In situ remediation of chlorinated solvents-contaminated groundwater: from site characterisation to the Monitoring of Natural Attenuation (MNA) by biomolecular and isotopic tools.

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Abstract

The present work describes the assessment of applying a biological-based treatment for the remediation of a site contaminated by chlorinated aliphatic hydrocarbons (CAHs) and, to a lesser extent, by BTEXs. A chemical and microbiological characterisation was performed to define the biodegradative potential for the mentioned contaminants, including the quantification of marker genes (*tceA*, *vcrA*, *bvcA*, *etnC*, *bssA*, *todC* and the bacterial 16S rRNA gene) and Next Generation Sequencing (NGS) for the functional and taxonomical definition of the microbial community. Results highlighted the predominance of an anaerobic potential for the degradation of CAHs and the co-presence of both aerobic and anaerobic degradation potential for BTEXs. From NGS, microbial populations with specific metabolic capabilities were identified. Considering these biomolecular results and the decreasing of contaminant concentration over time, Monitored Natural Attenuation (MNA) strategy was applied. The monitoring plan included chemical, biomolecular and isotopic analyses on groundwater every six months, for three years. Specifically, biomolecular analyses, including the quantification of total bacteria, *Dehalococcoides spp*., *pceA*, *tceA* and *vcrA* genes and the bacterial 16S rRNA NGS, and Compound Specific Isotope Analysis (^{13}C-CSIA) of target CAHs were used for the monitoring of natural attenuation processes. Integrating chemical, isotopic and biomolecular results, the abatement of contaminant concentrations through natural biological processes was showed, suggesting the effectiveness of the selected strategy. This case study shows how to use innovative methodologies to design an efficient site-specific bioremediation strategy and to monitor the process.

Keywords *In situ* bioremediation · Environmental microbiology · Chlorinated solvents · Biomolecular analyses · Isotopic analyses · Groundwater remediation · Monitoring of Natural Attenuation (MNA)

1. Introduction

Chlorinated aliphatic hydrocarbons (CAHs), a class of contaminants that includes chlorinated methanes, ethanes and ethylenes, are listed as priority pollutants in EU, US and China, for their toxicity, teratogenicity and carcinogenicity (Benekos, 2007; Zhang, 2024; Chen Z. , 2024). Due to improper management during their production, transportation and storage, they enter the aquifer and accumulate as dense non-aqueous liquids (DNAPL) on the bottom, generating a plume that causes persistent groundwater contamination (Barbee, 1994; Wu, 2020; Zhao, 2023; Tang, 2022). Their wide and growing use as chemical materials and organic solvents on large-scale industrial processes, mainly in plastic, cleaning, pesticides and drugs sectors, led to the need to find efficient solutions for their removal (Bonse, 1976; Murray, 1973).

In nature, many microorganisms are able to biodegrade these compounds, in particular chlorinated ethylenes, mainly through three processes (Xing, 2022). The first is the anaerobic reductive dechlorination, in which chlorinated ethylenes are reduced for their use as terminal electron acceptor using molecular hydrogen as electron donor, undergoing the transformation into low chlorinated compounds (Němeček, 2020; Nijenhuis, 2016). The enzyme, responsible for the substitution of Cl with H, is a reductive dehalogenase specific for each step of the pathway: PceA for Tetrachloroethylene (PCE) and Trichloroethylene (TCE), TceA for TCE and cis-1,2-Dichloroethylene (cis-1,2-DCE), vcrA and bvcA for cis-1,2-DCE and vinyl chloride (VCM) (Dolinová, 2017; Bertolini, 2023; Behrens, 2008; Clark, 2018). For chlorinated ethylenes, the only known anaerobic bacteria able to perform the complete dechlorination from PCE to ethylene belong to the *Dehalococcoides* genus, but many other anaerobic bacteria can partially dechlorinate CAHs, such as *Dehalobacter, Dehalospirillum, Dehalogenimonas, Desulfuromonas Desulfitobacterium, Sulfurospirillum, Geobacter, Clostridium* species (Friis, 2007; Duhamel, 2006; Dutta, 2022). A partial degradation led to the conversion of higher chlorinated ethylenes to less chlorinated ones, as DCE and VCM, that are more hazardous and more toxic, tending to accumulate (Chambon, 2013). The other two degradative pathways are mediated by oxygenase enzymes and use oxygen as terminal electron acceptor. One is the aerobic metabolic degradation, where CAHs are directly oxidated being used as a source of energy and as a growth substrate. The reaction is activated by an alkene monooxygenase (EtnABCD) and an epoxyalkane-coenzyme enzyme (EtnE), with the formation of an epoxide as intermediate, to obtain the complete mineralisation into chloride ions and carbon dioxide (Cortés-Albayay, 2021; Moratti, 2022; Dolinová, 2017). The epoxide is unstable and

highly reactive, but microorganisms involved in this reaction possess epoxide-transforming enzymes to detoxify it (Weatherill, 2018). This process is more efficient on lower chlorinated compounds, with consequently lower accumulation of intermediates (Richards, 2022; Jesus, 2016). The third process is the aerobic co-metabolic degradation, where CAHs are not the target substrates and thus are not used for energy production, but are fortuitously degraded by non-specific monooxygenases/dioxygenase expressed for a second compound, i.e. the growth substrate (Xingda, 2021). The main disadvantage of this pathway is that the primary target substrate, even if it competes with the CAHs for the active site, must be present to sustain oxygenase activity and cell growth (Frascari, 2012). Moreover, chlorinated epoxides or aldehyde toxic substances produced as intermediates are toxic and difficult to metabolise for most of non-specialised microorganisms (Mattes, 2010). The most studied co-metabolisms involve toluene and methane, with their respective monooxygenases (Chen S. , 2020; Chang, 1996; Deng, 2020; Yoshikawa, 2017).

Every process has its own advantages and disadvantages and to improve their effectiveness it is possible to design specific bioremediation strategies. The simplest bioremediation strategy is the Monitoring of Natural Attenuation processes (MNA), which consists in monitoring the natural removal of contaminants over time, thanks to a series of naturally occurring biological, physical and chemical processes, among which biodegradation is generally considered to be the key process (Zanini, 2021). The other most used bioremediation strategy is biostimulation, that consists in the addition of nutrients, electron donors/acceptors or other products to stimulate and support natural processes. Moreover, pure or mixed cultures of well-known biodegradative microorganisms can be added to the environmental matrix, a strategy called bioaugmentation (Pérez-de-Mora, 2014; Duhamel, 2006). With respect to other strategies applied for CAHs contamination, bioremediation results a very flexible, eco-friendly and cost-effective strategy, with the possibility to completely restore the impacted aquifers (Luo, 2024; Syafiuddin, 2020; Fan, 2022). To evaluate its possible application and for the selection of the most suitable site-specific remediation techniques, the determination of the biodegradative potential of the site is crucial.

The first step required is a deep characterisation of the site, both under a chemical and a biological point of view, to evaluate both the level of contamination and the presence of degradative metabolisms and consequently to evaluate the intrinsic potential in removing the contamination (Thornton, 2016; Ottosen, 2021). Regarding the chemical analyses, in addition to conventional methodologies, an in-depth investigations could be performed through isotopic analyses, aimed at assessing the evolution of the natural attenuation processes, the contribution of biological processes and their nature (e.g., aerobic or anaerobic degradation) (Mundle, 2012; Meckenstock, 2004). On the other hand, for the microbial community studies, the biomolecular approach is the most effective, allowing the study of all the microorganisms present in the sample without disturbing their conditions, thus obtaining a realistic and reliable representation. In particular, the use of the quantitative PCR technique (qPCR), which allows the estimation of the copy number of a target marker gene within the sample, enables to quantify specific genes related to a taxonomical group or a metabolic pathway of interest. Moreover, for taxonomical studies, Next Generation Sequencing (NGS) of taxonomical marker genes is useful to obtain information about the microorganisms and their relative abundances in the sample.

The same methodologies could be used during the monitoring process to evaluate the microbial evolution over time, assessing the effectiveness of the selected bio-based strategy (Blázquez-Pallí, 2019; Jendrzejewski, 2001). Combining isotopic analyses and biomolecular ones, it is possible to obtain detailed information about on-going processes and their effectiveness, allowing to first define and then monitor the best site-specific strategy for the contaminant removal. This work deals with the feasibility of applying a biological-based treatment for the remediation of a site contaminated mostly by chlorinated solvents and, to a lesser extent, by BTEXs, from the characterisation study of the site to the monitoring of the applied technology, using an innovative combination of isotopic-biomolecular tools.

2. Materials and methods

2.1. Background and Site description

The site is located in Lombardy, in the south of Milano city. The area, which extends for approximately 11.500 m^2, undergone a succession of activities that led to groundwater contamination, as storage of petroleum products, waste management and production of paraffins and waxes. During the preliminary investigation phase, some contaminants revealed concentration values over the threshold limits imposed by the Italian law. In 2008, the characterisation plan was approved and, in 2009, a piezometric monitoring network was created to define groundwater state. The characterisation highlighted that the concentration limits exceeded for C>12, in soil, and for CAHs, BTEXs, Mn, Fe and chlorobenzenes in groundwater. Consequently, a site-specific Risk Analysis (RA) was required and done in 2010 to determine the risk threshold concentrations and verify compliance. In the third revision of the RA, a soil gas monitoring plan was also proposed, then implemented in 2014. The last revision of the RA dates to 2017 and defined that surface and deep soil and groundwater were not contaminated, while an unacceptable risk for groundwater was present at the Point of Conformity (POC). POC was represented by the border line of the area in the hydrogeological direction (S-SW border), where the PZB, PZK, PZD and PZA monitoring piezometers are located. For this reason, an emergency safety action was active in correspondence of these piezometers, consisting of a *pump&stock* hydraulic barrier. However, the low economic and environmental sustainability of this system led to the evaluation of the feasibility of an alternative treatment technology, based on bioremediation.

2.2. Groundwater sampling

For the chemical and microbial characterisation analyses, performed in 2017-2018 to design the remediation project, the same piezometers analysed during the first chemical characterisation in 2009 were used. In May 2018, groundwater samples for chemical and biomolecular analyses were collected, and n. 8 piezometers were selected: PZ2 (upstream), PZG, PZJ, PZF, PZE (central area), PZB, PZK, PZD (downstream) (Fig. 1). The sampling was preceded by purging each piezometer, for a volume of water equal to 5 times the volume of water contained in the piezometer itself. The purge time used for each piezometer was variable, depending on its characteristics and on the average pumping flow rate (approximately 10 L/min). Water from the PZE and PZK piezometers was sampled with a submersible electric pump.

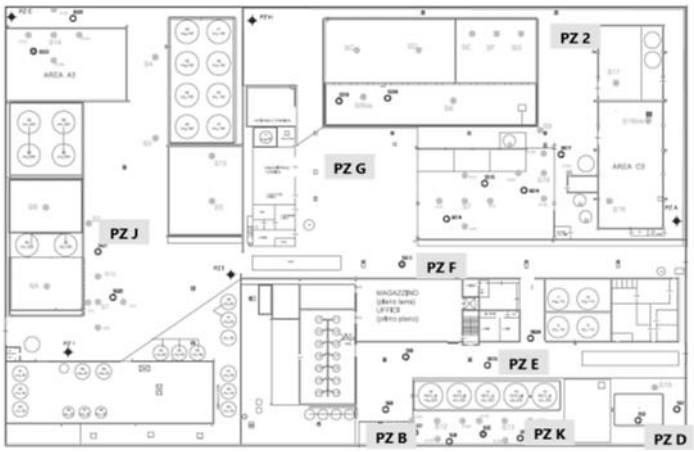

Fig. 1 Map of the site

2.3. Chemical and physical analyses

The physico-chemical parameters of temperature, pH, electrical conductivity, oxidation-reduction potential and dissolved oxygen were detected directly in the field, using a multiparametric probe. The piezometric level was measured with a phreatimeter. Analysis of nitrates, sulfates, sulfides and methane were carried out to have a complete view of the available electron acceptors and their reduction products. For the previous investigations performed from 2009, water samples were analysed for total hydrocarbons, aromatic organic compounds (BTEXs and PAHs), carcinogenic and non-carcinogenic CAHs, chlorobenzenes, chloroaniline, methylaniline, ethylaniline, ethylaniline, dichloroaniline, other aromatic amines (Aniline, Diphenylamine, p-toluidine) and metals (Al, Sb, Ag, As, Be, Cd, Co, Cr, Cr^{VI}, Fe, Hg, Ni, Pb, Cu, Se, Mn, Tl, Zn). In this case, an integration of the analytical set was done for ethylene and the 1,2-trans-DCE isomer.

For BTEXs and CAHs, chemical results from 2011 were processed to define the contamination level over time.

2.4. Inverse Distance Weighting (IDW) interpolation

Chemical data acquired in the field were interpolated through the Inverse Distance Weighting (IDW) method. The choice of this interpolation method is justified by the limited data available, corresponding to the number of piezometers present in the area, arranged in such a way that it does not allow the estimation of a model of spatial correlation (e.g. semi-variogram) of sufficient quality. In addition, analysing the stratigraphies, below the topsoil are layers of silt and sand with horizontally uniform structure: this allows the assumption that the transport and diffusion of pollutants occur under conditions of spatial uniformity and that the three-dimensional concentration fields, downstream of a release volume, have a shape that is mathematically regular (i.e. continuous and differentiable up to at least the second order). This last aspect suggests the possibility of using, in the absence of more detailed information, a deterministic method. Mathematically, the IDW method is a weighted average for the inverse of distance. The assumption behind this method is as follows: the interpolated value, at a given unknown point, will be more determined by the known values at the nearest neighbours than at the most distant. This method, although it tends to generate "bull's-eye", honors the range of actual variability of the experimental data by avoiding overshoot phenomena (Golden Software, 2014).

In the present work, the software Surfer® was used to perform interpolation and obtain concentration maps, expressed in µg/L, for each hydrocarbon of interest. For this purpose, the data relating to each sampling were analysed. The mean (m) and median (M) of the concentrations detected in each of the 13 piezometers, the interquartile range (IQR) and the absolute value of the difference between mean and median (|m-M|) were calculated for all the 26 samplings performed. In this way, it was possible to evaluate the symmetry of the data distribution; in particular, the minimum value of |m-M| corresponds to the most symmetrical distribution that exists. The IQR value, however, provided indications regarding the dispersion of the data: the maximum IQR value corresponds to the maximum variation in the data.

2.5. DNA extraction and preparation

For biomolecular analyses, 2 L of groundwater sample collected from each piezometer was filtered on nitrocellulose filters with a pore size of 0,45 µm. The filters were used as extraction matrix, using the kit FastDNA™ Spin Kit for Soil (MP Biomedicals). To evaluate the quality of obtained DNA samples , a 492 bp fragment of the bacterial 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 519R (5'-WATTACCGCGGCKGCTG-3'), with the following program: initial denaturation at 95°C for 4min, and 29 cycles of 95°C for 30s, 55°C for 45s, 72°C for 45s, and a final elongation of 72°C for 5min. The amplified DNA was loaded on 1% agarose electrophoresis gel and visualized by UV lamp. The genomic DNA was then used for qPCR and NGS analysis.

2.6. qPCR analyses

The extracted DNA was used as template for quantitative analysis on specific genes involved in the target degradation pathways. The Fluocycle II™ Sybr Green Master Mix (EuroClone) and Eco48 (PCRMax) instrument were used for qPCR analysis,. Primer (concentration of 0,3 µM) sequences are reported in Table 1, while amplification programs in Supplementary Information (Table S1). For the characterisation of the site, total bacteria number was assessed by the quantification of 16S rRNA gene, whereas both aerobic and anaerobic pathways for BTEXs and CAHs were investigated by quantifying *todC* and *bssA* genes for BTEXs, while *etnC* and *tceA, vcrA, bvcA* genes for CAHs. For the monitoring of Natural Attenuation processes, specifically of anaerobic dehalogenation, were instead quantified the bacterial 16S rRNA gene as biomarker of total bacteria and the 16S rRNA gene specific for *Dehalococcoides spp*., together with *pceA, tceA,* and *vcrA* genes.

Table 1 Primer sequences for qPCR analyses.

Gene	Biological meaning	Primer	Sequence (5'-3')	Reference
16S rRNA	Total bacteria	331F	TCCTACGGGAGGCAGCAGT	(Marchesi, 2018)
		797R	GCACTACCAGGGTATCTAATCCTGTT	
16S rRNA *Dehalococcoides spp.*	CAHs degradation model bacterial genus	Dhc1083F	AGACTGCCCCGCGAAACG	(Cupples, 2008)
		Dhc1220R	AGCTTTGGGGATTAGCTCCAG	
pceA	Anaerobic degradation of PCE	pceAF	ACCGAAACCAGTTACGAACG	(Popat, 2012)
		pceAR	GACTATTGTTGCCGGCACTT	
tceA	Anaerobic degradation of TCE and DCE	TceA1270F	ATCCAGATTATGACCCTGGTGAA	(Ritalahti, 2006)
		TceA1336R	GCGGCATATATTAGGGCATCTT	
vcrA	Anaerobic degradation of DCE and VCM	VCMr1022F	CGGGCGGATGCACTATTTT	(Ritalahti, 2006)
		VCMr1093R	GAATAGTCCGTGCCCTTCCTC	
bvcA	Anaerobic degradation of DCE and VCM	BVC925F	AAAAGCACTTGGCTATCAAGGAC	(Ritalahti, 2006)
		BVC1017R	CCAAAAGCACCACCAGGTC	
etnC	Aerobic degradation of VCM	RTC_F	ACCCTGGTCGGTGTKSTYTC	(Jin, 2010)
		RTC_R	TCATGTAMGAGCCGACGAAGTC	
bssA	Anaerobic degradaion of BTEXs	7772f	GACATGACCGACGCSATYCT	(Netzer, 2013)
		8546r	TCGTCGTCRTTGCCCCAYTT	
todC	Aerobic degradaion of BTEXs	TODC1-F	CAGTGCCGCCAYCGTGGYATG	(Hendrickx, 2006)
		TODC1-R	GCCACTTCCATGYCCRCCCCA	

2.7. Next Generation Sequencing (NG) analysis

Next Generation Sequencing (NGS) analysis was performed to study the structure of microbial community and its changes over time using Illumina MiSeq platform. The V5-V6 hypervariable regions of 16S rRNA gene were amplified for all DNA samples. The amplification reaction was performed using Green Taq Master Mix 2x (Promega), 1 µM of primers (783F: 5'-CAGGATTAGATACCC-3', 1027R: 5'-CGACRRCCATGCANCACCT-3') and 2,5 µL genomic DNA. The amplification program was an initial denaturation at 94°C for 5min, followed by 27 cycles with 94°C for 50s, 47°C for 30s and 72°C for 30s, with a final extension of 72°C for 5min. An electrophoresis on 1% agarose gel stained with GelStar (Lonza) was prepared to purify the specific 282 bp fragment, using Wizard SV Gel and PCR Clean-up System kit (Promega) following manufacturer's protocol. For library preparation, the concentration of the purified DNA was determined using a Qubit® 2.0 Fluorimeter (Invitrogen). The sequencing was carried out following Illumina protocols in the Department of Earth and Environmental Sciences of the University of Milano-Bicocca (Milano, Italy). The obtained sequences in .fastq format were taxonomically classified by RDP classifier (confidence > 80%).

2.8. Isotopic analyses

To identify the presence and the nature of biodegradation processes for chlorinated solvents, samples were analysed using ^{13}C-CSIA (Compound Specific Isotope Analysis) technique. These analyses were carried out atIT^2E laboratories (Milan, Italy). Analyses were performed for VCM, 1,1-DCE, 1,2- trans-DCE, 1,2- cis-DCE, TCE and PCE. Results were analysed individually for each compound and, as a whole, with an isotopic balance analysis relative to the respective molar fractions.

3. Results and discussion

3.1. Characterisation

To in deep understand the chemical and biological processes present in the site and define its biodegradative potential, a chemical and microbiological characterisation was performed in relation to both CAHs and BTEXs.

3.1.1. Chemical, physical and IDW analyses

From chemical analyses performed between 2011 and 2019, a generalized and progressive reduction of CAHs emerged, but with some sporadic increase in their concentration. CAHs concentrations, in fact, showed a reduction going from values of 70 µg/L, measured in 2012, to values never exceeding 10 µg/L (Italian law concentration limit), in the same piezometers from 2017. In detail, PCE (Italian law concentration limit = 1.1 µg/L) underwent the most evident reduction, from concentrations over 40 µg/L, detected in PZE before 2014, to values that still exceeded the above-mentioned law limit, but in the order of few µg/L, widely detected in the site, even in piezometers located hydrogeologically upstream (Fig. 2). Moreover, in PZE, piezometer in which the highest values of CAHs were recorded over time, the concentrations of PCE by-products, as TCE, 1,2-cis-DCE and VCM, showed a coherent and constant decreasing as well.

Regarding the other contaminants (i.e., BTEXs and metals (Fe and Mn)), the removal efficiency resulted less marked. In particular, the presence of BTEXs was episodic, quite limited and with compositional profiles difficult to interpret: the PZE piezometer is the only one at which all BTEXs were detected. Benzene is the compound with the highest concentration, that constantly exceeded concentration limit in PZE, almost constantly in PZK and occasionally in PZJ.

Metals showed a widespread background contamination in the site, with higher values for Mn in the hydrogeological upstream piezometers, probably due to locally more reducing aquifer conditions, while Fe was detected mainly in the central area of the site, with values in the order of thousands of µg/L.

In May 2018, integrative analyses were performed to better understand the site conditions, as a IDW study. Results of IDW are showed in Supplementary Information (Figure S2).

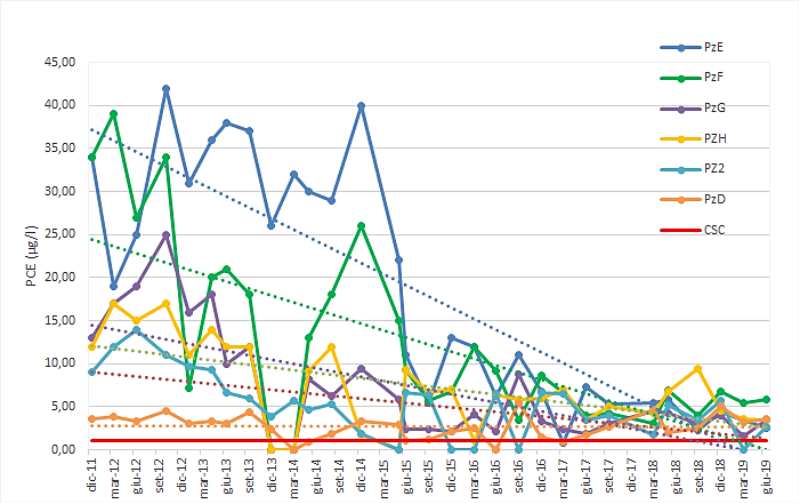

Fig. 2 PCE concentration trend between 2011 and 2019. CSC= Italian law concentration limit. Sampling was carried out each December (dic), March (mar), June (giu) and September (set)

3.1.2. Biomolecular analyses

Piezometers	16S rRNA n°copies/L	*tceA* n°copies/L	*TCE µg/L*	*bvcA* n°copies/L	*VCM µg/L*	*todC* n°copies/L	*Benzene µg/L*
PZ2	3,47 x 10^7	b.d.l.	< 0.50	b.d.l.	< 0.10	b.d.l.	< 0.10
PZG	1,73 x 10^8	1,85 x 10^7	0,88	b.d.l.	< 0.10	b.d.l.	< 0.10
PZF	8,81 x 10^8	2,14 x 10^7	1,3	b.d.l.	0,22	b.d.l.	0,16
PZE	3,09 x 10^8	1,48 x 10^8	1,2	b.d.l.	0,41	2,65 x 10^8	**9,3**
PZJ	5,14 x 10^9	b.d.l.	-	b.d.l.	-	4,86 x 10^9	-
PZK	1,34 x 10^8	b.d.l.	0,75	1,3 x 10^7	**1,6**	b.d.l.	< 0.10
PZD	5,22 x 10^6	9,07 x 10^6	< 0.50	b.d.l.	< 0.10	b.d.l.	< 0.10
PZB	2,08 x 10^8	b.d.l.	-	b.d.l.	-	b.d.l.	-

Table 2 Chemical results of the last characterisation campaign (18/06/2019) for TCE, VC and benzene and qPCR results for related degradation genes. B.d.l. = below detection limit. Chemical values over Italian law concentration limits are underlined with bold font.

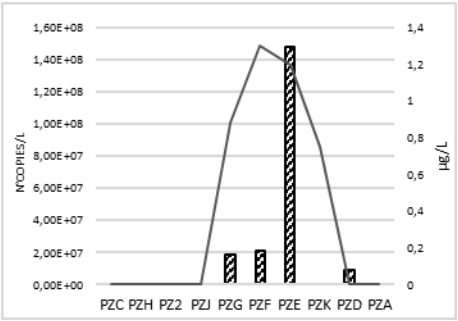

Fig. 3 TCE concentrations (µg/L) of the last characterisation campaign (18/06/2019) and qPCR results for the marker gene of its degradation, *tceA* (n° of gene copies/L)

The bacterial 16S rRNA was used as the marker gene of total bacteria, with results that suggest the lowest biomass in PZD and the highest in PZJ, with average values of 10^8 number of gene copy numbers/L. The catabolic genes *tceA*, *vcrA* and *bvcA* were selected as biomarkers for reductive dechlorination of CAHs. All the results of qPCR analysis, for the selected genes, are reported in Supplementary Information (Table S3). Comparing the biomolecular data with the chemical one (Tab.2 and Fig. 3), a well-defined correspondence is noted, suggested by the strict correlation between the presence of contaminants and the detection of marker genes involved in their anaerobic degradation. In particular, *tceA* gene, encoding the reductive dehalogenase TceA, showed the highest values in PZE, where the highest concentration of TCE was detected, followed by PZG, PZF and PZD. Regarding marker genes encoding the reductive dehalogenases which catalyse the dechlorination of VCM, *vcrA* is present in almost all piezometers, while *bvcA* was detected only in the piezometer PZK, the one with the highest concentration of VCM. The catabolic gene *etnC,* marker gene for the aerobic oxidation of low chlorinated ethylenes, was not detected in any piezometer. This result agrees with the anoxic conditions of the site. Regarding BTEXs, the catabolic gene *bssA* was selected for the anaerobic degradation and *todC* for the aerobic one. Results revealed the presence of anaerobic potential for degradation of BTEXs in almost all piezometers, especially in the central area of the site. In the most impacted piezometers, both anaerobic and aerobic marker genes for BTEXs degradation were found. In fact, *todC* was detected only in PZE and PZJ.

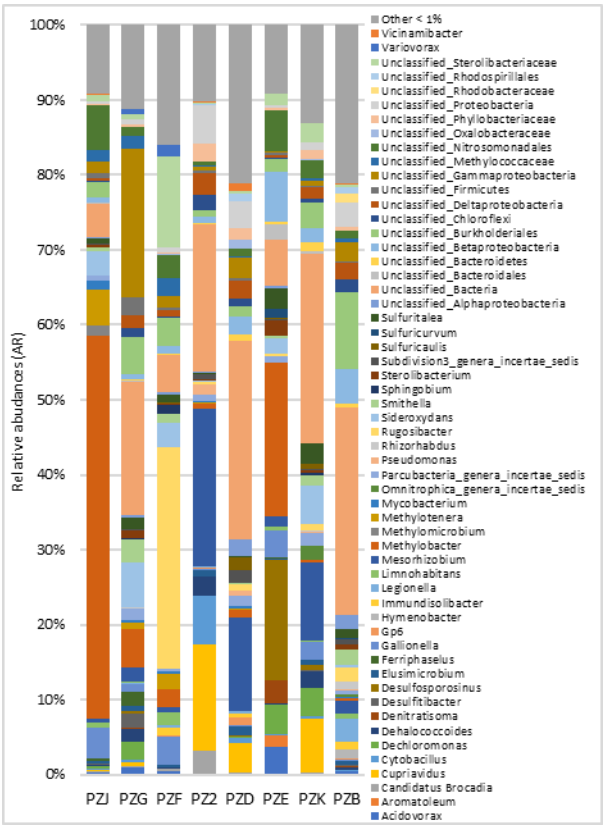

Fig. 4 NGS results of site characterisation expressed as relative abundances (AR). Data are shown at Genus taxonomical level, genera with AR<1% are grouped under "Other < 1%"

From sequencing analyses (Fig. 4), a selected community in most piezometers emerged, with few bacterial genera representing most of the population. Several bacteria able to anaerobically metabolize CAHs were identified, both obligate halorespiring microorganisms involved in the complete degradation of CAHs, as *Dehalococcoides* (PZ2, PZK, PZG), and facultative halorespiring microorganisms involved in their partial degradation, as *Desulfosporosinus* (PZE), *Dechloromonas* (PZK, PZE, PZG), *Geobacter* (PZE), *Desulfuvibrio* (PZE, PZK) (Friis, 2007; Duhamel, 2006; Dutta, 2022). Moreover, bacteria able of aerobic co-metabolic degradation were found, as *Methylococcales*, a bacterial genus with methanotrophic metabolism (Kotik, 2013). On the contrary, for BTEXs degradation, mainly anaerobic degradative community was detected through NGS, including *Georgfuchsia* (PZF, PZE), *Dechloromonas* (PZK, PZE, PZG) and *Azoarcus* (PZE) (Sperfeld, 2018; Aburto-Medina, 2014; Chakraborty).

Quantitative and qualitative microbiological analyses indicated the presence of bacterial populations (NGS) and metabolisms (qPCR) for the degradation of target contaminants. Regarding BTEXs, a natural potential for the aerobic degradation in the further downstream areas (PZE and PZK), and for the anaerobic one in the central area of the site, was found. For CAHs degradation, the site tendency to anaerobic dechlorination processes and the absence of aerobic degradation potential were observed. Thus, these analyses confirmed the possibility to apply a biological remediation strategy. In detail, two different strategies were selected: Monitoring of Natural Attenuation (MNA) in the central area of the site, where CAHs concentrations were higher and anaerobic degradation processes active, and Bio-Sparging in the downstream area of

the site, where higher concentrations of metals, BTEXs and VCM were found, in order to sustain and stimulate aerobic removal of these compounds. This paper is focused on MNA application.

3.2. Monitoring of Natural Attenuation (MNA)

Considering the characterisation results, showing the presence of specific microorganisms and metabolisms potentially involved in the anaerobic reductive dechlorination and the contaminant removal over time, the Monitoring of Natural Attenuation (MNA) strategy was selected for the central area of the site, for the remediation of the contaminated plume. MNA lasted 3 years and involved a monitoring plan consisting in chemical, isotopic and biomolecular analyses applied every six months between 2021 and 2024, with a total of 7 monitoring campaigns (t0-t6): March 2021 (t0), December 2021 (t1), March 2022 (t2), December 2022 (t3), March 2023 (t4), December 2023 (t5), March 2024 (t6). For MNA analyses, 4 piezometers were selected: PZE, PZF, PZG and PZH.

3.2.1. Chemical and Isotopic analyses

Physico-chemical analyses were performed as described in 2.2.2, but, differently from characterisation, in this case chemical analyses were performed only on target contaminants: BTEXs (benzene, ethylbenzene, toluene, p-xylene, o-xylene, m-xylene), styrene, CAHs (PCE, TCE, 1,2- cis-DCE, 1,2- trans-DCE, 1,1-DCE, VCM), Fe and Mn. Results are shown in Fig. 5.

Contaminant concentrations decreased in all piezometers, except for PZH, which resulted contaminated only by PCE, with values around 4 µg/L, and, at the end of the monitoring, it resulted around the same values. Isotopic measurements of 1,2-cis-DCE and VCM were not carried out as their concentrations were below the detection limit.

On the other hand, the other piezometers had an evident decrease, especially in PZE, that underwent the most effective removal for PCE and 1,2- cis-DCE. In fact, from values of 3.2 µg/L for PCE and 2.4 µg/L for DCE, both compounds were below the detection limit at the end of MNA. In particular, the general decrease in concentrations was probably due to biological processes since, at final sampling campaigns, the isotopic enrichment of 1,2- cis-DCE and the increase in the mole fraction of VCM indicated the possibility of degradation of 1,2- cis-DCE through reductive dehalogenation processes (Figure S4). As regard PZG, after an increase in the concentration of PCE and TCE at t2 and t3, a reduction in their concentrations at t4 and t5, along with isotopic enrichment, confirmed the on-going biodegradation processes. On the contrary, the isotopic values of 1,2-cis-DCE are similar to those of the precursors, indicating that degradation did not go further this step. In PZF, PCE was totally removed, while 1,2- cis-DCE, after a rapid decrease from t2 to t5, in the last monitoring campaign increased again. It is therefore evident an increase in biodegradation processes only for PCE.

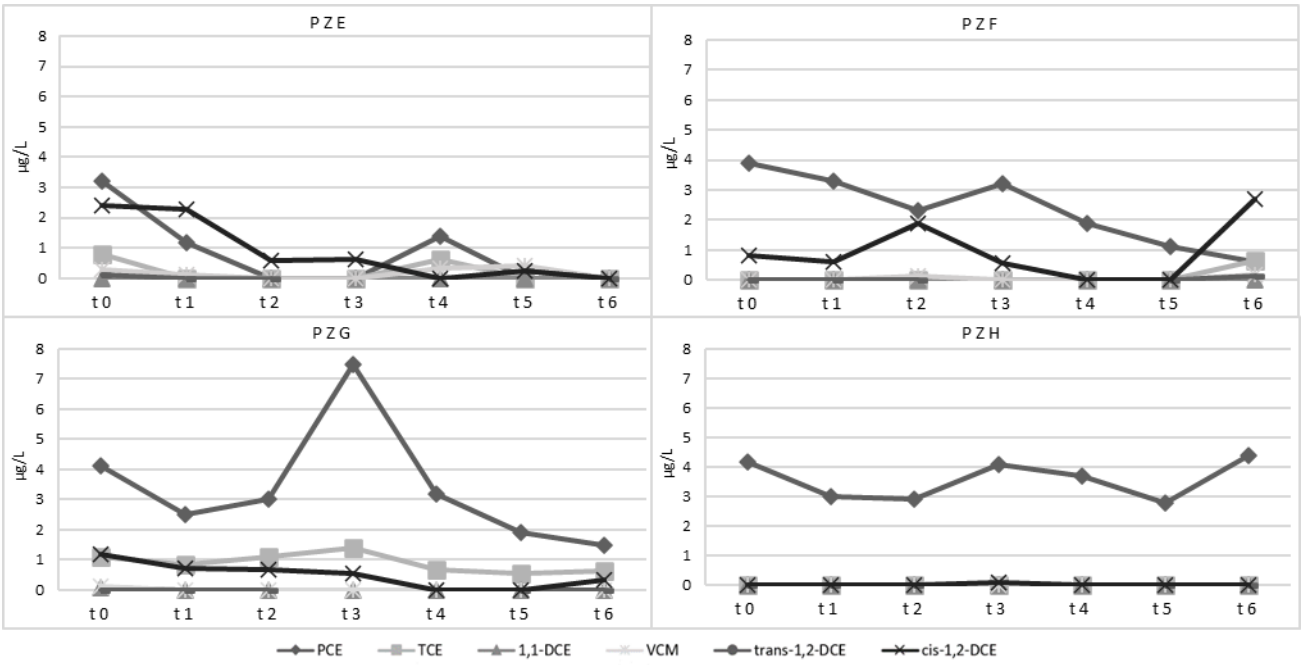

Fig. 5 CAHs concentrations during the monitoring, from March 2021 (t0) to March 2024 (t6)

3.2.2. *qPCR analyses*

Concerning qPCR analyses (Fig. 6 and Figure S5), the total number of bacteria slightly increased from $10^{8/9}$ copy numbers of the bacterial 16S rRNA gene/L at t0 up to $10^{9/10}$ at t4-t5 in all samples. Specifically, the bacterial genus *Dehalococcoides* was detected at t0 in all samples, with values of 10^5-10^6 gene copy numbers/L. From t2, an increase occurred in all samples, followed by a decrease from t4, reaching values, especially for the PZF and PZH samples, below the detection limit (10^2 gene copy numbers/L). PZG sample showed the highest values over time, from t0 to t6. For the degradation potential assessment of higher chlorinated ethylenes, *pceA* and *tceA* marker genes copy numbers increased only in PZE up to 10^4 gene copy numbers /L at t6. It should be underlined that, from the chemical analyses, the PZE sample is the only one in which PCE is below the detection limit at t5. The *vcrA* gene, marker gene for lower chlorinated ethylenes degradation potential, was also detected, at t5, in PZG and PZH samples. At t6, the presence of a greater degradation potential in the PZE sample, compared to the other samples, is evident, due to the presence of all the genes that encode the enzymes involved in the reductive dechlorination process of CAHs. This result is in line with the chemical and isotopic ones that showed the highest PCE removal extent in this piezometer. The total removal of PCE and the presence of this metabolic potential indicate the occurrence of biological removal processes in this part of the site.

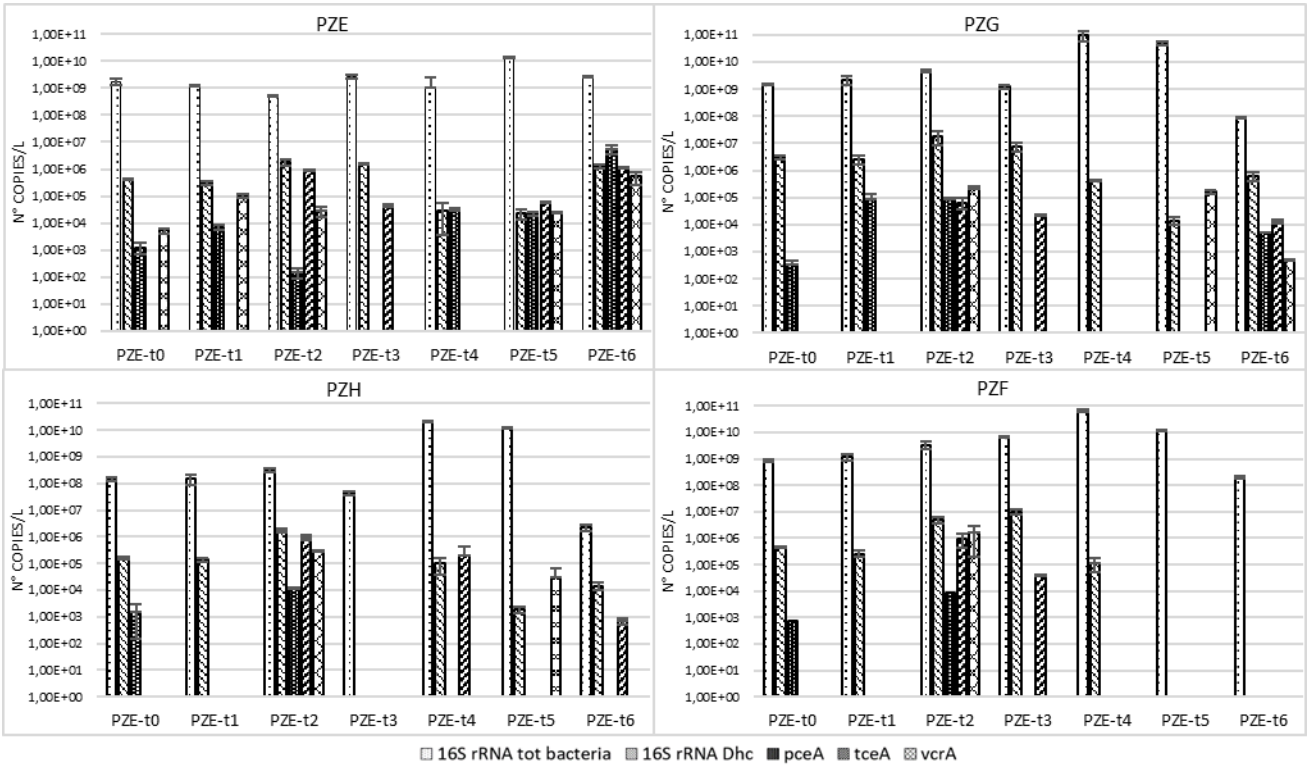

Fig. 6 qPCR analyses results during the monitoring, from March 2021 (t0) to March 2024 (t6), expressed as n° of gene copies/L of groundwater

3.2.3. *Next Generation Sequencing analysis*

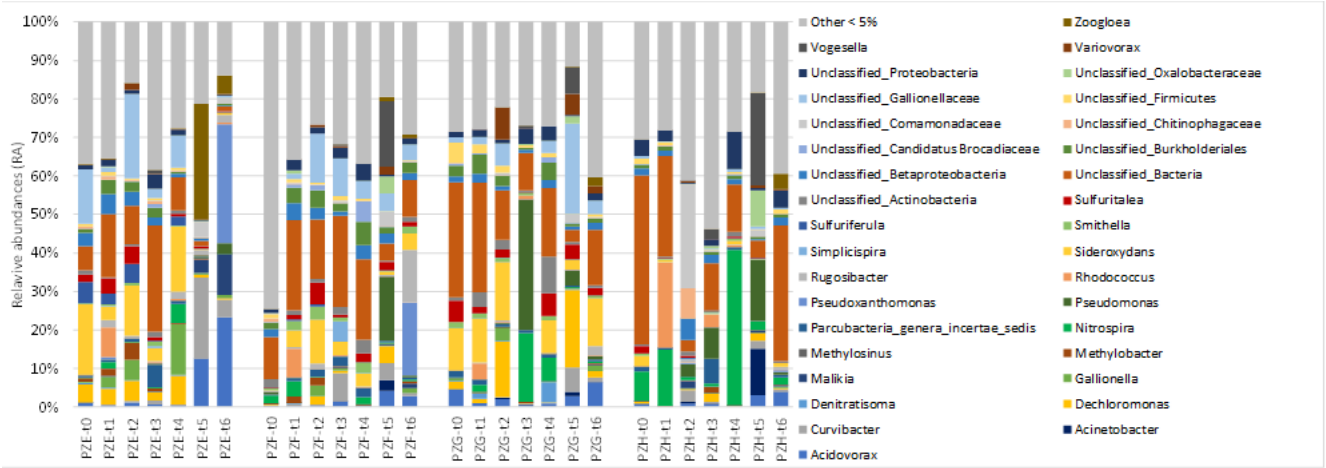

Fig. 7 NGS analyses results during the monitoring phase, from March 2021 (t0) to March 2024 (t6), expressed as relative abundances (AR). Data are shown at Genus taxonomical level, genera with AR<5% are grouped under "Other < 5%"

NGS results (Fig. 7) showed, at t0, the presence of the genus *Dehalococcoides spp.* in all samples (RA < 5%) with the highest relative abundances in PZG, in agreement with qPCR results. Other anaerobic bacterial genera capable of using chlorinated solvents as final electron acceptors, but limiting to their partial dechlorination, as *Geobacter* (Futagami, 2008), *Dechloromonas* (Kittelmann, 2008) and *Desulfosporosinus* (Robertson, 2001) spp., were also identified and particularly abundant in PZE (RA 2.58%, 4.7% and 2.41%, respectively). In this piezometer, all CAHs were found, including 1,2-cis-DCE and VCM, indicating a possible accumulation of these intermediates and therefore confirming the occurence of partial reductive dechlorination processes at the beginning, as proved during the characterisation of the site. There is also evidence of the presence of genera involved in the cycles of metals (e.g. Fe and Mn), such as *Cupriavidus spp., Georgfuchsia spp., Rhodoferax spp.*, *Ferribacterium spp*. and *Sideroxydans spp*., all detected with the highest relative abundances in PZE, piezometer with the highest concentrations of Fe (6150 µg/l) and Mn (1437 µg/l) (Vandamme, 2004; Tiwari, 2017; Weelink, 2009; Zaa, 2010; Cummings, 1999). During the monitoring, the communities evolved differently, however always maintaining a very selected community for the target contaminants, especially for Fe and Mn (*Acinetobacter, Dechloromonas, Denitratisoma, Ferribacterium, Gallionella, Geobacter, Georgfuchsia, Pseudomonas, Rhodoferax, Sideroxydans, Simplicispira*), but also for chlorinated compounds. In particular, the community is increasingly anaerobic, both for the bacterial groups linked to Fe cycle and for the metabolisms linked to the nitrogen cycle (*Acidovorax, Dechloromonas, Zoogloea, Denitratisoma, Nistrospira, Acinetobacter*). Despite this, conditions did not appear to be strictly anaerobic, given the presence of aerobic or facultative anaerobic bacteria (Figure S6); an evolution is observed with a greater percentage of aerobic metabolisms in PZH compared to the other samples, and the relative abundances of genera involved in the aerobic metabolisms of chlorinated compounds processes increased over time, as well as co-metabolic processes or direct oxidation (*Curvibacter , Nitrospira, Rhodococcus, Acidovorax, Hydrogenophaga, Pseudomonas*). However, at the same time, bacterial genera abundances linked to reductive dechlorination (*Aquabacterium, Dechloromonas, Dehalococcoides, Desulfosporosinus, Geobacter*) slightly increased too. At t6, the bacterial community of PZE sample was dominated by *Acidovorax spp.* (RA 23%), *Malikia spp.* (RA 10%) and *Pseudoxanthomonas spp*. (RA 31%). *Acidovorax* genus is composed of facultative anaerobic bacteria, known degraders of aromatic hydrocarbons (Singleton, 2009; Benedek, 2018); it has been found in environments co-contaminated by TCE and petroleum hydrocarbons (Lien, 2016) and it may be involved in the co-degradation of BTEXs and dichloromethane (DCM) (Yoshikawa, 2017). Bacteria belonging to the genus *Malikia* are aerobic, involved in the degradation of BTEXs as well (Benedek, 2018); they were abundantly found in a community selected for the aerobic degradation of TCE (Fujii, 2020). Lastly, *Pseudoxanthomonas* genus includes, like the previous ones, bacteria that have been studied for their degradation capabilities towards total hydrocarbons and BTEXs (Sheng, 2016; Choi, 2013). Although no degradation ability towards chlorinated hydrocarbons has been detected for this genus, it is interesting to underline that one of the most common co-metabolisms for chlorinated ethylenes is mediated by toluene monooxygenase, and therefore by BTEX-degrading bacteria. PZF sample is mostly represented by the genera *Pseudoxanthomonas* (RA 19%) and *Rugosibacter spp.* (RA 14%) at t6. *Rugosibacter* genus is represented by aerobic bacteria capable of degrading BTEXs, thanks to the presence, among others, of the *tmoA* gene encoding toluene monooxygenase, potentially involved in the co-metabolic processes above mentioned (Somee, 2022). In PZG, significant abundances of the genera *Acidovorax* (RA 6%) and *Sideroxydans* (RA 12%) are observed at t6. *Sideroxydans* genus, as well as *Gallionella* genus, belong to Gallionellaceae family: they both are iron-oxidizing microaerophilic bacteria that act as main players in the iron cycle in groundwater environments (Fabisch, 2013; Bethencourt, 2020). At last, PZH is the piezometer with the highest biodiversity: 40% of bacterial genera, with abundances lower than 1%, and many unclassified bacterial genera (35%) were found in this sample. Moreover, PZH is the piezometer that evolved more over time. These results are confirmed by a Principal component analysis (PCA, Fig. 8), a linear dimensionality reduction technique useful to simplify a large data set and emphasize significant trends (Kurita, 2020; Ali, 2017)

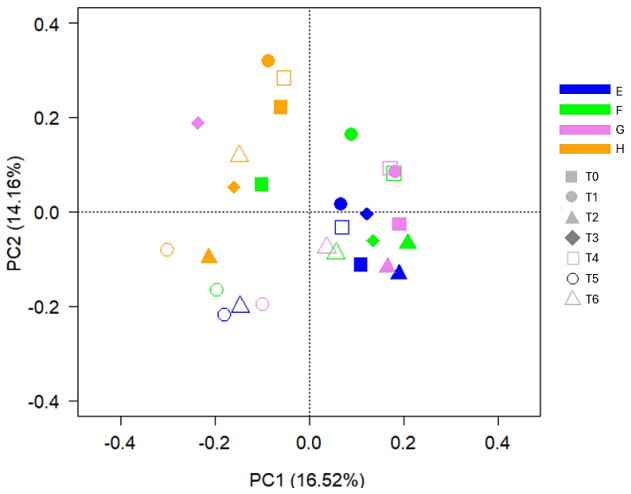

Fig. 8 PCA analyses of NGS results of MNA, from March 2021 (t0) to March 2024 (t6)

4. Conclusions

In this work, a first physico-chemical and biomolecular characterisation of the site was carried out to evaluate the feasibility of applying a biological-based treatment as an eco-friendly, cost effective and sustainable strategy to remediate a contaminated site. In this regard, the assessment of the biodegradative potential of the native microbial community is crucial to identify the best site-specific bio-based approach. Biomolecular qPCR analysis showed a great correspondence between the presence of CAHs and the presence of the marker genes involved in their anaerobic degradation, suggesting a great degradative potential. The same potential was observed also for BTEXs as proved by the quantification of biomarkers for their aerobic and anaerobic degradation. Concerning the contamination, a decrease in CAHs concentration, particularly for PCE, was observed from 2011 to 2019, while a less marked removal of BTEXs and metals was observed. But for the choice of the most suitable bioremediation technique, some aspects should be considered. First, anaerobic and aerobic processes are chemically, physically and biologically opposite, implying the choice of one strategy at the time. On one hand, the stimulation of anoxic conditions could have accelerated the on-going CAHs reductive dechlorination but could have cause a higher mobilization of metals (Fe and Mn). On the other hand, the stimulation of oxic conditions could stimulate aerobic biodegradation of BTEXs and VCM and favour metals precipitation, but inhibits the natural reductive dechlorination of CAHs. Based on these considerations, MNA and Biosparging were chosen for different areas of the site, with different physico-chemical and microbiological characteristics. In particular, MNA was applied, in the central area of the site, for the remediation of the CAHs contamination plume, where the natural anaerobic biodegradative potential resulted higher than in other areas. Anyway, MNA must be supported by data that demonstrate that it is a technology capable of achieving site remediation objectives within a reasonable timeframe: chemical analyses are needed for the assessment of contaminant concentration decreasing, while biomolecular and isotopic ones for the identification of microorganisms and biodegradation pathways involved in the natural attenuation process and for the evaluation of the biodegradation rate. For these reasons, biomolecular analyses of reductive dechlorination marker genes were carried out for the selected piezometers (PZE, PZF, PZG, PZH), while chemical and isotopic analyses were performed on target CAHs. From chemical and isotopic analyses carried out between t0 and t6, it is evident an increase in degradation processes, especially for PZE piezometer, secondarily for PZF and PZG. Despite the fluctuations observed in some monitoring campaigns, concentrations decreased in all piezometers, except in PZH. The isotopic analysis proved that the contaminant decrease can be ascribed to biodegradation processes, mainly reductive dechlorination in PZE, while probably both aerobic and anaerobic in PZG and PZF. Indeed, data from qPCR analyses showed an increase in the anaerobic biodegradative genetic potential during the monitoring, especially in PZE, while NGS sequencing showed a strong influence of contaminants on the microbial community structure, resulting in the presence of a bacterial population related to the removal of chlorinated compounds, both anaerobically (reductive dechlorination processes) and aerobically (direct oxidation or co-metabolism), and of the metals present, even in this case mainly in PZE. However, non-strictly reducing conditions were present at the site, because, apart from diverse anaerobic bacterial, the growth of aerobic or facultative anaerobic bacteria was favoured in some areas of the site over time. The comprehensive evaluation of all these data proved the effectiveness and efficiency of the selected remediation strategy (MNA) and allowed the remediation of the site using an economically and environmentally sustainable biological approach.

Supplementary Information The online version contains supplementary material available at …

Acknowledgments This project was committed by Systema Ambiente SpA and managed by TAUW Italia s.r.l. The authors want to thank all the staff of IT^{2}E laboratories (Milan, Italy) for isotopic analyses and data interpretation, a crucial point for the completion of this research paper. A special thanks to Paolo Pacetti, environmental engineer at the company TREEC s.r.l., for the consultancy during the remediation….. Thanks to Crc Centro Ricerche Chimiche S.r.l. for the chemical analyses.

Author contributions All authors contributed to the project as their role and affiliation concerned. Systema Ambiente e Tauw. The project for the characterisation phase was first proposed and designed by Insubria University, within the master thesis of Luca Colombo, under the supervision of Cristiana Morosini, professor in environmental engineering, and Elisabetta Zanardini, professor in environmental microbiology. Microbiological analyses of the characterisation phase were performed and elaborated at the University of Milano-Bicocca, in the laboratories of Andrea Franzetti, associate professor in environmental microbiology. The Operative Project and the consecutive monitoring were defined by Tauw and M3R s.r.l., respectively with Fabio de Palma, project manager, and Tatiana Stella, CEO of the company. The monitoring phase is included in the PhD project of Francesca Formicola, PhD student in environmental sciences at the University of Milano-Bicocca, with a high apprenticeship contract with M3R, with Andrea Franzetti and Tatiana Stella as academic and external tutors. Sampling, analysis and data collection for the microbiological monitoring were performed in M3R. The first draft of the manuscript was written by Francesca Formicola and revised by all authors. All authors read and approved the final manuscript.

Funding This research did not use any grant provided by funding agencies in the public, commercial, or not-for-profit sectors.

Data availability Data will be made available on reasonable request.

Declarations

Ethical approval Not applicable.

Consent to participate All authors agreed to contribute in this study.

Consent to publish The authors agreed to publish.

Competing interests The authors declare

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