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Toluene-driven anaerobic biodegradation of chloroform in a continuous-flow bioelectrochemical reactor

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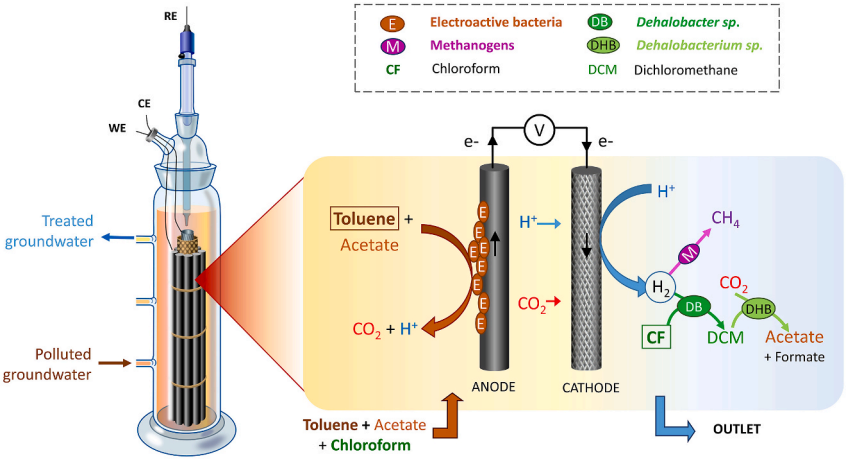
HIGHLIGHTS

- Toluene and chloroform biodegradation in single-stage bioelectrochemical reactor.
- Nearly complete removal of the target contaminants via distinct pathways.
- Chloroform metabolite dichloromethane was also removed via fermentation.
- Acetate negatively impacted the removal of the contaminants.
- Taxonomical and functional gene-based analysis revealed a competent microbiome.

GRAPHICAL ABSTRACT



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ABSTRACT

Subsurface co-contamination by multiple pollutants can be challenging for the design of bioremediation strategies since it may require promoting different and often antagonistic degradation pathways. Here, we investigated the simultaneous degradation of toluene and chloroform (CF) in a continuous-flow anaerobic bioelectrochemical reactor. As a result, 47 μmol L^{-1} d^{-1} of toluene and 60 μmol L^{-1} d^{-1} of CF were concurrently removed, when the anode was polarized at +0.4 V vs. Standard Hydrogen Electrode (SHE). Analysis of the

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Toluene
Chloroform

microbial community structure and key functional genes allowed to identify the involved degradation pathways. Interestingly, when acetate was supplied along with toluene, to simulate the impact of a readily biodegradable substrate on process performance, toluene degradation was adversely affected, likely due to competitive inhibition effects. Overall, this study proved the efficacy of the developed bioelectrochemical system in simultaneously treating multiple groundwater contaminants, paving the way for the application in real-world scenarios.

1. Introduction

Due to their widespread usage, chlorinated compounds and aromatic hydrocarbons are among the most frequent groundwater and soil contaminants (Rivett et al., 2011). Their occurrence in subsurface environments is mainly caused by industrial activities, surface runoff, accidental spills from tanks and pipes, improper handling or disposal practices (Blázquez-Pallí et al., 2019; Collins et al., 2002). Due to their toxicity and persistence in the environment, these contaminants pose high risks to human health and the ecosystem (Chary and Fernandez-Alba, 2012).

Bioremediation is considered a very effective approach to treat these kinds of pollutants, having in general lower requirements in terms of costs, equipment, labor and energy, and a lower environmental impact compared with conventional physicochemical techniques, such as air sparging and activated carbon adsorption, which also typically result in a phase transfer of contaminants without an effective degradation (Lhotský et al., 2017). However, the presence of multiple types of contaminants in the same site may complicate operations and increase the costs of bioremediation, since different treatments need to be implemented in different times and space to meet the distinct metabolic needs of the different microbial degraders (Megharaj et al., 2011; Rabus et al., 2016). As aromatic hydrocarbons are preferentially degraded via oxidative pathways, the microbial community requires availability of high potential electron acceptors such as oxygen or nitrate (Allard and Neilson, 1997; Jabbar et al., 2022). On the contrary, the reductive dechlorination of chlorinated compounds, carried out by organohalide-respiring bacteria (OHRB), requires an electron donor (*i.e.* molecular hydrogen) while the contaminant serves as the respiratory electron acceptor (Farhadian et al., 2008).

Theoretically, it would be possible to exploit the oxidation of aromatic hydrocarbons as source of electrons for the reduction of chlorinated compounds. However, since most OHRB are restricted to using H_2 as electron donor, the possibility of carrying out the simultaneous removal of both types of pollutants in a single-step reaction is very limited (Soder-Walz et al., 2022).

Microbial electrochemical systems (MESs) are being proposed as a promising alternative to conventional bioremediation strategies. They are able to provide inexhaustible source and/or sink of electrons to sustain microbial metabolism in form of a solid electrode, thus avoiding the drawbacks connected to the injection of air, oxygen or other chemicals in the aquifer (He and Su, 2015). Many studies on bioremediation with MESs focused on Microbial Fuel Cells, which can spontaneously oxidize organic compounds at the anode by using oxygen as the terminal electron acceptor at the cathode (Kronenberg et al., 2017). However, in contaminated subsurface environments, the access to O_2 is strongly limited due to rapid depletion, thus hindering the biodegradation of pollutants (Wartell et al., 2021).

In Microbial Electrolysis Cells (MECs), a potential difference is applied to the electrodes in order to catalyze sluggish or thermodynamically unfavorable oxidation reactions at the (bio)anode and/or reduction reactions at the (bio)cathode (Kadier et al., 2014). Several studies demonstrated the possibility to use MECs to treat aromatic hydrocarbons at the anode (Friman et al., 2013; Marzocchi et al., 2020; Palma et al., 2018a, 2019; Tucci et al., 2021, 2022c, 2022a; Zhang et al., 2010) or chlorinated compounds at the cathode (Aulenta et al., 2011; Lai et al., 2017; Leitão et al., 2015; Verdini et al., 2015) in anaerobic conditions. Nevertheless, the research on coupling aromatic hydrocarbon oxidation at the anode and chlorinated compounds reduction at the cathode with MECs is still very limited (Tucci et al., 2022b).

Recently, we proposed a prototype of MEC named "Bioelectric well" (Palma et al., 2018b): its tubular design allows insertion in groundwater wells and piezometers for in-situ groundwater treatment. Moreover, the absence of membrane between anode and cathode minimizes the ohmic losses and facilitates its upscaling. Recent works proved that the bioelectric well is able to remove at the same time oxidable (toluene, TPH) and reducible (sulfate, TCE) contaminants from a synthetic groundwater (Tucci et al., 2021; Viggi et al., 2022).

In this study we performed the simultaneous degradation of toluene, as a model aromatic hydrocarbon, and chloroform (CF), a highly toxic and possibly carcinogen chlorinated pollutant (Yamamoto et al., 2002), in a continuously fed bioelectric well operated for over 190 days. Importantly, CF biodegradation has never been previously studies in a continuous-flow bioelectrochemical reactor. Here, toluene was oxidized by the electroactive microbial consortium at the anode, while CF was converted to harmless end products via a two-step process involving first a reductive hydrogenolysis step leading to dichloromethane (DCM) followed by the fermentation to acetate and formate.

2. Materials and methods

2.1. Chemicals and electrode potentials

All chemicals used for the experiments were of analytical grade. All of them were purchased from Merck KGaA (Germany). De-ionized water (Millipore, Germany) was used to prepare the mineral medium and all other solutions. All potentials reported in this work are referred to the standard hydrogen electrode (SHE).

2.2. Reactor setup and operations

The bioelectric well was set up as previously reported by Tucci et al. (2021): the anode consisted of a cylinder made of 8 contiguous graphite rods (purity: 99.995%, length: 30 cm, ø: 0.6 cm; Merck KGaA, Germany), whereas the cathode was a stainless steel mesh (dimensions: 3 × 30 cm; type 304, Alpha Aesar, USA) (Fig. 1A). Anode and cathode were contained in a 250 mL glass cylinder and were kept separated by a polyethylene mesh (ø: 1 cm, length 30 cm; Fig. 1B), which allowed free circulation of the electrolyte. The distance between the anodic and the cathodic surfaces was 2 mm. The two electrodes were connected to an external circuit with titanium wires (ø: 0.81 mm Alfa Aesar, USA). During the experiments, the anode was polarized at +0.4 V vs. SHE by means of an IVIUMnSTAT potentiostat (IVIUM Technologies, The Netherlands). This potential was chosen according to the findings of previous studies with the bioelectric well, where the bioelectrochemical oxidation peak of toluene on graphite ranged between 0.2 and 0.4 V (Tucci et al., 2022c). The counter electrode potential was periodically measured throughout the experiment. A saturated Ag/AgCl electrode (+0.198 V vs. SHE; AMEL, Italy) was used as reference electrode.

To ensure the presence of microbes able to degrade toluene, the reactor was inoculated with 0.25 L of real groundwater contaminated with petroleum hydrocarbons collected from a petrochemical site in Italy.

During the experiment, the reactor was fed in continuous mode with mineral medium (pH 7) in which O_2 was eliminated through N_2 sparging. The medium composition is reported in the supporting

information (Table S1). The medium was spiked with the contaminants toluene and CF as summarized in Table 1. Acetate was also added to the medium to study the impact of a readily available substrate on the reactor performances. The inlet was stored in 5 L collapsible Tedlar® gas bags and pumped in the reactor through the bottom port (flow rate: 0.63 L d^{-1}, HRT 11 h) by means of a peristaltic pump (120 S, Watson Marlow, Falmouth, UK). The treated effluent was discharged from the upper port by passive overflow. Flow-through sampling cells (volume: 25 mL) were installed at the inlet and at the outlet of the reactor for liquid and gas monitoring. The liquid phase of the reactor was constantly recycled with another peristaltic pump (flow rate: 192 mL min^{-1}; model: 323, Watson Marlow, Falmouth, UK) to avoid concentration gradients of substrates, products and/or biomass. The tubings were made of Viton® (Merk KGaA, Germany) to minimize volatilization losses and adsorption of organic contaminants. During the whole study the system was maintained at room temperature (*i.e.,* 24 ± 3 °C).

The experiment lasted 190 days, during which different conditions were tested (**Table 1**). The first run was an acclimation phase for the toluene degrading consortium in absence of CF. Then, CF was added to the mineral medium at a concentration of 14 mg L^{-1} and the reactor was inoculated (10% vol/vol) with two microbial cultures: one enriched with *Dehalobacter* spp. and the other with *Dehalobacterium formicoaceticum*, capable to perform the reductive hydrogenolysis of CF to DCM and the subsequent fermentation of DCM to acetate and formate, respectively (Fernández-Verdejo et al., 2022; Trueba-Santiso et al., 2017, 2020).

At this point three different concentrations of toluene (in the range of 15–36 mg L^{-1}) were tested from runs 2 to 4. In runs 5 and 6 the electrodes were disconnected from the potentiostat to study the removal of contaminants at open circuit potential (OCP). Finally, in runs 6 and 7 acetate was omitted from the mineral medium and during run 7 the system was reconnected to the potentiostat and polarized.

Table 1
Operational conditions for all the different runs operated with the bioelectric well.

Run	Days	Toluene (μmol L^{-1})	Chloroform (μmol L^{-1})	Acetate (μmol L^{-1})	Polarization (V vs. SHE)
1	0–13	260	/	920	+0.4
2	14–36	260	120	920	+0.4
3	37–46	390	120	920	+0.4
4	47–80	160	120	920	+0.4
5	81–114	130	120	920	OCP
6	115–127	100	120	/	OCP
7	128–190	100	120	/	+0.4

2.3. Cyclic voltammetries

The bioanode and (bio)cathode developments were monitored with Cyclic voltammetries (CVs, scan rate: 1 mV s^{-1}) using an IVIUMnSTAT potentiostat (IVIUM Technologies, The Netherlands). The stainless-steel cathode and the graphite anode were used as counter electrode during the anodic and cathodic CVs, respectively. For all CVs, an Ag/AgCl electrode (+0.198 V vs. SHE; AMEL, Italy) was used as reference.

2.4. Gas-chromatographic analyses

The quantification of O_2, H_2 and CH_4 was performed using a gas-chromatograph (Agilent 8860, GC system USA) equipped with a thermal conductivity detector (TCD); the quantification of toluene, CF and acetate was performed with a gas-chromatograph (Agilent 8860, GC system USA) equipped with a flame ionization detector (FID). The

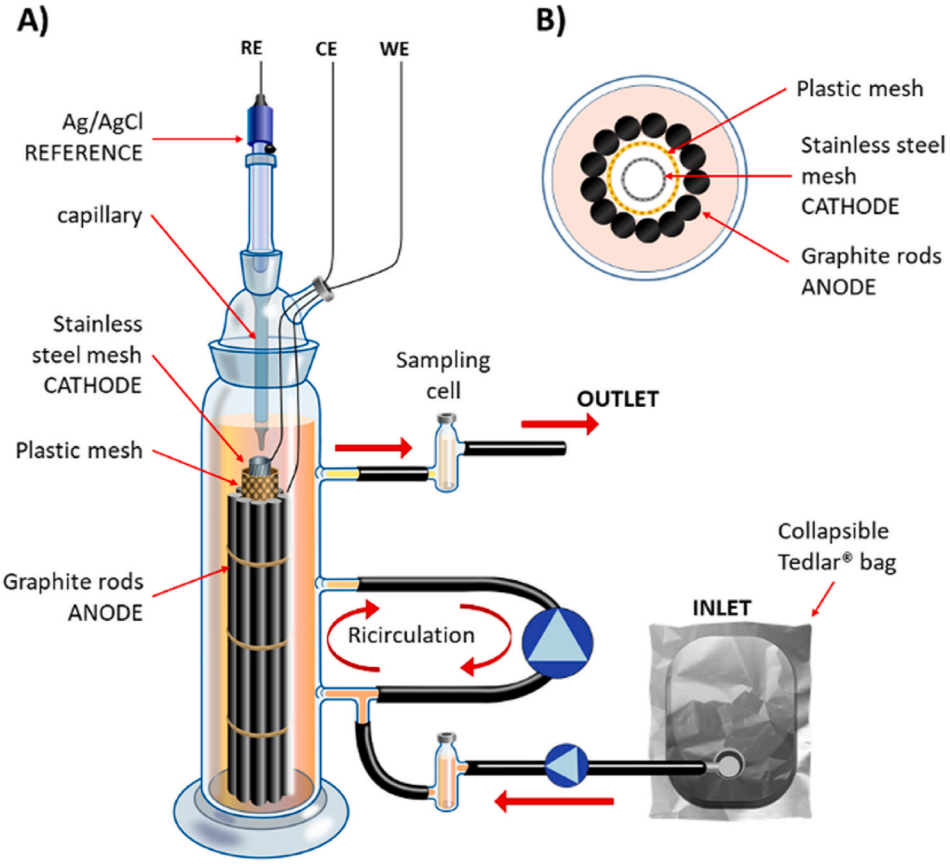

Fig. 1. The bioelectric well: A) schematic representation of the setup used for the experiments, where WE is the working electrode, CE is the counter electrode and RE is the reference electrode; B) cross-sectional view showing the relative position of electrodes.

methods for the GC, the calibration ranges and detection limits (LOD) are reported in the Supporting Information (**Table S2**). Gas-phase concentrations were converted into liquid-phase concentrations using tabulated Henry's Law constants (Sander, 2015).

2.5. High-throughput rRNA gene sequencing and bioinformatic analysis

The liquid effluent (15 mL) and the biofilm grown on the graphite rods were collected at the end of the operation, filtered through polycarbonate membranes (pore size 0.2 mm, 25 mm diameter, Nuclepore) and immediately stored at −20 °C. The DNA extraction was performed by using the DNeasy PowerSoil Pro Kit (QIAGEN - Germantown, MD) following manufacturer's instructions. The genomic DNA was utilized as template for the amplification of the V1–V3 region of 16 S rRNA gene of *Bacteria* (27F 5′-AGAGTTTGATCCTGGCTCAG-3'; 534R 5′-ATTACCGCGGCTGCTGG-3′) following the procedure for library preparation and sequencing described elsewhere (Crognale et al., 2019). The samples were paired end sequenced (2 × 301bp) on a MiSeq platform (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina, USA) following the standard guidelines for preparing and loading samples. Phix control library was spiked at a concentration of 20%. Bioinformatics analysis were performed using QIIME2 v. 2018.2 (Bolyen et al., 2019) following the procedure reported elsewhere (Crognale et al., 2021). High-throughput sequencing of the V1–V3 region of the bacterial 16 S rRNA gene yielded a total of 16,934 sequence reads after quality control and bioinformatics processing that resolved into 150 amplicon sequence variants (ASVs).

2.6. Quantification of key-functional genes

The genomic DNA was also used as template for the quantification of functional genes involved in the upper pathway of anaerobic toluene degradation (*bssA, bcrC, bzdN, bamB*), CF to DCM reduction (*cfrA*) and DCM fermentation (*mecE, mecF*). 16 S rRNA of *Dehalobacter* spp. and *D. formicoaceticum* were also quantified. Absolute quantification assays were performed via Digital Droplet PCR (ddPCR) with the QX200™ Droplet Digital™ PCR System (Bio-rad, United States) as describe elsewhere (Di Franca et al., 2022). The primers used have been listed in Table S3.

The steps for the quantification assays included: i) preparation of the PCR reaction mixture for each targeted gene (22 μl total volume: ddPCR EvaGreen Supermix ® (Bio-Rad, United States), 3 μl of DNA as a template, and 900 nM of each primer); ii) droplets generation (20 μl of PCR mixture and 70 μl of Droplet Generation Oil® (Bio-Rad, United States) with the QX200 Droplet Generator (Bio-Rad, United States); iii) PCR amplification on 40 μl of the droplets' mixture with a T100 thermal cycler (Bio-Rad, United States) (cycling conditions: 5 min at 95 °C, 39 cycles for 30 s at 95 °C and 1 min at 60 °C (ramping rate set to 2 °C/s), 5 min at 4 °C, 5 min at 90 °C, ending at 4 °C); iv) quantitative data reading with QX200 Droplet Reader (Bio-Rad, United States) to determine the positive and negative fluorescent droplets and calculate the targeted gene concentrations. The data were analyzed using the QuantaSoft® software (Bio-Rad, United States) by calculating the ratio of the positive droplets over the total droplets in each sample. Quantitative data have been reported as gene copies L^{-1} of liquid effluent or gene copies cm^{-1} of graphite rod (95% confidence intervals).

3. Calculations

The removal rate r (μmol L^{-1} d^{-1}) of toluene and CF were calculated using the following equation:

$$r = \frac{\Delta Conc.}{V_r} Q \quad (1)$$

where $\Delta Conc.$ (μmol L^{-1}) is the difference between the concentration of toluene or CF measured in the influent (C_{In}) and the concentration measured in the effluent (C_{out}), V_r (L) is the empty volume of the reactor and Q (L d^{-1}) is the flow rate of the influent.

The relative removal q% of toluene and CF were calculated as follows:

$$q_{\%} = \frac{\Delta Conc}{C_{in}} \times 100 \quad (2)$$

4. Results and discussion

4.1. Reactor performances

The bioelectric well was operated for 190 days in different conditions (**Table 1**). In Fig. 2A the current profile for all the different runs is reported. After a start-up phase with toluene as the only contaminant (run 1), the current started to increase and CF was added (run 2). Then, the current reached a steady level with an average value of 3.4 mA (runs 3 and 4). During run 5 and 6 the system was kept in OCP, and thereafter reconnected in run 7. It can be noticed how, as soon as the electrodes were polarized again, the current increased until it reached values similar to those of the previous polarized runs. The recorded CVs clearly point to a gradual formation of an electroactive biofilm on the anode, starting from T0 to the end of run 3 (Fig. 2B). Indeed, it can be noticed the formation of peaks associated with a redox couple (red line) and a time-dependent increase of the oxidative current. The cathodic reductive current also increased overtime, probably due to the accumulation of hydrogenase-containing microorganisms at the electrode, which can facilitate H_2 formation at the cathode (Fig. 2C) (Aulenta et al., 2012). Furthermore, the measured cathodic potential maintained a value of 0.77 ± 0.07 V vs. SHE throughout the experiment, which is suitable for H_2 generation.

The current generation is strictly linked to the degradation of toluene, as it can be observed in Fig. 3A. At the beginning of the experiment, electroactive bacteria started to degrade toluene at the anode in presence of acetate. However, even at the lowest influent toluene concentration (i.e. 160 μmol L^{-1}), only a fraction of the toluene load was removed. Conversely, during run 7, where acetate was not present in the inlet, almost 100% of toluene was degraded. During OCP (runs 5 and 6), as expected, negligible toluene degradation was observed.

The consumption of acetate was also monitored (Fig. S1): acetate was completely consumed during the polarized runs (1–4), while almost no removal was observed during OCP (run 5). When acetate was omitted from the inlet (runs 6 and 7), no residual amounts were detected in the reactor.

Regarding CF, the degradation started quickly as soon as it was added to the reactor (Fig. 3B). As a result, the dechlorination product DCM (deriving from the reductive hydrogenolysis of CF) started to being produced. At the same time, DCM fermentation commenced, hence slowly decreasing its concentration in the reactor. During OCP, the dechlorination of CF slowed down but it was still present, being probably sustained by DCM fermentation and the endogenous decay of biomass. Indeed, it is well documented that the slow release of electron donors generated by fermentation processes is effective in sustaining reductive dechlorination processes (Amanat et al., 2022; Yang and McCarty, 2000). When the system was polarized again (run 7), the previous level of CF removal was restored. It is worth noticing that, towards the end, almost all CF and its metabolite DCM were removed. Furthermore, no adverse effects on toluene degradation were observed with the addition of CF.

Methanogenesis started at the beginning of run 1, sustained by electrochemically produced H_2 (Fig. 3C). However, methane generation decreased over time after CF addition, probably due to the inhibitory effect caused by this contaminant on methanogens (Yu and Smith, 2000). During OCP runs (5 and 6) no hydrogen was generated, and thus no CH_4 was produced. Once almost all CF was removed by the

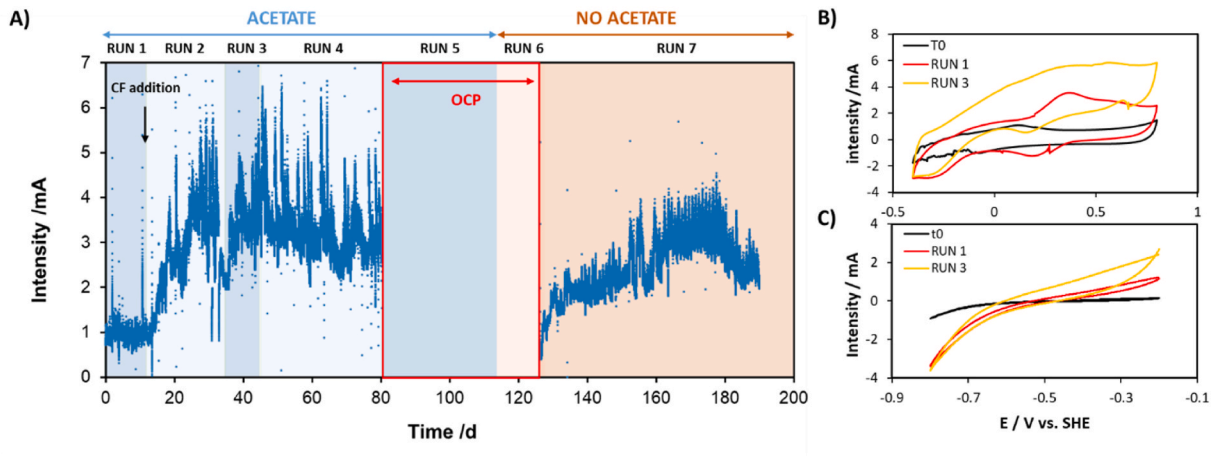

Fig. 2. A) Current trend of the bioelectric well during the different runs. B) CVs of the (bio)anode at T0, in run 1 and 3. C) CVs of the (bio)cathode at T0, in run 1 and 3.

dehalogenating bacteria (run 7), methanogenesis started again.

During the polarized run, in absence of acetate (run 7), 47 μmol L^{-1} d^{-1} of toluene and 60 μmol^{-1} d^{-1} of CF were removed, corresponding to 96% and 94% of the contaminant load, respectively (**Table 2**). This proves the effectiveness of the bioelectrochemical system, since only 0.1 μmol L^{-1} d^{-1} of toluene (i.e. 0,2%) and 47 μmol L^{-1} d^{-1} of chloroform (i.e. 77%) were removed during OCP. Nevertheless, the degradation rate of toluene obtained in this work is considerably lower than the ones obtained in previous studies with the bioelectric well, which were as high as 336 and 150 μmol L^{-1} d^{-1} when sulfate and TCE were used as co-contaminants, respectively (Tucci et al., 2021; Viggi et al., 2022). It is likely that the co-contamination with acetate and CF limited the presence of toluene-degrading microorganisms within the microbial community, the first one through competition and the second through inhibition. As a matter of fact, CF has well documented toxic effects on bacteria, including anaerobic consortia (Pollice et al., 2001). As a result, the microbial community showed a reduced toluene degradation potential and thus a lower removal rate.

The maximum CF consumption rate obtained in this experiment (60 μmol L^{-1} d^{-1}) was lower as compared to the one obtained in previous experiments conducted with a two-vessel BES (132 μmol L^{-1} d^{-1}) (Fernández-Verdejo et al., 2022). The reason behind this decrease in CF degradation rate could be the lower supplied concentration of CF in the system, leading to overall lower reaction kinetics. A similar phenomenon was observed with toluene in previous studies conducted with the bioelectric well where a clear dependency of bioreaction rates on substrate concentration, in the mg/L range, was observed (Tucci et al., 2022c).

4.2. Effects of toluene concentration and acetate addition

The influent toluene concentration is a key-factor influencing the kinetics of the anodic bio-oxidation (Fig. 4A). In this study, toluene removal increased with the inlet concentration up to 280 μmol L^{-1}, where the system became apparently saturated. This is in contrast with previous studies, where a direct correlation between the amount of toluene and its removal rate was observed in a similar system up to 434 μmol L^{-1} (Tucci et al., 2022c). Once more, the effect of the co-contamination with CF may be the cause for the limitations in the toluene removal efficacy. Conversely, CF removal rate did not seem correlated to the concentration of toluene (Fig. 4B). This is likely due to the fact that, during cycles 2, 3 and 4, electrons were also generated by the oxidation of acetate, thus masking a possible correlation with the concentration of toluene.

In Fig. 5 it is possible to see how the degradation of toluene was lower when acetate was added to the inlet, both in absolute terms and as percentage of the contaminant load in the inlet. This phenomenon is probably caused by the fact that both toluene and acetate competed for the active catalytic sites at the anodic biofilm, with acetate likely being preferentially metabolized compared to toluene (Edwards and Grbic-Galic, 1994; Zhang et al., 2010). It is worth noticing that higher toluene degradation in absence of acetate indirectly resulted in higher CF removal. However, the mechanism behind this phenomenon is unclear and warrants further investigation. It is worth noticing that only in the absence of acetate (run 7), it was possible to reach an almost complete degradation of both contaminants.

4.3. Characterization of the mixed microbial communities

The amplicon sequencing of the 16 S rRNA gene revealed a highly selected microbial community both in the liquid effluent and in the biofilm taken at the end of the operation of the reactor (Fig. 6). In particular, the liquid effluent was mostly constituted by members of the genus *Propionicicella* (~ 2% of total reads) capable to produce acetic and propionic acids. Some *Propionicicella* strains (i.e. *P. superfundia* (Bae et al., 2006)), have been isolated from groundwater contaminated with chlorinated ethanes and vinyl chloride and are facultative anaerobe capable of utilizing fermentative metabolic strategies. *Candidatus Endomicrobium*, unidentified members of family *Lentimicrobiaceae* and *Acetobacterium* were also found (≥8%, each), the latter known to produce acetate and formate when grown in mixed bacterial culture with DCM and H_2 (Trueba-Santiso et al., 2017).

Furthermore, reads affiliated with families *Anaerolineaceae*, *Burkholderiaceae*, *Geobacteraceae*, and *Rikenellaceae* counted together up to 6.5% of total reads suggesting the potentialities of the effluent's microbiome in hydrocarbon degradation (Weelink et al., 2010). *Burkholderiaceae* is considered a key family of toluene degradation with several members, as for example *Ralstonia pickettii*, involved in the complete toluene degradation pathway via two successive ring-hydroxylating reactions (Lünsmann et al., 2016). The dominant role of this family can be due to occurrence of toluene monooxygenase and dioxygenases-encoding genes, which would allow the microorganisms harboring this type of enzymes to be adapted to grow on some aromatic compounds (Martínez-Lavanchy et al., 2015).

Differently from the liquid effluent, the microbiome composition of the biofilm grown on graphite rods was mostly represented by unidentified members of *Actinobacteria* order OPB41 (41.0%). The direct involvement of members of this order in toluene degradation was not discussed so far even though they were reported as a component of microbial community in previous works concerning hydrocarbons

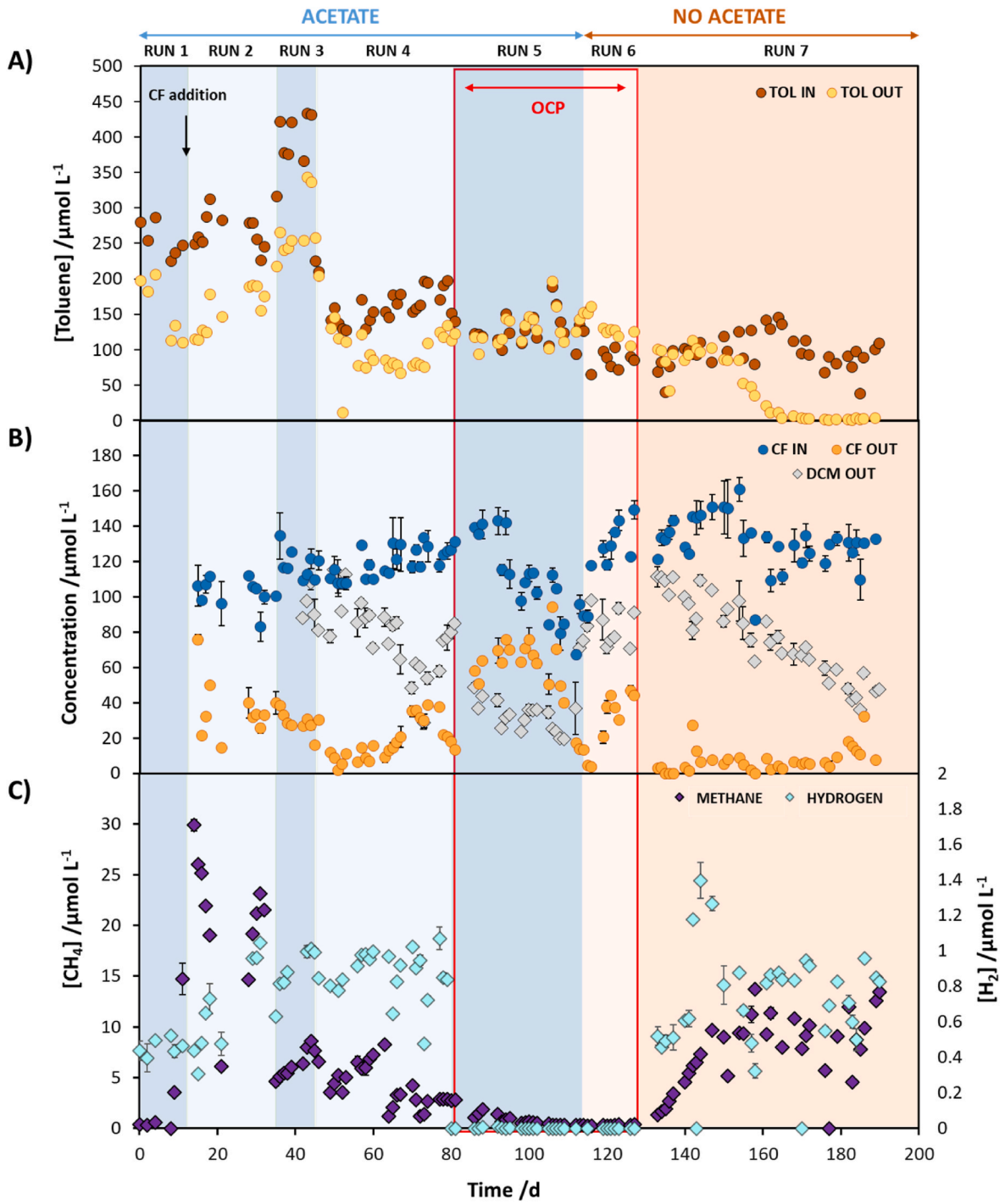

Fig. 3. A) Trend of toluene concentration in the inlet and in the outlet of the reactor. B) Trend of CF concentration in the inlet and in the outlet of the reactor and DCM formation in the outlet. C) Trend of H_2 and CH_4 formation in the outlet of the reactor.

Table 2
Reactor performances in terms of toluene and CF removal during polarized mode vs. OCP mode in absence of acetate.

	Polarized (Run 7)	OCP (Run 6)
Tol. rem. Rate (µmol L^{-1} d^{-1})	47 ± 3	0.1 ± 0.2
Tol. rem. %	96 ± 1	0.2 ± 0.2
CF rem. Rate (µmol L^{-1} d^{-1})	60 ± 1	47 ± 2
CF rem. %	94 ± 1	77 ± 1

degradation (Chen et al., 2016; Laso-Pérez et al., 2019). In line with previous evidence in similar bioelectrochemical studies (Tucci et al., 2022a, 2022c), the enrichment of the electroactive microbiome in the biofilm was suggested by the presence of members of family *Anaerolinaceae* (19.0%) and genus *Geobacter* (8.6%). Furthermore, 6.5% of total reads were affiliated with family *Lentimicrobiaceae*, comprising strictly anaerobic gram-negative bacteria able to form a consortium with other electroactive microorganisms to convert acetate into electric energy (Xiao et al., 2015; Zhu et al., 2022).

The key functional enzymes involved in the initial two steps of the anaerobic toluene and CF degradation pathways (Lueders, 2017; Tang

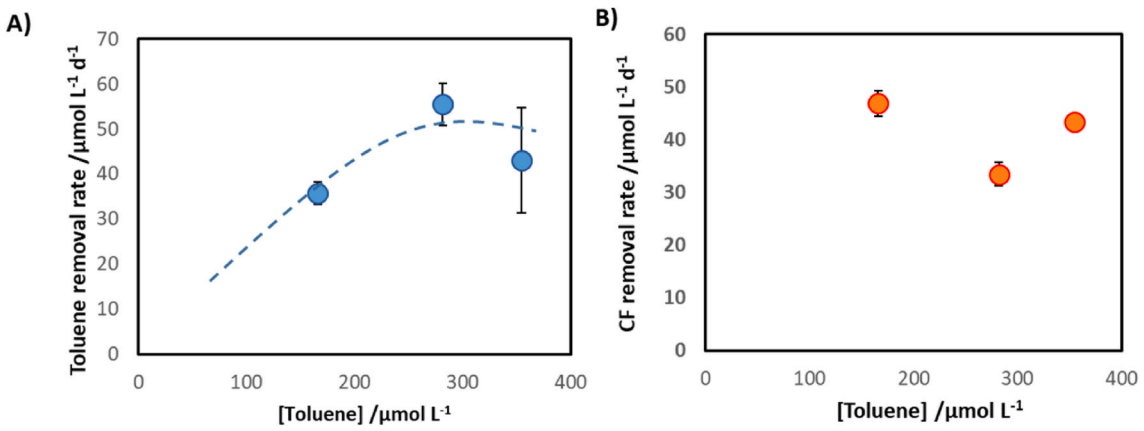

Fig. 4. Effect of the toluene concentration on the toluene A) and CF B) removal rates calculated as averages of the cycles 2,3 and 4.

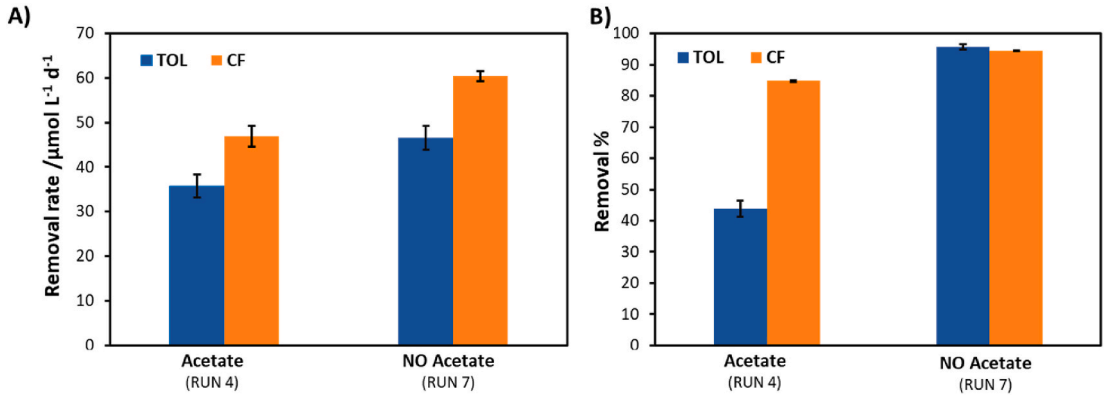

Fig. 5. Effect of acetate on the removal of toluene and CF in terms of rate A) and percentage B) of the contaminant load in the influent.

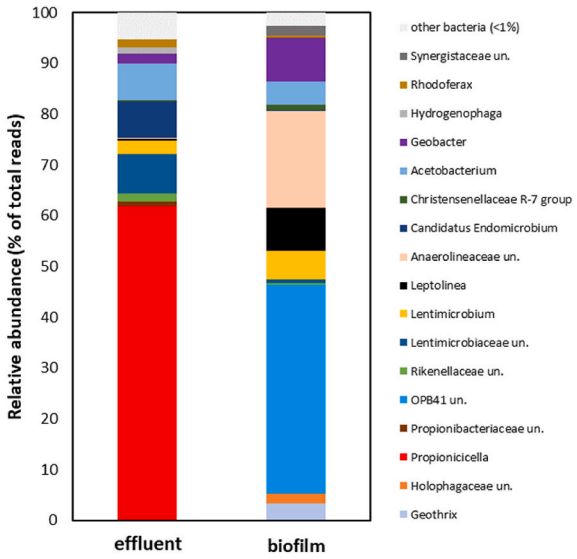

Fig. 6. - Bacterial community composition revealed by the 16 S rRNA gene amplicon sequencing. Data are expressed as relative abundance (% of total reads) of genera (≥1% in at least one sample) in the liquid effluent of the reactor and biofilm grown on graphite rods.

and Edwards, 2013; Von Netzer et al., 2016) have been quantified both in the liquid effluent and on the biofilm established on the graphite rods (Fig. 7).

The functional genes involved in the anaerobic toluene degradation are the benzylsuccinate synthase (*bssA*), encoding for the enzyme that catalyzes the anaerobic toluene activation by fumarate addition (Winderl et al., 2007); the *bcrC*, *bzdN* genes encoding for the ATP-dependent class I benzoyl-CoA reductases (Kuntze et al., 2011); the *bamB* gene encoding for the ATP-independent class II benzoyl CoA reductase (Löffler et al., 2011). Tese genes were highly abundant both in the effluent and in the biofilm of the graphite rods (Fig. 7A). In the liquid effluent, genes involved in the toluene degradation accounted for 1,73E+09 (*bcrC*), 4,62E+08 (*bzdN*), 4,95E+08 (*bamB*) and 2,75E+08 (*bssA*) gene copies L^{-1} (Fig. 7A). Similarly, in the biofilm of the graphite rods, the same concentration trend was observed, with *bcrC* gene as the most abundant one (1,1E+07 gene copies cm^{-1} graphite rod).

Regarding the CF degradation, the presence of the anaerobic bacteria *Dehalobacter* spp., responsible for the reductive hydrogenolysis of CF to DCM, and of *D. formicoaceticum* involved in the fermentation of DCM to acetate and formate, were also ascertained both in the liquid effluent and on the graphite surface, although not spotted by the 16 S rRNA gene amplicon sequencing (Grostern et al., 2010; Justicia-Leon et al., 2014). Moreover, process-specific biomarker genes were quantified to track CF to DCM (*cfrA* gene) transformation and DCM fermentation (*mecE* and *mecF* homologous genes belonging to the methylene chloride catabolism gene cassette, recently identified as prognostic and diagnostic tools supporting bioremediation of matrices impacted by DCM (Murdoch et al., 2022)).

16 S rRNA of *Dehalobacter* (*dre* gene in Fig. 7B) was found with 1,63E+09 16 S rRNA gene copies L^{-1} effluent and 4,15E+06 gene copies cm^{-1} graphite. *cfrA* gene was also found at high concentration in the liquid effluent (1.74E+09 gene copies L^{-1}) and on the graphite surface (1,18E+06 gene copies cm^{-1}). These results are in line with the observed performances of the reactor and previous evidence (Tucci

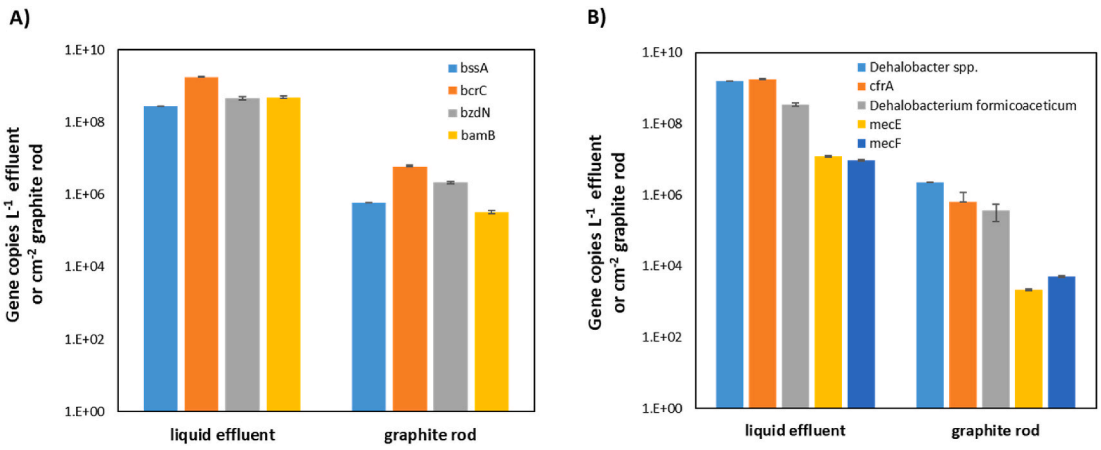

Fig. 7. Quantification of functional genes involved in toluene degradation (bssA, bcrC, bzdN, bamB) (7A), in CF to DCM degradation (*Dehalobacter* spp., cfrA) and in DCM fermentation (*D. formicoaceticum*, mecE and mecF genes).

et al., 2022a, 2022c) suggesting that, though likely catalyzed by different microorganisms, toluene utilization in the liquid medium and on the graphite's biofilm shared the same initial degradation steps likely commencing with fumarate addition and involving benzoyl-CoA as a central intermediate. Moreover, *D. formicoaceticum* was highly abundant in the liquid effluent (3,54E+08 gene copies L^{-1}) and in the biofilm (4, 15E+06 gene copies L˙cm). Similarly, *mecE* and *mecF* homologous genes were found both in the liquid effluent and on the graphite surface with high concentrations (Fig. 7B).

5. Conclusion

For the first time, the removal of toluene was coupled with CF degradation in a single-stage bioelectrochemical reactor. The bioelectric well proved once again to be effective in treating complex mixtures of contaminants and exploiting both the oxidation and the reduction reaction simultaneously. Furthermore, under optimal conditions, the reactor was able to achieve almost complete removal of the target contaminants. However, the presence of CF reduced the reactor's capability of degrading toluene as compared to previous studies. Indeed, it is likely that CF partially inhibited the activity of the anodic toluene-degrading microbial community. Another important finding is that a readily biodegradable substrate such as acetate has an adverse effect on the degradation of toluene, probably due to its competitive inhibition effects for the catalytic sites of the electroactive biofilm.

Taken as a whole, these results highlight the importance of studying the effect of multiple contaminants on the performance of novel bioremediation technologies, in order to properly address real-world scenarios. Thus, our findings are an important stepping-stone towards the application of the bioelectric well for in-situ bioremediation of multi-contaminated subsurface environments.

CRediT authorship contribution statement

Matteo Tucci: Conceptualization, Validation, Visualization, Writing – original draft. **David Fernández-Verdejo:** Investigation, Formal analysis, Data curation. **Marco Resitano:** Investigation, Formal analysis, Data curation. **Pamela Ciacia:** Investigation, Formal analysis, Data curation. **Albert Guisasola:** Conceptualization, Supervision. **Paqui Blánquez:** Conceptualization, Supervision. **Ernest Marco-Urrea:** Conceptualization, Supervision. **Carolina Cruz Viggi:** Conceptualization, Validation, Visualization, Writing – original draft. **Bruna Matturro:** Investigation, Formal analysis, Data curation. **Simona Crognale:** Investigation, Formal analysis, Data curation. **Federico Aulenta:** Conceptualization, Supervision, Writing – original draft, Project administration.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.139467.

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