was dominant in all C and M sediments, archaeal sequences strongly affected each microbial community, as Ca. Bathyarchaeota consistently represented one of the most abundant phyla among the samples analyzed. Besides, it was evident that the contaminants represented one of the main factors that affected the microbial community structure present in the Caretti site. In line with our finding, a significant presence of *Archaea* in the microbial communities active in the dechlorination of halogenated hydrocarbons has also been reported in the past (Lee et al. 2012; Richardson et al. 2002). Most of them are related to different groups of methanogens belonging to both the *Euryarchaeota* and Ca. Bathyarchaeota phyla that may be co-localized together with the dehalogenating bacteria to form bioflocules (Rowe et al. 2008). It has been shown that some methanogens (e.g., *Methanosarcina* spp.) produce corrinoids that can be used as reductive dehalogenase cofactors by bacteria such as *Dehalococcoides* spp. (Mazumder et al. 1987). In the present work, the majority of the *Archaea* belonged to the Ca. Bathyarchaeota phylum. Members of Ca. Bathyarchaeota (formerly referred to as the “Miscellaneous Crenarchaeota



**Fig. 6** Phylogenetic tree of the dominant archaeal ASVs retrieved in the sediments under analysis. Colored dots represent the samples where each ASV is present. The dot size indicates the abundance of each ASV in the microbial community composition

Group,” Inagaki et al. 2003) are very frequently found in marine environments, particularly in the subsurface region

and also in wetland and peat soils (Hawkins et al. 2014; Fillol et al. 2016).

Numerous reports have shown that *Bathyarchaeota* spp. can use a broad set of complex organic compounds as growth media, including proteins, cellulose, chitin, and aromatic compounds through fermentation (Lloyd et al. 2013), acetogenesis (He et al. 2016), and methanogenesis (Evans et al. 2015; Lloyd et al. 2013; Meng et al. 2014; Lazar et al. 2016). Recently, organoautotrophic growth of *Bathyarchaeota* with lignin as an energy source and bicarbonate as a carbon source has also been reported (Yu et al. 2018). Genetic and metabolic studies have also demonstrated that *Bathyarchaeota* spp. have the genetic potential for inorganic carbon fixation via the reductive acetyl-CoA Wood-Ljungdahl pathway (WLP) and some species also possess the carbon monoxide dehydrogenase/acetyl-CoA synthase complex (Cdh/Acs) along with the capacity to produce acetate and H<sub>2</sub> (He et al. 2016). Functional features from the most abundant Ca. Bathyarchaeota ASVs found in PCE and TCE contaminated samples were predicted. According to the NSTI values, Ca. Bathyarchaeota ASV functional profiles were inferred from uncultured *Crenarchaeota* identified in geothermal systems and from a coalbed methane well (Table S2). According to the energy metabolism, all Ca. Bathyarchaeota ASVs shared functions involved in the utilization of methylated compounds for methanogenesis and associated with the carbonyl and methyl-branch of the WLP. Additionally, acetate biosynthetic pathways from CO<sub>2</sub> fixation and from glycolysis via pyruvate were also inferred from all Ca. Bathyarchaeota ASVs (Fig. 7 and Fig. 9).

Sequences affiliated to the *Anaerolineae* class of the *Chloroflexi* phylum were abundant in all analyzed samples. These bacteria are considered typical fermenting microorganisms catalyzing the anaerobic digestion of organic matter (Rivière et al. 2009). Recently, the capacity of *Anaerolineae* spp. to grow on starch (Yamada et al. 2006) and cellulose (Podosokorskaya et al. 2013) has been demonstrated. Furthermore, analysis of fully sequenced genomes in combination with meta-transcriptomics data demonstrated that acetate and lactate are likely the main products of glycolytic metabolic pathway of this bacterial class (Xia et al. 2016). In respect to the *Anaerolineae* ASVs, the functional prediction was inferred from uncultured bacteria identified in deep oceanic hydrothermal fluid (Table S2). While the functional profile associated to *Anaerolineae* ASVs indicated the presence of pathways involved in the degradation of starch and cellulose, the lack of genes linking the glycolytic pathways to the synthesis of acetate and lactate indicated that this microbial group does not contribute to the degradation of chlorinated solvents. The presence of [Ni-Fe]-hydrogenase was also reported in several *Chloroflexi* confirming the metabolic ability to produce hydrogen during fermentation (Nunoura et al. 2013; Imachi et al. 2014). In this respect, members of the *Anaerolineae* lineage of *Chloroflexi* are capable of autotrophy with molecular hydrogen and carbon dioxide as substrates

since they encode both the WLP and a sodium-motive ferredoxin:NAD oxidoreductase complex used for energy conservation (Schuchmann and Müller 2016). However, in the functional profiling of *Anaerolineae* ASV, both [Ni-Fe]-hydrogenase and enzymes of the WLP were missing (Fig. 7). Core functions involved in the synthesis of fermentation products such as acetate and CO<sub>2</sub> were inferred for ASVs affiliated to Ca. *Aminicenantes* (Fig. 7). Specifically, CO<sub>2</sub> synthesis via formate dehydrogenase and functions involved in the conversion of acetyl-CoA to acetate were predicted from an uncultured *Aminicenantes* isolated from Sakinaw Lake.

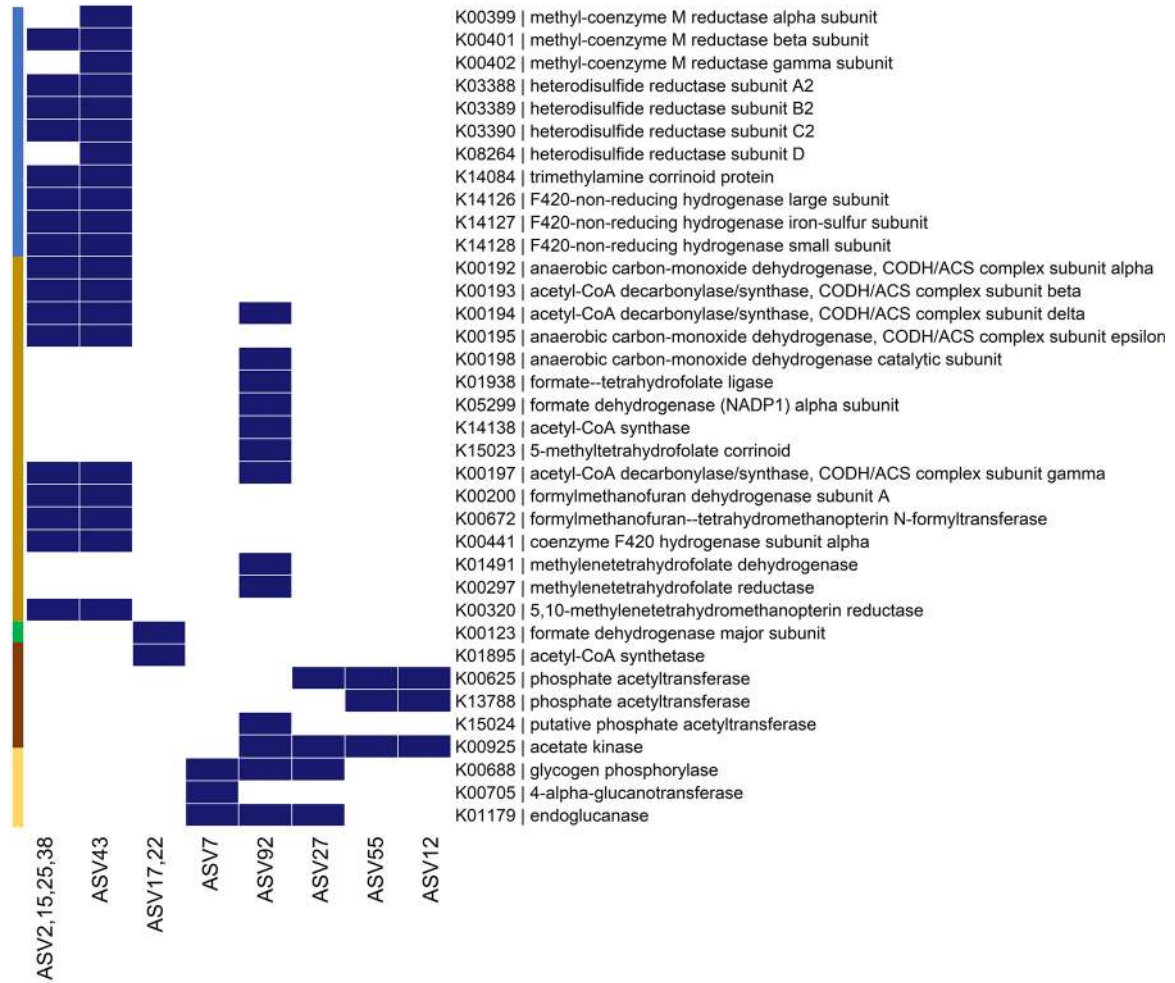
*Elusimicrobia* spp. were also found in abundance in all samples tested. Until recently, bacteria belonging to the *Elusimicrobia* class have only been associated with the intestinal tract of termites, but phylogenetic analyses of environmental sequences have also revealed their presence in different habitats such as groundwater, soil, and sediments (Herlemann et al. 2007). Groundwater-associated *Elusimicrobia* bacteria have considerable metabolic versatility, and most of their genomes have the potential for expressing genes involved in the fermentation of sugars to acetate, butyrate, and ethanol with the generation of ATP by substrate phosphorylation. Despite being identified among the most abundant ASVs, NSTI values associated with *Elusimicrobia* were far above the confidence threshold chosen as an accurate prediction of the functional profile.

Methanogenic bacteria were also detected in the C sediments but in small amounts. Most of them were found in sample C1.2 and belonged to the *Methanocellaceae* family and the genus *Methanocella*. Isolated strains of this latter genus were shown to utilize H<sub>2</sub>/CO<sub>2</sub> and formate for growth and methane production (Sakai et al. 2008).

Based on our observation, it is therefore likely that part of H<sub>2</sub>, CO<sub>2</sub>, and acetate generated by acetogenic bacteria are converted into CH<sub>4</sub> through methanogenesis. On the other hand, methanogens may be necessary, together with other *Firmicutes* and *Deltaproteobacteria* syntrophic partners, to provide the appropriate cobamide lower base of vitamin B<sub>12</sub> which is vital for *Dehalococcoides* dechlorination (Men et al. 2014).

Sequences identified as uncultured *Deltaproteobacteria* Sva0485 were found in all samples analyzed and represented between 5.3 and 7.2% of all sequences classified at the order level. Recently, uncultured *Deltaproteobacteria* Sva0485 genomes were retrieved from a metagenomics study of an artificial acid mine drainage system and analyzed in-depth to characterize the metabolism of this clade (Tan et al. 2019). Reconstructed metabolic pathways and gene expression profiles showed that they were likely facultative anaerobic autotrophs capable of both nitrogen fixation and fermentation of simple sugars into ethanol and lactate. The reconstruction of metabolic pathways and gene expression profiles also showed that these microorganisms are able to perform dissimilatory

- Methanogenesis
- Methyl and Carbonyl-branch of the Wood-Ljungdahl pathway and acetate biosynthesis
- CO<sub>2</sub> synthesis via formate
- Acetate synthesis via Acetyl-CoA
- Cellulose and starch degradation



ASV	C1.2	C2.1	C2.2	C2.3	Taxonomy
ASV2	5.78	5.43	7.29	1.67	p_Ca. Bathyarchaeota
ASV7	2.74	1.26	0.51	0.06	p_Chloroflexi; c_Anaerolineae
ASV12	1.70	0.91	0.70	1.93	p_Proteobacteria; g_Halomonas
ASV15	1.65	0.88	0.71	0.81	p_Ca. Bathyarchaeota
ASV17	2.09	0.32	1.24	0.03	p_Ca. Aminicenantes
ASV22	1.10	0.08	0.75	0.11	p_Ca. Aminicenantes
ASV25	0.23	0.31	1.03	0.21	p_Ca. Bathyarchaeota
ASV27	1.16	0.28	0.67	0.86	p_Spirochaetes; o_Spirochaetales
ASV38	0.30	0.27	1.30	0	p_Ca. Bathyarchaeota
ASV43	1.23	0.41	0.38	0.03	p_Ca. Bathyarchaeota
ASV55	0.14	0.52	1.06	0	p_Calditrichaeota
ASV92	1.16	0	0.01	0	p_Firmicutes; g_Desulfitobacterium



◀ **Fig. 7** Heatmap displaying the predicted functional profile of ASVs involved in the synthesis of reducing equivalents and reducing equivalent and carbohydrates digestion. Upper panel, presence/absence of functional orthologues is encoded in midnight blue and white, respectively. Lower panel, ASVs relative abundance in C samples

sulfate reduction and/or oxidize sulfide depending on the oxygen concentration present.

*Dehalococcoidia* spp. were found in all characterized samples. It should be noted that in contaminated samples, their percentage decreased from the shallowest C1.2 sample (12%) to the deepest C2.3 (2.3%) collected in the peaty zone. Members of the *Dehalococcoidia* class have the peculiar ability to use halogenated organic compounds (PCE and TCE) as terminal electron acceptors in the respiratory chain coupled to H<sub>2</sub> as the sole electron donor and acetate as carbon source. Furthermore, some species of *Dehalococcoidia*, such as *Dehalococcoides mccartyi*, are known to metabolically respire PCE to the non-toxic ethylene (Maymó-Gatell et al. 1997) using reductive dehalogenase enzymes BvcA and VcrA (encoded by genes *bvcA* and *vcrA*) (Krajmalnik-Brown et al. 2004; Müller et al. 2004; Sung et al. 2006). On the other hand, the consumption of H<sub>2</sub> by *Dehalogenomonas* spp. can create a thermodynamically favorable environment for fermenting microorganisms that could be inhibited by high concentrations of hydrogen (Lee et al. 2012).

*Dehalobacter* members were highly present in sample C2.3. The genus *Dehalobacter* is phylogenetically closely related to the *Desulfitobacterium* genus (Villemur et al. 2006). However, compared to the latter, most strains of *Dehalobacter* are specialized for organohalide respiration, a lifestyle similar to *Dehalococcoides* or *Dehalogenimonas* in the *Chloroflexi* phylum (Siddaramappa et al. 2012). Furthermore, sample C2.3 was also rich in other bacteria of the *Clostridia* class which can ferment numerous compounds, including glucose, arabinose, fructose, pectin, glycerol, starch, fumarate with ethanol, acetate, and hydrogen production (Blouzard et al. 2010; Giallo et al. 1985; Liu et al. 2014; Ravachol et al. 2016). Their presence might favor the biodegradation of chlorinated organics by *Dehalobacter* spp. (Shan et al. 2010). Indeed, the mixed acid fermentation activity is likely to lower the ambient redox potential, thereby creating conditions for a better reductive dechlorination activity by *Dehalobacter* (Shan et al. 2010).

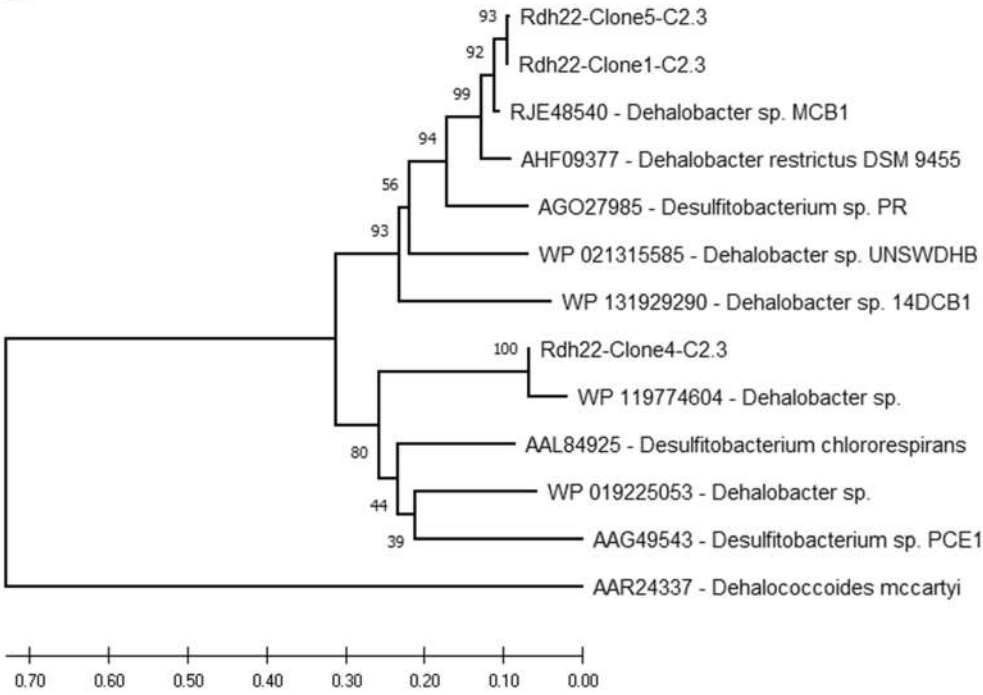
The *vcrA* metabolic genes for reductive VC dechlorination were amplified in samples C1.2 and C2.2, while the Rdh22 genes were found only in sample C2.3. The *vcrA* genes encode for the PCE, DCE, and VC, to ethylene-reducing dehalogenase and are mainly found in *Dehalococcoides* spp. Their presence in C1.2 and C2.2 samples was correlated with the abundance of 16S rRNA amplicons belonging to *Dehalococcoides*, along with the concentration of VC detected in samples C2.2. In this respect, a positive correlation

between the abundance of *Dehalococcoides* spp., *vcrA* gene amplification, and VC transformation to ethylene has previously been reported in an anoxic dechlorinating bioreactor, anaerobic microcosms containing superficial river sediments, and sediment samples collected from sites contaminated initially with PCE (Maphosa et al. 2010; Yoshikawa et al. 2017; Atashgahi et al. 2013). However, as reported by Takeuchi et al. (2011), numerous reductive dehalogenase genes in sediment might also be affected by multiple chemical properties, such as natural organic matter, and not only VC concentration. This latter observation may explain why, in our study, we failed in amplifying complementary Rdhase genes such as *tceA* and *bvcA* from all the contaminated samples we tested.

The genes encoding reductive dehalogenases that were amplified using the Rdhase group 22 primers were related to those of the *Dehalobacter* genus. They were amplified only from the C2.3 sample according to the prevalence of 16S rRNA sequences affiliated to the genus *Dehalobacter* found exclusively in this contaminated sample. *Dehalobacter* spp. are known for respiring both aliphatic, e.g., PCE (Holliger et al. 1998), and aromatic-like dichlorobenzoate, and they can dechlorinate PCE and TCE to cDCE (Grostern and Edwards 2006; Tang and Edwards 2013). Notably, no further reductive dechlorination of cDCE to VC and ethylene has been found in species of this genus (Regeard et al. 2004). These results, combined with data on the distribution of 16S rRNA sequences of *Dehalococcoides* and *Dehalobacter* in contaminated samples, allowed us to conclude that PCE and TCE were mainly dehalogenated in sample C2.3. Concurrently, VC dehalogenation is likely to occur in samples closest to the surface where *Dehalococcoides* are more numerous as compared to sample C2.3, and where *vcrA* genes have been detected (Fig. 8).

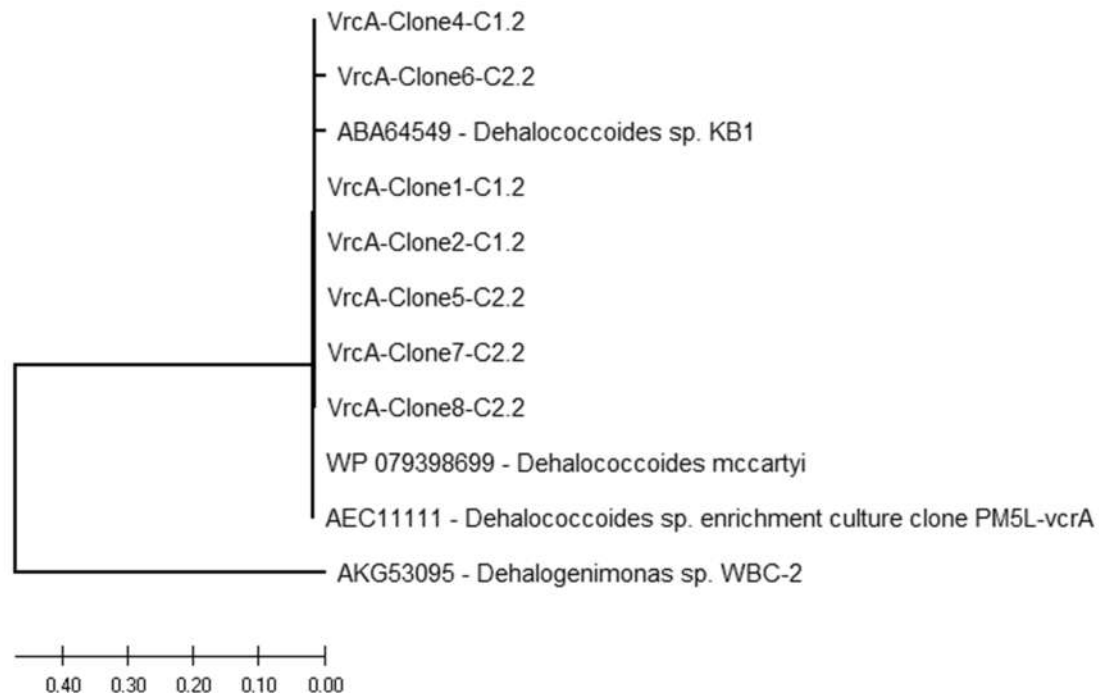
Taken all together, based on the present and past observations describing the contaminated samples of the Caretti site, we propose a working model of the possible metabolic interactions within the microbial communities involved in the reductive dehalogenation process analyzed here (Fig. 9). Such model also takes in consideration the functional profiling of ASVs affiliated to taxa involved in the synthesis of fermentation products that supports the dechlorination process. In our tentative scheme, several groups of *Anaerolineae* and Ca. Bathyarchaeota can transform some of the main organic components of peat, such as cellulose, hemicellulose, lignin, humic acids, fulvic acids, and mixed carbohydrates, into low molecular weight metabolites that can be further fermented by *Elusimicrobia*, Ca. Aminicenantes, and *Clostridia*, with organic acids, CO<sub>2</sub>, and hydrogen as final products. These compounds are essential to stimulate the reductive dechlorination of TCE and PCE to ethylene by *Dehalococcoides* that have been found predominant in C1.2, C2.1, and C2.2 samples, together with the presence of *vcrA* genes which code for the VcrA enzyme that dechlorinates vinyl chloride to

**a**



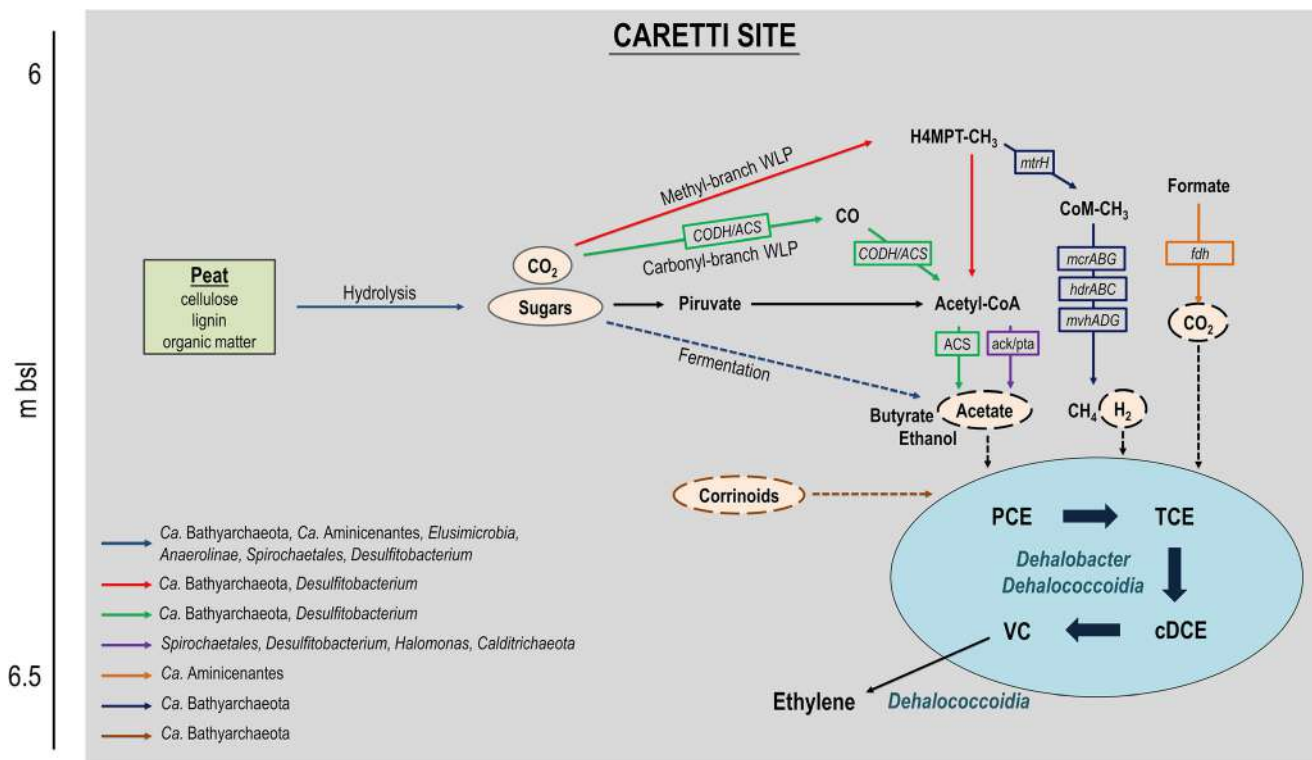
*Clostridia*  
(Firmicutes)

**b**



*Dehalococcoidia*  
(Chloroflexi)

**Fig. 8** Phylogenetic trees of the amino acid sequences retrieved from clone library analysis of **a** Rdh22 and **b** VrcA



**Fig. 9** Schematic overview of the metabolic processes which are proposed to take place within the dechlorinating microbial consortia at the Caretti site aquifer. Metabolic pathways are indicated with different colors and the microorganism contributing to each metabolic pathway are reported in the figure legend. Dashed lines indicate pathways non

predicted from the functional profiling of ASVs. Gene names are *mcr* (methyl coenzyme M reductase); *hdr* (heterodisulfide reductase subunit); *mvh* (F420 non reducing hydrogenase); CODH/ACS (carbon monoxide dehydrogenase/acetyl CoA synthase); *ack/pta* (acetate kinase/phosphate acetyltransferase); *fdh* (formate dehydrogenase)

ethylene, thus avoiding its accumulation. In the deepest sample C2.3, members of the *Dehalobacter* genus contribute to the reductive dechlorination of TCE to cDCE and VC, together with the syntrophic fermentation activity of other microorganisms of the *Firmicutes* phylum.

Numerous reports describe the enrichment and isolation of anaerobic microbial consortia in microcosms or bioreactors amended with organic acids or alcohols and in the presence of halogenated aliphatic compounds (Yohda et al. 2015; Hug et al. 2012). It is interesting to note that these consortia's microbial composition resembles the natural microbial population structure present in the Caretti site. The peat layers provide the organic material for fermentation and methanogenesis metabolisms assisting the reductive dechlorination process carried out by *Dehalococcoidia* and *Dehalobacter* and naturally created an involved microbial selection community capable of dehalogenating organic halogenated compounds. The implementation of metagenomic analyses with the monitoring of the expression of reductive dehalogenases and quantification of the main actors involved in the dechlorination process by qPCR along with the comparison with adjacent uncontaminated sites, where dechlorination fails, will provide indications to understand and

optimize the natural microbial consortium activity. This information will be critical to promote and stimulate the processes of reductive dechlorination in natural environments.

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**Authors' contributions** Conceptualization: Stefano Fedi, Daniele Ghezzi, Maria Filippini, Martina Cappelletti; methodology: Stefano Fedi, Daniele Ghezzi, Martina Cappelletti; formal analysis and investigation: Stefano Fedi, Daniele Ghezzi, Maria Filippini, Andrea Firrincieli; writing original draft preparation: Stefano Fedi, Daniele Ghezzi, Maria Filippini; Andrea Firrincieli; writing review and editing: Stefano Fedi, Daniele Ghezzi, Maria Filippini, Davide Zannoni; funding acquisition: Davide Zannoni, Alessandro Gargini; supervision: Stefano Fedi, Alessandro Gargini, Davide Zannoni

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**Data availability** All data generated or analyzed during this study are included in this published article (and its supplementary information files).

## Compliance with ethical standards

**Competing interests** The authors declare that they have no competing interests.

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