Bioelectrochemical BTEX removal at different voltages: assessment of the degradation and characterization of the microbial communities

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Abstract

BTEX compounds (Benzene, Toluene, Ethylbenzene and Xylenes) are toxic hydrocarbons that can be found in groundwater due to accidental spills. Bioelectrochemical systems (BES) are an innovative technology to stimulate the anaerobic degradation of hydrocarbons. In this work, single chamber BESs were used to assess the degradation of a BTEX mixture at different applied voltages (0.8 V, 1.0 V, 1.2 V) between the electrodes. Hydrocarbon degradation was linked to current production and to sulfate reduction, at all the tested potentials. The highest current densities (about 200 mA/m^2 with a maximum peak at 480 mA/m^2) were observed when 0.8 V were applied. The application of an external voltage increased the removal of toluene, *m*-xylene and *p*-xylene. The highest removal rate constants at 0.8 V were: 0.4 ± 0.1 $days^{-1}$, 0.34 ± 0.09 $days^{-1}$ and 0.16 ± 0.02 $days^{-1}$, respectively.
At the end of the experiment, the microbial communities were characterized by high throughput sequencing of the 16S rRNA gene. Microorganisms belonging to the families *Desulfobulbaceae*, *Desulfuromonadaceae* and *Geobacteraceae* were enriched on the anodes suggesting that both direct electron transfer and sulfur cycling occurred. The cathodic communities were dominated by the family *Desulfomicrobiaceae* that may be involved in hydrogen production.

1. Introduction

Due to accidental spills BTEX compounds (Benzene, Toluene, Ethylbenzene and Xylenes) can contaminate soil and water, raising concern because they are recalcitrant, toxic for human health and relatively mobile in water [1].

Chemical, physical and biological strategies (bioremediation) are currently used for the remediation of BTEX contaminated environments [2]. Bioremediation relies on the ability of different groups of microorganisms to degrade organic contaminants which are used as a source of energy and carbon [3]. The goal of the biological technologies is to overcome the limitations to the microbial activity and to stimulate the biodegradation of the contaminants [3]. This can be achieved by supplying oxygen or other water soluble electron acceptors but it can be, in some cases, expensive and technically difficult [4,5]. Indeed, oxygen is rapidly depleted in groundwater and could diffuse away from the contaminated area [3]. Moreover, oxygen can react with reduced compounds such as Fe^{2+} and Mn^{2+}, which are normally present in hydrocarbon contaminated groundwater [6]. The limitations of the aerobic approaches for the stimulation of the microbial degradation may be overcome by developing new technologies. A strategy can be the use of bioelectrochemical systems (BESs) [7]. A BES is a device in which microorganisms degrade the organic matter in anaerobic conditions by using an electrode (anode) as final electron acceptor and generating an electrical signal [8]. Several advantages can be obtained by using BESs for the bioremediation. BES-based approaches can be cost effective because the anode is a virtually inexhaustible electron acceptor and the electron flux can be maintained constant over time [6]. When graphite electrodes are used the contaminants can be

adsorbed on the electron acceptor generating an area with high metabolic activity [6]. The electrical signal produced can be used in biosensors for the real time monitoring of the microbial activity [9,10].

Bioelectrochemical enhanced bioremediation could be particularly suitable for creating reactive zones in the aquifer where contaminants are efficiently removed. Particularly BESs could contribute to overcome some current limitations of biobarriers (BBs) applications. Indeed, BBs are an innovative strategy for *in-situ* bioremediation of contaminated groundwater. A BB is a passive system built by placing a permeable material in the contaminated aquifer to intercept the contaminated plume [11]. The support material (e.g. volcanic pumice) is colonized by microorganisms able to degrade the contaminants [12]. Oxygen can be supplied to stimulate the aerobic degradation in BBs but, due to the porosity of the support material, microenvironments with a low oxygen concentration may be present [13]. An alternative approach may be the stimulation of the microbial metabolism in anaerobic BBs by placing electrodes directly in the support material.

Electrodes have been shown to effectively stimulate the biodegradation of toluene and benzene in marine sediments and groundwater [6,14,15], however mixtures of hydrocarbons (e.g. gasoline), rather than single compounds, are often present in contaminated environments. BTEX compounds are among the main constituents of gasoline [16] but, to our knowledge, no information about the degradability of BTEX mixtures in BESs is available so far [7].

The main goal of this study was to investigate the degradability of a BTEX mixture at different voltages by using an anode as solid electron acceptor. Furthermore, the

microbial communities enriched during the treatment were characterized by sequencing of the 16S rRNA gene.

2. Materials and methods

2.1. Bioelectrochemical reactor set-up, operation and experimental conditions

Single chamber BES reactors [17] were constructed by using 120 mL serum bottles. Each bottle was filled with 30 g of commercially available volcanic pumice (Euroterriflora) as support material for microbial growth. Pumice was grinded and sieved prior to use in order to select particle size in the range of 2-5 mm. Volcanic pumice was chosen because it is a suitable support material for BBs [12]. Reactors were flushed with N_2 for 30 minutes in order to remove oxygen. After flushing, each reactor was sealed with a thick butyl rubber stopper (Rubber BV, Hilversum, Netherlands) and refinery wastewater (85 mL, COD 720 mg O_2/L, total P 1 mg/L, total N 20 mg/L) was injected as microbial inoculum. Reactors were flushed prior to the addition of the microbial inoculum in order to avoid stripping of the organic contaminants in the refinery wastewater. Graphite plates (10 cm^2, geometric area) connected to stainless steel wire (1 mm) were used both as anode and cathode. No catalysts were used on the cathode surface. The stainless steel wire and the electrical connections were covered with a heat shrinkable tube to prevent corrosion. The experiment was performed by applying three voltages (0.8 V, 1.0 V, 1.2 V) in duplicate using an external self made power supply. The current production was monitored with a data logger (Squirrel

SQ2010, Grant, Cambridge, UK), data were recorded every 1 minute and the average calculated every 30 minutes was reported. Furthermore, two open circuit controls (OC) and one abiotic control for each external voltage applied (i.e. 0.8 V, 1.0 V and 1.2 V) were set up. The reactors were run for few days in order to allow microbial colonization, a mixture of BTEX was then spiked and the concentration of hydrocarbons was monitored over time. At the end of the experiment samples of both the electrodes and the pumice were collected and stored at -20 °C for the microbial communities characterizations.

2.2. Chemical analyses

BTEX concentration was monitored by a GC-FID (Agilent 6890N) equipped with a headspace autosampler (Agilent 7697A). BTEX removal rate was estimated by the first order kinetic constants calculated during the second and the third batch cycles in order to minimize the effect of adsorption onto the electrodes. The first order kinetics constants were calculated by linear regression of time *vs* $\ln(C/C_0)$ were C is the measured concentration of each compound at each sampling time and C_0 is the initial concentration. The R^2 values are summarized in Table S1.

The determination of sulfate concentration was performed by ion chromatography (Thermo Scientific Dionex ICS-1100) with a detection limit (DL) of 0.1 mg/L. Before the analysis, several samples were diluted with deionized water.

Total phosphorus and total nitrogen were measured in the refinery wastewater by spectrophotometry following standard methods [18].

2.3. Amplification of 16S rRNA genes, sequencing and sequence analyses

The microbial biofilm was aseptically removed from the electrodes and the genomic DNA was extracted using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The same extraction kit was used for the DNA extraction from the pumice collected from the reactors and from the refinery wastewater used as microbial inoculum. The V5-V6 hypervariable regions of the 16S rRNA gene were PCR-amplified and sequenced by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) using a 250 bp x 2 paired-end protocol. The multiplexed libraries were prepared using the 783F and 1046R primers [19,20] modified by adding external barcodes in order to allow the parallel processing of multiple samples. The PCR was performed in 2 x 50 μL reactions with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 μM of each primer. Amplification conditions were: 94 °C for 5 minutes, 29 cycles with 94 °C for 50 seconds, 47 °C for 30 seconds, 72 °C for 30 seconds and a final elongation step of 72 °C for 5 minutes. The amplicons were then purified with the Wizard® SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions and quantified using Qubit® (Life Technologies, Carlsbad, CA, USA). All DNA samples were tested for amplification inhibition by sample dilution. Reads from sequencing were demultiplexed according to the internal barcodes. The Uparse pipeline was used for the following elaborations [21]. Forward and reverse reads were merged with perfect overlapping and quality filtered with default parameters. Singleton sequences (i.e.

sequence appearing only one time in the whole data set) were removed both from the whole dataset and from each sample dataset. Operational Taxonomic Units (OTUs) were defined on the whole data set clustering the sequences at 97 % sequence identity and defining a representative sequence for each cluster [14]. A subset of 10000 random sequences was chosen from each sample and the abundance of each OTU was estimated by mapping the sequences of each sample against the representative sequence of each OTU at 97 % sequence identity. Classification of the sequences representative of each OTU at different taxonomic ranks was done using the RDP classifier (≥ 80% confidence) [22]. The sequences were deposited in the European Nucleotide Archive with accession number PRJEB14978.

A post-hoc comparison of mean abundances (Tukey-Kramer test) at family and OTU level (95 % confidence level) was performed using STAMP [23]. The post-hoc tests were performed by applying the Benjamini-Hochberg FDR correction [24].

3. Results

3.1. Current generation and hydrocarbon degradation

After a short lag phase (about 4 days) the current density raised up to 220 mA/m^2 (average of the replicates at 0.8 V) (Fig. 1A), 170 mA/m^2 (average of the replicates at 1.0 V) (Fig. 1B) and 113 mA/m^2 (average of the replicates at 1.2 V) (Fig. 1C). Since no BTEX were added in the reactors, the current peak is linked to the degradation of the organic carbon added with the refinery wastewater used as microbial inoculum. After 12

days of incubation a BTEX mixture was spiked in each reactor and the current slowly increased to the highest value (~ 200 mA/m^2) in the reactor 0.8Va (day 42) and in the reactor 0.8Vb (day 48). On day 82 the BTEX mixture was re-spiked and the current density immediately raised up to 480 mA/m^2 in the reactor 0.8Vb while in the other reactors it remained on currents comparable to the values observed during the previous batch cycle. After the following spike of the BTEX mixture (day 116) the current did not increase and a slow decreasing was observed. The current density in the abiotic control at 1.2 V was under 20 mA/m^2 during the whole experiment (Fig. 1D). Similar result were obtained in the abiotic controls when 0.8 V and 1.0 V were applied (Fig. S1). The cumulative charge was calculated (Fig. S2) and the highest values were 800 C (0.8Va) and 1148 C (0.8Vb). At potentials higher than 0.8 V (i.e. 1.0 V and 1.2 V), cumulative charges between 580 C and 630 C were calculated.

Sulfate depletion from the medium was also observed, suggesting that sulfate acted as electron acceptor as well as the anode. Sulfate concentration decreased from about 250 mg/L to 3-6 mg/L after 160 days of incubation (Fig. S3).

BTEX were immediately removed after the addition both in the inoculated reactors (Fig. 2) and in the abiotic controls (Fig. S1 and Fig. S4). Toluene was removed at the end of each batch cycle in all the reactors with the exception of the open circuit controls (Fig. 2B). In the open circuit controls the residual concentration was 1.4 ± 0.1 mg/L (second batch cycle) and 1.5 ± 0.8 mg/L (third batch cycle) from an initial concentration of 9 ± 4 mg/L and 8 ± 3 mg/L respectively. Similarly to toluene, *m*-xylene was removed from all the reactors at which a voltage was applied. *m*-Xylene removal was complete at 0.8 V and 1.2 V, while at 1.0 V less than 1 mg/L of *m*-xylene was measured at the end of the second batch cycle, although complete removal occurred during the first and the third

batch cycles (Fig. 2E). In the open circuit controls the residual concentration of *m*-xylene was 1.1 ± 0.3 mg/L (second batch cycle) and 1.81 ± 0.01 mg/L (third batch cycle) from an initial concentration of 7 ± 4 mg/L and 7.3 ± 0.2 mg/L respectively. Among the other xylenes, *p*-xylene was completely removed only when 0.8 V were applied (Fig. 2F) while *o*-xylene was removed in all the tested conditions (with and without applying a voltage – Fig. 2D). However, *o*-xylene was completely removed also from the abiotic control at 1.2 V during the third batch cycle (Fig. S4), therefore it was not possible to link its removal to the microbial activity. Benzene (Fig. 2A) and ethylbenzene (Fig. 2C) removals were slower compared to the other BTEX compounds and no complete removal was achieved during the experiment.

The first-order kinetics constants were calculated during the second and the third batch cycle for benzene, toluene, ethylbenzene, *m*-xylene and *p*-xylene (Fig. 3). The rate constant was not calculated for *o*-xylene since it was completely removed also in one of the abiotic controls (1.2 V). The constants calculated for benzene were similar within the tested conditions and ranged from 0.012 ± 0.002 $days^{-1}$ (1.2 V) to 0.018 ± 0.002 $days^{-1}$ (OC). Similarly, the first order rate constants ranged from 0.038 ± 0.004 $days^{-1}$ (1.0 V) to 0.044 ± 0.004 $days^{-1}$ (OC) for ethylbenzene. Toluene, *m*-xylene and *p*-xylene showed a higher removal rate at 0.8 V (0.4 ± 0.1 $days^{-1}$, 0.34 ± 0.09 $days^{-1}$ and 0.16 ± 0.02 $days^{-1}$ respectively) compared to the other conditions.

3.2. Characterization of the microbial communities

The bacterial DNA was extracted from the electrodes surface, from the bulk of each reactor, and from the microbial inoculum. Microbial communities were characterized by High Throughput Sequencing (HTS) of the 16S rRNA gene.

A strong change in the composition of the microbial communities after the treatment was observed (Fig. 4 and Fig. S5). In the microbial inoculum, the most abundant families were the *Peptostreptococcaceae* (22%) and the *Pseudomonadaceae* (22%) whereas in the samples collected from the reactors these microorganisms were not enriched. The family *Desulfobulbaceae* was significantly more abundant on the anodes (from 46% on the anode of 1.0Vb to 81 % on the anode of 0.8Va) and in the bulk of the reactors when the voltage was applied (from 21% in the bulk of 1.0Vb to 47% in the bulk of 0.8Va) (Fig. S6). The enrichment of *Desulfobulbaceae* was lower on the samples collected from the open circuit controls (both from the graphite and from the bulk) and was negligible on the cathodic surface and in the microbial inoculum. The sequences of the most abundant OTUs (Fig. S7) were used to determine the best match to sequences contained in the RDP database. Within the family *Desulfobulbaceae* most of the sequences were phylogenetically close to *Desulfurivibrio alkaliphilus* but also microorganisms close to *Desulfobulbus rhabdoformis* were detected on the anodes (Fig. S7). Also the family *Desulfuromonadaceae* was enriched in the reactors in comparison with the inoculum, but the amount of sequences belonging to this family was similar in the samples collected from the open circuit controls, from the bulk and from the anodes of the other reactors. During the treatment, the family *Geobacteraceae* was also enriched on the anodes. However, the abundance of *Geobacteraceae* in the bulk of the open circuit controls was higher compared to the anodes (Fig. S6). A more in depth

analysis at OTU level revealed that members of this family close to *Geobacter lovleyi* were selected on the anodes as well as in the bulk of the open circuit controls (Fig. S7). The communities enriched on the cathodes were dominated by the family *Desulfomicrobiaceae* that ranged from 89% (1.2Va) to 99% (0.8Va) (Fig. 4 and Fig. S6). Most of the sequences of the family *Desulfomicrobiaceae* belong to a microorganism close to *Desulfomicrobium baculatum* (Fig. S7).

4. Discussion

The degradation of a BTEX mixture in BES reactors was studied by applying a range of voltages (0.8 V, 1.0 V, 1.2 V). The best efficiency, both in term of bioelectrochemical performances (i.e. maximum current and cumulative charge) and hydrocarbon removal, was observed in the reactors where the lower voltage (0.8 V) was applied. Although the electrode potentials were not measured in this study, the anode potential should be higher when higher voltages are applied. The degradation (i.e. oxidation) is therefore expected to increase when 1.2 V are applied instead of 0.8 V. Several studies have investigated the effect of the anode potential in BESs and, despite the use of more positive potentials generally increases the performances, in some case the best results were obtained at the lower potentials [25]. This discrepancy suggests that the potential of the terminal respiratory proteins, rather than the anode potential, influences the performances of the reactors [25]. Different terminal respiratory proteins may be produced not only when different microbial populations grow on the anode, but

also when the same pure culture grows on anodes polarized at different potentials as observed with *Geobacter sulfurreducens* [26].

BTEX removal started without a lag phase because of the adsorption on the electrodes as previously shown when graphite electrodes are used for the stimulation of hydrocarbon removal in BESs [6,14]. When the external voltage was applied, BTEX removal was improved compared to the open circuit controls. The degradation rate of toluene and *m*-xylene increased at all the tested potentials compared with OC whereas the enhancement of the degradation of *p*-xylene was observed only in the reactors at 0.8 V. Conversely, the application of a potential did not improve the removal of benzene and ethylbenzene. These data are consistent with the reported microbial degradability of BTEX compounds in anaerobic conditions. Benzene is the most recalcitrant compound among BTEX [27]. Toluene degradation was extensively observed both by isolates and pure cultures with several electron acceptors (i.e. NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-} and CO_2) and *m*-xylene is reported to be the most degradable among the xylenes [27]. Ethylbenzene has a structure similar to toluene but it is usually degraded using a different pathway, thus the biodegradability can be different [27].

The presence of multiple compounds can lead to interactions (i.e. stimulation or inhibition) during the degradation [28]. In the present study the degradation rate of toluene, *m*-xylene, and partially of *p*-xylene was higher compared to the other hydrocarbons in the mixture. However, it is not possible to disentangle if the lower degradation rate of other compounds (i.e. benzene and ethylbenzene) is due to inhibition or to the lack of specific pathways.

BTEX removal was coupled both to current production and to sulfate reduction. Maximum current densities of about 200 mA/m^2 with a peak at 480 mA/m^2 were

observed during the experiment and were coupled to hydrocarbon removal. Similar current densities (283 to 341 mA/m^2) were achieved during toluene degradation via sulfur cycling in dual chamber BES reactors inoculated with marine sediments, when the anode was polarized (0 and + 300 mV *vs* Ag/AgCl) [14]. Lower current densities were obtained during bioelectrochemical toluene degradation with a pure culture of *Geobacter metallireducens* (~ 150 mA/m^2) [6] and during total petroleum hydrocarbon degradation in soil with an electrode serving as electron acceptor (86 mA/m^2) [29]. Because the anode and the cathode were placed in the same chamber, they both potentially influenced the evolution of microbial communities as well as the generated current density. Currents up to 2.1 ± 0.2 A/m^2 (corresponding to about 190 mA/m^3) were obtained in single chamber BES reactors during refinery wastewater treatment when 0.7 V were applied [30] but, in another work, lower values (~ 10 mA/m^3) were achieved when a dual chamber microbial fuel cell was used [31]. The total amount of BTEX removed during the experiment ranged between 110 mg/L and 125 mg/L during the three batch cycles. Hypothesizing that toluene was the sole electron donor, between 3.7 mmol and 4.1 mmol of electrons should have passed in the circuit from the first to the third batch cycle. Actually, the total amount of electrons passed in the circuit ranged between 5.7 mmol and 11.6 mmol. It is thus reasonable that other oxidation reactions occurred at the anode, increasing the electron transfer. It was demonstrated that, when an anode is used as electron acceptor in a single chamber BES, H_2 can be produced at the cathode and then consumed in the bulk by sulfate reducing bacteria. Indeed, it is well known that sulfate reducing bacteria are able to use H_2 as electron donor [32]. However, previous investigations also hypothesized that, depending on the balance between H_2 production and consumption rates in the bulk, hydrogen could reach the

anode where it could be eventually used as electron donor, thus enhancing the current production in a process called hydrogen recycling [17,33,34]. Similarly to hydrogen recycling, sulfur cycling may have influenced the current production. Hydrocarbon degradation can proceed by sulfate reduction to sulfide. Sulfide are then oxidized to elemental sulfur on the anode surface by a chemical reaction, or by a biological reaction mediated by members of the family *Desulfobulbaceae*, and further re-reduced to sulfide by members of the family *Desulfuromonadaceae* leading to internal sulfur cycling and increasing the current production [14]. A possible role of sulfur cycle has been suggested also in the electron transfer process on the air cathode of single chamber microbial fuel cells [35,36]. Indeed, sulfide produced by sulfate reducers can be oxidized to elemental sulfur by purple non-sulfur bacteria and then back oxidized to sulfate [35,36].

Microorganisms linked to the sulfur cycle were enriched during the treatment. The higher abundance of the family *Desulfobulbaceae* was observed in the samples collected from the reactors where the voltage was applied. Most of the sequences classified as *Desulfobulbaceae* were close to *Desulfurivibrio alkaliphilus* (score 0.712). Filamentous bacteria close to *Desulfurivibrio alkaliphilus* were found to perform long range electron transfer in marine sediment and to couple sulfide oxidation and oxygen reduction [37]. Interestingly, in this study, microorganisms close to *Desulfurivibrio alkaliphilus* were abundant both on the anodes and in the bulk when the voltage was applied. It is thus reasonable to hypothesize that putative cable bacteria were involved in the electron transfer from the bulk to the anode. The presence of microorganisms close to *Desulfurivibrio alkaliphilus* has been observed also in sediment cores collected at the oxic-anoxic interface in an hydrocarbon-contaminated aquifer [38]. Members of the

family *Desulfobulbaceae* have been identified by stable isotope probing as putative toluene degraders in toluene degrading microbial communities in sulfate reducing conditions [39,40]. Despite it is not possible to clearly identify the microorganisms involved in hydrocarbon removal in this study, the involvement of the family *Desulfobulbaceae* could be put forward.

Also microorganisms of the family *Desulfuromonadaceae* were found on the anodes, enforcing the hypothesis that internal sulfur cycling occurred with sulfide acting as mediator of the electron transfer to the anode. Members of the families *Desulfobulbaceae* and *Desulfuromonadaceae* (i.e. *Desulfobulbus propionicus* and *Desulfuromonas* strain TZ1) were also able to oxidize elemental sulfur to sulfate with an electrode serving as electron acceptor [41]. Thus, their role in the back oxidation of elemental sulfur to sulfate during internal sulfur cycling in this study can be hypothesized. The presence of the family *Geobacteraceae* however suggested that also direct electron transfer may have occurred. Members of this family belonging to the genus *Geobacter* have been extensively described both as hydrocarbon degraders [42,43] and bioelectrogenic [44,45]. Furthermore, the ability of *Geobacter metallireducens* to degrade toluene in a BES using the anode as electron acceptor has been demonstrated [6]. In this experiment the family *Geobacteraceae* was enriched also in the bulk of the open circuit controls. This result may be linked to the stimulation of the microbial metabolism due to the presence of a conductive material (i.e. graphite) that facilitates the electron transfer processes [46].

The cathodic communities were dominated by members of the family *Desulfomicrobiaceae* phylogenetically close to *Desulfomicrobium baculatum*. The family *Desulfomicrobiaceae* belong to the order *Desulfovibrionales*, the same order of

the genus *Desulfovibiro*. *Desulfovibrio paquesii* is able to use an electrode as electron donor and to produce H_2 [47]. Furthermore, the presence of NiFe hydrogenases was reported in *Desulfomicrobium baculatum* and it was demonstrated that, when an electrode is poised at a negative potential, NiFe hydrogenases are able to catalyze H_2 production [48]. It is therefore likely that the family *Desulfomicrobiaceae* in this study is linked to H_2 production at the cathode. However, the investigation of the electrode potentials in future experiments may be helpful for a deeper characterization of the complex reactions in BESs during removal of hydrocarbons.

5. Conclusions

BES reactors were used for the stimulation of the degradation of a BTEX mixture and three voltages were tested (0.8 V, 1.0 V and 1.2 V). The bioelectrochemical treatment improved the degradation of toluene, *m*-xylene and *p*-xylene compared to the open circuit controls. The best performances were observed by applying a voltage of 0.8 V. Complex electron transfer mechanisms involving hydrogen and sulfur cycling have been hypothesized (Fig. 5), but further experiments have to be performed in order to clearly elucidate the interactions between the microbial populations and to identify the key hydrocarbon degraders.

It is crucial to consider that *in-situ* conditions (e.g. concentrations of the contaminants, groundwater conductivity, mass transport, presence of alternative electron acceptors) can influence the process and that observations made in simple batch reactors cannot be immediately transferred to large scale plants. Data presented here suggest that BES-BBs

may be an effective strategy for the treatment of gasoline contaminated groundwater. A possible application for BES-BBs may involve the placing of electrodes in the support material (e.g. volcanic pumice), or the use of conductive support materials (e.g. graphite granules), with configurations similar to the “Lasagna Process” [49], as already hypothesized for the bioelectrochemical treatment of groundwater contaminated by chlorinated solvents [7]. Alternatively, the use concentric electrodes, may overcome some of the limitations of the above mentioned configuration (e.g. electrode spacing) [7]. Future studies have to be directed to the progressive scale-up of the technology.

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Figure legends

Fig. 1 - Current density measured in the reactors at 0.8 V (A), 1.0 V (B), 1.2 V (C) and in the abiotic control at 1.2 V (D). The arrows indicate when the BTEX mixture was spiked. Current production was observed regardless of the applied voltage.

Fig. 2 - Concentration of benzene (A), toluene (B), ethylbenzene (C), *o*-xylene (D), *m*-xylene (E) and *p*-xylene (F) measured in the reactors when different voltages (0.8 V, 1.0 V, 1.2 V) were applied and in the open circuit controls. The removal of toluene, *m*-xylene and *p*-xylene was stimulated when the voltage was applied compared to the open circuit control.

Fig. 3 - Average first order kinetic constants and standard errors calculated for the hydrocarbons in the mixture during the second and the third batch cycle. The highest kinetic constants were obtained when 0.8 V were applied between the electrodes.

Fig. 4 - Taxonomic composition at family level of the microbial communities enriched during the treatment. The families with a relative abundance of 5% (or higher) in at least one sample are reported. The family *Desulfobulbaceae* was enriched on the anodes and the family *Desulfomicrobiaceae* dominated the communities enriched on the cathodes.

Fig. 5 - Possible electron transfer mechanisms involved during bioelectrochemical BTEX removal. Hydrocarbon degradation can occur by direct electron transfer to the electrode involving the *Geobacteraceae* (A) and by sulfate reduction (e.g. by members of the family *Desulfobulbaceae*) (B). Sulfide produced by sulfate reduction is oxidized to elemental sulfur on the anode by members of the family *Desulfobulbaceae* (C). The family *Desulfuromonadaceae* can be involved in the reduction of elemental sulfur to sulfide (D). Members of the families *Desulfobulbaceae* and *Desulfuromonadaceae* can be also involved in the back oxidation of elemental sulfur to sulfate (D and E). The family *Desulfomicrobiaceae* can be involved in hydrogen cycling (F). Hydrogen can be produced at the cathode and can be used as electron donor by microorganisms (i.e. by sulfate reducers) (G).