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Evaluation of a bioelectrochemical reductive/oxidative sequential process for chlorinated aliphatic hydrocarbons (CAHs) removal from a real contaminated groundwater

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ABSTRACT

In the present study, the sequential reductive/oxidative bioelectrochemical process has been tested with real groundwater from a contaminated site in Northern Italy for chlorinated aliphatic hydrocarbons (CAHs) removal. The sequential system was developed by connecting in series two membrane-less microbial electrolysis cells (MECs) equipped with an internal graphite counter electrode. The first MEC aimed at the CAHs reductive dechlorination (RD) and was constituted of a granular graphite working electrode. In the second MEC, a mixed metal oxide working electrode stimulated the oxidative dechlorination of the low chlorinated RD's by-products through oxygen production. The sequential process allowed complete mineralization of the CAHs contained in the real groundwater. A complete reduction of the perchloroethylene into vinyl chloride (VC) was observed in the first MEC polarized at −450 mV vs SHE, while the resulting VC was oxidized with a 92 ± 2 % efficiency in the second MEC due to the HRT increment from 0.7 to 1.7 days. Biomarkers of the reductive (*Dehalococcoides mccartyi* 16S rRNA and reductive dehalogenase genes) and oxidative (*etnE*, *etnC* genes) dechlorination have been monitored in the two MECs along with the ecotoxicity tests. Overall, they provide information on the efficiency of the applied technology and allow to assess the potential adverse effects. According to the *Tetrahymena pyriformis* reproduction inhibition test and *Panagrellus* redivivus mortality tests, showed a significant ecotoxicity reduction with respect its initial inhibitory effect at the tested concentrations.

1. Introduction

Chlorinated aliphatic hydrocarbons (CAHs) are widely diffused groundwater contaminants, and due to their physio-chemical properties they accumulate in the lower part of the aquifer causing the contamination of huge amounts of groundwater [1,2]. As CAHs are toxic and carcinogenic compounds, their removal from the natural environments is required for human and environmental safety. Due to their extremely low solubility, conventional remediation approaches based on chemical-physical techniques usually result in cost intensive interventions that require important capital and maintenance costs. In this context, the utilization of bioremediation strategies not only allows to reduce the remediation costs but results highly efficient when the treatment is addressed to the removal of a residual concentration of contaminants after the primary source of contamination removal. The stimulation of the remediation capacity of indigenous microorganisms, also named enhanced in situ biostimulation, consists in the stimulation of the indigenous microbial communities by supplying specific nutrients/conditions for their growth [3].

In particular, specialized microorganisms (i.e. organohalide respiring bacteria, OHRB) are known to perform the anaerobic reductive dechlorination (RD) process, showed in Fig. 1, by respiring the CAHs in

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the presence of electron donors (hydrogen [4,5] or fermentable organic compounds [6–9]). Among the OHRB, only *Dehalococcoides mccartyi* is capable to complete the reduction of chlorinated compounds (i.e. perchloroethylene, PCE; trichloroethylene, TCE; 1,2 cis-dichloroethylene, cisDCE; vinyl chloride, VC) to the harmless ethene through a step-by-step reduction led by specialized reductive dehalogenases (i.e. *TceA*, *BvcA*, *VcrA*) [10–14]. Therefore *D. mccartyi* and the genes encoding of the reductive dehalogenases are robust biomarkers for monitoring the RD process, both at laboratory and field scale.

Usually, the RD is stimulated by the injection of fermentable organic matter directly in the aquifer which promote an in situ hydrogen release, however, due to the decreasing of chlorine atoms in the carbon backbone, RD reaction results incomplete leading to cis-DCE and VC accumulation [15,16]. Low chlorinated RD by-products (i.e. *cis*-DCE and VC) are more easily oxidized via biological aerobic pathways as refining steps which can be stimulated providing oxygen or stimulating the in situ oxygen production (Fig. 1) [17–19]. Several microorganisms are known to perform metabolic [20,21] and co-metabolic VC oxidative dechlorination [22] with dioxygenases and monooxygenases that initiate the biodegradation by converting aliphatic substrates into epoxides. Among them, the functional genes involved in the VC oxidation are *etnC*, which encodes the alpha subunit of alkene monooxygenase (AkMO) and *etnE*, which encodes the epoxyalkane coenzymeM transferase (EaCoMT) subunit leading to the complete mineralization [23].

Microbial electrochemical technologies (METs) are innovative processes which involved the use of electroactive microorganisms for environmental purposes [24–27]. Indeed, METs gained the interest in several environmental applications in which an electron acceptor (i.e. aerobic and anoxic respiration) or an electron donor are required [28,29]. METs based on the microbial electrolysis cells (MECs) requires the utilization of an electric potential to overcome the thermodynamic and kinetic limitations of non-spontaneous reactions and are able to stimulate oxidation and reduction reactions [30,31]. MECs have been successfully adopted in the last years for the stimulation of the reductive and the oxidative dechlorination of CAHs through the polarization of a biocathode or a bioanode [32–34]. More in details, a sequential reductive and oxidative environment has been obtained by the utilization of different MEC configurations including the utilization of an ion exchange membrane as ionic separator [35] or, the adoption of membrane-less bioelectrochemical reactors [36]. Recently, as reported in previous paper [19], our research group adopted a new membrane-less MEC configuration consisting in a tubular reactor provided with an internal graphite counterelectrode. The membrane-less MEC concept has been tested opening a new perspective for bio-electro remediation allowing a simple and cheap design of the reactors, particularly advantageous for the scale up of the technology [37]. Previous studies conducted on the membrane-less reductive reactor showed the crucial role of nitrate and sulphate load rate on current production [38,39], which was directly correlated with the increase of the process energy consumption [40,41]. In this study, the validation of the sequential reductive/oxidative process with real contaminated groundwater is presented using chemical, genomics and ecotoxicological tools.

Ecotoxicological test batteries are regularly used for the monitoring of the efficiency of (bio)remediation technology applications for groundwater, soil and waste treatment [42,43]. However, in the case of microbial electrochemical remediation applied to CAHs, such ecotoxicological test batteries have been scarcely used besides chemical analysis and microbiological studies. As previously reported [35], an ecotoxicity test with algae to assess the reduction of toxicity in a continuous-flow bioelectrochemical reactor with sequential reductive-oxidative treatment of groundwater contaminated by TCE and VC was applied. The alga test confirmed the progressive toxicity reduction from inlet to cathodic and anodic effluent. In some other studies biodegradation of CAHs was followed by plant ecotoxicity tests [44,45]. Due to the limited number of examples for the integration of ecotoxicity testing into the monitoring strategy for microbial electrochemical systems, our study shows a novel application of various acute ecotoxicological tests with test organisms from different trophic levels (bacterium, protozoon, plant, aquatic invertebrate, microworm) together with genotoxicity assessment to assess technological performance of the sequential bio-electrochemical process.

2. Materials and methods

The schematic representation of the experimental work performed on the sequential reductive/oxidative bioelectrochemical process characterization is reported in Fig. 2 which summarises the methodological sequence of the different performed operations.

2.1. Sequential processes setup, operation, and analytical characterization

The sequential bioelectrochemical reductive/oxidative process consisted in two separate microbial electrolysis cells named reductive and oxidative reactors, respectively. In the reductive reactor (empty volume 8.24 L), the working electrode (i.e. the cathode) was constituted by

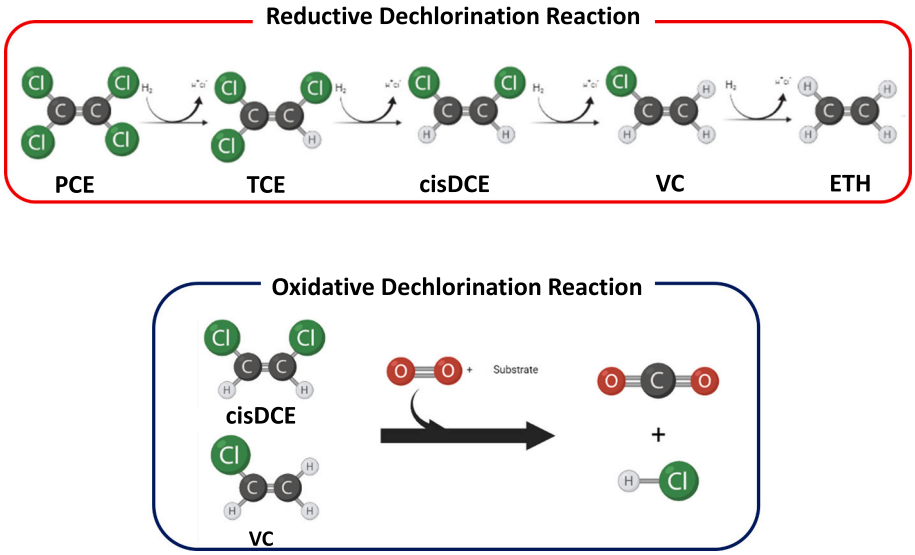

Fig. 1. Mechanism diagram for the reductive and oxidative dechlorination biological reactions.

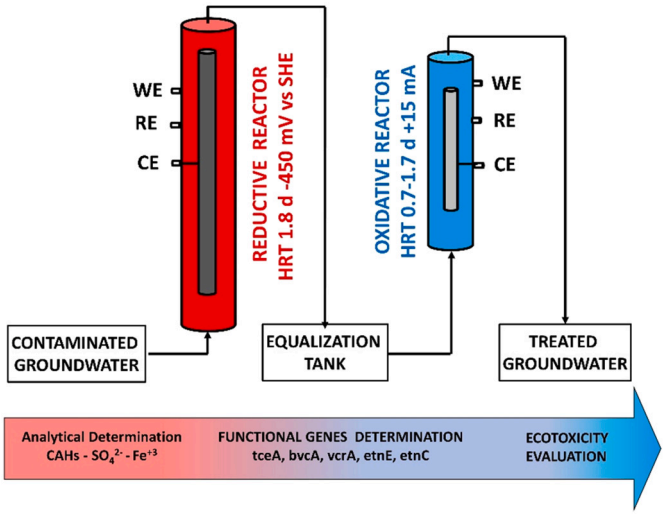

Fig. 2. Schematic representation of the experimental set up and of the multi-disciplinary analysis conducted during the reactor operations.

graphite granules to stimulate the reductive dechlorination of high chlorinated CAHs, while the oxidative reactor (empty volume 3.14 L) adopted a commercial mixed metal oxides (MMO) electrode (Magneto special anodes, The Netherlands) as working electrode (i.e. the anode) placed in a silica bed. Reductive reactor and oxidative reactors have been inoculated with specialized microbial consortium coming from "fill and draw" reactors. Both reactors were set up with an internal graphite counter electrode separated by a non-ion selective plastic mesh which avoid the use of ion exchange membrane [16]. The sequential process was fed by a peristaltic pump with the real groundwater which was stored in 25 L plastic tanks without any pre-treatment and transferred anaerobically in a plastic auto collapsing bag. During all the operation the reductive reactor working electrode was polarized at −450 mV vs SHE, while a galvanostatic condition at +15 mA was adopted in the oxidative reactor. The potentiostatic condition at −450 mV vs SHE was chosen in the reductive reactor to balance the reaction rates between reductive dechlorination and competing reaction (i.e. methanogenesis and sulphate reduction), on the contrary, in the oxidative reactor, the galvanostatic condition at +15 mA was adopted to ensure oxygen evolution on the MMO electrodes in a less conductive environment, consisting in the untreated real groundwater. The sequential bioelectrochemical process has been daily monitored with several analytical techniques for the determinations of the in the influent and effluent CAHs concentrations, and in terms of SO_4^{2-} and Fe^{3+}concentrations. The detailed description of the analytical methods and the elaboration of the results are reported in the supplementary material session [46].

Table 1
Elaboration of the analytical results related to the competitive mechanisms in the reductive reactor.

Reductive reactor competitive mechanisms evaluation	
Sulphate (RD) removal rate RS (μeq/Ld)	$RS\ (\mu eq/Ld) = Q_{liquid} / V_{reductive} * [SO_4^{-2}]_{removed} * 8$ $RS\ (mA) = RS\ (\mu eq/Ld) * V_{reductive} * F / 86{,}400$
Fe^{+3} removal rate RFe^{+3} (μeq/Ld)	$RFe^{+3}\ (\mu eq/Ld) = Q_{liquid} / V_{reductive} * [Fe^{+3}]_{removed} * 5$ $RFe^{+3}(mA) = RFe^{+3}\ (\mu eq/Ld) * V_{reductive} * F / 86{,}400$
Methane production rate ($rCH_{4(eq)}$)	$RCH_4\ (\mu eq/Ld) = Q_{gas} / V_{reductive} * [CH_4] * 8$ $RCH_4\ (mA) = RCH_4\ (\mu eq/Ld) * V_{reductive} * F / 86400 / 1000$
RS Coulombic efficiency (CE_{RS}, %)	$CE_{RS} = RS\ (mA) / I_{redcutive}\ (mA) * 100$
RFe^{+3} Coulombic efficiency (CE_{RFe+3}, %)	$CE_{RN} = RFe^{+3}\ (mA) / I_{redcutive}\ (mA) * 100$
RCH_4 Coulombic efficiency (CE_{CH4}, %)	$CE_{CH4} = RCH_4\ (mA) / I_{redcutive}\ (mA) * 100$

F = 96,485 C/mol e^-, 86,400 = s / d, Q_{liquid} = liquid flow rate, Q_{gas} = gaseous flow rate.

2.2. Calculations

The mass balances of the chlorinated species determined in the reductive reactor was calculated considering the Vinyl Chloride as final product of the RD reaction, using the following equation:

$$\text{Removal Efficiency}\ (\%) = \frac{[VC_{Out}] - [VC_{In}]}{\{([PCE_{In}] - [PCE_{Out}]) + ([TCE_{In}] - [TCE_{Out}]) + ([cDCE_{In}] - [cDCE_{Out}])\}} \times 100 \quad (1)$$

The RD Reaction rate were calculated consequentially using the following equation:

$$RD_{Rate}\left(\frac{\mu eq}{Ld}\right) = \frac{([PCE_{In}] - [PCE_{Out}])*6 + ([TCE_{In}] - [TCE_{Out}])*4 + ([cDCE_{In}] - [cDCE_{Out}])*2}{V_{reductive}} \quad (2)$$

In which the CAHs concentration are expressed as μmol/L, and $V_{reductive}$ represents the empty volume of reductive reactor, 8.24 L. The Coulombic efficiency for reductive reactor was calculated starting from the RD rate using the following equation:

$$CE_{RD}(\%) = \frac{RD_{Rate}\left(\frac{\mu eq}{Ld}\right) * \frac{F}{86400}}{-i_{reductive}\ (mA)} * 100 \quad (3)$$

In which $i_{reductive}$ is the flowing current in the reductive reactor and F is the Faraday's constant (96,485 C/mol^-.

For the oxidative reactor, the oxidative dechlorination rate (OD, μmol/Ld) was calculated by the following equation

$$OD(CAHs)\ (\mu mol/Ld) = Q_{liquid}/V_{oxidative} * [CAHs]_{in} - [CAHs]_{out} \quad (4)$$

while the oxidative removal efficiency (OD, %) of each CAHs (cisDCE and VC) was calculated by the following equation:

Table 2
Composition of the tested groundwater.

Compound	Concentration (mg/L)
PCE	0.035 ± 0.001
TCE	0.047 ± 0.004
cis-DCE	0.129 ± 0.01
VC	0.075 ± 0.008
Organic carbon	47 ± 4
Inorganic carbon	119 ± 5
TDS	0.65
pH	6.79
EC (μS/cm)	1005±
Fe^{3+}	20 ± 2
SO_4^{2-}	59 ± 7

$$OD(CAHs)\ (\mu mol/Ld) = \left([CAHs]_{in} - [CAHs]_{out}\right)/[CAHs]_{in} * 100$$

All the calculations involved in side-reactions and competitive mechanisms in the reductive reactor are reported in Table 1.

2.3. Groundwater sampling and composition

The real groundwater used to test the sequential bioelectrochemical process (i.e. almost 200 L) was collected in two different sampling campaigns which were necessary for the maintenance of the process for 60 days with an operational HRT of 1.8 days for the reductive and oxidative reactor respectively. The collected groundwater initial composition, reported in Table 2, showed the initial concentration of the main species contained in the groundwater.

As reported, all the chlorinated ethenes (i.e. PCE, TCE, cisDCE, VC) were present with a total concentration of 200 μg/L. The presence of several RD by-products suggested an existing dechlorination potential in the contaminated site. Moreover, as also reported in Table 2, the organic carbon (47 ± 4 mg/L) was mainly composed by acetate. The presence of acetate in the groundwater resulted from a previous experimental test conducted on the site after the injection of an organic substrate in the aquifer during a field scale test [47]. Moreover, Fe^{+3} and SO_4^{-2} were detected in the groundwater at the concentrations of 20 ± 2 and 59 ± 7 mg/L, respectively.

2.4. Functional genes quantification

The quantification of *D. mccartyi* 16S rRNA, reductive dehalogenase genes *tceA*, *bvcA*, *vcrA* and oxidative genes *etnE* and *etnC* involved in the oxidative dechlorination was performed on reductive and oxidative reactor effluents, respectively. Details of the methods adopted [20,48–50] are included in the supplementary material.

2.5. Ecotoxicological tests

All the samples, i.e. fresh groundwater and reductive and oxidative reactor effluents, have been tested using a problem-specific ecotoxicity toolkit which includes the use of the following organisms: bacteria, unicellular organisms, plant and animals. Samples were tested in 3–6 parallels with two-fold serial dilutions, the complete information about the ecotoxicity tests are reported in section S1.4 of the supplementary material.

3. Results and discussion

3.1. Performance of the reductive reactor

The reductive reactor influent CAHs concentration is reported in Fig. 3A. During the first 65 days of operation, the main species present in the groundwater were cis-DCE and VC with an average concentration of 47 ± 4 and 75 ± 8 μg/L, respectively, while TCE and PCE, were present at an average concentration 11 ± 3 and 5 ± 1 μg/L, respectively. The different CAHs concentrations detected in the contaminated groundwater used as reactor influent was probably due to the biodegradative activity of the autochthonous microorganisms present in the contaminated site. Indeed, while high chlorinated PCE and TCE concentrations respectively decreased from 35 to 5 μg/L and from 47 to 11 μg/L, VC concentration increased from 43 to 75 μg/L indicating some RD in the groundwater storage tank.

The reductive reactor was polarized with a cathodic potential of −450 mV vs SHE and it was fed with an average flow rate of 4.6 ± 0.5 L/d, which corresponded to a hydraulic retention time (HRT) of 1.8 days. The reductive reactor effluent composition showed in Fig. 3B, was mainly constituted by VC and cis-DCE with an average concentration of 134 ± 16 and 8 ± 1 μg/L. Considering the CAHs mass balance between the influent and the effluent solutions, the RD rate of 0.51 ± 0.04 μeq/Ld was estimated. Moreover, considering the average current of −35 ± 1 mA flowed in the circuit (Fig. S2) during the 65 days of operation, the Coulombic efficiency (i.e. the amount of electrons involved in the CAHs reduction) resulted 0.013 ± 0.001 %. The low value of the CE for the RD reaction, which in other previous published work resulted considerably higher [51,52], was probably due to the low CAHs concentration in the groundwater, which is in the typical range of concentrations from aged-contaminated sites.

As reported in Table 2, the groundwater contained SO_4^{2-} and Fe^{3+} that under the adopted operating conditions can be reduced either by bioelectrochemical or abiotic conditions [37,53]. More in details, previous study performed with a synthetic groundwater in the same reactor, showed the predominance of the bioelectrochemical sulphate reduction which resulted the predominant process responsible for current production [37,54]. The presence of Fe^{3+} was carefully considered in the reductive reactor because Fe^{+3} reduction standard potential is considerably higher with respect the adopted in the reductive reactor (i.e. +0.77 V vs SHE vs −0.45 V vs SHE). Due to the membrane-less configuration of the reactor, the Fe^{+3} reduction to Fe^{+2}, would create an electrons loop between the working (i.e. the cathode) and the internal

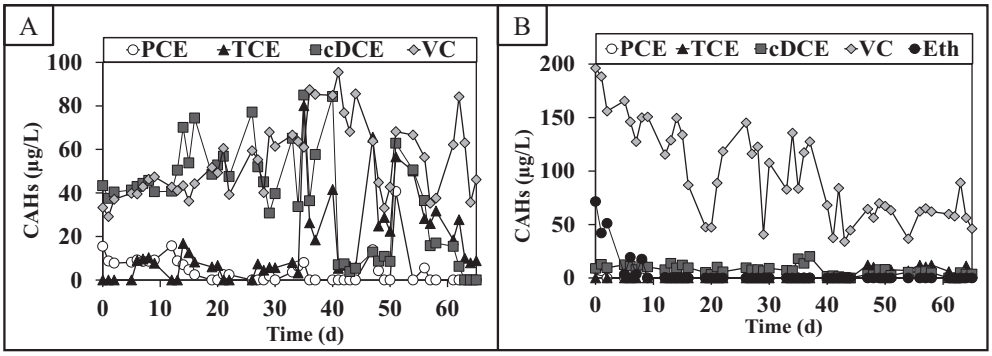

Fig. 3. Time course of CAHs in the reductive reactor influent (A) and effluent (B).

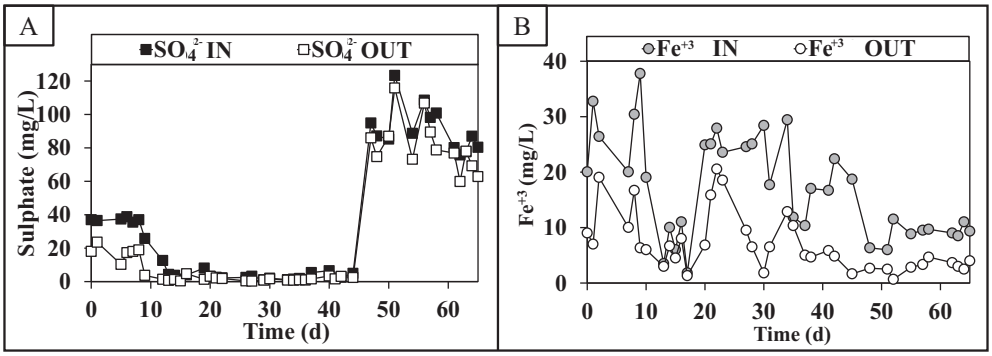

Fig. 4. Time course of the SO_4^{2-} (A) and Fe^{+3} (B) in the reductive reactor Outlet.

counter electrode (i.e. the anode) with a potential increase of the current flowing in the circuit that promote an increase of the process energy consumption.

As reported in Fig. 4A, which showed the SO_4^{2-} time course in the inlet and in the outlet of the reductive reactor, the SO_4^{2-} concentration in the influent solution decreased after day 12 dropping down from an average concentration of 34 ± 2 mg/L to 11 ± 3 mg/L. This decrement can be either explained by the presence and the consumption of organic carbon (Fig. S2) in the groundwater storage tank which was caused the heterotrophic SO_4^{2-} reduction under anoxic conditions. Furthermore, as also showed in Fig. 4A, from day 47 the influent SO_4^{2-} concentration increased to the average value of 91 ± 4 mg/L due to the utilization of the groundwater coming from the second sample campaign which contained a higher SO_4^{2-} concentration. Otherwise, a significant SO_4^{2-} removal in the reductive reactor was obtained during the first 12 days of operation with an average removal of 5 ± 1 mg/Ld while, between day 13 and 65, the average removal rate resulted equal to 1 ± 1 mg/Ld. As a consequence, considering the complete reduction of SO_4^{2-} into sulphide (i.e. 8 electrons), the current produced from SO_4^{2-} reduction accounted for 5 ± 1 and 1 ± 1 mA, which corresponded to an average Coulombic efficiency (for the overall operational period) for SO_4^{2-} reduction of 6 ± 1 %.

As reported in Fig. 4B, the Fe^{3+} concentration resulted less affected by the organic carbon presence and consumption in the storage tank or by the different sample campaign, indeed, an almost stable Fe^{+3} concentration of 17 ± 2 mg/L and 7 ± 1 mg/L was observed in the influent and effluent groundwater, respectively. Considering the reduction of Fe^{+3} to Fe^{+2} (i.e. 1 electron), a Fe^{+3} reduction rate of 5.6 ± 0.9 mg/Ld (i.e. 0.10 meq/Ld) was estimated in the reductive rector which accounted for the consumption of 1 ± 1 mA, indicating a Coulombic efficiency (i.e. the current involved in the Fe^{+3} reduction) for Fe^{+3} reduction of 2 ± 1 %. Despite the notable Fe^{+3} concentration in the groundwater, which derived from previous experimental activities on the contaminated site, current generation in the reductive reactor resulted not significantly affected by Fe^{+3} reduction. Although the favourable reducing condition provided by the cathodic potential of the reductive reactor (i.e., −450 mV vs SHE), Fe^{+3} resulted not available for abiotic reduction due to its colloidal form, which caused the typical dark brownish colour of the groundwater.

Considering a negligible methane production (data not shown), the identified reduction processes, i.e. RD reaction, SO_4^{2-} reduction and Fe^{+3} reduction, accounted only for the 9 % of the overall current flowing in the circuit (Table 3). This condition can be explained by the presence of electron loops of reduced and oxidized species that can migrate across the plastic HDPE membrane utilized as physical separator. Indeed, using an internal counter electrode and a membrane-less configuration the contribution of the reductive and the oxidative reactions is not exactly evaluable being the entire volume of reactor crossed by the liquid flow, as determined by the tracer test in previous studies [53].

Table 3
Performances of the reductive reactor.

−450 mV vs SHE HRT 1.8 d	
RD rate (μeq/Ld)	0.51 ± 0.04
RS rate (μeq/Ld)	250 ± 45
RFe^{+3} rate (μeq/Ld)	103 ± 42
CE_{RD} (%)	0.013 ± 0.001
CE_{RS} (%)	7 ± 1
CE_{RFe} (%)	2 ± 1

3.2. Performance of the oxidative reactor

The oxidative reactor received the reductive reactor effluent as feeding solution. During the first period of the oxidative reactor operation, the use of the same peristaltic pump with an average flow rate of 4.6 ± 0.5 L/d, caused the application of an HRT of 0.7 days to the oxidative reactor. As shown in Fig. 5A and B, the oxidative removal of the cis-DCE and VC resulted 1.0 ± 0.7 % and the 28 ± 10 %, respectively. Moreover, VC influent concentration in the oxidative reactor was affected by the previous sequential process operation with the synthetic groundwater, in which the CAHs concentration resulted two orders of magnitude higher [56]. In order to use an HRT similar to previous experiments in which the VC removal efficiency reached 99 % [37], an equalization tank between reductive and oxidative reactor was introduced while, through the utilization of another peristaltic pump the HRT of 1.8 d was set to the oxidative reactor. Moreover, the polarization of the oxidative reactor was conducted by a galvanostatic method in which the current flowing between working and counter electrode was controlled at a value of 15 mA (Fig. S2-B). The current value was chosen accordingly to the previous experiments in which a similar current value ensured the efficient VC removal.

After the setup of the 1.8 d HRT, performed at day 29, Fig. 5 a substantial increase of the VC and cis-DCE removal efficiencies was obtained, with an increase of the average removal efficiencies of 92 ± 2 and 100 ± 6 % respectively. Even though the substantial VC removal, its residual average concentration of 7 ± 4 μg/L, resulted higher with respect the Italian legislation limit of 0.5 μg/L [55]. Table 4 summarized the main performances of the oxidative reactor under the two explored HRTs.

3.3. Energetic consumption of the process

The energy consumption of the reductive and oxidative reactor has been calculated by the current and cell voltage product considering 24 h of operation, the consequent kWh/d was expressed as energy consumption to treat a certain flow rate of groundwater. As reported in Table 5, energy consumption (pumping not included) of the sequential bioelectrochemical process was 0.97 ± 0.09 kWh/m^3 treated water of which 0.56 ± 0.05 kWh/m^3 was consumed by the reductive reactor and 0.41 ± 0.08 kWh/m^3 by the oxidative reactor.

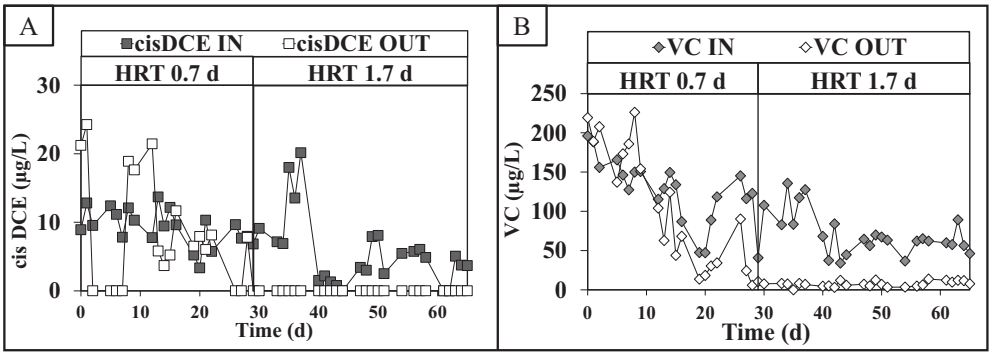

Fig. 5. Time course of the inlet and Outlet cisDCE and VC for the oxidative reactor.

Table 4
Performances of the oxidative reactor at the two HRT explored.

HRT (d)	0.7	1.7
cisDCE removal rate (µg/Ld)	3 ± 2	4 ± 1
VC removal rate (µg/Ld)	34 ± 15	55 ± 7
$VC_{removal\ efficiency}$ (%)	28 ± 10	92 ± 2
$cisDCE_{removal\ efficiency}$ (%)	1.0 ± 0.7	100 ± 6

Table 5
Energetic consumption evaluation of the sequential bioelectrochemical process.

Reactor	Reductive	Oxidative
Average current (mA)	−35 ± 1	15 ± 1
Average cell voltage (ΔV)	−3.13 ± 0.11	−2.76 ± 0.12
Energy consumption ($kWh/m^3_{groundwater}$)	0.56 ± 0.05	0.41 ± 0.08

3.4. Quantification of biomarkers in the reductive and oxidative reactor

Functional genes involved in the reductive or oxidative dechlorination, were quantified by Droplet Digital PCR (ddPCR) assays in the real contaminated groundwater used for the reactor, and in the outlet samples of the reductive and oxidative compartments at the end of the reactor operations. In detail, *D. mccartyi* 16S rRNA and reductive dehalogenase genes *tceA*, *bvcA*, *vcrA* were quantified as biomarkers for the RD process. Instead, *etnC* and *etnE* genes were determined as biomarkers for the oxidative dechlorination.

In the real contaminated groundwater all the biomarkers analyzed were found (Fig. 6A). *D. mccartyi* 16S rRNA accounted for 1.17E+07 gene copies/L, including 3.41E+05, 7.75E+06 and 9.98E+06 gene copies/L of *tceA*, *vcrA* and *bvcA*, respectively (Fig. 6A). These findings are in line with the presence of the RD intermediates found in the contaminated groundwater collected from the real site, indicating the occurrence of an existing dechlorinating potential in the groundwater used for the bioelectrochemical reactor. Interestingly, also *etnE* and *etnC* genes were detected in the real groundwater (4.5E+05 and 2.35E+05 gene copies/L), suggesting a potential for the oxidative dechlorination.

In line with the RD performances (Table 3), *D. mccartyi* was also detected (8.78E+06 16S rRNA gene copies/L) at the outlet of the reductive reactor collected at the end of the reactor operations (Fig. 6B). Accordingly, *tceA* (6E+06 gene copies/L) was the most abundant reductive dehalogenase gene found, while *bvcA* (3.56E+04 gene copies/

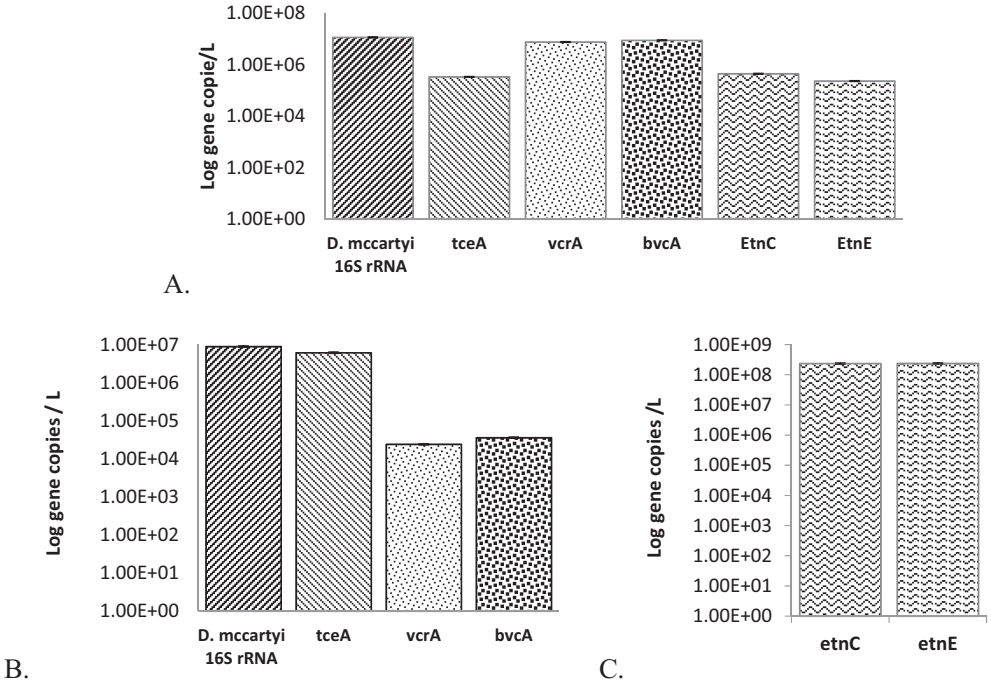

Fig. 6. Biomarkers for the reductive (*D. mccartyi* 16S rRNA, *tceA*, *vcrA* and *bvcA* genes) and oxidative (*etnC*, *etnE*) dechlorination quantified in the real contaminated groundwater used in the bioelectrochemical system (A) and at the outlet of the reductive (B) and oxidative (C) compartments at the end of the reactor operations.

Table 6
The effect on *Tetrahymena pyriformis* reproduction determined by tetrazolium reduction assay.

Reproduction inhibition[a] of *Tetrahymena pyriformis* [%]			
	GW (influent)	Reductive reactor effluent	Oxidative reactor effluent
24 h	0 (±0)	0 (±0)	0 (±0)
48 h	18.8 (±0.2)*	10.6 (±0.2)	12.2 (±0.8)

[a] Inhibition of reproduction at 4× dilution.
* Statistically significant inhibition compared to the control.

L) and *vcrA* (2.39E+04 gene copies/L) were detected at the minor extent. The occurrence of *tceA* is in line with kinetic data reporting PCE dechlorination up to cis-DCE or VC in the reductive reactor. Indeed, as already reported in the literature, *D. mccartyi* strains carrying *tceA* gene are capable of metabolic dechlorination up to cis-DCE and/or VC while *D. mccartyi* strains carrying *bvcA* or *vcrA* genes are capable of cis-DCE or VC dechlorination to the harmless ethene [15–19].

Also, at the outlet of the oxidative reactor, both *etnC* (2.36E+08) and *etnE* (2.38E+08) were found at high abundances (Fig. 6C), in line with the VC oxidation occurring in the oxidative compartment of the reactor, where VC and cis-DCE were quite completely removed (Table 4).

3.5. Ecotoxicity evaluation of the sequential process

The results of the *Aliivibrio fischeri* bioluminescence inhibition test displayed only slight non-significant inhibitory effect for the samples even at the highest tested concentration (data not shown).

According to the results of the *Tetrahymena pyriformis* reproduction inhibition assay determined by tetrazolium reduction (Table 6), the effluent samples from reductive and oxidative reactors did not cause significant toxic effect even at the highest tested concentration (4× dilution), while the influent groundwater showed slight significant inhibition compared to control after 48 h exposure (~19 %). The 32×, 16× and 8× dilutions of the influent (GW) and effluent samples from the reductive and oxidative reactors had no inhibitory effect on the protozoon.

Based on the results of the *Lemna minor* (duckweed) frond number (Fig. S5), the samples displayed slight toxicity. The influent groundwater sample showed no toxic effect compared to the control, neither in leaf number nor in chlorophyll content. There was also no significant decrease in the number of the leaves in case of the treated effluent samples compared to the control (<10 % inhibition). The total chlorophyll content (Table S2) as an endpoint showed a higher sensitivity than the frond number; however, no significant differences were observed in this case either.

Interestingly, the treated effluent samples from oxidative reactor exhibited higher toxicity than the influent GW based on the total chlorophyll content values. Presumably, this phenomenon may be attributed to the degradation products or increased SO_4^{2-} concentration. The results of plant tests (Table 7 and Table S2) showed different levels of ecotoxicity, as well as different responses of *Sinapis alba* and *Triticum aestivum*. The results of *Sinapis alba* (white mustard) root- and shoot growth inhibition test (Table S3) did not show any significant adverse effect. No significant inhibition was shown compared to either the control or the influent sample. The plant growth test with common wheat (*Triticum aestivum*) displayed higher sensitivity. Table 7 shows the effect of samples on common wheat shoot and root growth at the highest tested concentration (without dilution).

Table 7
The effect on *Triticum aestivum* plant growth.

Shoot and root length of *Triticum aestivum* after 72 h exposure time [mm]				
	Control (DW)	GW (influent)	Reductive reactor effluent	Oxidative reactor effluent
Shoot length	11.4 (±0.3)	7.0 (±0.7)*	7.8 (±1.5)*	9.0 (±2.1)
Root length	21.9 (±3.7)	21.5 (±0.8)	18.4 (±1.2)	19.5 (±2.2)

* Statistically significant inhibition compared to the control.

Significant decrease in shoot length compared to control was demonstrated by the influent and the reductive reactor effluent samples. The highest tested concentration of the influent (without dilution) resulted in a significant inhibitory effect of 38 % after 3 days exposure; while reductive reactor effluent sample displayed 31 % inhibition of shoot growth, compared to control. According to the results of the root length as endpoint, the influent GW sample was not toxic. However, the treated effluent samples showed slight but non-significant inhibition (6–16 %) on common wheat root growth compared to control.

The *Daphnia magna* test organism was not sensitive to the influent groundwater sample and the treated effluent samples from reductive and oxidative reactors. The samples did not prove to be toxic to the aquatic invertebrate even at the highest applied concentration (2× dilution) (Table S4).

Table 8
The effect on *Panagrellus redivivus* mortality after 48 h.

Mortality of *Panagrellus redivivus* [%]			
Dilution level	GW (influent)	Reductive reactor effluent	Oxidative reactor effluent
8×	4.6 (±6.6)	0.8 (±1.4)	0 (±0)
4×	7.9 (±7.0)	0.9 (±1.7)	2.0 (±3.5)
2×	22.3 (±7.5)*	0.6 (±1.3)	4.7 (±6.3)
1×	47.8 (±2.0)*	36.2 (±9.1)*	28.4 (±14.1)*

* Statistically significant inhibition compared to the control.

According to the results of the *Panagrellus redivivus* mortality test, reported in Table 8, the nematode test organism demonstrated to be sensitive. The tested influent groundwater sample proved to be toxic to the microworm at the highest tested concentrations (without dilution and at 2× dilution). The treated samples from reductive and oxidative reactors (without dilution, 1×) exhibited lower but significant inhibitions compared to control. No significant difference was found between the effluents form reductive and oxidative reactors. The results of the *Panagrellus redivivus* mortality test showed that the sequential bio-electrochemical reductive/oxidative process was efficient for the removal of chlorinated aliphatic hydrocarbons.

According to the SOS Chromotest none of the tested samples were genotoxic since the induction factors (IF) were lower than 1.5 (GW: 1.1, reductive effl. 1.0 and oxidative effl. 0.9). The scientific literature shows contradictory results on the mutagenicity of CAHs [56–60]. Based on the ECHA registration dossiers PCE, TCE, cisDCE and ethylene are not mutagenic, while VC is considered to be mutagenic. However, according to the literature, VC was not genotoxic according to the SOS Chromotest.

The ecotoxicological test systems of our study showed that the untreated influent and the treated groundwater samples were slightly and moderately toxic. The ecotoxicity assessment of the influent and treated effluent samples of this study proved that the applied test batteries should encompass a spectrum of test organisms from different trophic levels.

The results obtained have clearly demonstrated the difference in the sensitivity of the applied test organisms. The protozoon *Tetrahymena pyriformis* and the microworm *Panagrellus redivivus* were sensitive to the organic contaminants (chlorinated aliphatic hydrocarbons, CAHs) in the samples, while bacterial and plant test systems exhibited no significant effects. Taking into account the entire ecotoxicological assessment of the influent groundwater and the effluent treated samples, it can be concluded that the protozoon and nematode organisms are suitable indicators of technological efficiency.

Reviewing the international literature, there is a lack in ecotoxicity studies about the effect of CAHs on the aquatic environment particularly

at environmentally relevant concentrations. Based on ecotoxicity data of ECHA registration dossiers [56–60] the lowest EC_{50} (Effective Concentration causing 50 % inhibition of the measured parameter) values (based on acute freshwater fish, algae and *Daphnia magna* tests) are higher than 1 mg/L for CAHs (e.g. 3.6 mg/L for PCE by algae, 20.8 mg/L for TCE by *D. magna*, 160 mg/L for cisDCE by *D. magna*, 210 mg/L for VC by fish, 40.5 mg/L for ethylene by algae) which is 400–1200 times higher than the concentrations measured in the groundwater.

Literature reference [56,57], showed that TCE and VC between 0.1 and 1 μg/L concentrations effected genes and proteins related to metabolism, reproduction, and growth in *D. magna*. However, other studies with green algae and cyanobacteria [58], freshwater bivalves [59,60] applied higher (1.5–100 mg/L), environmentally not relevant concentrations. This indicates that more sensitive test species and methodologies are needed to assess the potential toxicity of CAHs occurring at low concentrations in freshwaters.

To increase sensitivity of less sensitive species chronic tests with longer exposure times may be necessary in accordance with the observations of [57], who highlighted the importance of chronic exposure for testing impacts of TCE and VC.

However, the results of the *Tetrahymena pyriformis* reproduction inhibition assay and *Panagrellus redivivus* mortality test proved that the sequential bioelectrochemical reductive/oxidative process had good performance and was suitable for the complete mineralization of the CAHs present in the groundwater. Our ecotoxicity study reveals promising possibilities for the future use of protozoan and nematodes in ecotoxicological studies, testing the impact of CAHs on aquatic environmental systems.

3.6. Outlook and perspectives

The experimental activity performed on the real contaminated groundwater validated the sequential bioelectrochemical technology for CAHs removal. The characterization of the target reductive and oxidation reactions on CAHs in the reductive and oxidative reactor, as well as the characterization of the main side reactions, gives a complete overview of the processes involved in the process. The characterization of the biomarkers allowed for the quantification of specific dechlorinating species corroborating the establishment of effective dechlorinating communities in both, reductive and oxidative compartments. On the other hand, the ecotoxicity evaluation confirmed the decrease of the water ecotoxicity after the treatment in the bioelectrochemical sequential process, highlighting no undesired side reactions harmful for living microorganisms. The perspective of the presented study results extremely favourable for a process scale up and application in real environment. It is important to underline that the reactor configuration adopted in this study, i.e. the membrane-less configuration with an internal graphite counterelectrode, allowed for a cheap and flexible scale up of the technology. Moreover, being the bioelectrochemical technology an innovative approach in which the stimulation of the microbial activity is performed by the use of electric potential, no chemicals are required in the process but only electric energy which is an accessible and flexible source of energy, including the potential use of renewable electric energy from a photovoltaic system.

4. Conclusions

The sequential reductive oxidative bioelectrochemical process has been successfully validated by testing a CAHs contaminated groundwater coming from a real contaminated site located in the northern Italy. The sequential process was continuously operated for 65 days with a global HRT of 3.6 days (considering the empty volume of both reductive and oxidative reactor) which corresponded to a number of 18 HRT. The sequential process showed the capability to mineralize all the CAHs contained in the groundwater by the biological reduction of the high and medium chlorinated CAHs into VC, which was successfully removed by an oxidative process with an average removal efficiency of 92 ± 2 %. The VC oxidation required an HRT of 1.8 days which ensured a sufficient time for the biological VC oxidation. Although the Coulombic efficiency for the RD reaction resulted only 0.013 %, an important result was obtained by the analysis of the side reaction which potentially affected the reductive process, indeed, SO_4^{2-} and Fe^{+3} reduction accounted for a limited current production (i.e. 7 and 1). The energy consumption of the whole reductive oxidative bioelectrochemical process results <1.0 $kWh/m^3_{treated\ water}$ which resulted an interesting energy consumption in terms of energy investments. The biomarkers involved in the reductive (*D. mccartyi* and reductive dehalogenase genes) and oxidative (*etnE*, *etnC*) dechlorination were found in the real contaminated groundwater used for the reactor, suggesting the occurrence of an existing dechlorination potential in the real contaminated sample, thus prompting the biological dechlorination activity in the bioelectrochemical system during the operations of the reductive and oxidative compartments. Moreover, the quantification of the biomarkers involved in the reductive and oxidative dechlorination, as well as the ecotoxicity assessment showed important information related to the main microbial species and functional genes involved in reductive and oxidative processes as well as the environmental impact of the bioelectrochemical technology. Indeed, the analysis of the ecotoxicological results on the real groundwater showed an important toxicity reduction in the untreated groundwater samples.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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