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Polyhydroxyalkanoate as a slow-release carbon source for *in situ* bioremediation of contaminated aquifers: From laboratory investigation to pilot-scale testing in the field

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ABSTRACT

A pilot-scale study aiming to evaluate the potential use of poly-3-hydroxy-butyrate (PHB) as an electron donor source for *in situ* bioremediation of chlorinated hydrocarbons in groundwater was conducted. Compared with commercially available electron donors, PHB offers a restricted fermentation pathway (i.e., through acetic acid and molecular hydrogen) by avoiding the formation of any residual carbon that could potentially spoil groundwater quality. The pilot study was carried out at an industrial site in Italy, heavily contaminated by different chlorinated aliphatic hydrocarbons (CAHs). Prior to field testing, PHB was experimentally verified as a suitable electron donor for biological reductive dechlorination processes at the investigated site by microcosm studies carried out on site aquifer material and measuring the quantitative transformation of detected CAHs to ethene. Owing to the complex geological characteristics of the aquifer, the use of a groundwater circulation well (GCW) was identified as a potential strategy to enable effective delivery and distribution of electron donors in less permeable layers and to mobilise contaminants. A 3-screened, 30-m-deep GCW coupled with an external treatment unit was installed at the site. The effect of PHB fermentation products on the *in situ* reductive dechlorination processes were evaluated by quantitative real-time polymerase chain reaction (qPCR). The results from the first 4 months of operation clearly demonstrated that the PHB fermentation products were effectively delivered to the aquifer and positively influenced the biological dechlorination activity. Indeed, an increased abundance of *Dehalococcoides mccartyi* (up to 6.6 fold) and reduced CAH concentrations at the installed monitoring wells were observed.

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Introduction

Poly-3-hydroxy-butyrate (PHB) is a stereoregular polyester belonging to the family of polyhydroxyalkanoates, intracellular energy-storage materials used by a wide variety of bacterial strains [1,2]. PHB has the particular feature of being a biodegradable solid which is made by a single monomer (i.e., 3-hydroxybutyric acid, HB) whose fermentation gives a completely defined and restricted spectrum of end-products. Indeed, PHB is enzymatically hydrolysed to HB and then converted to butyric acid via hydrogenation. Butyric acid is further converted into acetate and H_2 through beta-oxidation and acetate can eventually be further fermented into H_2 and carbon dioxide [3]. Previous laboratory-scale studies [4] have clearly demonstrated that PHB fermentation products can be used as electron donors by specialised anaerobic bacteria that are able to reduce chlorinated solvents into less toxic or harmless forms. To date, only members of *Dehalococcoides mccartyi* (*Dhc*) are known to completely degrade chlorinated compounds to harmless ethene through the activity of key enzymes coded by reductive dehalogenase genes [5,6]. Among these, the *tceA* (involved in the degradation of tetrachloroethene or trichloroethene to vinyl chloride and ethene), *bvcA* and *vcrA* (involved in the degradation of *cis*-dichloroethene or vinyl chloride to ethene) reductive dehalogenase genes are biomarkers for specific *Dhc* strains [7].

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Several different electron donors have been reported, and a variety of products are commercially available in the remediation market for application. Compared with PHB, most of these products are not fermented through strict and defined pathways and thus can potentially release undesirable substances other than effective electron donors. Furthermore, PHB is a solid and the fermentation rate can be easily controlled by its shape and size (e.g powder, granules or pellets) and by the operating conditions (i.e. temperature, residence time). As an example, mixing PHB powder and PHB pellets makes it possible to obtain both quick start up and long-term stable supply of fermentation products.

At present, PHB is produced mainly for biomedical applications and food packaging via biotechnological processes from pure cultures and single substrates, at a cost of approximately 10 €/kg. This is comparable with the costs of the commercial products already available that are used for *in situ* bioremediation by reductive dechlorination. Moreover, extensive research has been carried out to minimise PHB production costs by using mixed cultures and waste streams as substrate, thus making the polymer a reasonable alternative to available products.

In this study, the use of PHB for the remediation of a site contaminated by chlorinated aliphatic hydrocarbons (CAHs) was evaluated in the field using a long-term large pilot test, the first of this type at the international level. A preliminary site evaluation suggested that an active dechlorinating microbial community was established, the lack of electron donor being the limiting factor for the complete conversion to non-chlorinated final compounds (i.e. ethene and ethane). Moreover, the results from a site specific laboratory-scale microcosm study, as reported in the Results section, clearly demonstrated the effectiveness of PHB as a suitable electron donor source for enhancing biological reductive processes up to non-toxic ethene.

When *in situ* biological reductive dechlorination (RD) is considered as a remediation strategy, a homogeneous electron donor distribution is typically one of the most relevant requirements for success. Conventional injection approaches (either continuous or pulsed) were not suitable at the investigated site owing to complex aquifer geological characteristics. Injected fluids would preferentially migrate through easily permeable zones and thereby prevent fluids from reaching less permeable layers where significant masses of contaminants could have potentially accumulated, especially in aged contamination sites. The use of groundwater circulation well (GCW) technology could advantageously improve the distribution of soluble electron donors by creating an effective three-dimensional circulation cell in the aquifer. This three-dimensional water flow is established by installing a multiple screened well, where a packer is inserted to hydraulically isolate the screen intervals. Groundwater is extracted from one screen and, after a generic treatment, is circulated back into the aquifer through another screen thereby, creating the circulation cell. The pressure gradient between the hydraulically separated screen sections induces a circulation flow in the aquifer, forcing water through less permeable layers not usually affected by conventional pumping and injection systems [8,9].

Based on the specific hydrogeological characteristics of the particular site studied, where most of the CAH are retained in low permeable lens (aged contamination), a GCW based pilot-scale plant was designed and realised in order to evaluate the possibility of delivering PHB fermentation products directly through the low k zones (*in situ* enhanced reductive dechlorination) and at the same time increasing CAH mobilisation (enhancement of mass flux from the source area). During plant operation, chlorinated solvents concentration, dissolved organic carbon, abundance of *Dhc* and the identification of *Dhc* strains [10] were monitored to evaluate the effectiveness of the treatment.

Materials and methods

Site characterization

The site under investigation was characterised by groundwater contaminated by CAHs at levels significantly above the limits imposed by Italian legislation (a few micrograms per litre) due to unregulated industrial degreasing operations. A routine analysis of the groundwater indicated that the area was significantly impacted by *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC), probably as a result of partial biological reductive dechlorination processes of high-chlorinated solvents (i.e. trichloroethene) used in the industrial degreasing activities. In this regard, higher chlorinated compounds probably used as solvent at the site (tethrachloroethene, trichloroethene and tetrachloroethane) were detected only at very low concentrations. In addition, geological surveys of the site demonstrated a complex hydrogeological situation (i.e. fine to middle sands intercalated with layers of less permeable sandy silts to clayey silts with 10^{-7}–10^{-4} m/s permeability values, as shown in Fig. 1, with residual CAHs mostly associated with the low-permeability layers. Clayey silts act as a persistent slow-releasing secondary contamination source kinetically controlled by slow

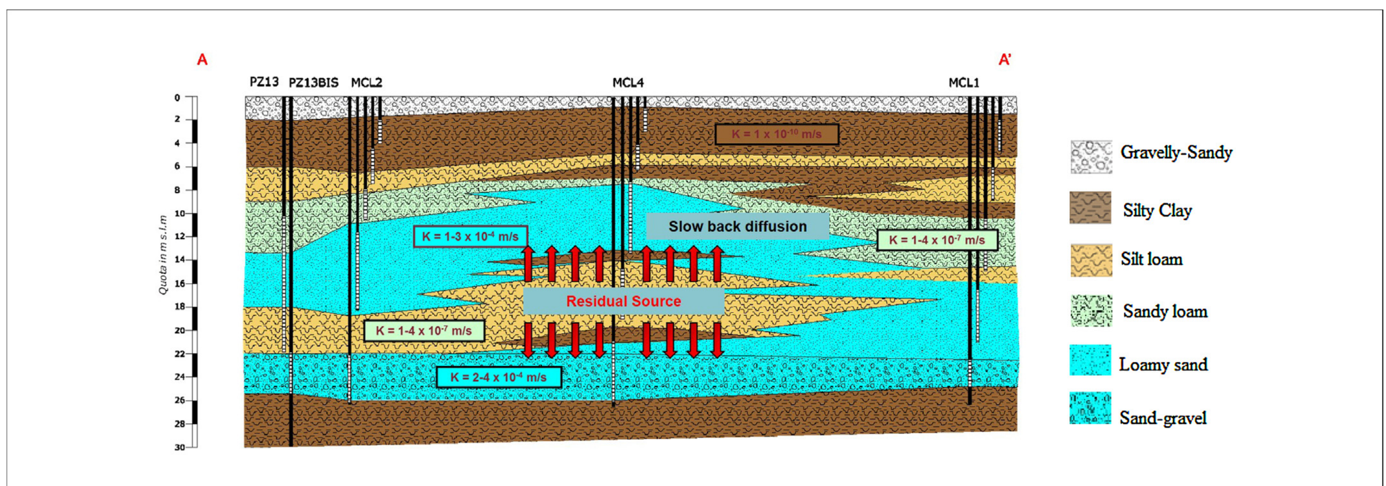

Fig. 1. Typical lithological cross-section of site illustrating the locations of monitoring wells (MCL and PZ) across the pilot test area.

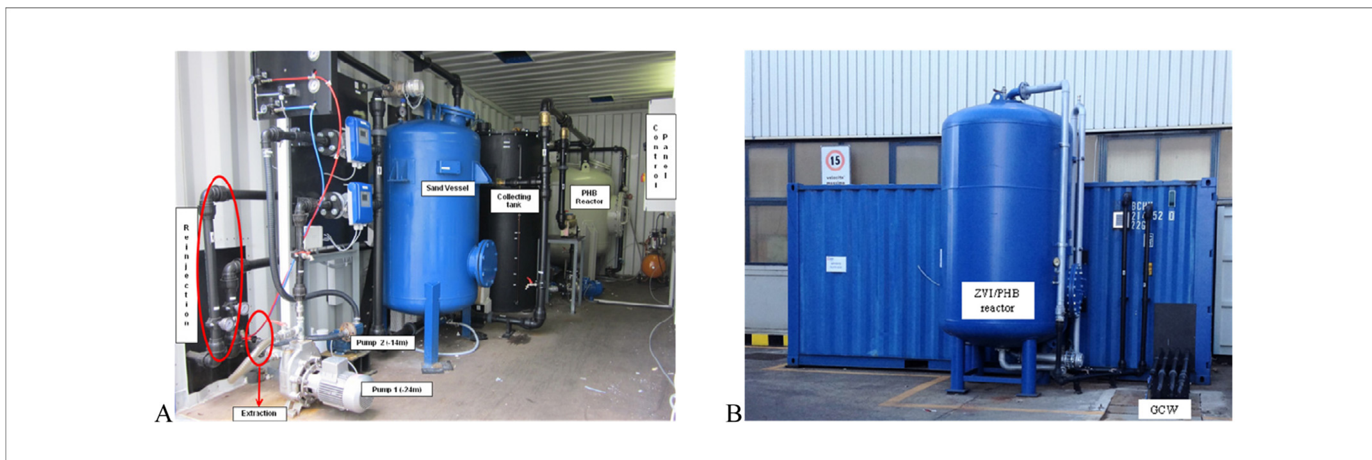

Fig. 2. External treatment unit: [A] sand tank and PHB-reactor inside the container, [B] ZVI/PHB-reactor outside the container.

back-diffusion mechanisms. The concentrations of dissolved CAHs (mainly *cis*-DCE e VC) were as high as 100 mg/L despite the use of an intensive pump-and-treat system. This system, which was activated to prevent contaminants from spreading out of the site, already removed nearly 7 tons of chlorinated compounds from the extracted groundwater.

Laboratory scale treatability: microcosm studies

Biological reductive dechlorination was recognised as an active mechanism after extensive preliminary microbiological characterisation of the site based on the presence of *cis*-DCE and VC (data not shown). The accumulation of *cis*-DCE and VC is often related to

Fig. 3. Piping and instrumentation diagram (P&ID) of the pilot plant (GCW and external treatment unit). The red lines depict the process in which the extracted groundwater is treated through the PHB-reactor and ZVI/PHB-reactor in series.

limited electron donor availability [11]. Consequently, laboratory microcosm experiments were set up to assess the potential for enhancing the biodegradative capabilities of autochthonous dechlorinating microorganisms to the point of complete dechlorination with the addition of electron donors. Furthermore, these experiments were used to identify potential interactions between the dechlorinating microbial population with other microbial populations that compete for the supplied electron donors [12–15].

Soil core and groundwater samples for the microcosm studies were collected during drilling operations at two representative contamination zones in order to represent the different contaminant distribution and geological characteristics of the site. One was located at the outer boundary of the site (lower CAHs concentration); the other was below the industrial warehouse where the degreasing machine was operated (highest CAHs concentration). Groundwater samples were collected in amber glass bottles completely filled to leave almost no headspace. Soil samples were collected from two different horizons (15–16 and 19–20 m below ground surface) representative of layers, holding the aquifer, with the highest transmissivity (medium sand and sandy silt, respectively). Samples were placed in glass jars and completely filled with groundwater in order to avoid headspace. All samples were immediately transferred to the laboratory and stored at 4 °C until use.

Microcosm reactors were prepared in 240-mL autoclaved serum bottles in an anaerobic glove box. The serum bottles were first filled with soil (30 g) and an electron donor source (PHB powder and lactate at concentrations of 180 and 270 mg/L, respectively, corresponding to 300 mg-COD), then sealed with Teflon-faced butyl rubber stoppers and aluminium crimp caps. The amount of electron donor source was calculated on the basis of sulphate concentration, being the major electron acceptor in the system. It was calculated as the stoichiometric need for complete sulphate reduction and doubling this value to establish an excess sufficient to quantitatively reduce the lower CAH concentrations. Next, 4 mL of anaerobic mineral medium (containing NH_4Cl 500 mg/L, $MgCl_2 \cdot 6H_2O$ 100 mg/L, $CaCl_2 \cdot 6H_2O$ 50 mg/L, and K_2HPO_4 400 mg/L) and 180 mL site groundwater were added, leaving a headspace volume of 50 mL. Reactors were prepared in duplicate to ensure reproducibility and transferred to a Dubnoff water bath (BSD/D, ISCO, Italy) for incubation in the dark at 25 °C to simulate real field conditions. Additionally, an intrinsic control microcosm with no exogenous electron donor was established to investigate the role of the electron donor and to verify the presence of natural attenuation mechanisms. Every 10–12 days, the concentrations of chloroethene, ethene and methane were quantified by sampling 100 μL of serum bottle headspace (with a gas-tight Hamilton syringe) into a Varian3400 gas chromatograph (glass column packed with 60/80 mesh Carbopack B/1% SP-1000, He carrier gas, oven temperature from 50 °C to 210 °C and 300 °C flame ionization detector temperature). The corresponding headspace CAHs and ethane concentrations were converted to nominal concentrations (i.e., total mass divided by the liquid volume) using tabulated Henry's Law constants [16].

Pilot (Field) test

The pilot test was located close to the old degreasing activities and consisted of a 30-m GCW equipped with an external treatment unit. The GCW was installed using a 0.60-m-diameter hollow-stem auger. Next, a well casing with an inside diameter of 0.39 m and containing three screened intervals was placed in the augured hole. The upper screen extended from 8 to 12 m bgs (below the ground surface), and the intermediate and lower screens extended, respectively, from 15 to 19 m bgs and from 22 to 26 m bgs. To hydraulically isolate the screen intervals, packers were placed at depths between from the ground surface to 8 m bgs, from 12 to 15 m bgs and from 19 to 22 m bgs. The GCW circulation system was operated using two centrifugal pumps installed on the surface, to draw out groundwater from the lower and intermediate screened sections and to circulate extracted groundwater to an in-ground treatment system at the wellhead (the external treatment unit). The external treatment unit was composed of a sand tank (for suspended solid filtration), a PHB reactor (electron donor supply) consisting of a 2-m^3 tank filled with a mixture of sand and PHB powder (approximately 300 kg) purchased from Biomer

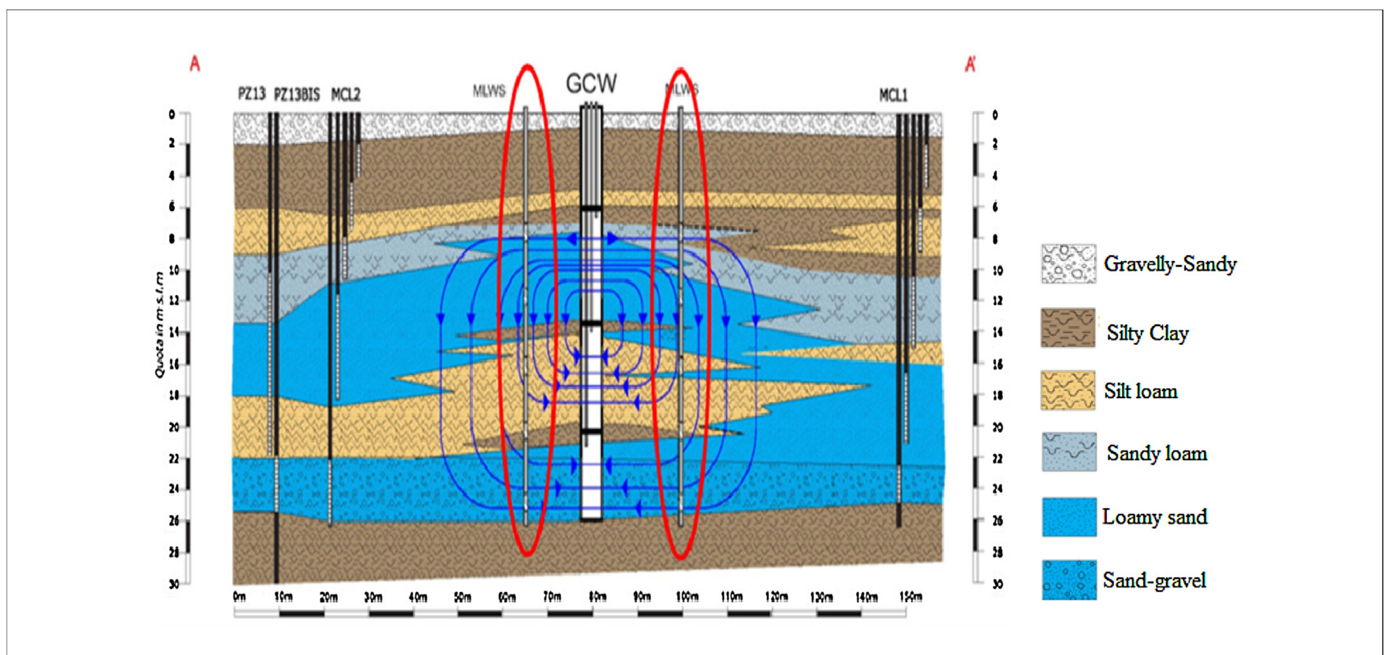

Fig. 4. Schematic illustration showing modelled hydraulic scheme of flow groundwater pattern (blue lines) around the operating GCW and locations of monitor wells (red ovals).

(Germany) (Fig. 2A), and a zero-valent iron (ZVI)/PHB reactor (for chlorinated solvents removal, [17,18]) (Fig. 2B). The system was designed to re-infiltrate groundwater enriched with PHB fermentation products into the aquifer in order to stimulate the autochthonous dechlorinating microorganisms in the low permeability zones and at the same time improve CAH mobilisation. The (ZVI)/PHB reactor allowed the continuous removal of extracted CAH as a part of source zone remediation; moreover, it was

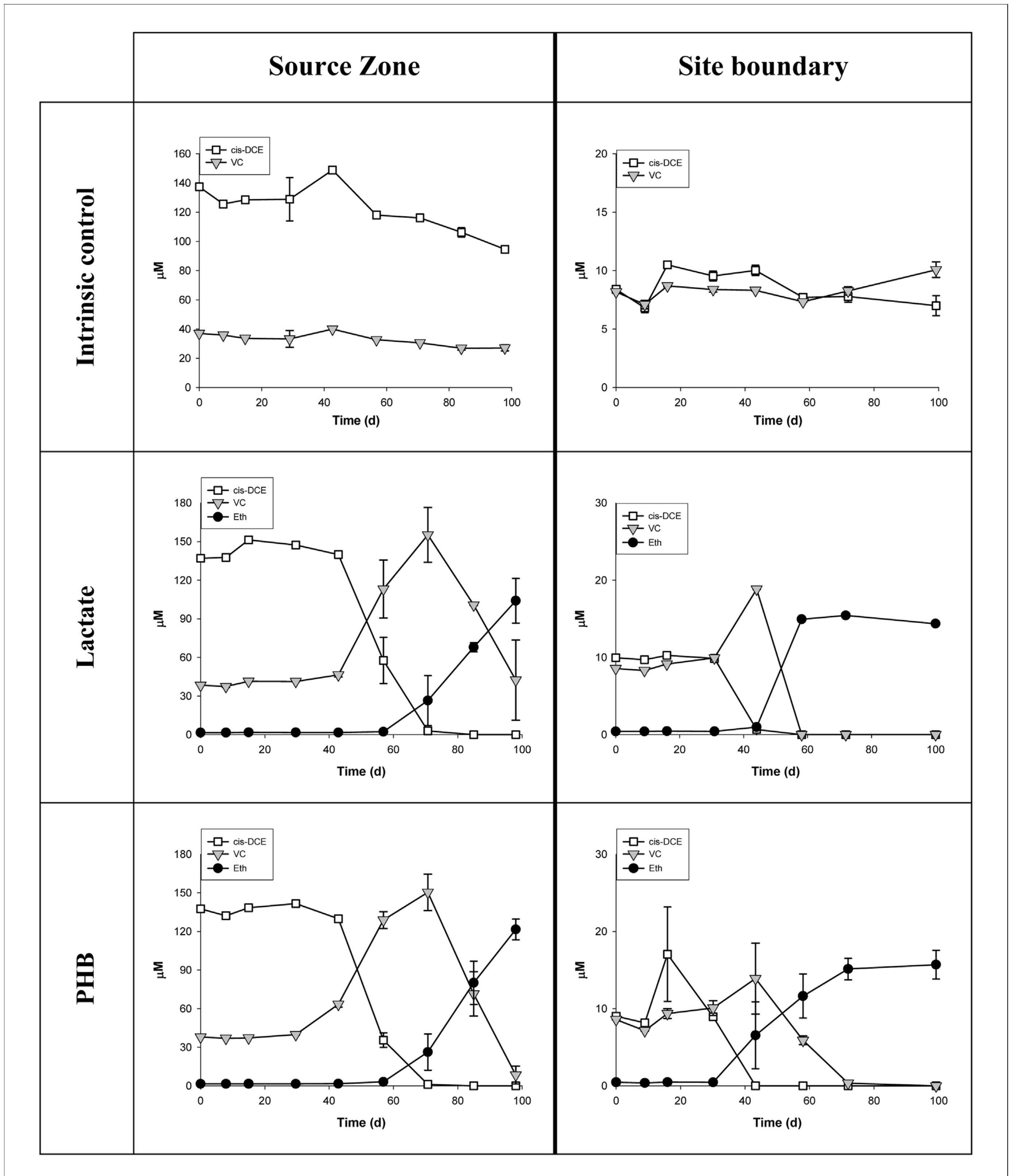

Fig. 5. Reductive dehalogenation of chlorinated solvents in microcosms carried out on samples from two selected areas without (intrinsic control) and with external electron donors (lactate and PHB). Error bars indicate standard deviations of the duplicate microcosms.

requested by local administration in order to allow re-infiltration of the extracted groundwater (not allowed in the presence of high CAH concentration). As illustrated in the piping and instrumentation diagram (PI&D) of the test plant (Fig. 3), the treatment groundwater was discharged into the aquifer by the upper screened section, creating two circulation water cells. One well was between the lower and upper screened sections; the other was between the intermediate and upper screened sections (Fig. 4). Taking into account the modelled groundwater flow around the operating GCW, two multilevel-well systems (MLWSs) were designed and installed inside the radius of influence of the GCW for monitoring and control at different depths. The MLWS consisted of a single casing in which packers were inserted to separate the screen areas and to create small sampling chambers. The MLWS groundwater samples were extracted via a mechanism consisting of a piston drive, operating within the cylinders and pumped to the surface through flexible hoses. In detail, the installed MLWSs were designed with five screened sections at different depths (i.e., −7.6, −11.6, −16.6, −20.6 and 24.6 m bgs), according to the geological conditions.

Chemical analysis

During the pilot test, groundwater samples were collected in a dynamic regime from all depths of the MLWSs. To monitor the effluent and influent of each reactor of the external treatment unit, aqueous samples were collected directly from gate valves positioned along piping (named PNS in P&ID, Fig. 3). Chlorinated solvent analyses were performed in accordance with EPA Method 5020C 2003 + EPA Method 8260C 2006 using a Purge and Trap Atomx instrument (GC–MS 5975 Agilent and GC–MS ISQ Thermo). Volatile fatty acids (VFAs, i.e., acetic, propionic and butyric acids) were measured using a Dani Master chromatograph equipped with a FID (flame ionization detector) and a glass column packed with 60/80 mesh Carbopack B/1% SP-1000. Additionally, total organic carbon (TOC) was analysed on a TOC-CSV analyser (Shimadzu®). The groundwater samples for VFA and TOC analyses were filtered through a 0.45-μm pore membrane.

Biomolecular analysis (real-time PCR)

In the pilot-scale study, groundwater samples collected from the MLWS monitoring wells were used for qPCR analyses to evaluate the occurrence of the main reductive dehalogenase genes (*tceA*, *bvcA* and *vcrA)* and abundances of *Dhc* (16S rRNA) responsible for the reductive dechlorination of TCE, *cis*-DCE and VC before and after the bioremediation process. On day 15 and on day 120 after the onset of the pilot test, 1 L of groundwater collected at the different depths of MLWS1 and MLWS2 was immediately stored in sterile bottles, kept at 4 °C and processed within 24 h. Groundwater from each sampling point was filtered through a 0.2-μm surfactant-free cellulose filter (Ø 47 mm, Millipore). Each filter was rolled with sterile tweezers and placed carefully into the bead beating tube provided by PowerSoil DNA Isolation kit (MoBio, Italy). The kit was used for DNA extraction

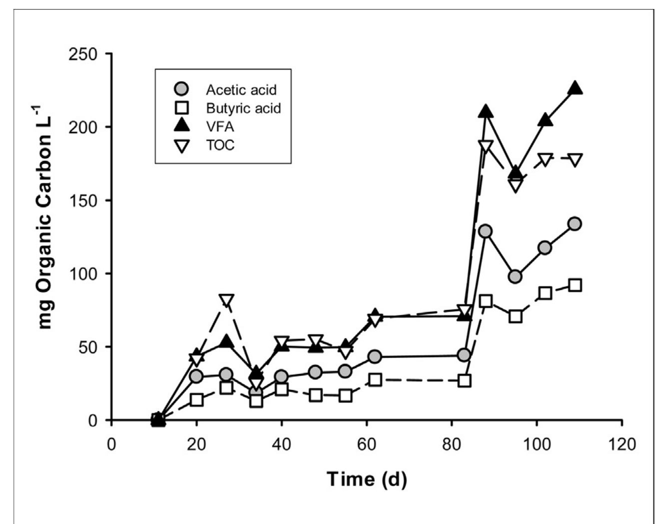

Fig. 7. Dissolved organic carbon concentration in groundwater samples collected from the PHB reactor effluent during operational period.

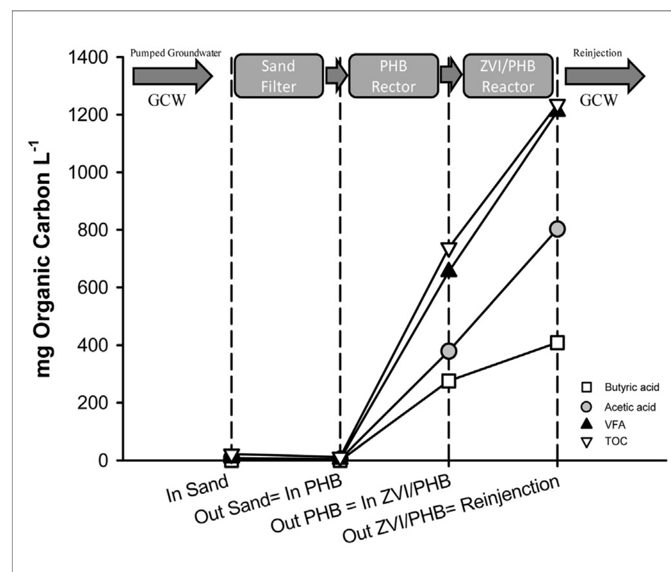

Fig. 6. Dissolved organic carbon concentration profile trough the external treatment unit; the extracted groundwater is filtered (sand filter) and passed in series through the PHB-reactor and the ZVI/PHB reactor.

according to the manufacturer's instructions with a slight modification: in the first step of the procedure, the filter with the harvested biomass was used instead of the soil sample. Extracted DNA was stored at −20 °C. The DNA extracted from groundwater samples was diluted 100 times and used as a template for qPCR targeting 16S rRNA, *tceA*, *vcrA* and *bvcA Dhc* genes. Reactions were performed in 20 μL total volume including 3 μL of the DNA template, 300 nM of forward primer and reverse primer and 300 nM of a TaqMan® probe comprising a 6-carboxyfluoresceine (FAM) 5′ end reporter fluorophore and an *N*,*N*',*N*'-tetramethyl-6-carboxyrhodamine (TAMRA) as the 3′ end quencher. The primers and probes were previously used by Ritalahti and colleagues [19]. Each qPCR reaction was performed in triplicate with a CFX96 Touch™ Real-Time PCR Detection System (Biorad, Italy). Standard curves for absolute quantification were constructed by using the long amplicons method previously reported in Matturro et al. [20]. Data were analysed using CFX Manager™ Software 3.0 (Biorad, Italy). Each data point was expressed as the number of gene copies per L of groundwater (gene copies L^{-1}). Averages and standard deviations were calculated for the three reactions for each sample.

Results and discussion

Biological dechlorination in microcosm reactors

The ability of PHB to enhance the biological reductive dechlorination of chlorinated solvents was evaluated, comparing CAH removal in PHB-amended reactors with that observed in anaerobic reactors without external electron donors and lactate-amended reactors. Lactate was used as a reference carbon and electron donor because it is widely recognised to promote reductive dechlorination processes [21,22]. The reactors were monitored over a period of 80–100 days, and the average CAH concentrations of the replicates were plotted as a function of time (Fig. 5). Reductive dechlorination was stimulated until the formation of ethene, via transient VC formation, in the lactate-amended microcosm, indicating that the process was limited by

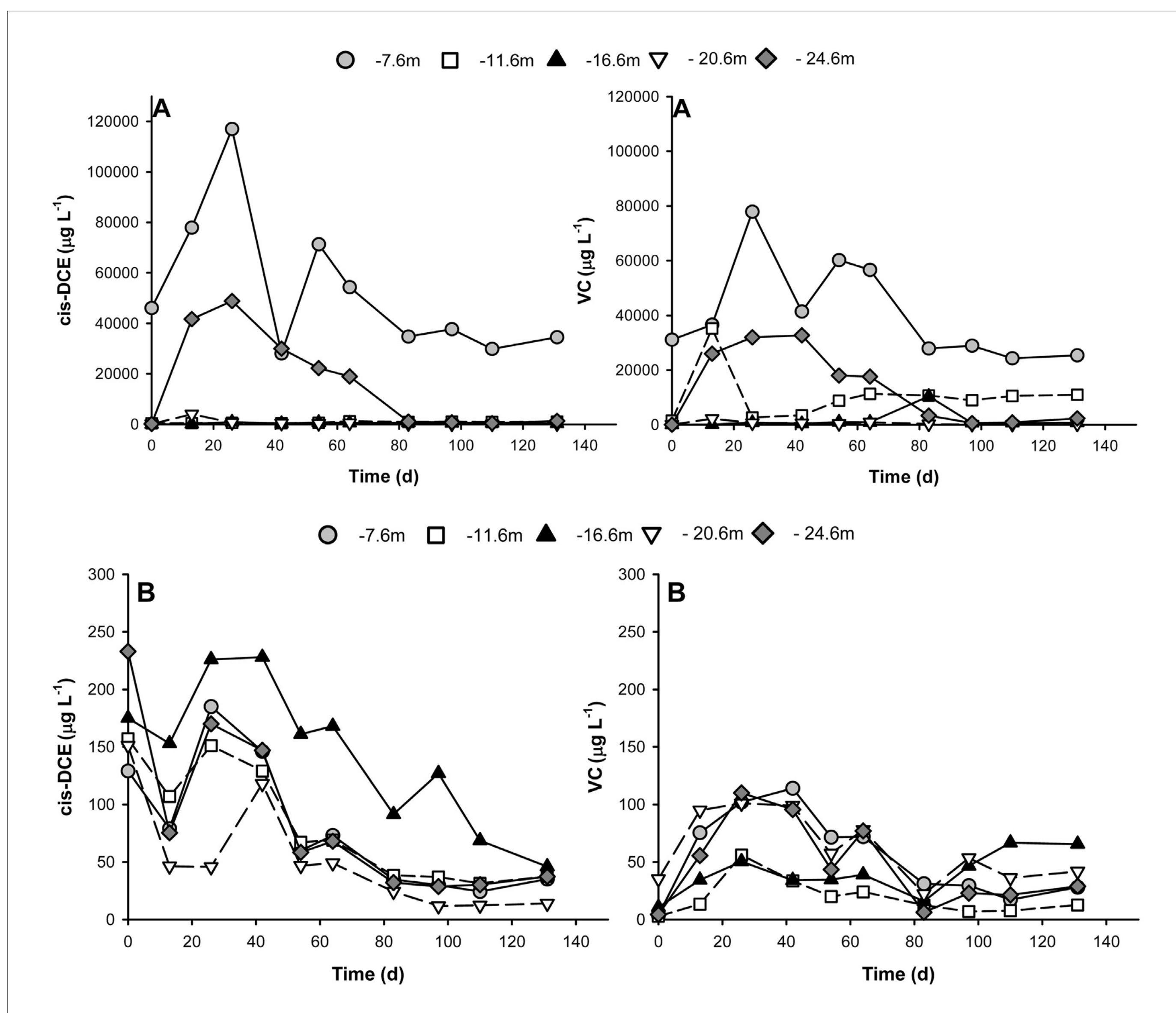

Fig. 8. Molar concentration of *cis*-DCE and VC in the groundwater samples collected at all depths of MLWS1 (A) and MLWS2 (B) over time.

the lack of electron donors. Similar results were obtained in the PHB amended microcosms, thus confirming PHB as an effective source of electron donors. A negligible variation in the *cis*-DCE and VC concentrations in the non-amended control demonstrated that the natural attenuation process mediated by indigenous dechlorinating bacteria appeared ineffective without the addition of electron donors. Additionally, the results of the microcosm study indicated how an approach based on the delivery of electron donors in an area characterised by very high concentrations of chlorinated solvents (source zone) could be successful in quantitatively degrading contaminants to harmless compounds.

Field test results

A primary goal of this pilot test was to assess the possibility of enriching the contaminated groundwater with PHB fermentation products (electron donors and carbon sources) and achieving the *in situ* biostimulation of dechlorinating microorganisms, especially in the less permeable layers where residual masses of CAHs act as persistent sources of contamination The installed plant pumped the extracted groundwater through the external treatment unit, which includes the reactor filled with the PHB powder and sand (which produces dissolved electron donors and carbon sources) and the reactor containing the ZVI/PHB powder (which removes mobilised CAHs). A typical TOC and VFA profile along the external treatment unit is depicted in Fig. 6 in terms of the dissolved organic carbon concentration. The increases in the TOC and VFA concentration at the outlets of the PHB and ZVI/PHB reactors clearly indicated an effective fermentation activity. The presence of PHB fermentation products (i.e., acetate and butyrate acids) was due to the microbial fermentation of PHB, which started spontaneously upon self-colonisation of the reactors by ubiquitous microorganisms. The acetate and butyric acid (VFA) content and the total organic carbon content were similar; this indicated that the use of PHB carried no potential risk in deteriorating aquifer quality by releasing unknown amounts of dissolved organic carbon. It is noteworthy that the groundwater flux rate through the reactors can be easily adjusted based on the progress of the PHB fermentation. Consequently, the distribution of electron donors in the subsurface can be controlled during the operation. Fig. 7 shows the dissolved organic carbon concentration in the PHB reactor effluent under different operating conditions (i.e., different flow rates). The dissolved organic carbon concentration varied from 50 mg L^{-1} to 200 mg L^{-1} depending on the hydraulic residence time of the groundwater in the PHB reactor. Because PHB is insoluble but easily biodegradable in water, groundwater recirculation through the PHB reactor allowed continuous delivery of electron donors in the contaminated aquifer. After 130 days, a significant and constant concentration of dissolved PHB electron donors was still observed.

The stimulation of the *in situ* biological reductive dechlorination by the distribution of the electron donors in the contaminated aquifer could be assessed by the *cis*-DCE and VC concentration trend in the samples collected from the MLWSs (Fig. 8). Due to the significant heterogeneity in the contaminant distribution and hydrogeological settings, different behaviours were observed for the two MLWSs. MLWS1 exhibited the highest CAH concentrations with a clear initial significant increases in *cis*-DCE and VC, probably due to the recirculation inducing mobilisation. A subsequent reduction in these concentrations could be ascribed to the stimulation of the *in situ* reductive dechlorination. MLWS2 is characterised by a significantly lower concentration and the effect of the recirculation appears less evident. On the other hand, the reduction of *cis*-DCE corresponds to the increase in the VC concentration, thus supporting the effectiveness of stimulation of the biological reductive process.

This hypothesis was confirmed by monitoring the presence and dynamics of the *Dhc* strains after two weeks and after four months of plant operation. In particular, the *Dhc* 16S rRNA gene and the strain-specific reductive dehalogenase genes, *tceA*, *bvcA* and *vcrA*, were quantified in samples collected at different depths for MLWS1 (Fig. 9a) and for MLWS2 (Fig. 9b).

Dhc was found in MLWS1 and MLWS2 at all analysed depths, and among the reductive dehalogenase genes screened only *tceA* and *vcrA* were found, while no *bvcA* gene was detected. The abundances of the *Dhc* 16S rRNA gene were similar in MLWS1 and MLWS2. In the samples collected from MLWS1, the abundance of the *Dhc* 16S rRNA gene ranged from 1.16E + 07 to 3.64E + 07 gene

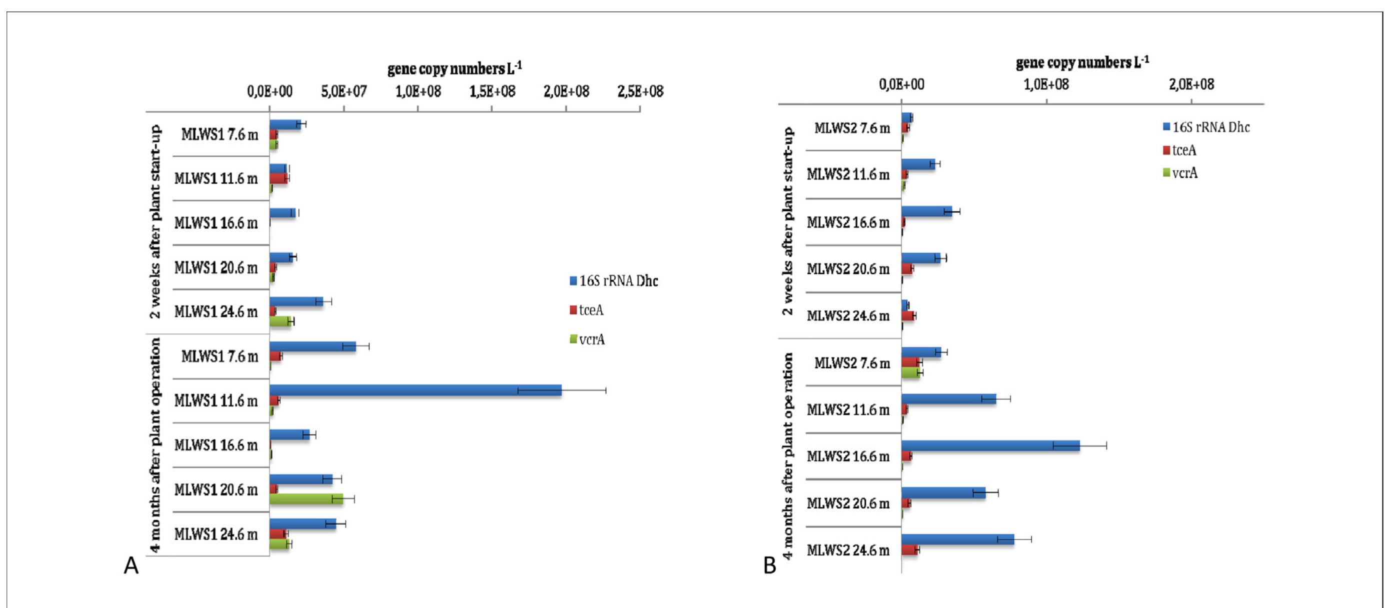

Fig. 9. Abundances of *Dhc* 16S rRNA and reductive dehalogenase genes *tceA* and *vcrA* estimated by qPCR in the groundwater samples collected at all depths of MLWS1 (A) and MLWS2 (B). Data are reported as gene copy numbers per L of groundwater.

copies L^{-1} (Fig. 9a). Similarly, in the samples collected from MLWS2, the *Dhc* 16S rRNA gene ranged from 4.46E + 06 to 3.50E + 07 gene copies L^{-1} (Fig. 9b).

The *Dhc* strains carrying the *tceA* gene were the most abundant, ranging from 3.08E + 05 to 1.17E + 07 gene copies L^{-1} in MLWS1 and 2.08E + 06-8.70E + 06 gene copies L^{-1} in MLWS2. However, the *Dhc* strains carrying *vcrA* were also present, and their abundance was higher in the MLWS1 samples (1.68E + 06–1.43E + 07 gene copies L^{-1}) than in the MLWS2 samples (5.30E + 05–2.06E + 06 gene copies L^{-1}).

Following the decreases in *cis*-DCE and VC concentrations, the *Dhc* 16S rRNA gene copies increased after four months of operation in the MLWS1 and MLWS2 wells (Fig. 9). Indeed, after four months of plant operation, 16S rRNA gene copies quantified at different depths were 3.7–4.6 and 3.4–6.6 fold higher than those estimated during the first sampling in MLWS1 and MLWS2 wells, respectively. Among these, the *Dhc* strains carrying *tceA* and *vcrA* were dominant.

Conclusion

In this study, the effectiveness of PHB in enhancing *in situ* biological reductive dechlorination (RD) was evaluated at a site heavily contaminated by chlorinated solvents. The results from a laboratory treatability study indicated that RD could be potentially enhanced by PHB amendments as a carbon and an electron donor source for the *in situ* removal of chlorinated solvents from groundwater. The possibility for scaling up the process was evaluated using a pilot test, the first of this type at the international level, located in one of the most contaminated areas of the investigated site. Our findings clearly showed that PHB fermentation positively influenced *Dhc* growth as demonstrated by the increase of the *Dhc* 16S rRNA gene copy numbers during the treatment. Additionally, the presence of biomarkers and the occurrence of reductive dehalogenase genes (i.e., *tceA* and *vcrA*) were in line with the kinetic performance of the reductive dechlorination process, as indicated by the monitoring results at the two MLWSs.

The coupling of the biological reductive processes with groundwater recirculation by GCW, which allows a vertical circulation flow field into disconnected pore volumes where most of the contamination occurred in aged source zones, could potentially enhance the mobilisation of pollutants and thereby reduce the remediation time.

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