UNIVERSITY OF MILANO-BICOCCA

Department of Earth and Environmental Sciences
PhD in Environmental Sciences (Cycle XXVII)

HYDROCARBON DEGRADATION WITH SOLID ELECTRON ACCEPTORS. MICROBIAL COMMUNITIES CHARACTERIZATION AND POTENTIAL APPLICATIONS

Tutor: Dr. Andrea Franzetti
Co-tutor: Prof. Korneel Rabaey

FINAL DISSERTATION
Matteo Daghio
Matr. 072165

If you can't fly then run, if you can't run then walk, if you can't walk then crawl, but whatever you do you have to keep moving forward.

Martin Luther King Jr.

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Bioelectrochemical based technologies for petrochemical

Abstract

Petroleum hydrocarbons are widely used both for fossil fuel and chemicals production. Due to their large use they can be accidentally released in the environment causing concern due to their toxic effects. Over 2 million potential contaminated sites were estimated only in Europe, and huge efforts have been made during the past years to develop strategies for the remediation of hydrocarbon contaminated sites. Both chemico-physical strategies and biological strategies have been used. While the former approach often allows reaching the goal of remediation more quickly, the latter has two main advantages: (i) the contaminant is completely mineralized, or at least transformed into a less harmful compound, instead then transferred into another matrix; (ii) a biological approach is often cheaper than a chemico-physical approach. Bioremediation relies on the ability of a wide range of microorganisms to degrade hydrocarbons by using them as carbon source. The degradation is stimulated by overcoming the limitations to the microbial activity usually supplying nutrients or electron acceptors. The most widely used approach is supplying oxygen. Oxygen can act both as a key reagent during the first step of the hydrocarbon degradation and as a thermodynamically favorable electron acceptor but is quickly consumed in contaminated sites. Faster degradation rates can be achieved in aerobic condition compared to the anaerobic conditions. However, supplying oxygen could be expensive and technically difficult: its solubility in water is low; oxygen can diffuse away from the contaminated area or being depleted by reactions with reduced species, such as Fe^{2+} or Mn^{2+}, requiring thus continuous amendment.

During the 80's the ability of microorganisms to degrade hydrocarbons in anaerobic conditions was described. Since then many anaerobic approaches to stimulate the microbial degradation have been reported.

Recently, the use of bioelectrochemical systems (BESs) was proposed as an effective technology for the bioremediation of anaerobic matrixes contaminated both by aromatic and aliphatic hydrocarbons. BESs are devices in which an electrode (anode) serves as external electron acceptor during the anaerobic biodegradation of organic compounds. The electrons transferred to the solid anode pass throughout a circuit producing current and reaching a second electrode (cathode). The circuit is closed by using the electrons and the protons produced during the degradation of the organic matter for the cathodic reduction of a final electron acceptor. The anode is thus a virtually inexhaustible electron acceptor. Furthermore, when graphite or carbon anodes are used, hydrocarbons are adsorbed concentrating the electron donor, the electron acceptor and the degradative microorganisms.

The goal of this PhD thesis was to give new insights for the application of BES technology during hydrocarbon biodegradation.

Hydrocarbon degrading microbial communities in bench scale aerobic biobarriers for gasoline contaminated groundwater treatment. Biobarriers are a relatively new *in situ* technology based on the placement of a permeable medium into the aquifer to intercept the contaminants and promote the biodegradation by stimulating the microbial activity. A detailed comprehension of the mechanisms involved during hydrocarbon biodegradation in such systems will be helpful in determining the applicability of BES-based biobarriers. BTEX compounds (benzene, toluene, ethylbenzene and xylenes) and methyl *tert*-butyl ether (MTBE) are widespread groundwater contaminants. In this work the microbial communities in a bench scale aerobic biobarrier for gasoline contaminated groundwater treatment were studied by 16S rRNA gene Illumina sequencing and catabolic genes quantification by qPCR. Two different bench scale columns, with and without a selected microbial inoculum were studied. The microbial inoluculum was composed by using three pure cultures (*Rhodococcus* sp. CE461, *Rhodococcus* sp. CT451 and *Methylibium petroleiphilum* LMG 22953). No differences were observed for the two tested conditions in term of removal efficiency, although microbial communities were different in the two experiments. The microbial communities detected in the uninoculated test were dominated by the genus *Thauera*, suggesting the presence of microenvironments with low dissolved oxygen concentration. Conversely, the inoculated column was dominated by aerobic bacteria and *Rhodococcus* was the most abundant genus both before and after the treatment, although it significantly decreased during the column operation. Both the 16S rRNA gene sequencing and the qPCR results clearly showed that the microbial inoculum was outcompeted by the native microorganisms of the pumice, and the bioaugmentation did not increase the degradative potential. The use of an aerobic biobarrier is an effective strategy to remediate gasoline contaminated groundwater and the addition of a microbial inoculum may be not necessary to improve the degradative potential of the indigenous microorganisms. However, different oxygen concentrations along the simulated biobarrier affected the microbial community and the biodegradation rate of pollutants suggesting that controlling oxygen concentration is crucial to allow a high efficiency of the biobarrier.

Bioelectrochemical toluene degradation in marine environments. Accidental oil spills can lead to considerable release of toxic petroleum hydrocarbons in marine environments. Benthic BESs have been already described as a suitable technology to power oceanographic devices on the ocean floor, by harvesting energy from anaerobic sediments and their applicability for the bioremediation of marine environment have been proposed. In this work an alternative anaerobic strategy to remove

monoaromatic hydrocarbons from marine environments by stimulating the biodegradation with an electrode as solid electron acceptor was investigated. Using toluene as a model compound, degradation was accomplished in BES with simultaneous current production. Glass BES reactors were set up using sediment collected from an hydrocarbon contaminated marine site as microbial inoculum, and artificial ocean water as growth medium. Different replicates with anode potentials of 0 mV and +300 mV (vs Ag/AgCl) were tested in order to check their influence on current production, enrichment of electrocatalytically active microorganisms and hydrocarbon degradation rate. At the end of the experiment, microbial characterization of the communities attached to the electrodes and in the bulk of each reactor was performed by Illumina sequencing of the 16S rRNA in order to get further insights into the relationship between the enriched communities and metabolic processes, particularly focusing on the sulfur cycle. Degradation of toluene was directly linked to current generation up to 301 mA m^{-2} for the reactors with the anode polarized at 0 mV (vs Ag/AgCl) and up to 431 mA m^{-2} for the reactors with the anode polarized at +300 mV (vs Ag/AgCl), however over time decreasing peak currents were obtained upon renewed spiking. In both the conditions a degradation rate of ~1 mg L^{-1} d^{-1} was observed. Monitoring of sulfate/sulfide concentrations during bioelectrochemical experiments suggested that sulfur metabolism might play an important role in toluene degradation on a bio-anode, potentially leading to passivation over time. The microbial communities were dominated by sulfate reducing microorganisms, particularly the family *Desulfobulbaceae* appeared to be linked both with current production and toluene degradation. The results allowed putting forward two mechanisms for toluene degradation in BESs: (i) by direct electron transfer to the anode, or (ii) by sulfate reduction with sulfide acting like an electron shuttle to the electrode. The competition between the two

metabolisms might be crucial to determine the applicability of BES based technologies for hydrocarbons degradation in sulfate rich environments.

Development of BES-based biobarriers for gasoline contaminated groundwater treatment. In order to achieve a successful bioremediation, controlling the electron acceptor delivery is crucial. Bioelectrochemical systems (BES) could be an alternative approach to stimulate the anaerobic degradation in biobarriers for gasoline contaminated groundwater treatment. BES-based biobarriers could be developed by placing the anode (reactive area) in the anaerobic contaminated aquifer, while the cathode could be buried in the surface soil where aerobic conditions occur and oxygen can be used as electron scavenger. Several engineering parameters (i.e. electrode material, electrode spacing, voltage applied, potential applied) might strongly affect the overall performance of the biobarrier. Furthermore, hydrocarbon degradation might be different when mixtures (i.e. gasoline) or single compounds are present due to interactions between the contaminants. In this preliminary work a high throughput BES reactor was used to stimulate the degradation of a BTEX and MTBE mixture by applying 1 V between the electrodes. Current production up to 420 mA m^{-2} coupled to hydrocarbon degradation was observed. However only toluene (1.1 ± 0.2 mg L^{-1} d^{-1}) and *p*-xylene (0.25 ± 0.04 mg L^{-1} d^{-1}) were degraded while the other contaminants in the mixtures appeared to be more recalcitrant to microbial degradation. Further studies will be helpful to assess the effect of substrate interaction and of different voltages on the degradation efficiency and on the microbial community composition.

Introduction

CONTAMINATED SITES IN EUROPE

The world demand of petroleum for fuel and chemicals production grew by 1.5 million barrels per day in 2013, 0.23 million barrels per day more than July 2012. This growth was led particularly by developing countries, such as China, that increased its petroleum demand by 0.33 million barrels per day both in 2012 and 2013. Conversely, a negative trend was observed in Europe. The European petroleum demand decreased by 0.17 million barrels per day in 2013 following the same trend of the past years. This reduction was particularly due to the continuous increasing in the taxation policies against oil usage (1).

Petroleum is a complex mixture containing more than 17,000 chemical components. Four main groups of chemicals can be identified: the saturated hydrocarbons and the aromatic hydrocarbons, the more polar fraction, and the non-hydrocarbon components the resins and the asphaltenes (2). Petroleum hydrocarbons are widely used for fossil fuel production. Monoaromatic hydrocarbons, such as BTEX compounds (benzene, toluene, ethylbenzene and xylenes), and additives, such as methyl *tert*-butyl ether (MTBE) and ethyl *tert*-butyl ether (ETBE) are some of the main constituents of gasoline (3, 4) and due to incidents can be released in the environment and contaminate soil and water (5, 6).

In addition to petroleum hydrocarbons, metals and halogenated compounds can be released in the environment causing concern. For example chlorinated solvents (such as perchloroethene and tricholoethene) are widely used compounds and are frequently detected in groundwater (7).

According to the European Environment Agency (EEA) the following definitions can be provided (8):

- contaminated site: a well-defined area in which the presence of contaminants has been proven and represents a potential risk for the human health and for the environment;
- potentially contaminated site: an area in which an unacceptable contamination is suspected but not verified and further investigations are needed.

A review of the current situation in Europe has been recently provided and lead to an estimation of 2,553,000 potentially contaminated sites and 342,000 contaminated sites of which only 58,336 have been already remediated (8). The same study investigated also the sectors that contribute more to site contamination reporting that the main source is the waste disposal and treatment sector (37%), followed by the commercial activities (33%), the storage (11%) and the transport (8%) (Fig. 1A). Focusing on the industrial and commercial sector about 60% of contaminations derives from the production sector and 33% from the service sector, while only 7% of contaminations is due to the mining sector (8) (Fig. 1B).

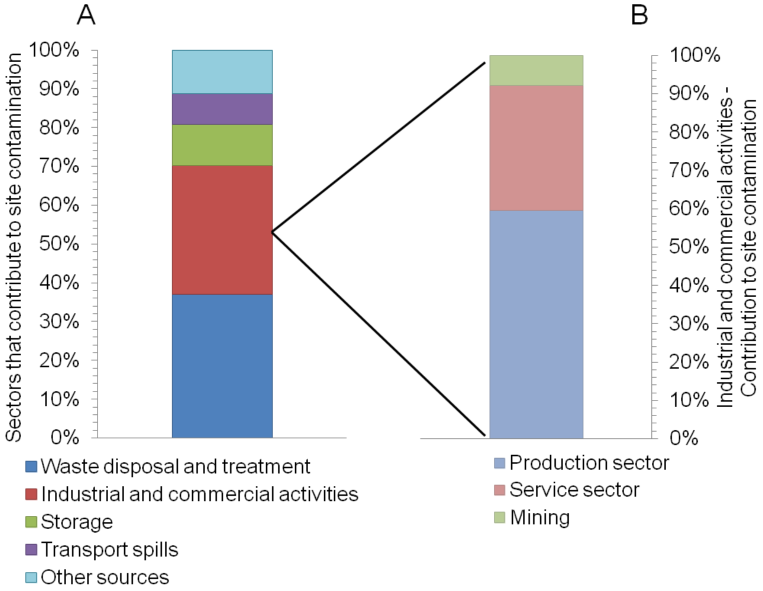

Fig. 1 – Sectors contributing to site contamination in Europe. Adapted from Panagos et al. 2013 (8).

The main contaminants in soil and groundwater are heavy metals and mineral oil, followed by BTEX compounds, polycylic aromatic hydrocarbons (PAHs) and chlorinated hydrocarbons (8) (Fig. 2).

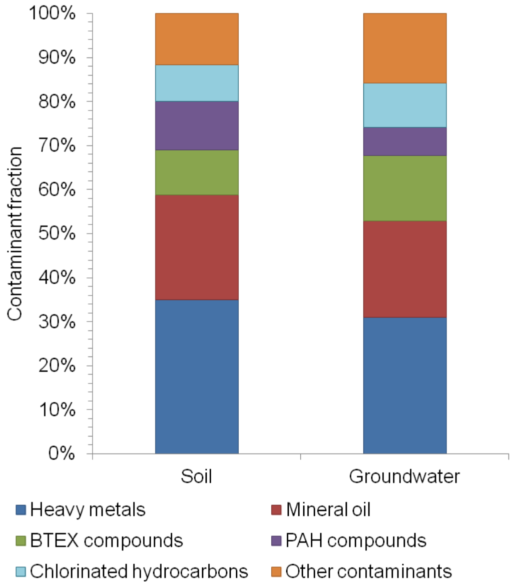

Fig. 2 – Contaminants in soil and groundwater in Europe. Adapted from Panagos et al. 2013 (8).

These contaminants can have a broad range of toxic effects affecting both the human health and the environment. Non-halogenated hydrocarbons, such as BTEX compounds, are hazardous pollutants that can cause severe diseases as for example respiratory irritation, central nervous system damages and cancer (9). Epidemiological evidences proved that the exposure to chlorinated hydrocarbons can be correlated to a multitude of human cancers, such as lymphoma, leukemia, liver and breast cancer (10).

For these reasons during the last decades huge efforts were carried out to develop strategies to remove hazardous contaminants from the environment and remediate contaminated sites.

BIOLOGICAL HYDROCARBONS DEGRADATION

Biological degradation of aromatic hydrocarbons has been reported both in aerobic and anaerobic conditions (11).

Aerobic degradation

The aerobic degradation of hydrocarbons is an easier process compared to the anaerobic degradation. During this process oxygen is both a thermodynamically favorable electron acceptor (the redox potential of the O_2/H_2O couple is +820 mV vs SHE) and a key reagent that allows the activation of the hydrocarbons and consequently the degradation.

The aerobic degradation of BTEX compounds involves two multicomponent enzyme systems:

- monooxygenases, that catalyze the incorporation of an hydroxyl group on the aromatic ring or on the alkyl group (Fig. 3A);
- dioxygenases, that catalyze the incorporation of two hydroxyl group on the aromatic ring (Fig. 3B).

This activation step (called upper pathway) leads to the formation of a substituted catechol that undergoes the ring cleavage carried out by a dioxygenase in the lower pathway (Fig. 3C). When the aromatic ring is open the molecule can be completely oxidized in the central metabolic pathways (11).

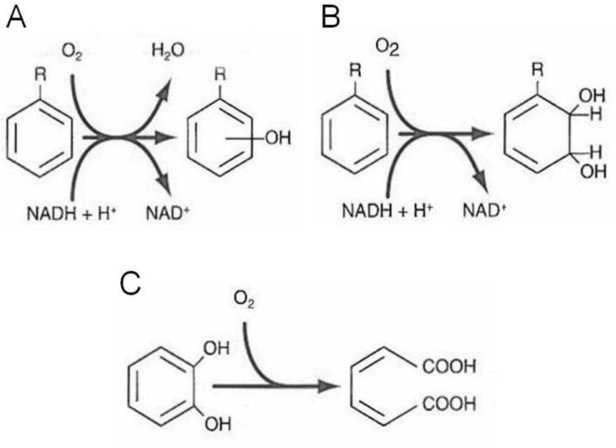

Fig. 3 – Oxygenases involved in the aerobic degradation of BTEX compounds. A) hydroxylation catalyzed by a monooxygense, B) hydroxylation catalyzed by a dioxygense, C) rig cleavage catalyzed by a dioxygense. Adapted from Barbieri et al. 2008 (12).

While lower pathways for the degradation of the monoaromatic hydrocarbons are conserved, several upper pathways can be used affecting thus the degradability of the different BTEX (12). Toluene is known to be the most degradable BTEX and several microorganisms able to mineralize it both in pure cultures and in consortia have been described; conversely, benzene appears to be the most recalcitrant BTEX. The three xylenes isomers have different degradability, while *o*-xylene is described to be the most recalcitrant, *m*-xylene and *p*-xylene are more easily degraded, often by using the same metabolic pathways involved in toluene degradation (11, 12).

Due to its chemical structure the biodegradation of MTBE is a very slow process. The ether bond and the steric effect due to the presence of the three methyl groups impede the activation step. The first attack to the MTBE molecule is carried out by a monooxygenase that hydroxylates the methoxy carbon, the product of this reaction is rapidly and abiotically decomposed to *tert*-butyl alchol (TBA) and formaldehyde. TBA often accumulates and is only slowly degraded through a series of reaction that lead first to the formation of 2-hydroxyisobutyric acid and then to the complete mineralization (Fig. 4) (13).

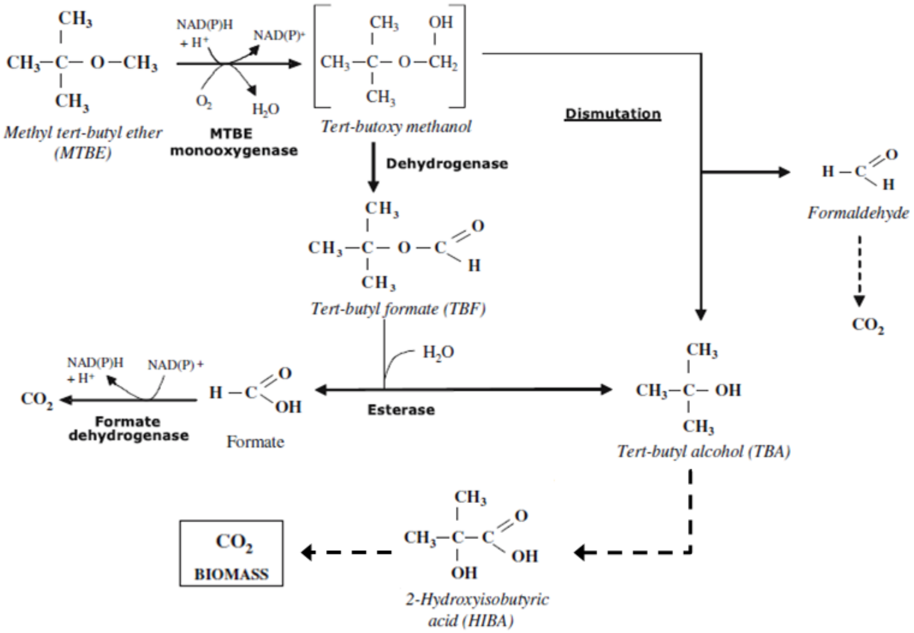

Fig. 4 – Aerobic degradation of MTBE. Adapted from Lopes-Ferreira et al. 2006 (13).

The monooxygenases involved in MTBE degradation include different forms of alkane hydroxylases and cytochrome P450s (14).

Anaerobic degradation

Starting from the 80’s the improvement of the cultivation methods in anaerobic conditions led the discovery of hydrocarbon degradation mechanisms that did not involve oxygen, neither for the activation step nor as electron acceptor. The anaerobic hydrocarbon degradation however is a very slow process that often requires the use of energy in the form of ATP during the first steps (12, 15).

During the anaerobic degradation the aromatic compounds are converted into a small number of central intermediates, among which the most common one is benzoil-CoA. Benzoil-CoA is then further degraded through an upper pathway, that leads to an aliphatic C_7-dicarboxyl-CoA derivative, and a lower pathway that generates three acetyl-CoAs and CO_2 (Fig. 5) (15).

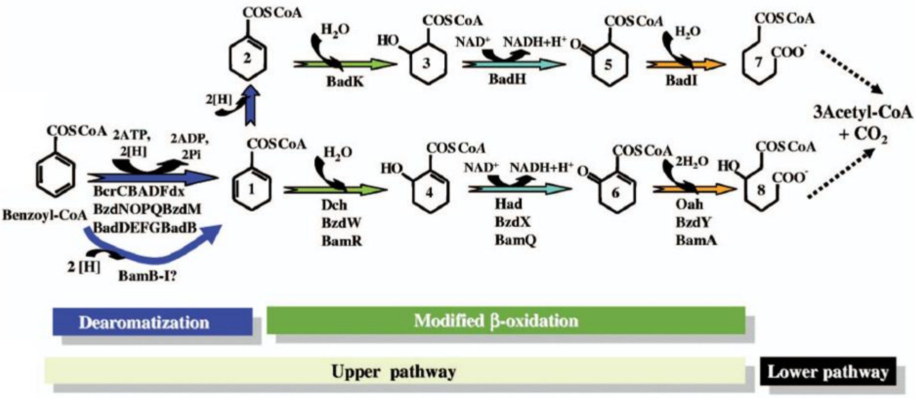

Fig. 5 – Benzoyl-CoA degradation pathway. Adapted from Carmona et al. 2009 (15).

Several metabolic pathways bring to the formation of the central intermediates. The anaerobic degradation of toluene is the most extensively studied because this compound is the most easily degradable among the BTEX. In anaerobic conditions toluene can be degraded with NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-} and CO_2 as terminal electron acceptors both by isolates or mixed cultures (16). The key step during toluene degradation is the addition of fumarate to form benzylsuccinate. This reaction is catalyzed by the benzylsuccinate syntethase (*bssABC*). Bezylsuccinate is then converted to benzoyl-CoA (Fig. 6) (16).

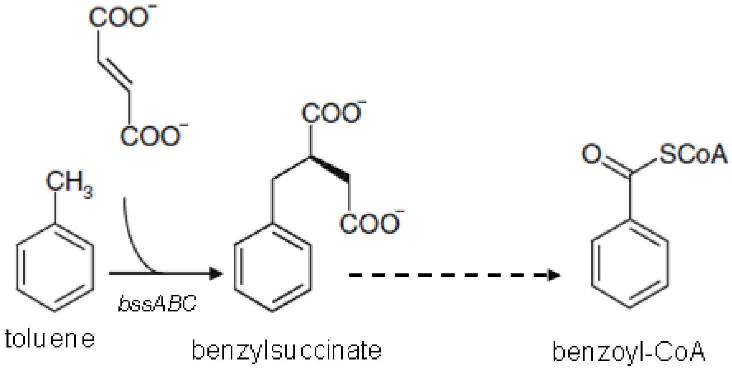

Fig. 6 – Toluene degrdadation through fumarate addition pathway. Adapted from Weelink et al. 2010 (16).

The anaerobic degradation of the three xylenes seems to proceed through the same pathway previously described for toluene, although different susceptibilities to the biodegradation have been observed and *m*-xylene appear to be the most degradable (16). The mechanism for benzene degradation still needs further elucidation although it has been observed to be degraded in several redox conditions. Three putative pathways have been proposed: methylation, hydroxylation and carboxylation (Fig. 7) (17).

So far, little information is available about MTBE degradation in anaerobic conditions. MTBE degradation has been observed by using several electron acceptors (NO_3^-, Fe^{3+}, Mn^{4+}, SO_4^{2-} and CO_2). These studies however reported a poor reproducibility indicating thus that the microorganisms able to degrade MTBE in such redox conditions are rare. The degradation pathway in anaerobic conditions is still unknown but it has been hypothesized that it could occur through the cleavage of the ether bond with the formation of TBA as well as during the aerobic degradation (18).

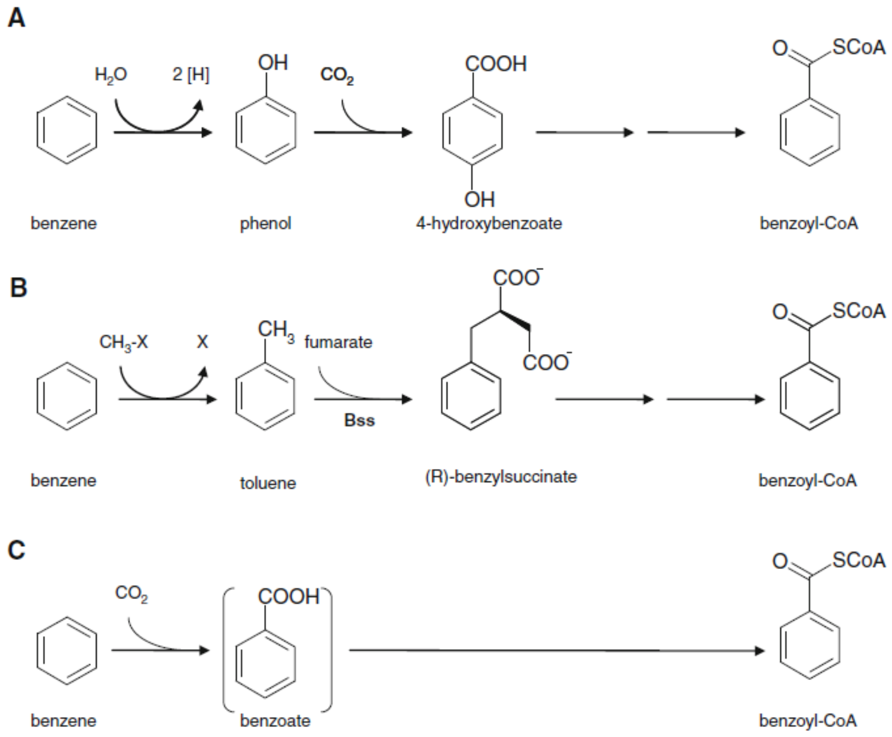

Fig. 7 – Possible mechanisms of the first steps during benzene degradation in anaerobic conditions. A) hydroxylation, B) methylation, C) carboxylation (16).

CONVENTIONAL REMEDIATION STRATEGIES

A wide range of remediation technologies can be applied to remove contaminants from a polluted site. These strategies can be grouped into two main categories that can be applied individually or in synergy: chemical-physical technologies, and biological technologies. The main activities that can be undertaken are (19):

- physical removal of the contaminants by excavation, extraction and washing of the polluted matrix;
- chemical treatment by addition of chemical oxidants or dispersants;

- extraction of contaminated groundwater and subsequent treatment by adsorption on activated carbon, filtration, electrodeposition, air stripping or biological reactors;
- biological treatment directly in the contaminated site;
- Monitoring of Natural Attenuation (MNA).

Biological methods

The use of biological technologies for the remediation (bioremediation) relies on the ability of microorganisms to degrade contaminants, or at least to transform them into less harmful compounds (20). The most common approach involves the stimulation of the indigenous microorganisms by acting on the factors that are limiting their metabolism, as for example adding electron acceptors or nutrients (biostimulation) (21). Another strategy, that is often applied when the native populations do not have the required metabolic abilities to remove the contaminants, is the addition of selected strains or consortia, to improve the degradation ability of the microbial community (bioaugmentation) (22). The successful application of the bioaugmentation is however highly site specific and relies also on the strategy chosen to add the microbial inoculum in the contaminated site, requiring thus an accurate feasibility study of the intervention (21, 22).

Bioremediation technologies can be further classified referring to the location where the process is performed, indeed it is possible to distinguish between (19):

- bioremediation *ex situ*, which involves the extraction of the contaminated matrix (soil or groundwater) and the treatment aboveground;
- bioremediation *in situ*, which involves the treatment directly into the site, without extraction.

A biological approach is often less expensive compared to the traditional chemical-physical methods but requires more time to achieve the complete removal of the pollutants (20). Furthermore, using a biological approach it is possible to completely destroy the contaminants, rather than transferring the pollutants from one phase to another one (like for example by stripping or by adsorption processes) (19). However, before applying bioremediation, it is necessary to perform a complete characterization of the site in order to assess the feasibility of this approach and choose the best available technology (21).

The *ex situ* bioremediation is generally a more expensive and difficult approach. This allowed *in situ* technologies to gain more interest for the treatment of contaminated matrixes (23).

The most common biostimulation technologies involve: bioventing, water recirculation, air sparging, and biobarriers (19).

Bioventing. This method is used to enhance the aeration in the vadose zone in order to stimulate the aerobic degradation of the pollutants. The treatment is performed by directly injecting air or by using vacuum pumps to pull the air out from the unsaturated zone, and thus inducing air with an higher content of oxygen to flow in it (4). With this approach is possible to treat the vadose zone and the surface of the saturated area (Fig.8).

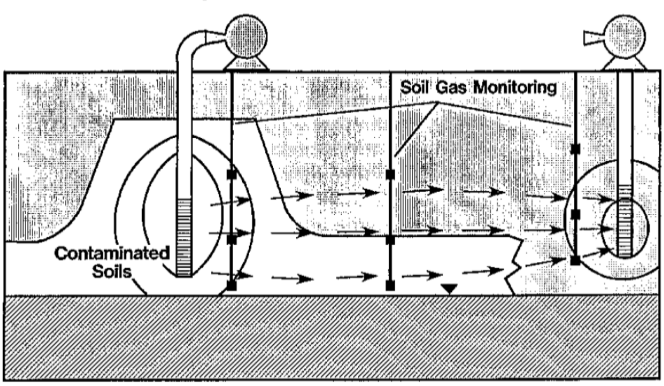

Fig. 8 – Scheme of a bioventing plant for bioremediation of the unsaturated zone (24).

Water recirculation. This technology is used for groundwater treatment. It consists of the extraction of groundwater that is treated aboveground and re-injected upstream. During this process is also possible to amend the water with electron acceptors and nutrients. One of the main drawbacks of this method is the clogging near the injection wells that could occur due to the high microbial growth in these areas. This phenomenon can be prevented by injecting nutrients and electron acceptors in pulsing mode (Fig. 9) (19).

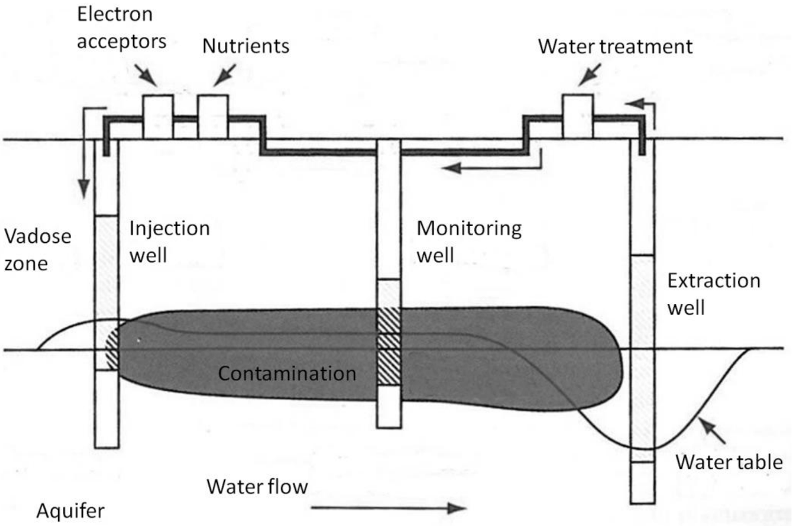

Fig. 9 – Scheme of a water recirculation plant. Adapted from Barbieri et al. 2008 (12).

Air sparging. This system involves the injection of compressed air (or O_2) in the saturated zone. This allow a double action, the most volatile contaminants are stripped and thus physically removed. Meanwhile both the saturated and the unsaturated areas are oxygenated and the biodegradation of the contaminants is enhanced (Fig. 10) (19).

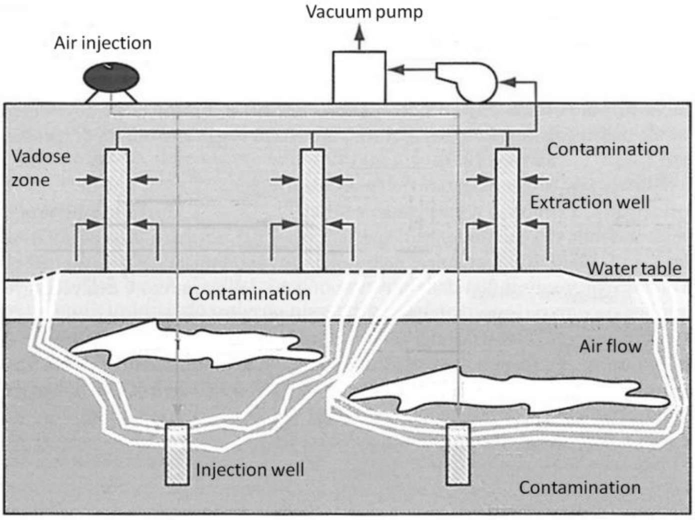

Fig. 10 – Combination of bioventing and air sparging for the treatment of both the saturated and the unsaturated zone. Adapted from Barbieri et al. 2008 (12).

Biobarriers. This is a new technology that can be successfully used for *in situ* bioremediation. It involves the placement of a support material into the contamination plume in order to allow the colonization of microorganisms. The microbial activity can be stimulated by adding both nutrients and electron acceptors. This lead to the formation of a biologically active zone that promote the degradation of the contaminants (Fig. 11) (25).

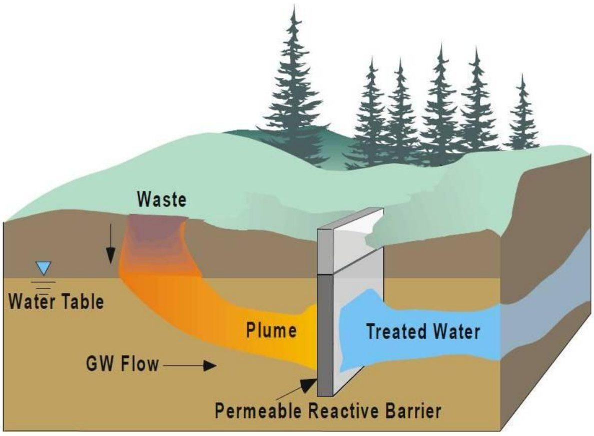

Fig. 11 – Scheme of a biobarrier (26).

The main goal of these strategies is to overcome the limitations that can affect the microbial activity, mainly by adding oxygen in order to stimulate the aerobic metabolism. The suitable electron acceptors for the microbial growth are used in the following order: $O_2 > NO_3^- > Mn^{4+} > Fe^{3+} > SO_4^{2-} > CO_2$. When an hydrocarbon contamination occurs oxygen is quickly depleted due to high concentration of degradable organic matter and anaerobic conditions are established near the contamination source (27). The aerobic degradation is faster compared to the anaerobic degradation due to the high redox potential of the couple O_2/H_2O and to the role of oxygen during the activation of the hydrocarbon molecules (19). Although the use of aerobic technologies allows reaching the remediation goals more quickly compared to anaerobic strategies, it is often expensive and technically difficult. In order to overcome these drawbacks new anaerobic strategies have been proposed during the last years, among which the use of bioelectrochemical systems (BES).

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Bioelectrochemical based technologies for petrochemical compounds removal

INTRODUCTION

Bioelectrochemical systems (BES) are an attractive technology for the bioremediation of petrochemical compounds, but still in its infancy. Many progresses have been obtained during the last years both regarding the contaminants that can be treated and the key parameters involved in the bioremediation process. In this chapter the state of the art in this field will be reported highlighting the main advantages of the BES-based stimulation compared to the existing bioremediation technologies. The main contaminants and the parameters involved in the degradation will be outlined focusing on the current literature. Furthermore, a description of the microorganisms involved in the bioelectrochemical removal of petrochemical contaminants will be provided.

BIOELECTROCHEMICAL SYSTEMS

In a BES specific microorganisms are used to catalyse redox reactions on or near the electrodes (1, 2). A typical reactor design entails the use of an anode and a cathode electrode separated by a ion conductive matrix such as a salt bridge or an ion exchange membrane (1). The first uses of this technology have been directed towards conversion of chemical energy into electrical energy. The anaerobic oxidation of the organic matter is microbially oxidized in anaerobic conditions. The electrons obtained are then transferred to the anode, which is a solid electron acceptor and flow through a circuit producing an electrical signal. When the electrons reach the cathode a final electron acceptor is reduced and the electrical circuit is closed (1) (Fig. 1). In the first studies a chemical cathodic reaction was exploited, often making use of expensive catalysts such as platinum. However, during the last years, biologically catalyzed cathodic reactions were described opening new perspectives for the application of BES technologies (2).

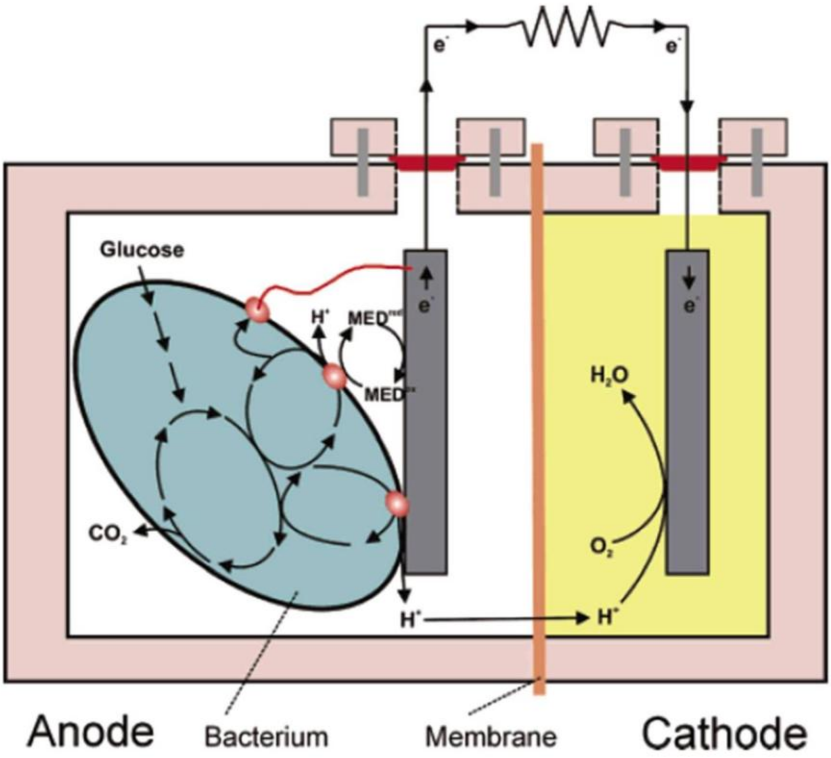

Fig. 1 – Scheme of a BES reactor for glucose oxidation. Adapted from Rabaey and Verstraete 2005 (3).

If the redox potential of the oxidation reaction is lower than the potential of the reduction reaction at the cathode, a nett energy gain can be achieved. This system is termed a microbial fuel cell (MFC) (1). A BES that is being used without a nett energy gain but where there is an electrical input into the system can be used to drive a desired reaction, and is called microbial electrolysis cell (MEC) (2). When operating a MEC there are two options: it is possible to use a fixed potential or a fixed current. Operate at a fixed potential has the advantage that a desired reaction can be driven or favourable conditions for a certain biocatalyst can be created. The drawback is that reaction rates are controlled by the catalyst and not by the operator. Operate a MEC at a fixed current allows the operator to control the reaction rates but not the type of reaction that occurs.

BESs rely on the ability of some microorganism to transfer electron by a process called external electron transfer. Different strategies can be used by bacteria for the external electron transfer (Fig. 2).

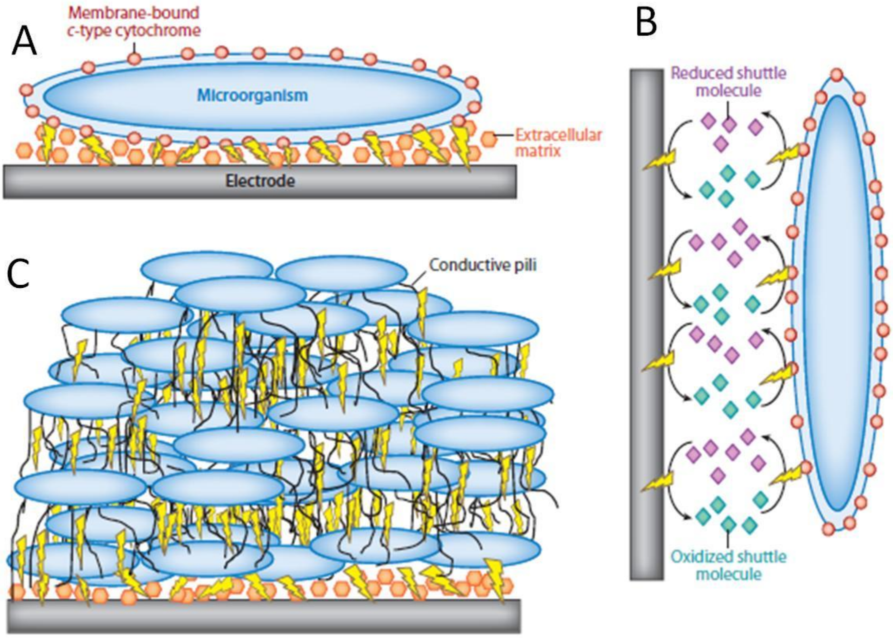

Fig. 2 - Principal external electron transfer mechanisms described for the anodic reaction. Direct contact by outer membrane cytochromes (A). Electron transfer by redox mediators (B). Long range transport by conductive pili coupled to short range transport by cytochromes (C). Adapted from Lovley 2012 (4).

Microorganisms in close contact with the electrode can accomplish a short range electron transfer by redox active proteins, such as *c*-type outer membrane cytochromes (4). Another strategy involves soluble exogenous, or endogenous, redox mediators that act as electron transfer shuttles. A redox mediator is a molecule that assists the electron transfer between the electrode and the cytochromes. When the electron shuttle is oxidized can be reduced at the outer cell surface. The reduced molecule diffuses to the anode and donates the electrons to the electrode (4). Long-range electron transport can occur also through a conductive biofilm via electrically conductive pili, accompained by short-range electron transfer from the biofilm to the electrode mediated by extracellular cytochromes that are released in the biofilm matrix and accumulate near the anode surface (5). Electron transfer by outer membrane *c*-type cytochromes and shuttles have been proposed as suitable pathways also for the electron uptake from the cathode (2). Recent studies showed that microbial communities adapted to the anodic electron transfer can be turned into biocathodic biofilms (2), however experimental evidences showed that different *c*-type cytochromes are involved in the latter process (4). Furthermore, biocathodic electron transfer can involve also hydrogenases which can accept the electrons via mediators or being linked to outer membrane cytochromes (2).

ANODIC REACTIONS

The anode of BES can be used to collect electrons derived from the oxidation of organic contaminants such as hydrocarbons. The current bioremediation technologies usually try to overcome the limitations to the biodegradation process by supplying electron acceptors. The aerobic metabolism is often stimulated by adding oxygen through the aeration of the contaminated matrix, or by using oxygen releasing compounds (6). Providing oxygen has the benefit of allowing a faster microbial metabolism compared to the anaerobic strategies. Furthermore oxygen is an important reagent for hydrocarbon activation (7). However introduction of oxygen could be difficult due to its low solubility, and its continuous depletion by reactions with reduced species, such as Fe^{2+} or Mn^{2+}, usually abundant in hydrocarbon contaminated matrixes (8, 9). In addition to the aerobic stimulation, the anaerobic metabolism can be effectively stimulated with the addition of chelators, which solubilize Fe^{3+}, or with the addition of humic substances or other soluble electron shuttles able to promote the electron transfer to insoluble electron acceptors, such as Fe^{3+} or Mn^{4+} oxides (10, 11). The anaerobic biodegradation could also be stimulated by adding sulfate or nitrate which can be used as soluble electron acceptors form a variety of hydrocarbon degrading microorganisms (12–14). The main drawback of the strategies mentioned above is due to quick diffusion of the supplemented species away from the contaminated area. Continuous amendment with the depleted reagents or electron acceptors is therefore required, increasing thus the overall cost of the bioremediation (15). An alternative strategy to promote the degradation of organic contaminants could be offered by BES technology.

Contaminants treated

The microbially mediated anodic oxidation of organic compounds in BES was initially suggested as a technology to reduce the Chemical Oxygen Demand (COD) and the Biochemical Oxygen Demand (BOD) in domestic wastewater, with concomitant energy production (1). During the last years an increasing number of studies started to propose the opportunity to exploit BES as an attractive technology to stimulate the anaerobic oxidation of petroleum hydrocarbons (Table 1). The first works in this field reported the use of complex mixtures of highly contaminated refinery wastewater and diesel contaminated groundwater as suitable electron donors in MFCs, coupling hydrocarbon removal with current production

(16, 17). Using an anode as electron acceptor diesel range organics (DRO) removal was 82% while in the open circuit control DRO removal was only 31% (17). As well as alkanes also aromatic hydrocarbons have been proved to be degraded in BES. Toluene is known to be the most easy degradable among BTEX compounds, and its degradation has been studied under a variety of anode potentials, both with pure cultures and consortia (15, 18, 19) (Chapter 2). Benzene degradation in BES was observed in mixed cultures enriched from contaminated sediments (15), groundwater (20) and wastewater (21). Polycyclic aromatic hydrocarbons (PAHs) degradation was reported in several studies with different inocula and reactors set up (15, 22, 23). Phenol has been bioelectrochemically degraded both by a pure culture of *Cupriavidus basilensis* and by a mixed culture (24, 25). In contaminated sites however, mixtures of different hydrocarbons rather than single compounds are often present. For this reason the possibility of stimulating the degradation of hydrocarbon mixtures other than diesel have been addressed. Total petroleum hydrocarbons (TPH) degradation was extensively assessed by different authors (26–31) demonstrating that BES is an effective technology to stimulate the degradation of this complex mixture (30, 31). Although the increasing number of studies about non-halogenated hydrocarbons oxidation in BES, the degradation of widespread mixtures such as gasoline still have to be assessed. In addition to the studies about the oxidation of non-halogenated hydrocarbons, the anodic oxidation of 1,2-dichloroethane (1,2-DCA) was studied with different microbial inocula. The authors proved that the bioelectrochemical oxidation is an attractive technology also for the bioremediation of chlorinated compounds (32).

Contaminant	Main microorganisms	Redox mediator	Working electrode material	Working electrode potential (vs SHE)	Reference
Diesel	Mixed culture dominated by NO_3^- - reducing bacteria	Not detected	Stainless steel scrubber	N.A.	(16)
	Mixed culture	Not detected	Stainless steel scrubber	N.A.	(17)
Toluene	*Geobacter metallireducens*	Not detected	Unpolished graphite rod	+500 mV	(15)
	Mixed culture	Not detected	Unpolished graphite rod	+500 mV	(15)
	Pseudomonas putida F1	Not detected [a]	Graphite rod	+325 mV	(18)
	Mixed culture	Not detected	Carbon cloth	N.A.	(19)
	Mixed culture	Neutral red	Carbon cloth	N.A.	(19)
	Mixed culture	Ferricyanide	Carbon cloth	N.A.	(19)
	Mixed culture dominated by SO_4^{2-} reducers	Not detected	Graphite plate	+200 mV	Chapter 2
	Mixed culture dominated by SO_4^{2-} reducers	Self produced mediator (+400 mV vs SHE)	Graphite plate	+500 mV	Chapter 2
Benzene	Mixed culture	Not detected	Unpolished graphite rod	+500 mV	(15)
	Mixed culture dominated by δ-Proteobacteria	Not detected	Graphite fibers	N.A.	(20)
	Mixed culture	Not detected	Carbon cloth	N.A.	(21)

Continued

Naphtalene	Mixed culture	Not detected	Unpolished graphite rod	+500 mV	(15)
TPH	Mixed culture	Not detected	Graphite plate	N.A.	(26)
	Mixed culture	Not detected	Graphite plate	N.A.	(27)
	Mixed culture	Not detected	Stainless steel brush	N.A.	(28)
	Mixed culture	Not detected	Carbon mesh	N.A.	(29)
	Mixed culture dominated by β-*Proteobacteria* and γ-*Proteobacteria*	Not detected	Carbon cloth	N.A.	(31)
	Mixed culture dominated by β-*Proteobacteria* and γ-*Proteobacteria*	Not detected	Biochar	N.A.	(31)
	Mixed culture	Not detected	Graphite granules	N.A.	(30)
	Mixed culture	Not detected	Biochar	N.A.	(30)
Phenantrene	Mixed culture dominated by *Nitrospira* sp and *Chloroflexi*	Not detected	Stainless steel (mesh)	N.A.	(22)
	Pseudomonas aeruginosa	Not detected	Carbon felt	N.A.	(23)
	Shewanella oneidensis	Not detected	Carbon felt	N.A.	(23)
	Mixed culture	Not detected	Carbon felt	N.A.	(23)
Pyrene	Mixed culture dominated by *Nitrospira* sp and *Chloroflexi*	Not detected	Stainless steel (mesh)	N.A.	(22)
Phenol	Mixed culture	Not detected	Carbon felt	N.A.	(24)

Continued

	Cupriavidus basilensis	Self produced mediator (+140 mV vs SHE)	Graphite rod	+325 mV	(25)
1,2-DCA	Enrichment form MFCs reactors	Not detected	Graphite plate	N.A.	(32)

[a] The presence of cathecol was inferred by the authors on the basis of cyclic voltammetry results

Table 1 - Summary of the key studies about petrochemical compound oxidation in BES.

Advantages of BES-based oxidation

The anode can be buried in the anoxic sediment and electrically connected to the cathode placed in the upper water layer (33). The anode placed in the sediment is rapidly colonized by the bioelectrogenic microorganisms and can collect the electrons derived from the anaerobic oxidation of the contaminant. The electrons can flow to the cathode in the aerobic subsurface were they could be used to reduce oxygen (5). The anode can therefore serve as a virtually inexhaustible electron acceptor, lowering the maintenance costs (16). Furthermore, if a graphite (or carbon) anode is used, hydrocarbons could be adsorbed, concentrating thus, in a high metabolic active area, the contaminant, the electron acceptor and the degrading microorganisms (15). In addition the electric signal could be used as a real time measure of the *in situ* microbial activity in order to gain information about the degradation rate in the contaminated site (34). The energy harvested could also be used to power electrical devices for *in situ* monitoring (35, 36). A similar set up could be used also to stimulate the bioremediation in hydrocarbon contaminated aquifers. It has been demonstrated that a borehole anode can serve as electron acceptor also when the cathode is embedded at the ground surface meters above (37). A simpler configuration can be obtained by using the so-called “electrochemical snorkels”. In this strategy the electrode material (a conductive rod) spans the aerobic and the anaerobic zones, functioning both as a cathode and as an anode. With this configuration however current is not harvested (38).

Electrode material

The choice of an appropriate anodic material is a key parameter that can affect the hydrocarbon removal efficiency. Few studies used stainless steel anodes (16, 17, 22, 28) but the most extensively used are made with carbon and graphite. Using the latter materials hydrocarbon contaminants are adsorbed on the electrode. The pollutants and the final electron acceptor are thus colocalized. The adsorption process usually affects the biodegradation of hydrocarbon by reducing the bioavailability of the contaminants. However, studies with [^{14}C]-toluene and [^{14}C]-benzene, and isotopic analysis during benzene degradation, showed that the contaminant can be metabolized also when adsorption on the electrode occurs (15, 20), but is unknown whether the process involved a direct degradation of the adsorbed pollutants, or whether hydrocarbons are first desorbed (15).

A comparison of the anodic materials in term of removal efficiency is really difficult because factors other than the electrode material could have affected the degradation rate in different studies. The effect of carbon cloth and biochar electrodes on TPH removal was recently assessed. A slightly higher removal efficiency (78.7%) was observed with the biochar anode compared to the carbon cloth anode (73.1%) over 64 days of operation starting from an initial concentration of 11.46 g kg^{-1}. The authors attributed the higher removal efficiency of the biochar to the higher sorption capabilities that facilitated hydrocarbon diffusion to the anode (31). The biochar electrode was also compared with a graphite granule anode. The two materials showed similar performances in term of TPH removal, but the graphite granule anode allowed an higher current generation (30).

Electrode potential

The anode in BES is the final electron acceptor of microbial metabolism. Usually microbial metabolism is faster using electron acceptors with a more positive potential (39), thus it is reasonable hypothesizing that a more positive anodic potential can enhance the hydrocarbon oxidation in BES. However, the role of the anodic potential during the bioelectrochemical oxidation of organic substrates is controversial. With easily degradable substrates a positive correlation between the anodic potential and the current production was observed, but some study showed the opposite (40, 41).

Most of the studies about hydrocarbon degradation in BES used an MFC configuration, without controlling the anodic potential. Few studies, however, assessed toluene degradation at different potentials. Toluene degradation with an anode poised at different potentials (+275-+700 mV vs SHE) was studied using a pure culture of *Pesudomonas putida* F1. The current production increased with the anodic potential but no information about the variation in the degradation rate were reported (18). Another study tested the ability of mixed cultures enriched form a marine contaminated sediment to degrade toluene, but no differences in the degradation rate were observed increasing the potential form +200 mV to +500 mV (vs SHE) (Chapter 2).

Redox mediators

The presence of redox mediators could facilitate the electron transfer to the anode, theoretically enhancing the degradation of the contaminant. The effect of exogenous redox mediators during toluene degradation was addressed. Neutral red and ferricyanide were used but toluene removal decreased in both the conditions compared to a control without redox mediators. The negative effect was probably due to the toxicity of the mediators (19). However, the use of mediatorless BES is desirable during bioremediation due to the toxicity of mediators like neutral red or ferricyanide.

Self-produced mediators were detected during toluene degradation. Firman and colleagues described the presence of a redox active compound with a potential of +470 mV (vs SHE). The authors hypothesized that the oxidation peak could have been due to the presence of 3-methyl cathecol in the medium (18). However, while the presence of cathecol (a typical intermediate during the aerobic toluene degradation) could be explained by the low oxygen concentration in the reactor (0.78 mg L^{-1}), it is unlikely that the redox active compound could have acted as a mediator of the electron transfer, since the anode was poised at +325 mV (vs SHE).

The production of an unidentified redox active moiety involved in the electron transfer to the anode was also described in a mixed culture dominated by sulfate reducers during toluene degradation. The midpoint potential of the redox active site was around +400 mV (vs SHE), but the nature of the redox mediator was unknown (Chapter 2).

Radius of influence

The extension of the radius of influence is one of the most important aspects to address before applying BES-based technology for the bioremediation of soil and sediment.

A first attempt in evaluating the radius of influence during TPH degradation in soil was conducted by using a U-tube MFC. The experiment was conducted by using a saline soil (conductivity 8.32 mS cm^{-1}). In spite of the high conductivity however an enhancement of the degradation rate after 25 days of incubation was observed only in samples collected close to the electrode (<1 cm), while the removal rate 1-2 cm and 2-3 cm far from the anode was similar to the open circuit controls removal (29). Opposite results were obtained by Lu and colleagues (31). TPH degradation was enhanced during 64 days of operation both at <1 cm and at 5 cm from the anode, compared to the controls (31).

A more recent study however showed that the measured radius of influence can be up to 34 cm from the electrode over a period of 120 days. Hydrocarbon degradation was initially observed close to the anode, but the influence of the bioelectrochemical stimulation increased over time. Assuming a linear correlation between the distance from the anode and the enhanced TPH removal, the authors predicted a maximum radius of influence of 90 cm after 45 days with a BES radius of 7.5 cm. This estimation however was not supported by experimental data at distance higher than 34 cm from the electrode (30). Other factors such as ohmic losses might lead to a non-linear correlation between TPH removal enhancement and the radius of influence for long distances (1), decreasing thus the extension of the predicted radius.

Microorganisms involved

Several pure cultures were studied for their ability of oxidizing hydrocarbons using an electrode as electron acceptor. Iron reducers have been used first as inoculum in MFC containing hydrocarbons as energy source. One of the first studies showed the ability of *Geobacter metallireducens* to use graphite electrodes as an electron acceptor for the degradation of toluene (15) while, recently, the degradation of phenanthrene by *Shewanella oneidensis* MR1 14063 coupled with current production in a MFC was reported (23). However, strict anaerobes are not the only microorganisms studied. In the abovecited work *Pseudomonas aeruginosa* NCTC 10662 resulted in higher degradation rates and biodegradation efficiencies than *S. oneidensis* both in pure cultures and in co-culture with undefined microbial consortia (23). Some reports seem to indicate that aerobes and facultative anaerobes are able of oxidizing petrochemical compounds in BES. *Cupriavidus bastilensis* was able to degrade phenol in BES via electron transfer mediated by a self-produced shuttle (+140 mV vs SHE) (25); while the catabolic intermediate catechol was identified during bioelectrochemical toluene degradation by *Pseudomonas putida* F1 (18). In this study the presence of catechol was due to the aerobic activation of toluene because of the low oxygen concentration in the reactor (0.78 mg O_2 L^{-1}) (18). This finding poses open questions regarding the degradation pathway and the role of oxygen in *Pseudomonas* sp. for hydrocarbon degradation in BES. Indeed, microorganisms belonging to this genus are well-described hydrocarbon degraders in aerobic conditions and the hydrocarbon activation proceeds by addition of OH group catalyzed by an oxygenase, requiring thus O_2 (42). It is therefore questionable whether aerobes and facultative anaerobes require low concentration of oxygen for coupling hydrocarbon degradation to current production in BES.

Many studies reported the anodic oxidation of hydrocarbon in BES by using mixed cultures as microbial inoculum. Different inoculum sources were used such as refinery wastewater (21), contaminated sediment (15, 22, 28–31), sludge (19, 23, 26, 27) and water (16, 17, 20). Although, few studies characterized the microbial communities developed in the reactors during the treatment, they suggest that both the presence of recalcitrant substrates and the availability of alternative electron acceptors may affect the selection of the microbial populations. Interestingly, microorganisms belonging to the genus *Geobacter*, that are often described in the anodic communities developed in reactors fed with more degradable substrates (i.e. acetate) (43, 44), seem to play only a marginal role during hydrocarbon degradation in BES. This suggests that mechanisms other than the ability to directly transfer the electrons to an electrode give an advantage when the substrate is recalcitrant (20, 31)

(Chapter 2). Morris and colleagues found that NO_3^- reducing bacteria dominated the anodic community in a single chamber MFC in which diesel was degraded with simultaneous current production (17). The high abundance of NO_3^- reducers however could be attributed to the MFC architecture used in this study. Indeed, the air cathode can allow a small amount of oxygen to diffuse into the reactor, advantaging thus facultative anaerobes like several NO_3^- reducers. In other investigations, microbial communities dominated by microorganisms belonging to the phylum *Chloroflexi* and to the genus *Nitrospira* were described during PAHs degradation (22). A recent detailed analysis performed after TPH soil remediation with BES highlighted that *Proteobacteria* (especially β-*Proteobacteria* and γ-*Proteobacteria*) was the most abundant phylum after the treatment. However, these microorganisms are often found to play an important role during hydrocarbon biodegrdation (31). Among β-*Proteobacteria* and γ-*Proteobacteira* the most abundant genera were *Bordetella*, *Comamonas* and *Pseudomonas*. While the last two genera were described as bioelectrogenic, microorganisms belonging to the genus *Bordetella* were not previously described in bioelectrogenic communities. This further indicates that with complex and recalcitrant contaminants factors other than the ability to actively transfer the electrons to the anode affect the development of specific microbial populations. The presence of sulfur species in the environments can also contribute to the assembly of the microbial community. In case of sulfide rich groundwater, the aerobic hydrocarbon degraders *Burkholderiales* where enriched due to a small amount of oxygen that diffused into the reactor and these process was supposed to be coupled with sulfide oxidation to sulfur and sulfate occurring at the anode (20). In case of a sulfate rich simulated marine environment, the *Desulfobulbaceae* and *Desulfobacteraceae* outcompeted other microbial populations owing to their potential to degrade hydrocarbons using both the anode and sulfates as electron acceptors (Chapter 2). Sulfates are reduced to sulfides which can be electrochemically oxidized to elemental sulfur on the anodic surface (Chapter 2).

CATHODIC REACTIONS

The cathodic reaction can be exploited to reduce a large number of oxidized compounds such as metals (45) or halogenated compounds (46, 47). The next paragraph is focused on the opportunities that BES technology offers for the bioelectrochemical removal of chlorinated compounds. Chlorinated hydrocarbons that contaminate water can be effectively remediated by reductive dechlorination. These compounds are used by bacteria as final electron acceptors during anaerobic respiration (48), the contaminant is thus dechlorinated into less chlorinated or non chlorinated compounds, which can be more easily biodegraded (49). Stimulation of the microbial reductive activity can be achieved by supplying electron donors, and a variety of strategies have been proposed. Although some study suggested that acetate can be used, the typical electron donor for reductive dechlorination is H_2 (50–52) which can be delivered directly or by passive dissolution using hollow-fibers membranes (53, 54). Another strategy to indirectly supply H_2 for the reductive dechlorination is by using organic substrates that can be fermented and accordingly release H_2. The main substrates that can be added include butyric acid, ethanol or lactic acid (55–57). These approaches can however require high costs due to the need of a continuous supplying of water soluble electron donors that can diffuse away from the contaminated site. Furthermore, controlling the addition rate of the electron donors can be a crucial step to avoid unwanted side reactions and the accumulation of fermentation products with deterioration of water quality. H_2 can be consumed not only by dechlorinating bacteria but also by other H_2 consuming microorganisms such as methanogens, homoacetogens and sulphate reducers. Experimental evidences suggested that dechlorinating microorganisms can outcompete other H_2 consumers at low H_2 concentrations (58–60).

Recently the production of H_2 by electrochemical systems has been proposed but the authors did not try to effectively control H_2 evolution rate in the system (61). The use of BES is an innovative approach to produce H_2 during the bioremediation. However, in order to make this bioelectrochemical reaction feasible thermodynamic constraints have to be overcome by artificially increasing the potential between the anode and the cathode. This goal could be achieved by setting the potential difference between the electrodes with an external power source or by lowering the cathodic potential by using a potentiostat (2). However recent advances in the description of the cathodic electron transfer mechanisms revealed that some dechlorinating bacteria are able to accept the electrons directly from a graphite electrode, without the addition of external mediators (62–64).

The use of BES has the advantage of allowing the control of H_2 delivery rate in the contaminated site by adjusting the current input and the potential (62, 63, 65). In addition the electrode colocalize the source of the electron donor and the contaminant in the same area, increasing the treatment efficiency (66). Moreover, since no chemicals injection is required the need for transport and storage is eliminated (66).

Contaminants treated

Several studies reported on the possibility of stimulating microbial dechlorination by using a cathode as direct electron donor or to bioelectrochemically produce H_2 (Table 2). The pioneer studies focused on trichloroethene (TCE) removal by using redox mediator to promote the electron transfer from the cathode to the final electron acceptors. TCE was dechlorinated all the way down to *cis*-dichloroethene (*cis*-DCE), vinyl chloride (VC) and ethene (46). Subsequent studies explored TCE dechlorination at different chatodic potential with and without the presence of redox mediators, and proved that TCE removal can be stimulated also by direct electron transfer from the cathode (62, 63, 65). Although TCE is the most studied, it is not the only alogenated hydrocarbon that was proved to be removed in BES. The more oxidized tetrachloroethene (PCE) was reduced to *cis*-DCE by a pure culture of *Geobacter lovleyi* able to use the electrode poised at -300 mV (vs SHE) as sole electron donor (64). Another innovative study used H_2 produced by water electrolysis to reduce PCE to ethene and simultaneously used electrochemically produced O_2 to stimulate the microbial oxidation of dechlorination products (67).

Contaminant	Main microorganisms	Redox mediator	Working electrode material	Working electrode potential (vs SHE)	Reference
TCE	Mixed culture containing *Dehalococcoides* spp.	Methyl viologen	Glassy carbon	-500 mV	(46)
	Mixed culture containing *Dehalococcoides* spp.	Methyl viologen	Glassy carbon	-800 mV	(46)
	Mixed culture containing *Desulfitobacterium* sp. and *Dehalococcoides* spp.	Methyl viologen	Glassy carbon	-450 mV	(68)
	Mixed culture containing *Dehalococcoides* spp.	Not detected	Unpolished graphite rod	-220 mV	(61)
	Mixed culture containing *Dehalococcoides* spp.	Not detected	Unpolished graphite rod	-650 mV	(61)
	Mixed culture containing *Desulfitobacterium* sp. and *Dehalococcoides* spp.	Self produced mediator (-400 mV vs SHE)	Carbon paper	-450 mV	(63)

Continued

	Geobacter lovleyi	Not detected	Carbon paper	-450 mV	(63)
	Mixed culture containing *Desulfitobacterium* sp. and *Dehalococcoides* spp.	AQDS	Glassy carbon	-250mV	(69)
	Mixed culture containing *Desulfitobacterium* sp. and *Dehalococcoides* spp.	Self produced mediator (-550 mV vs SHE)	Carbon paper	-550mV	(62)
	Mixed culture containing *Dehalococcoides* spp.	Not detected	Graphite granules	-250mV	(65, 70)
	Mixed culture containing *Dehalococcoides* spp.	Not detected	Graphite granules	-450mV	(65, 70)
	Mixed culture containing *Dehalococcoides* spp.	Not detected	Graphite granules	-550 mV	(65, 70)
	Mixed culture containing *Dehalococcoides* spp.	Not detected	Graphite granules	-650 mV	(65, 70)
	Mixed culture containing *Dehalococcoides* spp.	Not detected	Graphite granules	-750 mV	(65, 70)
PCE	*Geobacter lovleyi*	Not detected	Unpolished graphite rod	-300 mV	(64)
	Mixed culture containing *Desulfitobacterium* sp. and *Dehalococcoides* spp.	Not detected	Stainless steel mesh	NA	(67)
cis-DCE	Mixed culture containing *Desulfitobacterium* sp. and *Dehalococcoides* spp.	Self produced mediator (-550 mV vs SHE)	Carbon paper	-550mV	(62)

Table 2 - Summary of the key studies about petrochemical compound reduction in BES.

Redox mediators

The presence of redox mediators is a key parameter during the bioelectrochemical dehalogenation of chlorinated hydrocarbons. Aulenta and colleagues (46) used the redox mediator methyl viologen to promote the complete dehalogenation of TCE. It was demonstrated that with an electrode poised at -500 mV (vs SHE) the dechlorination was stimulated only when methyl viologen was in the medium. However when the potential was further decreased at -800 mV (vs SHE), H_2 was produced (46). Even though H_2 can be used as electron donor for the dehalogenation, it can reduce the efficiency of the process by stimulating other metabolisms and the formation of undesirable products, such as methane. Another study showed the effect of methyl viologen concentration (68). At low concentration (25-750 µM) only dechlorination was stimulated, but when the methyl viologen concentration was increased up to 5000 µM, H_2 was produced. It was suggested that at high methyl viologen concentrations the rate at which the electrons were transferred to the microorganisms exceeded the electron utilization for the dehalogenation process. The electrons were thus diverted to H_2 production via hydrogenase (68). An alternative mediator is the humic acid analogue antraquinone-2,6-disulfonate (AQDS). Humic acids are ubiquitous redox active compounds in the environment and several studies proved their role during the biodegradation processes (71). AQDS was successfully used to reduce TCE to *cis*-DCE but was unable to further stimulate the dechlorination to vinyl chloride and then to ethene (69).

Electrode potential

The cathode potential in another important parameter that could affect the performance of the dechlorination processes by controlling side reactions particularly methanogenesis due to H_2 evolution. It was demonstrated that cathodic potentials from -600 mV to -800 mV (vs SHE) can be applied to stimulate TCE dechlorination via H_2 production. The best performances were achieved around -650 mV (vs SHE). However also methane production was stimulated in the same range of potentials, reducing thus the efficiency of the dechlorination process (61). A strategy to better control the microbial reductive dechlorination and to eliminate side reactions could be by supplying the electrons directly from the electrode. The first evidence of this process was reported with a culture of *Geobacter lovleyi* able to reduce TCE to *cis*-DCE with an electrode poised at -300 mV (vs SHE) serving as electron donor (64). Further studies demonstrated that also mixed culture were able to use an electrode poised at -450 mV (vs SHE) as sole electron donor without supplying external redox mediators. An unidentified redox active moiety with a midpoint potential around -400 mV (vs SHE) was detected in the supernatant. This molecule was not detected when the culture was grown with H_2 as electron donor, therefore it was possible to hypothesize that this molecule had a role in the electron transfer from the solid electron donor (63). An higher dechlorination rate was obtained by further decreasing the cathodic potential to -550 mV (vs SHE). The complete dechlorination of TCE to ethene was described without the presence of external or self-produced mediators. An electrode active compound with a potential close to the cathodic potential was produced. However the electron transfer process appeared to be closely associated with components on the electrode surface, rather than mediated by soluble electron shuttles released in the medium (62). An accurate study of the effect of the electrode potential was recently performed using a continuous flow reactor. Five cathodic potentials from -250 mV to -750 mV were tested without exogenous nor endogenous redox mediators. At -250 mV (vs SHE) TCE dechlorination rate was low but rapidly increased when the cathodic potential was decreased at -450 mV. This was consistent with the reduction potential of the couple H_2/H^+ (-414 mV vs SHE at pH 7). Decreasing the potential increased H_2 production, increasing thus the dechlorination rate. However a negative correlation between the electrode potential and methane production was observed. The coulombic efficiency of the dechlorination process was nearly 100% at -250 mV but decreased to less than 1% at -750 mV because methanogenesis acted as an electron sink (65).

Microorganisms involved

In the first studies about the bioelectrochemically mediated reduction of chlorinated hydrocarbons pure cultures of *Geobacter lovleyi* were used as microbial inoculum (63, 64) showing the abilities of *Geobacter* spp. of using electrodes both as electron acceptors and electron donors. However, the most extensively tested microorganisms in dechlorinating BESs belong to the genus *Dehalococcoides* (Table2). Up to date *Dehalococcoides* spp. are the only known microorganisms able to completely dechlorinate TCE to ethene (72, 73); indeed the presence of these bacteria was confirmed by FISH analysis both in the communities attached to the anode and in the medium, thus demonstrating that the cells are likely active and involved in TCE dechlorination in BES (62, 63). The presence of *Dehalococcoides* on the cathode seems to be correlated with the electrode potential. The first enzymes involved in the electron transport chain in this microorganism during chloroethenes reduction were suggested to be hydrogenases (69). These enzymes typically work close to the redox potential of the couple H_2/H^+ (-414 mV vs SHE at pH 7) (74), consistently with experimental data in a *Dehalococcoides* enriched mixed community (63). At higher potentials, other electron transfer pathways might be involved leading to the enrichment of different groups such as *Desulfitobacterium* spp. (69). These findings were confirmed by the results of a recent study in which the authors performed a detailed characterization of the cathodic communities involved in TCE dechlorination by CARD-FISH. *Dehalococcoides* was the dominant genus in the range from -550 to -750 mV (vs SHE); conversely, an unidentified member of the *Chloroflexi* phylum outcompeted the *Dehalococcoides* when the cathode potential was increased in the range from -250 to -450 mV (70).

IN SITU **MONITORING**

The use of BES technology in the bioremediation field gives interesting outlooks not only for the stimulation of contaminants removal. Many studies tried to assess a correlation between the electrical output and the bioelectrochemical degradation of organic matter, and more in general of environmental contaminants. The electrical current is proportional to the number of electrons that flow into the circuit per time unit:

$$1A = 1C / 1s$$

where A = ampere; C = coulomb, s = second. The charge exchanged in the reaction (C) is given by the number of electrons transferred (n) and the Faraday's constant (F; 9.64853×10^4 C/mol):

$$C = n \times F$$

The current into the circuit should be therefore proportional to the extent at which the electrons are transferred to the electrode by the oxidation reaction (for the anodic process), or diverted by the reduction reaction (for the cathodic process). The current produced can thus be used as a real time parameter to quantify the rate of specific metabolic processes. Furthermore the current could be a measure of the substrate concentration available for microbial degradation. Previous studies demonstrated that the electricity production can be correlated to COD and BOD in batch fed and flow MFCs and that the relationship can be described by a Monod type kinetic (75–77). Tront and colleagues found that the acetate concentration in the range 0-2.3 mM was correlated with current production in an MFC inoculated with a pure culture of *Geobacter sulfurreducens*. Increasing acetate concentration above 2.3 mM did not lead to an increasing in the electrical signal, probably due to limitations attributed to system design parameters (34). The same relationship was observed in a similar system inoculated with *Shewanella oneidensis* and fed with lactate (0-41 mM) (78). These data indicate that, in the range of the concentrations tested, the substrate consumption is governed by a first order kinetic. However, further studies about the degradation kinetics will be required, in order to apply BES based biosensors for measuring the concentration of recalcitrant compounds (i.e. hydrocarbons). A recent study proved that an MFC based biosensor was an effective alternative to measure the extent of the bioremediation in field. Borehole graphite anodes were installed in an aquifer where U(VI) bioreduction was stimulated by supplying acetate. The authors demonstrated that the current was correlated with uranium removal and that the indigenous microorganisms in the aquifer were able to colonize the electrode responding quickly to the change in the electron donor concentration (37). The development of BES based technologies for *in situ* monitoring is an attractive field. BES based biosensors could be easily installed in the existing monitoring well networks and decrease both the number of analysis and the time needed to assess the microbial activity, lowering thus the monitoring cost (34).

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Aim

The main aim of this PhD thesis is to give new insight on the development of innovative bioremediation technologies for the treatment of hydrocarbon contaminated matrixes. The objectives were: (i) characterizing the microbial degradation in an aerobic biobarrier for treatment of gasoline contaminated groundwater; (ii) assessing the applicability of bioelectrochemical systems (BES) as an alternative approach to stimulate hydrocarbon removal; (iii) characterizing the microbial communities involved in the process and inferring their potential role during hydrocarbon bioelectrochemical degradation; (iv) proposing a strategy to develop biobarriers with an integrated BES stimulation of the microbial activity.

Chapter 1 - Hydrocarbon degrading microbial communities in bench scale aerobic biobarriers for gasoline contaminated groundwater treatment

ABSTRACT

BTEX compounds (benzene, toluene, ethylbenzene and xylenes) and methyl *tert*-butyl ether (MTBE) are some of the main constituents of gasoline and can be accidentally released in the environment, causing concern due to their recalcitrance and toxicity. In this work the effect of bioaugmentation on the microbial communities in a bench scale aerobic biobarrier for gasoline contaminated water treatment was studied by 16S rRNA gene Illumina sequencing. Catabolic genes (*tmoA* and *xylM*) were quantified by qPCR, in order to estimate the biodegradation potential, and the abundance of total bacteria was estimated by the quantification of the number of copies of the 16S rRNA gene. Hydrocarbon concentration was monitored over time and no difference in the removal efficiency for the tested conditions was observed, either with or without the microbial inoculum. In the column without the inoculum the most abundant genera were *Acidovorax*, *Bdellovibrio*, *Hydrogenophaga*, *Pseudoxanthomonas* and *Serpens* at the beginning of the column, while at the end of the column *Thauera* and *Georgfuchsia* became dominant. In the inoculated test the microbial inoculum, composed by *Rhodococcus* sp. CE461, *Rhodococcus* sp. CT451 and *Methylibium petroleiphilum* LMG 22953, was outcompeted and other genera (*Acidovorax*, *Azoarcus*, *Hydrogenophaga*, *Mycobacterium* and *Serpens*) were found to be abundant. Quantitative PCR results showed an increasing in *xylM* copy number, indicating that hydrocarbon degrading bacteria were selected during the treatment, although only a low increase of the total biomass

was observed. However, the bioaugmentation did not lead to an increase in the degradative potential of the microbial communities.

INTRODUCTION

Petroleum hydrocarbons are extensively used both as fossil fuel and for chemical production and can be accidentally released in the environment. Due to spills, hydrocarbons contaminate soil, sediments and groundwater (1, 2). Monoaromatic hydrocarbons, such as BTEX compounds (benzene, toluene, ethylbenzene and xylenes), and additives, such as methyl *tert*-butyl ether (MTBE) and ethyl *tert*-butyl ether (ETBE) are some of the main constituents of gasoline (3, 4) and cause concern because of their recalcitrance, toxicity and mobility in groundwater (5). When a contamination occurs, physical, chemical and biological strategies can be used to recover a polluted site (6).

Biological permeable reactive barriers, also known as “biobarriers” (BBs), are an *in situ* technology based on the placement of a permeable medium into the aquifer to intercept the contaminants and promote the biodegradation by stimulating the microbial activity (7). Biobarriers are currently a developing technology and little information is present in literature about the microbial communities developing and operating on the supporting material and in the nearby. Microorganisms have been largely described to degrade hydrocarbons with a variety of electron acceptors in both aerobic and anaerobic conditions (8–10). Bioaugmentation is a bioremediation strategy that involves the addition of specialized microorganisms to the contaminated site; it is especially used when the indigenous communities do not have the necessary metabolic abilities (11). A successful application of this strategy is however highly site specific, and requires a deep characterization of the system (12). Monitoring techniques, such as quantitative PCR (qPCR) for detection of catabolic genes, can be exploited to gain more insight into complex bioremediation processes by giving information about the abundance of bacteria with specific degradation potential (13). In a previous study, the genetic potential for aerobic hydrocarbon degradation was investigated and degradative functional genes were quantified by qPCR. Catabolic genes were ubiquitous in groundwater within the site but reverse transcription qPCR (RT-qPCR) showed that only in the areas where the degradation was actively observed the genes were expressed (14). Other works used qPCR to quantify aromatic oxygenase genes in gasoline contaminated sites to evaluate the effect of different remediation strategies, such as the use of oxygen releasing materials (15) and multiphase extraction (16). Furthermore, 16S rRNA gene sequencing has been broadly used to describe the taxonomic composition of the microbial communities in hydrocarbon contaminated sites (17). A cloning and sequencing approach was recently used to assess the bioremediation potential of the BP Deepwater Horizon spill in the Gulf of Mexico (18). In recent years, Next Generation Sequencing (NGS) technologies were used to characterize the microbial communities developed during polycyclic aromatic hydrocarbon bioremediation in column reactors (19), and the effect of rice straw biochar addition on petroleum contaminated soil bioremediation (20).

In this study we characterized the microbial communities developed in two bench scale aerobic biobarriers for gasoline contaminated groundwater treatment as previously described (21). Each column was filled with volcanic pumice as support material, and in one column an allochtonous bacterial consortium was added as microbial inoculum. At the end of the experiments, the communities selected throughout the column were thoroughly described by Illumina sequencing of the 16S rRNA gene and catabolic genes were quantified by qPCR. The aim of our study was to describe the microbial communities developed during the treatment in both the conditions and to understand whether i) the microbial inoculum persisted during the treatment, and ii) the bioaugmentation with selected bacteria improved the biodegradation potential.

MATERIALS AND METHODS

Column experiments

The column tests were carried out as previously described (21); below a brief description of the experimental set up is reported. A Teflon-coated basin (250 cm long, 10 cm wide) filled with a 15 cm layer of volcanic pumice was used to perform the experiments. The water-saturated layer was 10 cm high. Eight piezometers (‘A’ to ‘H’) were uniformly distributed along the column to collect water samples (a list of the abbreviations is reported in Table 1).

Abbreviation	Sample
P	Dry pumice
A	Piezometer A
B	Piezometer B
C	Piezometer C
D	Piezometer D
E	Piezometer E
F	Piezometer F
G	Piezometer G
H	Piezometer H
UN	Uninoculated column
IN	Inoculated column
t0	Before the treatment
tf	After the treatment

Table 1 - List of the abbreviations.

In the inoculated test (IN), an allochthonous microbial consortium containing *Rhodococcus* sp. CE461, *Rhodococcus* sp. CT451 and *Methylibium petroleiphilum* LMG 22953 (OD_{540} = 0.1 each, volume = 5 L each) was added into the saturated zone of the column and was recirculated for 3 days before starting the test. The microbial inoculum was selected as previously reported for its ability of efficiently degrading hydrocarbons in batch experiments (22). The cultures used as microbial inoculum for IN were incubated 24 hours in LB rich medium at 30°C under shaking, washed and resuspended in a phosphate buffer solution (PBS) to OD_{540} = 0.1 (23). The duration of the tests was 43 days for the uninoculated test (UN) and 85 days for IN. Tap water artificially contaminated with gasoline was used as the inflow solution at 3.0 L d^{-1}. BTEX, MTBE, ETBE and *tert*-Butyl Alcohol (TBA) were quantified under steady state conditions. To ensure aerobic conditions, a commercial oxygen-release compound (EHC-O, Adventus, 0.3% on dry weight basis of pumice) was uniformly distributed throughout the saturated layer of pumice before starting the tests. Further amounts of EHC-O (250 mg twice a week) were poured into the piezometers during the IN test. Nitrogen (27.7 g of NH_4NO_3) and phosphorus (2.1 g of H_2KPO_4 and 2.7 g of HK_2PO_4) were also added as nutrient source. Water samples were collected twice a week to measure the pollutant concentrations, temperature, the pH value (electronic pH-meter XS pH 6, Oakton), and the dissolved oxygen (DO) concentration (OXI 340i probe, WTW). Hydrocarbons in the water samples were extracted by solid-phase microextraction and quantified by GC-MS (UNICHIM 1210:1997). At the end of the experiments pumice samples were collected near each piezometer and stored at -20 °C for the microbial community characterization.

Amplification of the 16S rRNA gene, sequencing and sequence analyses

Total bacterial DNA was extracted using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) from pumice samples according to the manufacturer’s instructions. The presence of environmental inhibitors was investigated by performing serial dilutions of the extracted DNA and inhibition effect was not observed. 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 a dual PCR amplification protocol. The first PCR was performed in 3 x 75 µL volume reactions with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 µM of each primer. 783F and 1046R primers were used (24, 25) and the cycling conditions were: initial denaturation at 98°C for 30 s; 20 cycles at 98°C for 10 s, 47°C for 30 s, and 72°C for 5 s and a final extension at 72°C for 2 min. The second PCR was performed in 3 x 50 µL volume reactions by using 23 µL of the purified amplicons (Wizard® SV Gel and PCR Clean-up System, Promega Corporation, Madison, WI, USA) from the first step as template and 0.2 µM of each primer. Primers sequences contained the standard Nextera indexes (Illumina, Inc., San Diego, CA, USA) and the cycling conditions were: initial denaturation at 98°C for 30 s; 15 cycles at 98°C for 10 s, 62°C for 30 s, and 72°C for 6 s and a final extension at 72°C for 2 min. DNA quantity after the amplification was evaluated using Qubit® (Life Technologies, Carlsbad, CA, USA). The sequencing was carried out at the Parco Tecnologico Padano (Lodi, Italy). Each sequence was assigned to its original sample according to its barcode. Forward and reverse reads were merged with perfect overlapping and quality filtered with default parameters using Uparse pipeline (26). 6000 randomly selected sequences were retrieved from each library and taxonomically classified by RDP classifier (>80% confidence). The sequences were deposited in the European Nucleotide Archive with accession number PRJEB7329.

Catabolic genes and 16SrRNA gene quantification

The abundance of total bacteria was estimated by the quantification of the number of copies of the 16S rRNA gene as previously reported (27). A 466-bp fragment of the bacterial 16S rDNA (331–797 according to *Escherichia coli* position) was PCR-amplified with a universal primer set (Table 2) (28).

The PCR was performed in a total volume of 10 µL using the FluoCycle II Sybr reaction mix (Euroclone, Pero, Italy) with 0.3 µM (final concentration) of forward and reverse primers. The amplification was carried out under the following conditions: 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, with acquisition of the fluorescence at the end of each 72 °C elongation step.

The abundance of hydrocarbon-degrading bacteria was estimated by quantification of catabolic genes. Xylene monooxygenase (*xylM*) and toluene monooxygenase (*tmoA*) were chosen as target genes using respectively TOL and RDEG primers (Table 2) (29). The PCR mix was prepared as previously described for the 16S rRNA gene and the amplification was performed in both cases under the following conditions: 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s, 57 °C for 30 s and 72 °C for 45 s, with acquisition of the fluorescence at the end of each 72 °C elongation step. The fragments of interest were amplified from reference strains (*E. coli* K-12 substr. DH10B for 16S RNA gene and *Pseudomonas* sp. CXP452 for *xylM*) and cloned into the plasmid pCR2.1 (Life Technologies Italia, Monza, Italy) to prepare standards for calibration curves. Since *tmoA* was not detected in any sample, the analyses on this gene were not conducted further. The concentration of plasmidic DNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Serial dilutions of the plasmidic DNA were included in triplicate in each run together with the samples.

Gene amplified	Primer set	Fragment size	Reference
16S rRNA	F: 5′-TCCTACGGGAGGCAGCAGT-3′ R: 5′-GGACTACCAGGGTATCTAATCCTGTT-3′	466-bp	28
xylM	F: 5′-TGAGGCTGAAACTTTACGTAGA-3’ R: 5′-CTCACCTGGAGTTGCGTAC-3’	455-bp	29
tmoA	F: 5′-T(C/T)TC(A/C/G)AGCAT(A/C/T)CA(A/G)AC(A/C/G)GA(C/T)GA-3’ R: 5′-TT(A/G/T)TCG(A/G)T(A/G)AT(C/G/T)AC(A/G)TCCCA-3’	470-bp	29

Table 2 - Primer set used to perform the quantification of the 16S rRNA, *xylM* and *tmoA* genes by qPCR.

Statistical analysis

A Principal Component Analysis (PCA) of the genus relative abundance and the Welch’s t-test on the most abundant genera (95% of confidence) were performed using the STAMP software (30).

RESULTS

Column parameters

At the end of the experiments, DO concentration showed generally lower values compared to the beginning. Moreover, it decreased throughout the column (from piezometer A to piezometer H) in both UN and IN test (Fig. 1). After a fast decrease next to the inlet, however, UN showed a higher DO concentration (ranging from 4.8 mg L^{-1} to 5.4 mg L^{-1}) than IN (ranging from 2.1 mg L^{-1} to 4.8 mg L^{-1}). Since DO drop observed in IN occurred after only 6 days of operation, to ensure aerobic conditions during the experiment, ECH-O was periodically added into the column.

MTBE average concentrations in the inlet and in the outlet at the stationary state are reported in Fig. 2A. The removal efficiency observed in UN was about 78%, slightly higher than that in IN (63%), suggesting that the addition of BTEX degrading microorganisms did not improve the overall performance of the treatment system for MTBE. Toluene was almost completely removed from the column during the treatment (Fig. 2B). The removal efficiency in both IN and UN was about 96% despite the addition of a selected microbial inoculum in IN.

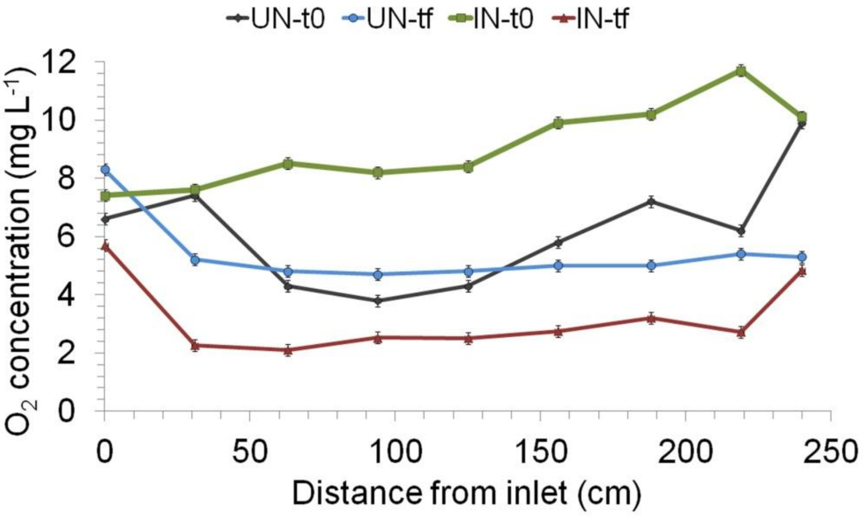

Fig. 1 - Dissolved oxygen profile during gasoline contaminated groundwater treatment. UN-t0 = uninoculated column before the treatment; UN-tf = uninoculated column after the treatment; IN-t0 = inoculated column before the treatment; IN-tf = inoculated column after the treatment.

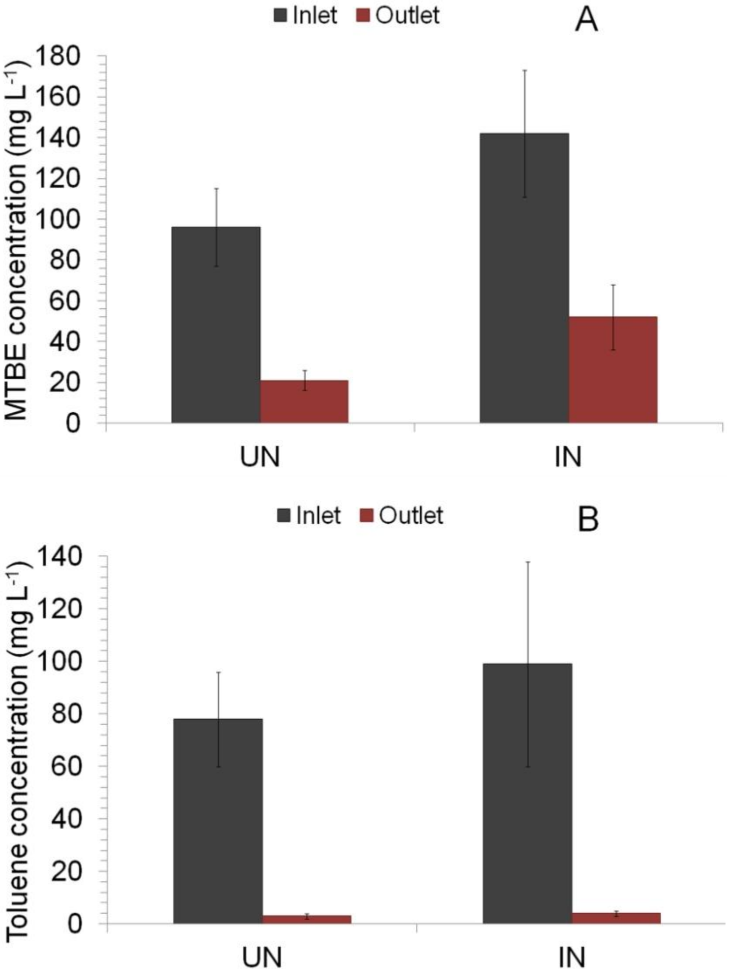

Fig. 2 - MTBE (A) and toluene (B) concentrations measured in the inlet and in the outlet in the uninoculated column (UN) and in the inoculated column (IN) in stationary conditions.

Taxonomic composition and biodiversity

The classification of the sequences was performed at the genus level and a comparison of the communities developed throughout the column was performed. The Shannon Index was calculated for each sample as an estimation of the alpha diversity. The alpha diversity observed for the pumice before the treatment (P) was 3.0 while the diversity value in the samples collected in the columns, both before and after the treatment, ranged from 1.0 to 3.1 (Fig. 3).

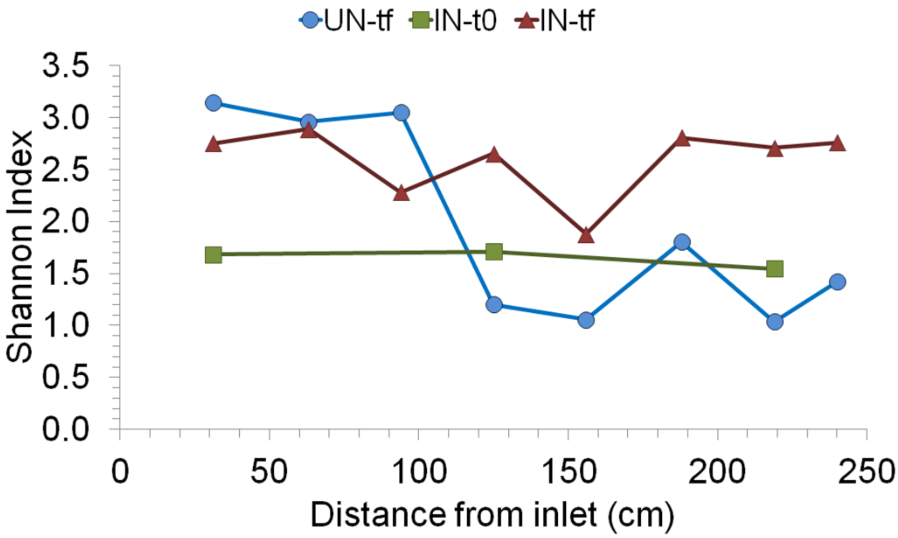

Fig. 3 - Shannon Index calculated on the basis of the genus relative abundance estimated by Illumina sequencing of the 16S rRNA gene. SI calculated for the dry pumice was 3.4. UN-tf = uninoculated column after the treatment; IN-t0 = inoculated column before the treatment; IN-tf = inoculated column after the treatment.

For UN a clear difference between the communities developed at the beginning of the column and those developed at the end was observed. The alpha diversity decreased in UN throughout the column and the Shannon Index reached the lowest values near the piezometers E and G, 1.0 and 1.1, respectively. IN showed a constant biodiversity before the treatment with the Shannon Index ranging from 1.5 to 1.7 and an increase of the alpha diversity was observed after the treatment. At the end of the experiment the diversity in the samples collected from IN ranged from 2.7 to 2.9 with the only exception of the piezometers C and E (the Shannon Index was respectively 2.3 and 1.9).

PCA revealed that pumice before the treatment, samples collected from UN, samples collected from IN before the treatment and samples collected from IN after the treatment formed separate clusters (Fig. 4). A clear separation between the microbial communities developed in the first part of the column (piezometers A-C, Cluster 1) and the second part of the column (piezometers D-H, Cluster 2) in UN was also observed (Fig. 4).

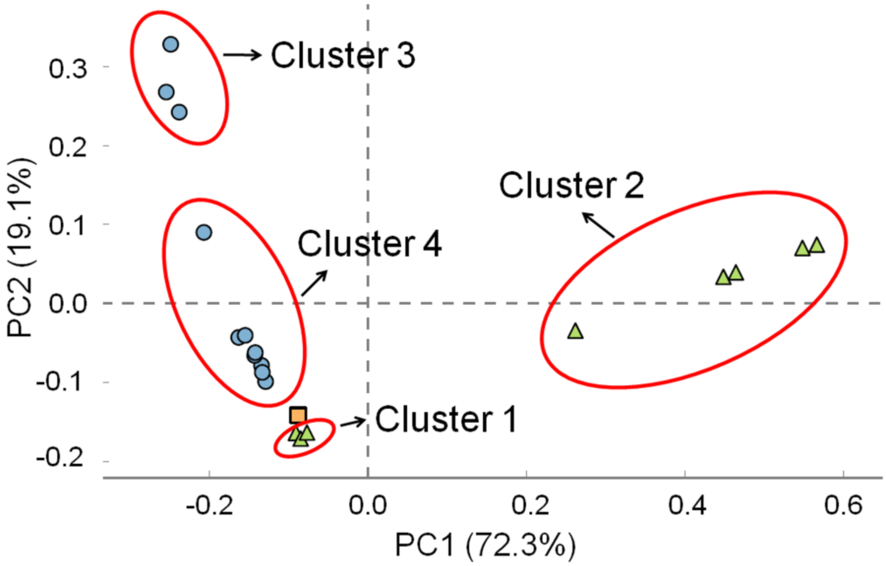

Fig. 4 - Principal Component Analysis of the genus relative abundance. Uninoculated column (UN-green triangles); inoculated column (IN-blue circles); dry pumice (P-orange square); t0 = sample collected before the treatment; tf = sample collected after the treatment; letters (A-H) indicate the sampling point in the column. Cluster 1 = A-UN-tf, B-UN-tf, C-UN-tf; Cluster 2 = D-UN-tf, E-UN-tf, F-UN-tf, G-UN-tf, H-UN-tf; Cluster 3 = A-IN-t0, D-IN-t0, G-IN-t0; Cluster 4 = A-IN-tf, B-IN-tf, C-IN-tf, D-IN-tf, E-IN-tf, F-IN-tf, G-IN-tf, H-IN-tf.

The taxonomic composition in UN changed heavily throughout the column, from piezometer A to piezometer H (Fig. 5). The most abundant genus in the first part of the column (samples A-UN-tf, B-UN-tf, and C-UN-tf) was *Acidovorax* (respectively 8.3%, 15.4% and 8.0%). *Hydrogenophaga* genus represented 11.3% (A-UN-tf), 14.7% (B-UN-tf) and 5.7% (C-UN-tf) of the sequences in the first part of the column (piezometers A-C).

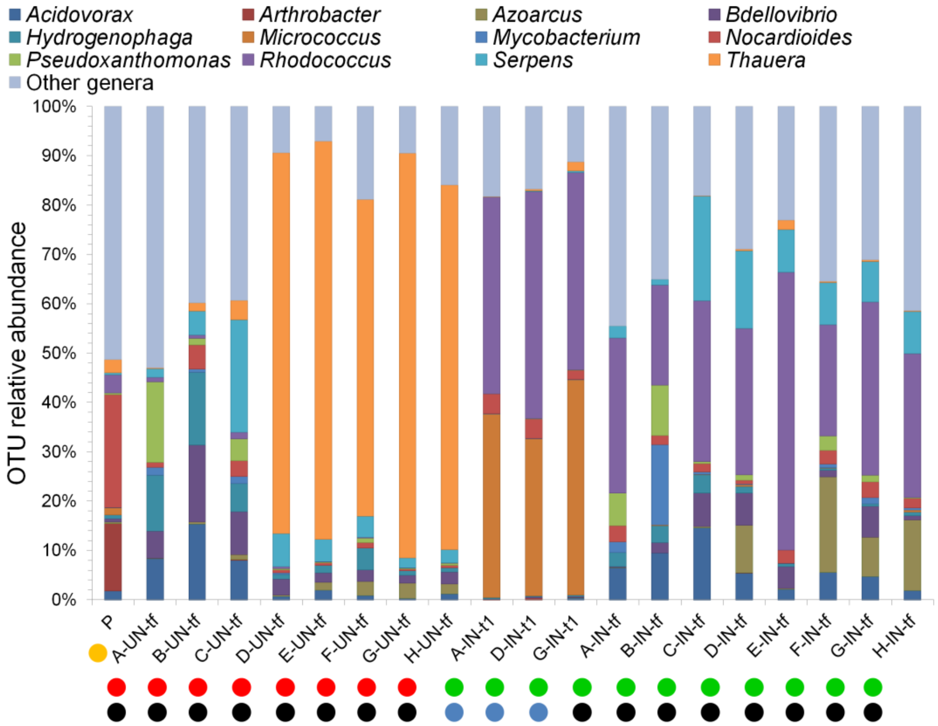

Fig. 5 - Taxonomic composition at genus level of the microbial communities selected into UN (red circles) and IN (green circles), before (t0) (blue circles) and after (tf) (black circles) the treatment and on the dry pumice (P) (yellow circle). The letters (A-H) indicate near which piezometer the sample was collected. Only the OTUs with a relative abundance of 10.0% (or higher) in at least one sample were reported.

Genera *Pseudoxanthomonas*, *Bdellovibrio* and *Serpens* were particularly abundant respectively in samples A-UN-tf (16.4% of the sequences), B-UN-tf (15.6% of the sequences), and C-UN-tf (22.8% of the sequences). In the second part of UN, from the piezometer D until the end of the column (piezometer H), the microbial communities were dominated by facultative anaerobes. *Thauera* was the most abundant genus (Fig. 6A) with a relative abundance of 64.2% (in F-UN-tf) up to 82.0% (in G-UN-tf).

Samples collected from IN before the treatment showed that genera *Rhodococcus* and *Micrococcus* were the most abundant with respectively 39.8%, 46.0%, 39.9% and 37.2%, 31.8%, 43.7% of the sequences near the piezometers A, D, and G. No sequences belonging to *Methylibium petroleiphilum* were detected. After the treatment, *Rhodococcus* was the most abundant genus in IN ranging from 20.2% (B-IN-tf) to 56.2% (E-IN-tf) but its abundance decreased during the treatment (Fig. 6C).

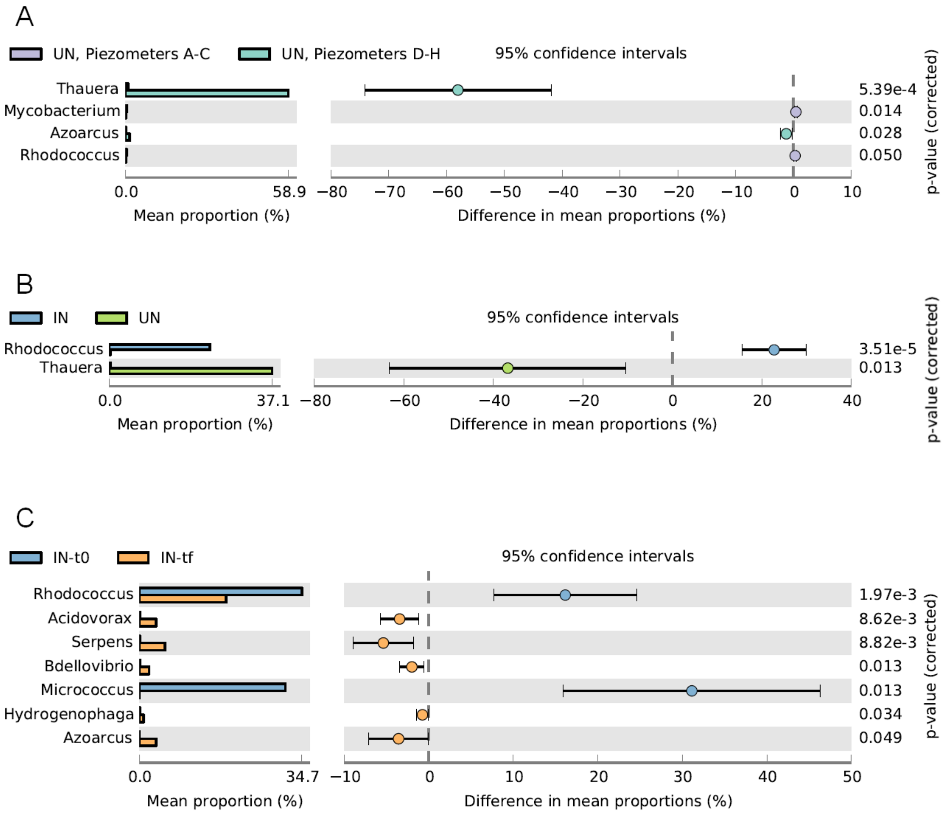

Fig. 6 - Welch's t-test results for the most abundant genera. A) Comparison between the microbial communities developed in the first part of the uninoculated column (UN, piezometers A-C) and in the second part of the uninoculated column (UN, piezometers D-H). B) Comparison between the microbial communities developed in the uninoculated column (UN) and in the inoculated column (IN) at the end of the treatment. C) Comparison between the microbial communities in the inoculated column before the treatment (IN-t0) and after the treatment (IN-tf).

Microorganisms belonging to *Serpens* were found throughout the whole column but they were more abundant near the piezometers from C to H. Similarly, *Acidovorax* represented up to 14.6% of the sequences near the piezometer C, but in lesser extent it was detected in all the samples. *Mycobacterium* was enriched in the first part of the column and represented 16.2% of the sequences in the sample B-IN-tf. *Hydrogenophaga* genus was detected in almost every community but it was not as abundant as in UN. The facultative anaerobe *Azoarcus* colonized the second part of the column, from piezometer D to piezometer H and represented up to 19.4% of the sequences.

Gene quantification

The number of ribosomal operons per g of pumice in sample P was about 10^7 while, in the samples collected from UN at the end of the treatment, it increased of an order of magnitude (Fig. 7).

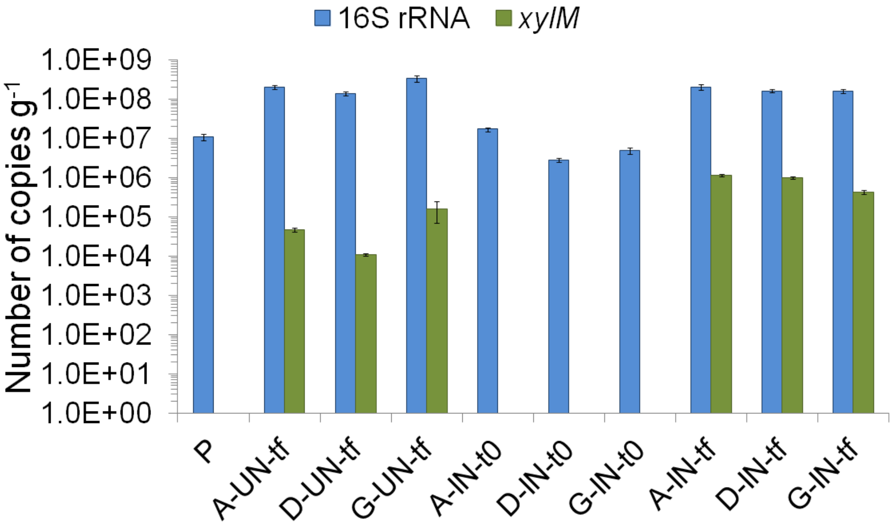

Fig. 7 - Number of copies per g of pumice of the 16S rRNA gene and *xylM* detected by qPCR into UN and IN, before (t0) and after (tf) the treatment and on the dry pumice (P). The letters (A-H) indicate near which piezometer the sample was collected. *XylM* was under the detection limit in samples P, A-IN-t0, D-IN-t0, G-IN-t0.

When the bacterial inoculum was added into the column, the number of ribosomal operons did not increase in samples A-IN-t0, D-IN-t0 and G-IN-t0 compared to P. Furthermore, after the treatment, the number of ribosomal copies in IN did not differ from that observed in UN and, similarly, it increased up to 10^8 compared to the beginning of the experiment.

In both IN and UN *tmoA* was not detected. Conversely, the presence of *xylM* was not observed before the treatment in both IN and UN but it became relevant at the end of the experiment (Fig. 7), Moreover, in the IN experiment, the number of copies of *xylM* was generally one order of magnitude higher compared to the UN test (10^5 vs 10^4 copies of *xylM* per g of pumice).

DISCUSSION

Gasoline contaminated water treatment was performed with a bench scale biobarrier. The removal efficiency was tested both with and without the addition of a selected microbial inoculum, however the addition of selected microorganisms did not lead to an increasing of hydrocarbon removal. These data are in contrast to what previously observed in a similar system. In a permeable reactive barrier with cell-immobilized beads for gasoline contaminated groundwater treatment, the bioaugmentation with a microbial inoculum composed by two pure cultures (*Mycobacterium* sp. CHXY119 and *Pseudomonas* sp. YATO411) improved the removal efficiency (31). The different behavior could be due to the immobilization of the microbial inoculum on the support material in this last case, allowing an easier competition with the indigenous microorganisms in the column. The use of support materials could enhance the degradative activity and promote the persistence of external inocula (32). In the present study pumice did not show the ability to promote neither the degradative activity nor the persistence of the microbial inoculum. However, native bacteria on the pumice were already able to degrade the contaminants and thus the stimulation of the native communities, rather than the addition of an external inoculum, is an attractive strategy.

The taxonomic composition of the microbial communities enriched during the treatment was performed at genus level. The Shannon Index showed an increasing of the diversity in IN after the treatment, which suggested that the microbial inoculum was outcompeted by the native microorganisms of the pumice. This observation was confirmed by the PCA because the samples collected from IN formed two different clusters. Furthermore, both the Shannon Index and the PCA showed that the microbial communities enriched in UN had a spatial separation throughout the column and changed from the first part of the column (piezometers A-C) to the second part of the column (piezometers D-H). These findings were also consistent with the difference in the degradation rate constants measured in the two parts of the column for some of the tested compounds (Table 3). This difference is clearly visible for *m*-, *p*-xylene on the one hand, and ethylbenzene on the other, that showed a higher degradation rate respectively in the first part of the column (piezometers A-C) and in the second part of the column (piezometers D-H) (22).

The most abundant genera in the first part of UN were *Acidovorax* and *Hydrogenophaga* respectively. Microorganisms belonging to the genus *Acidovorax* were described as PAH degraders, and were found in column reactors designed to stimulate the biodegradation in contaminated soil (19). The microbial characterization of the communities of a site contaminated with high concentrations of BTEX and MTBE showed that *Acidovorax* was abundant in the oxic samples (33). *Hydrogenophaga* genus have been previously described to include hydrocarbon degraders. *Hydrogenophaga flava* ENV735 is a gram negative bacterium isolated from enrichment cultures on MTBE and was able to degrade both TBA and MTBE in pure culture (34). This genus was also found in MTBE degrading communities that colonized bioreactors (35, 36), and in a mixed culture enriched from a groundwater sample from a gasoline contaminated site (37). The presence of *Hydrogenophaga* was also described in a microcosm set up using groundwater from a BTEX contaminated site as inoculum and a mixture of toluene and benzene as carbon source (38), and seemed to have a role in polycyclic aromatic hydrocarbon (PAHs) degrading communities (39). Other abundant bacteria enriched in UN (piezometers A-C) were *Pseudoxanthomonas*, *Bdellovibrio* and *Serpens*. *Pseudoxanthomonas* genus was broadly described as able to degrade PAH and several strains were isolated from contaminated soil and sediment (40–42), while *Pseudoxanthomonas spadix* BD-a59, a bacterium isolated from a gasoline contaminated sediment, was reported to be able to degrade all the BTEXs (43). *Bdellovibrio* was previously enriched in a BTEX-fed reactor for water treatment (44), while, to the best of our knowledge, this is the first time that *Serpens* genus is described in a hydrocarbon degrading community. *Thauera* was the dominant microorganism in UN from the piezometer D to the piezometer H. A member of this genus is *Thauera aromatica* K 172, a microorganism well known for its ability to degrade aromatic hydrocarbons both in aerobic and denitrifying conditions (45). *Thauera* was also found in a microcosm set up with toluene as sole carbon source in denitrifying conditions by using a sediment collected from a tidal flat as inoculum (46). Li and colleagues found the genus *Thauera* to be dominant in toluene and benzoate enrichment cultures amended with nitrate as sole electron acceptor (47).

The most abundant microorganisms in IN before the treatment were *Rhodococcus* and *Micrococcus*. While *Rhodococcus* was inoculated in the column with the artificial consortium used as inoculum, microorganisms belonging to the genus *Micrococcus* grew in the column during the colonization phase. *Methylibium petroleiphilum* was probably outcompeted by other bacteria during the 3-day recirculation phase. The high similarity among microbial communities detected in different piezometers of IN before the treatment confirmed that the recirculation phase was effective in the homogeneous distribution of the inoculum. However, since less than half of the sequences belong to microorganisms added with the inoculum, it is reasonable to speculate that most of the inoculated bacteria could not survive during the initial recirculation.

After the treatment *Rhodococcus* was the most abundant microorganism in IN, which was expected since *Rhodococcus* sp. CE461 and *Rhodococcus* sp. CT451 were monoaromatic degraders added in the column with the microbial inoculum. However, the abundance of this genus was significantly higher before the treatment than after the treatment, suggesting that the inoculum did not successfully colonize the pumice. Other taxonomic groups that were abundant in IN after the treatment were *Serpens, Acidovorax, Mycobacterium* and *Azoarcus*. *Mycobacterium austroafricanum* IFP 2015 was reported as MTBE degrader and was isolated from an MTBE degrading microcosm inoculated with drain water of an MTBE supplemented gasoline storage tank (48). In a previous work the microbial biodiversity in BTEX contaminated groundwater was studied and *Mycobacterium* sp. C3, able to degrade both toluene and *m*-xylene, was isolated (49). Several microorganisms belonging to the genus *Azoarcus* were reported to degrade hydrocarbons in nitrate reducing conditions. *Azoarcus evansii* KB 740 was able to use a variety of aromatic substrates as sole carbon source in the presence of nitrate (45), while *Azoarcus*-like strain EbN1, isolated from anoxic freshwater mud, was previously used as model microorganism to describe the oxidation mechanism of toluene and ethylbenzene in anaerobic conditions (50). Microorganisms belonging to *Azoarcus* genus were also isolated from a BTEX contaminated groundwater (49).

The enrichment of microbial communities dominated by facultative anaerobic microorganisms in UN (piezometers D-H) could be explained referring to the DO concentrations measured during the experiment. DO measured in the area next to every piezometer was approximately 5 mg L^{-1}, but the heterogeneity of the system could have caused the presence of microenvironments with a variable DO concentration, or even without oxygen. However, the available information about DO concentration in the system concern only the environment external to the pores of the pumice and oxygen diffusion into the pores could be difficult. Thus, bacteria able to grow both with and without oxygen as electron acceptor (i.e. *Thauera*), could have taken advantage over the aerobic microorganisms. The presence of *Thauera* was significantly higher in the second part of the column. Furthermore, sequences belonging to the facultative anaerobe, hydrocarbon degrader, *Azoarcus* were observed to be more abundant from the piezometer E to the piezometer H, although it represented less than 3% of the microbial communities. These findings enforce the hypothesis that microenvironments with low DO characterized the second part of the column in UN (piezometers D-H) leading to an increase in the hydrocarbon degradation using NO_3^- as electron acceptor. Thus, the NH_4NO_3 added served not only as a nutrient source but also as an electron sink.

The higher abundance of aerobic microorganisms in IN compared to UN explained the faster oxygen consumption in this experiment. Furthermore, aerobic microorganisms could have been advantaged from the periodical spiking with EHC-O during the treatment, leading to an homogeneous DO concentration in the column. In contrast, since

NH_4NO_3 was not further added in IN during the experiment, there was a lack of NO_3^- that could have been used as electron acceptor, limiting the growth of NO_3^--reducers, with the only exception of a small amount of *Azoarcus*. Finally, the taxonomic composition analysis of IN confirmed the findings of the Shannon Index and PCA results. In fact, the microbial inoculum persisted into the column only partially and was largely outcompeted by the native microorganisms of the pumice during the operation of the BB.

The taxonomic analysis performed on both columns revealed that, although the gasoline contaminated water treatment led to microbial communities with a completely different composition in the two cases, the measured degradation kinetic constants were similar in the two conditions for both toluene and MTBE (Table 3) (22).

	UN (piezometers A-C)	UN (piezometers D-H)	IN
Benzene	0.389 ± 0.123 d^{-1}	0.558 ± 0.122 d^{-1}	0.473 ± 0.025 d^{-1}
Toluene	0.536 ± 0.112 d^{-1}	0.780 ± 0.147 d^{-1}	0.505 ± 0.025 d^{-1}
Ethylbenzene	0.464 ± 0.181 d^{-1}	1.565 ± 0.205 d^{-1}	0.774 ± 0.035 d^{-1}
m-, *p*-Xylene	1.206 ± 0.038 d^{-1}	0.196 ± 0.042 d^{-1}	0.511 ± 0.021 d^{-1}
o-Xylene	0.240 ± 0.078 d^{-1}	0.643 ± 0.089 d^{-1}	0.457 ± 0.022 d^{-1}
MTBE	0.281 ± 0.106 d^{-1}	0.210 ± 0.033 d^{-1}	0.180 ± 0.018 d^{-1}

Table 3 - Degradation kinetic constants measured in the uninoculated column (UN) and in the inoculated column (IN). The constants in UN test were split because the values calculated in the first part of the column (piezometers A-C) were different from the values observed in the second part (piezometers D-H) (22).

The number of copies of the 16S rRNA gene was quantified by qPCR in order to compare the bacterial abundance at the beginning and at the end of the column experiments. This parameter is not directly linked to cell number due to the presence of multiple ribosomal operons in the bacterial genomes; however, it can be used to look at relative shifts in microbial biomass as the bias in copy number is likely constant (27).

It was observed that the number of ribosomal operons per g of pumice did not increase after the inoculum addition. Therefore, the addition of the inoculum did not lead to an increasing of the biomass into the columns, confirming that it was, at least partially, washed away not only after the treatment, but also after the 3-day recirculation phase. The microbial inoculum used for IN test was composed by two Gram-positive strains (*Rhodococcus* sp. CE461 and *Rhodococcus* sp. CT451). Gram-positive microorganisms are reported to use mainly dioxygenases to degrade monoaromatic hydrocarbons. However, these enzymes are mainly represented by naphthalene dioxygenases that can also degrade monoaromatic hydrocarbons with more aspecific mechanisms (51). However, *xylM*-like and *tmoA*-like genes were also occasionally detected in Gram-positive bacteria (i.e. *Rhodococcus* and *Arthrobacter* strains) probably because of horizontal gene transfer (52). For this reasons, in this study we focused our attention on monooxygenases that are specific for BTEX degradation. The presence of both *tmoA* and *xylM* genes was assessed, since these genes were suggested to be the most frequently retrieved BTEX catabolic genes in environmental samples (52). Increases in oxygenase copy number were previously found to be related to the catabolic ability to degrade hydrocarbons during bioremediation processes (16). In our experiments, *tmoA* was not detected in any sample, suggesting that this gene was not crucial during the BTEX degradation in this system. However, the increasing in the number of copies of *xylM* indicated that BTEX degrading microorganisms were selected. Furthermore, the higher number of copies of *xylM* observed in IN compared to the UN test may be due to the higher presence of aerobic bacteria in IN than in UN, thus indicating that the degradation mechanisms occurred in the two conditions were different.

CONCLUSIONS

Two columns for treatment of synthetic gasoline contaminated groundwater were set up, using pumice as filling material, in order to test their performance both with (IN) and without (UN) a selected microbial inoculum. The bacterial communities were described by in deep Illumina sequencing of the 16S rRNA gene. Furthermore 16S rRNA gene and catabolic genes (*tmoA* and *xylM*) were quantified by qPCR. No differences in the removal efficiency were observed for the two tested conditions, despite the addition of a selected inoculum. The microbial communities detected in UN at the end of the treatment were different from the community of the pumice before the treatment, with a dominance of *Thauera*, indicating the presence of microenvironments with low oxygen concentration despite the average DO was about 5.0 mg L^{-1} throughout the column. Conversely, IN was dominated by aerobic bacteria and *Rhodococcus* was the most abundant genus both before and after the treatment, although it significantly decreased during the column operation. This genus was added with the selected microbial inoculum, but both the 16S rRNA gene sequencing and the qPCR results clearly showed that i) it was outcompeted by the native microorganisms of the pumice and persisted only partially into the column, and ii) that the bioaugmentation did not lead to an increase in the degradative potential. The overall results of this study showed that the use of an aerobic BB is an effective strategy to remediate gasoline contaminated groundwater and that the addition of a microbial inoculum may be not necessary to reach a suitable removal efficiency. However, results also showed that different DO concentrations along the simulated biobarrier affected the microbial community composition and, subsequently, the biodegradation rate of pollutants.

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Chapter 2 - Bioelectrochemical toluene degradation in marine environments

ABSTRACT

Hydrocarbons released during oil spills often show high persistence in marine sediments due to the absence of suitable electron acceptors below the oxic zone. Here we investigated an anode of a bioelectrochemical system as alternative strategy to remove toluene as model monoaromatic hydrocarbon. With sediment collected from an hydrocarbon contaminated marine site as microbial inoculum and anode potentials of 0 mV and +300 mV (vs Ag/AgCl), enrichment of bioelectrogenic microorganisms was observed. Degradation of toluene was directly linked to current generation up to 301 mA m^{-2} and 431 mA m^{-2} for the bioanodes polarized at +0 mV and +300 mV, respectively. However, over time decreasing peak currents were obtained upon renewed spiking. Monitoring of sulfate concentrations during bioelectrochemical experiments suggested that sulfur metabolism is involved in toluene degradation at bioanodes. Microbial characterization of the bulk and anode samples by 16S rRNA-based Illumina sequencing conducted at the end of the experiments revealed the dominance of sulfate reducing microorganisms. In particular, the family *Desulfobulbaceae* appeared to be linked both with current production and toluene degradation. Based on our observations we propose two mechanisms for bioelectrochemical toluene degradation: (i) direct electron transfer to the anode, and/or (ii) sulfide mediated electron transfer.

INTRODUCTION

The large number of oil spills from offshore extraction platforms, ships or more diffuse sources pose major concerns to the aquatic ecosystems (1). Volatile organic compounds (VOCs) such as hexane, benzene, toluene, ethylbenzene and xylenes are a large fraction of petroleum hydrocarbons that represent up to 15% of the total hydrocarbon contaminants (2). These compounds can have a broad range of toxicity on a large number of organisms (3) affecting natural biota and ecosystems (4), fisheries (5) and human health (6). Although the most dramatic effects are usually observed when a large volume of oil is spilled, it has also been shown that small quantities of oil can lead to a severe impact on marine ecosystems (7).

When an oil spill occurs hydrocarbon removal strategies can be applied to avoid risk for the environment. Biological methods are nowadays widely used in this context (8). However, in confined environments such as sea sediments and aquifers, the excess of carbon source due to the presence of contaminants leads to the depletion of nutrients (9) and thermodynamically favorable terminal electron acceptors (10) thus reducing the biodegradation rate of pollutants. Biostimulation of biodegradation often aims at overcoming the limitations that inhibit the removal of the contaminants by naturally present microorganisms. Thus, bioremediation represent a cost-effective strategy compared to other remediation technologies (8). Furthermore, bioremediation potentially allows complete mineralization of the contaminants rather than transferring the pollutants from one phase to another one, like for example by stripping or adsorption processes (11). As a matter of fact one of the most used bioremediation approach is the stimulation of the aerobic metabolism by adding oxygen into the environment (12). However, oxygen diffusion is limited and oxygen losses can occur due to side reactions with reduced species, such as Fe^{2+} or Mn^{2+} (13, 14).

Furthermore, oxygen naturally escapes from the contaminated area. Continuous amendment with electron acceptors is therefore required, which however increases the overall process cost (15).

Bioelectrochemical systems (BESs) constitute an alternative approach to overcome electron acceptor limitations, also for oil contaminated sediments (16, 17). On the ocean floor, benthic BESs have been already described as a suitable technology to power oceanographic devices, by harvesting energy from anaerobic sediments (18, 19). A BES is a device in which electrogenic microorganisms oxidize an electron donor using an electrode as a sole electron acceptor (20). Recently, lab scale studies demonstrated that BES-based technologies can be successfully applied to remove both aliphatic and aromatic hydrocarbons from oil contaminated sediments (15, 21–23), groundwater (22) and refinery wastewater (24) with simultaneous current production. Very few studies have discussed the possibility to bioelectrochemically degrade toluene, either by inoculating BES reactors with hydrocarbon contaminated sediment (15) or pure cultures, i.e. of *Geobacter metallireducens* (15) and *Pseudomonas putida* (25). A characterization linking developing electrocatalysis to the involved microbial communities is lacking, as well as in-depth knowledge on microbial ecology and energy flows associated with hydrocarbon degradation using an anode. There are indications that iron reducing (22), nitrate reducing (17, 26) and aerobic bacteria (26) may play a significant role in hydrocarbon degradation, as they have been found in the biofilm formed on anodes. Earlier research also elucidated that sulfide can serve as shuttle to convey electrons from acetate to an anode (27). Considering the strong presence of sulfate in marine conditions, anaerobic hydrocarbon degradation linked to sulfate reduction may occur, leading to presence of sulfur intermediates after anodic partial oxidation. A later study speculated that sulfur cycle can play a role in the electron transfer between the anode and the electron donor during benzene degradation in BES (22).

In this study, bioelectrochemical toluene degradation in marine contaminated environments was assessed using BESs. The objectives of this study were to (i) investigate the biodegradability of toluene in BESs inoculated with marine contaminated sediment, (ii) evaluate the effect of anodic potential on toluene biodegradation; (iii) identify the most abundant microbial populations that are involved in bioelectrochemical toluene degradation process, and (iv) elucidate the role of sulfur cycle on toluene biodegradation in a BES.

MATERIALS AND METHODS

Bioelectrochemical reactor set-up, operation and experimental conditions

The bioelectrochemical experiments were carried out using custom-made two chamber glass BES reactors with five necks (250 mL) (28). The anodic chamber was filled with 190 mL of artificial ocean water (Instant Ocean® Sea Salt) as growth medium to simulate marine environment (pH 6.4) and 10 mL of sea sediment as microbial inoculum (Mokbaai, Netherlands) (Table 1). provided by the Royal Netherlands Institute for Sea Research. Before starting up electrochemical experiments, the anode chamber was first flushed with N_2:CO_2 (90:10) for 30 min to ensure anoxic conditions. Toluene, which acted as the sole source of carbon and energy (23 µL), was periodically spiked into the anode chamber using a microsyringe. The cathodic chamber was filled with diluted artificial ocean water (1:4 dilution with distilled water; pH 8.2) in order to avoid oxidation of the cathode material. Anodes were graphite plates (Mersen, Bay City, MI, USA) with a projected surface area of 8 cm^2. Titanium wire (1 mm diameter - Advent Research Materials, Oxford, England) was fixed in the top center of the graphite plate to serve as a current collector. It was covered with a heat shrinkable PTFE tube (Sigma-Aldrich) in order to prevent corrosion due to the high salinity of the medium. Cathodes were constructed using stainless steel mesh (4.5 cm x 5.0 cm) connected to titanium wire current collectors. The anodic chamber and the cathodic chamber were separated using a cation exchange membrane (Ultrex$^{\text{TM}}$) soaked before use in 5% NaCl at least for 24 hours.

Solid phase density (g cm^3)	2.53 ± 0.03
Porosity	0.54 ± 0.01
Median grain size (µM)	175 ± 8
Org C (%)	0.65 ± 0.07
N total (%)	0.085 ± 0.006
C/N ratio	9.0 ± 0.2
Ca (%)	0.81 ± 0.04
Mg (%)	0.32 ± 0.01
Fe (%)	0.71 ± 0.02
Mn (%)	0.0084 ± 0.0002
P (%)	0.022 ± 0.001
S (%)	0.23 ± 0.01

Table 1 - Chemical characterization of the sea sediment used as microbial inoculum.

If not stated otherwise, all potential values refer to the Ag/AgCl (3 M KCl) reference electrode (+200 mV vs SHE). All bioelectrochemical experiments were conducted at room temperature (22 ± 2 °C) in duplicates or triplicates with the aid of a potentiostat (VSP – BioLogic, France). Anodes were poised at 0 mV and +300 mV, the current profile was recorded using chronoamperometry. Two abiotic controls (ocean water + toluene, hereafter referred to as Ab0 and Ab300) and one control without toluene (sediment + ocean water, referred to as Sed300) were also set up. Further details about these experiments are provided in Table 2. Current density profiles, and concentrations of toluene, sulfate and sulfide were monitored over time. Cyclic voltammetry (CV) was conducted during substrate turnover and substrate non turnover conditions using a scan rate of 1 mV/s in order to detect redox active moieties in the electrochemical process. At the end of the experiments, bioanodes and bulk samples were collected and stored at -20 °C for the analysis of microbial communities.

Reactor	Anode potential (vs Ag/AgCl)	Inoculum	Toluene
R300a, R300b, R300c	+300 mV	Yes	Yes
R0a, R0b	0 mV	Yes	Yes
Ab300	+300 mV	No	Yes
Ab0	0 mV	No	Yes
Sed300	+300 mV	Yes	No

Table 2 - Reactor and experimental details.

Chemical analyses

Samples for toluene analysis were acidified (pH 2) and stored at 4°C until the analysis (EPA method 5035A) (29). The analysis was carried out by GC-FID (Agilent 6890N) equipped with a Headspace Autosampler (Agilent 7697A) (EPA method 5021A) (30). The toluene removal rate was estimated by the first order kinetic constant calculated during the second batch cycle.

In order to monitor sulfate, samples were filtered and stored at 4°C until the analysis by Ion Chromatograph (Metroohm 761 Compact IC). Sulfide was measured immediately after sampling with the NANOCOLOR® Sulphide Standard test (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions.

Microscopy analysis

Scanning Electron Microscopy (SEM-EDS) (JEOL JSM-7600F) and Fluorescent In Situ Hybridization (FISH) (Nikon A1R) analyses were performed on bioanode samples at the end of the experiments. For SEM-EDS, the bioanode was placed in 2% paraformaldehyde and 1.5% glutaraldehyde for 2 hours at 4°C, subsequently washed with Phosphate Buffer Solution (PBS) and fixed with 1% OsO_4 in PBS for 90 minutes. The treated bioanode was then washed twice with distilled water and 25% ethanol, and dehydrated in a series of ethanol solutions (25%, 40%, 50%, 60%, 70%, 80%, 90%, 100%) for 5-10 minutes each. Finally, the bioanode was critical point dried using a gradient series of ethanol/hexamethyldisilazane (HMDS) solutions (25%, 50%, 75%, 3x100%) 10 minutes each, dried overnight and covered with a thin layer of Au. Samples for FISH were fixed with 4% parafromaldehyde, washed in PBS and stored in PBS/ethanol (50%) at -20°C until the analysis. The hybridization was performed using a mixture of Cy3 labeled SRB385 and SRB385Db (31) probes for targeting sulfate reducing bacteria (SRB).

Amplification of the 16S rRNA gene, sequencing and sequence analyses

The microbial biofilm was aseptically removed from the anodes and total bacterial DNA was extracted using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA). DNA was also extracted from bulk collected from the reactors. 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 a dual PCR amplification protocol as previously described (Chapter 1). Briefly, the first PCR was performed in 3 x 75 µL volume reactions with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 µM of each primer. 783F and 1046R primers were used (32, 33). The second PCR was performed in 3 x 50 µL volume reactions by using 23 µL of the purified amplicons (Wizard® SV Gel and PCR Clean-up System, Promega Corporation, Madison, WI, USA) from the first step as template and 0.2 µM of each primer. Primer sequences contained the standard Nextera indexes (Illumina, Inc., San Diego, CA, USA). DNA quantity after the amplification was measured using Qubit® (Life Technologies, Carlsbad, CA, USA). The sequencing was carried out at the Parco Tecnologico Padano (Lodi, Italy). Reads from sequencing were demultiplexed according to the indexes. Uparse pipeline was used for the following elaborations (34). Forward and reverse reads were merged with perfect overlapping and quality filtered with default parameters. Singletons sequences (i.e. sequence appearing only one time in the whole data set) were removed both from the whole dataset and from each sample files. Operational Taxonomic Units (OTUs) were defined on the whole data set clustering the sequences at a 97% of similarity and defining a representative sequence for each cluster. A subset of 6000 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% of similarity. Taxonomic classification at different ranks of the OTUs' representative sequences was obtained by RDP classifier (35). The sequences were deposited in the European Nucleotide Archive with accession number PRJEB7900.

A Principal Component Analysis (PCA) based on Hellinger transform data of the family relative abundance and the post-hoc analysis at family and OTU level (95% of confidence) were performed using the STAMP software (36). The post-hoc tests were performed by applying the Benjamini-Hochberg FDR correction (37).

RESULTS

Toluene degradation and current production

Current production in reactors R0a and R0b with the anodes polarized at 0 mV increased after a short lag phase (Fig. 1A, 1B), albeit at low level and likely associated with residual carbon in the sediment. After 7 days of experiment, these reactors were spiked with toluene. A maximum current density of 283 mA m^{-2} (day 33) and 301 mA m^{-2} (day 32) was reached for reactors R0a and R0b, respectively. When the current decreased due to toluene depletion, the reactors were spiked again with toluene and a reproducible current production was observed. The current density increased immediately to 267 mA m^{-2} (R0a) and 250 mA m^{-2} (R0b) and decreased again after toluene depletion. After further toluene additions the current density did not increase to the same extent again, and after 90 days of operation the current density decreased to about 50 mA m^{-2} in both reactors. The decrease in the electrochemical performance correlated with the onset of sulfate reduction. Whereas the sulfate concentration decreased only slightly during the first 58 days of reactor operation, it dropped sharply when the current decreased on day 64 (R0a) and on day 61 (R0b).

Toluene measurements indicated that toluene in solution decreased immediately after addition, without a lag phase, which likely associates to adsorption on the graphite anode. This assumption was confirmed by the results of the abiotic controls where toluene concentration dropped immediately after addition, but no current production occurred (Fig. S1 - Annexes). These results confirm the link between toluene degradation and current production during the first two batch cycles, further toluene removal in the subsequent cycles was most likely linked to the microbial sulfate reduction. The toluene degradation rate was calculated during the second batch cycle at the highest current production at ~1 mg L^{-1} d^{-1} for both R0a and R0b reactors.

The bioanodes polarized at +300 mV in reactors R300a and R300b exhibited discrepancies in terms of current production (Fig. 1C, 1D). In these reactors, a lag phase of about ten days was observed before the current production started. After this lag phase, the current density increased to 90 mA m^{-2} (R300a) and 110 mA m^{-2} (R300b). When the reactors were spiked with toluene, the current in R300a increased up to 328 mA m^{-2} (day 29) but no further increase was observed in R300b. When toluene was consumed in R300a the current dropped down, but recovered up to 260 mA m^{-2} after toluene addition. However, further toluene additions did not result in current production. The deceasing trend in the electrochemical performance in both R300a and R300b could also here be attributed to microbial sulfate reduction as observed for the two replicates at 0 mV. In order to investigate which of the two profiles (R300a R300b) was reproducible, a third replicate (R300c, Fig. 1E) was set up. Like R300a and R300b, R300c showed a lag phase of approximately one week before the current density increased to 27 mA m^{-2}. When the current decreased, toluene was spiked in the reactor and the current increased up to 431 mA m^{-2} (day 34). The current dropped down when toluene was depleted and increased after refreshing the medium with toluene. Fluctuations thereafter in Fig 1E can be attributed partially to malfunctioning of magnetic stirrer and to sulfate reduction. Initial toluene removal uncoupled to current production could be related to adsorption onto the electrode, as observed for all the replicates at 300 mV. This was confirmed by the abiotic control results, where the same behavior in terms of toluene removal was found, but no current production was observed. The toluene degradation rate, calculated for the second batch cycle was ~1 mg L^{-1} d^{-1}, which is exactly the same as for the bioanodes polarized at 0 mV.

To further elucidate whether toluene was used for current production and to find out the role of sediment in current production, a control without toluene (Sed300) was set up, and the anode was polarized at +300 mV (Fig. S1 - Annexes). The current density remained under 50 mA m^{-2} over one month of operation, indicating that the presence of toluene was necessary for current production.

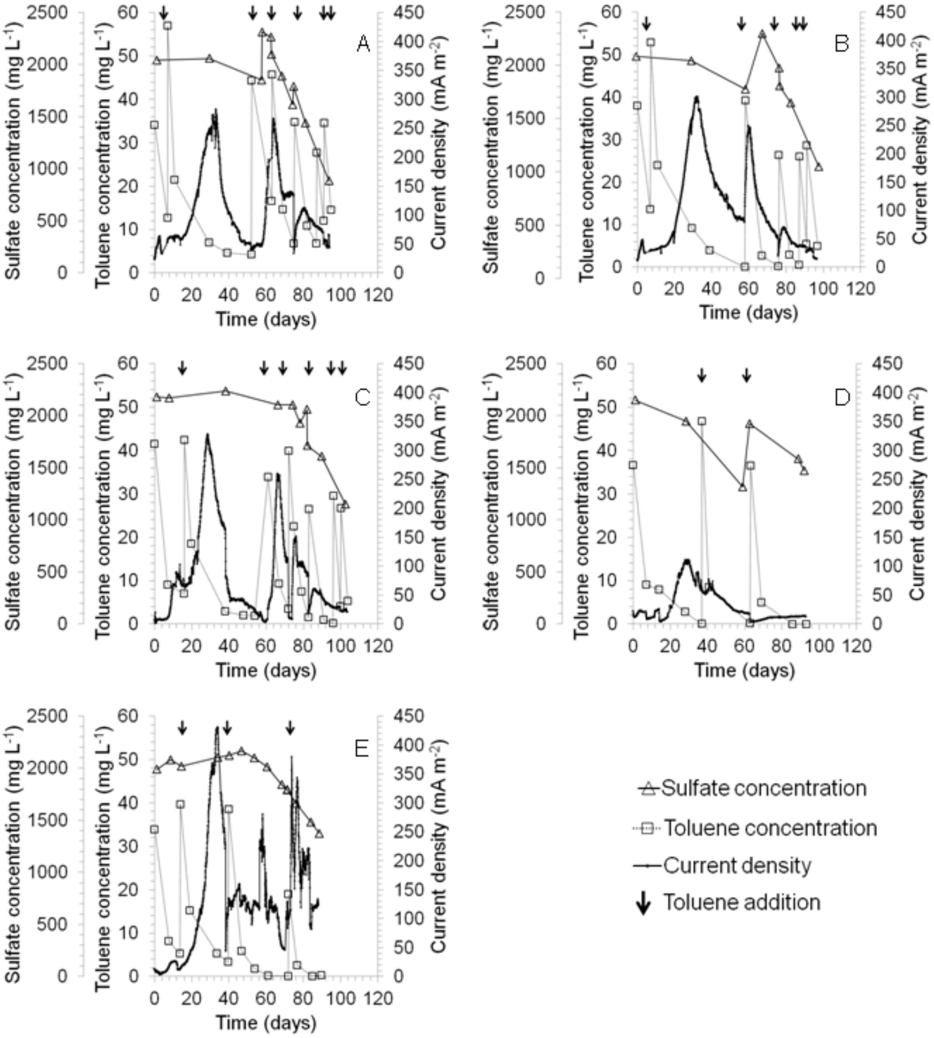

Fig. 1 - Current density, toluene concentration and sulfate concentration profiles in bioanodes polarized at 0 mV vs Ag/AgCl - (R0a (A), R0b (B)) - and at +300 mV - (R300a (C), R300b (D), R300c (E)).

Cyclic voltammograms were recorded on reactor R300c during substrate turnover conditions (~5 mg L^{-1}) (at maximum current density - day 35 - and during current decreasing - day 38) and non-turnover conditions (day 38). A catalytic wave was observed between +170 to +340 mV and an oxidation peak was detected at +200 mV (Fig. 2) indicating the presence of a redox active moiety with an oxidation potential just below the anode potential. No reduction current was observed in the backward scan, indicating a non reversible oxidation reaction at the anode.

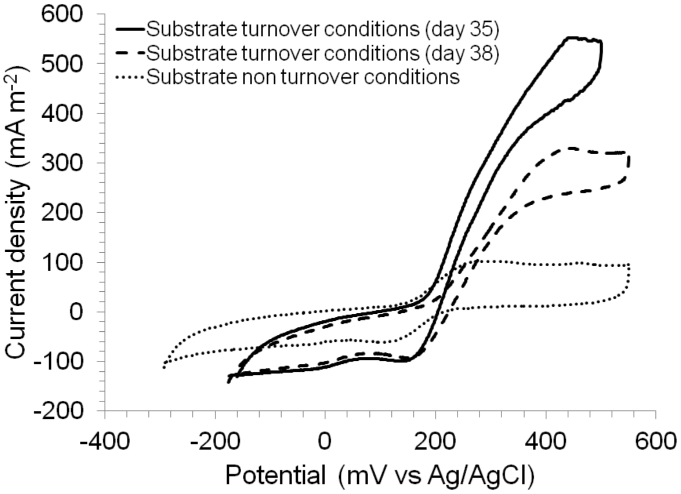

Fig. 2 - Cyclic voltammograms recorded with reactor R300c during substrate turnover (~5 mg L^{-1}) and non-turnover conditions. Scan rate: 1 mV s^{-1}.

Microscopy analysis

FISH analysis performed using SRB385 and SRB385Db probes on the anode of R300c revealed the presence of a biofilm containing SRB (Fig. 3). SRB were detected in all the observed fields confirming their importance in the process of toluene removal from the reactors. Samples collected from the same anode were analyzed by SEM-EDS to assess the extent of electrochemical oxidation of sulfide (produced from sulfate reduction) to elemental sulfur on the anode (38) and to quantify the amount of sulfur on the electrode surface. Sulfur deposition ranged from 1.1% to 2.0% and FeS particles were detected (Fig. S2 - Annexes).

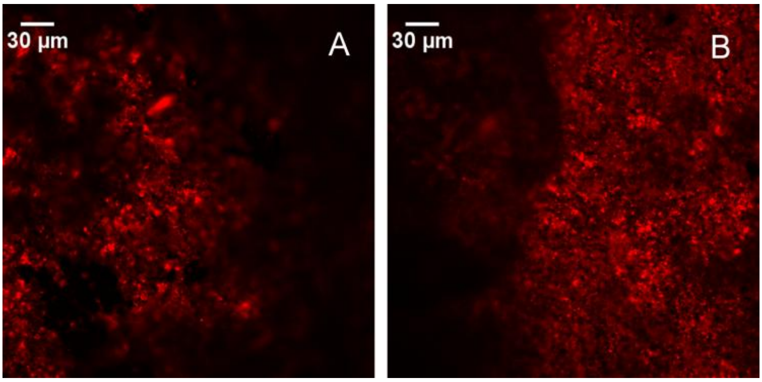

Fig. 3 - FISH images of the anode surface of the reactor R300c. The red fluorescence indicated the presence of sulfate reducing bacteria. The hybridization was performed using a mixture of Cy3 labelled SRB385 and SRB385Db probes.

Structure and taxonomic composition of the microbial communities

PCA based on Hellinger transform data of the family relative abundance showed two main clusters within the samples (Fig. 4).

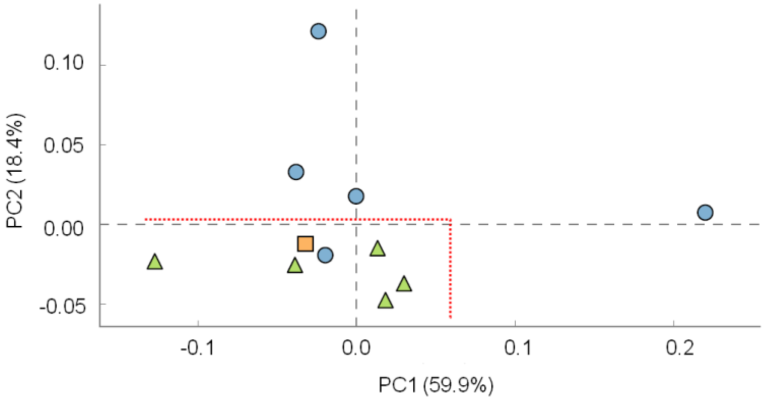

Fig. 4 - Principal Component Analysis of the family relative abundance. Blue circles (samples collected from the anodes), orange square (microbial inoculums), green triangles (samples collected from the sediment in the reactors at the end of the treatment).

Microbial communities in the bulk and from the inoculum formed a cluster in the lower part of the graph. The microbial communities grown on the anodes during the experiments positioned in the upper part of the graph, with the only exception of the microbial community on the anode of the reactor R300b that was close to the communities in the bulk and in the inoculum.

The microbial community in the sediment used as an inoculum showed greater biodiversity compared to the communities selected during toluene biodegradation (Fig. 5 and Table S1 - Annexes).

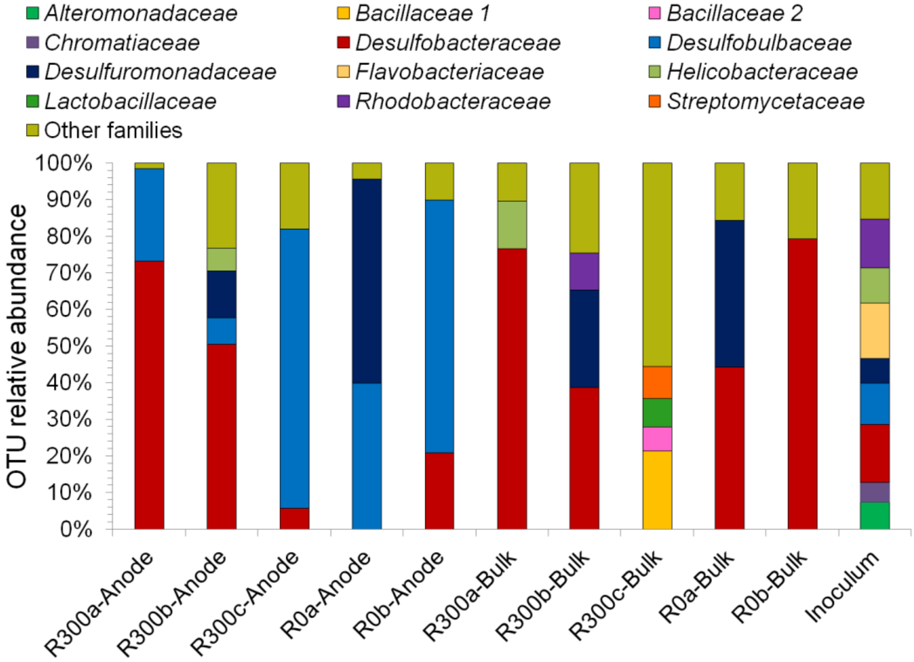

Fig. 5 - Taxonomic composition at family level of the microbial communities enriched/selected at the anodes, in the bulk of reactors polarized at 0 mV (vs Ag/AgCl) and +300 mV (vs Ag/AgCl), and in the start-up sediment inoculum. The families with a relative abundance of 5% (or higher) are reported.

The microbial communities in reactors were enriched abundantly with SRB. The *Desulfobulbaceae* were abundant on the anodes of the reactors R0a and R0b. The enrichment of *Desulfuromonadaceae* was observed in R0a both on the anode and in the bulk. The family *Desulfobacteraceae* was selected in the bulk of R0a and R0b, and in a lesser extent on the anode of R0b. Similarly, bioanodes polarized at +300 mV were dominated by sulfate reducers: the *Desulfobacteraceae* were the most abundant microorganisms on the anodes of R300a and R300b while the family *Desulfobulbaceae* dominated the anode of R300c. The variability in the composition of the microbial communities enriched in the bulk was high: while the *Desulfobacteraceae* were the most representative microorganisms in the samples collected form R300a and R300b, the most abundant microorganisms in the sample collected from R300c belonged to the family *Bacillaceae*.

A post-hoc test (confidence 95%) was performed in order to detect families associated to specific conditions. The conditions applied during bioelectrochemical toluene degradation led to the selection on the anodic biofilms of bacteria belonging to the family *Desulfobulbaceae*, since they were statistically more abundant (Fig. S3 - Annexes). The only anodic sample that showed a poor enrichment of *Desulfobulbaceae* was collected from the reactor R300b, which explains the low current production even after repeated toluene additions. Therefore, the presence of *Desulfobulbaceae* may be linked to the current production coupled with biodegradation of toluene.

The sequences of the most abundant OTUs (Fig. S4 - Annexes) were used further to perform the best match with the sequences contained in the RDP. The results showed that the microorganisms enriched in the reactors were phylogenetically close to the sulfate reducers and hydrocarbon degraders *Desulfosarcina ovata*, *Desulfococcus multivorans*, *Desulfobacterium catecholicum*, *Desulfotignum toluenicum* and *Desulfobacula toluolica* (39–42). Furthermore, the post-hoc test analysis showed that bacteria phylogenetically close to *Desulfobulbus rhabdoformis* and *Desulfobulbus propionicus* were statistically more abundant on the anodes, while bacteria phylogenetically close to *Desulfotignum balticum*, *Desulfotignum toluenicum* and *Desulfobacula toluolica* were statistically more abundant in the bulk (Fig. S5 - Annexes).

DISCUSSION

Current production and toluene degradation

Toluene degradation in marine environments was studied in batch reactors by applying different anodic potentials (0 mV and +300 mV). Regardless of the applied potential, toluene degradation coupled with current production was observed. So far, only one study examined the effect of the electrode potential on hydrocarbon degradation. Firman and colleagues assessed toluene degradation by inoculating a BES with a pure culture of *Pseudomonas putida* F1 and applying anode potentials in the range of 75-500 mV (25). The current production was directly correlated with the potential applied in a range between 11 mA m^{-2} and 94 mA m^{-2}, but no information about toluene degradation rates at different potentials was reported (25). Studies with more degradable substrates (i.e. acetate) showed a discrepancy in the results. In some cases a positive correlation between the anodic potential and current production was observed (43), while other works showed the opposite result (43, 44).

The maximum current outputs of 301 mA m^{-2} (0 mV) and 431 mA m^{-2} (+300 mV) obtained here were higher compared to the current densities obtained in other studies with the same carbon source. Lin and colleagues studied bioelectrochemical toluene degradation with neutral red and ferricyanide as redox mediators. The power generation was increased when the mediators were added and the maximum voltage obtained was 110.4 mV. The voltage harvested in that case corresponds to a current density of less than 2 mA m^{-2}, significantly lower that the values obtained in our experiment. In this study, however, the anode was not poised which could have affected the current production. A higher current density of about 150 mA m^{-2} has been reported with a pure culture of *Geobacter metallireducens* by using an electrode polarized at +300 mV as sole electron acceptor (15). Very low current densities have been reported with other hydrocarbons. For instance, in a recent study, a sediment microbial fuel cell was used to stimulate total petroleum hydrocarbons degradation and a peak current of 86 mA m^{-2} was reached by using a biochar anode (26).

The toluene removal rate was ~1 mg L^{-1} d^{-1} for both the anode potentials tested in this study. This value was slightly higher than the degradation rates observed in studies with other mixed cultures able to oxidize toluene by using anode as a sole electron acceptor, either with or without redox mediators (15, 45). The higher rate found in our experiments could be linked to the adsorption of toluene on graphite electrode. Adsorption of hydrocarbons occurs often when graphite electrodes are used; however it has been demonstrated that toluene mineralization is not restricted by adsorption onto the electrodes (15). To minimize adsorption effects, the degradation rate was therefore calculated based on the second batch cycle results.

The cyclic voltammograms obtained under substrate turnover conditions and under substrate non-turnover conditions with the reactor R300c (+300 mV) showed a non-reversible reaction. The mid-point potential of the redox active moiety in both turnover and non-turnover conditions is located at around 200 mV. This result suggests the involvement of this moiety in the electron exchange process. The microbial community in this reactor was dominated by members of the families *Bacillaceae* (bulk) and *Desulfobulbaceae* (anode). *Bacillus subtilis* (family *Bacillaceae*) has been reported to produce an undetermined electron shuttle with an oxidation peak at 40-60 mV, a potential lower than that observed in our study. The nature and the redox potential of the external electron transfer mediators in the family *Desulfobulbaceae* is not described in literature. The nature of the redox active moiety detected in R300c is also undetermined.

With the only exception of reactor R300b, current production was attributed to toluene degradation during the two batch cycles. After the third batch cycle the current density decreased and toluene removal was most probably coupled with sulfate reduction (although sulfide concentration was under the detection limit). This shift in the microbial metabolism in a sulfate rich medium was previously described during bioelectrochemical benzene and sulfide removal, but the current production did not decrease over time (22). The growth of sulfate reducers is usually slow, however, the low anode surface (8 cm^2) compared to the total working volume (200 mL) favors microorganisms not in direct contact with the electrode to use soluble electron acceptors such as sulfate, which was abundantly present (2 g L^{-1}) in the bulk. Sulfate reducers might further have been advantaged by (partial) anode passivation due to sulfur deposition on the electrode surface. Furthermore, the overgrowth of biofilm with sulfate reducers could have outcompeted electroactive microorganisms by limiting toluene diffusion to the anodic surface. Indeed, sulfide can be electrochemically oxidized to elemental sulfur on the anodic surface (38, 46). Elemental sulfur can be used as an alternative electron acceptor leading to sulfide formation which can be reoxidized enhancing the current production as long as the cycle is active (27). This hypothesis was confirmed by SEM-EDS analysis of the anode collected from reactor R300c that showed a high sulfur deposition on the electrode. On the basis of the amount of sulfate reduced in the reactors and assuming the complete reduction of sulfate to sulfide followed by the complete oxidation of sulfide to elemental sulfur on the electrode, between 60 and 120 mg of sulfur should have been produced in the reactors at +300 mV (R300a, R300b, R300c) and between 80 and 110 mg in the reactors at 0 mV (R0a, R0b).

Microbial communities

The reduction of sulfate to sulfide, which was subsequently oxidized to elemental sulfur, might have occurred both on the electrode surface and in the bulk of the reactor. The presence of SRB in the anodic biofilm was demonstrated by FISH results and confirmed by 16S rRNA gene sequencing. SRB, belonging to the families *Desulfobulbaceae* and *Desulfobacteraceae* were found in the microbial inoculum and were further enriched during the electrochemical experiments. Members of these families have been previously described as key players during hydrocarbon degradation in sulfate reducing conditions (47, 48). Furthermore, members of the families *Desulfobulbaceae* and *Desulfobacteraceae* have been previously enriched on the anode during bioelectrochemical benzene degradation in sulfide rich groundwater (22). Specifically, *Desulfobulbus propionicus* (member of the *Desulfobulbaceae*) was able to produce current in sulfide rich marine sediment (44, 45). The presence of the family *Desulfobulbaceae* was significantly higher in the microbial communities of the anodic biofilms than in the communities enriched in the bulk. This is a remarkable result since filamentous *Desulfobulbaceae*, the so called cable bacteria, have been recently reported to perform electron transfer over centimeter long distance in marine sediments, delivering the electrons obtained by sulfide oxidation (51, 52). The role of *Desulfobulbaceae* in current production is supported by the fact that the anode which had the lower abundance of *Desulfobulbaceae* was found in reactor R300b that did not show current production over the last month of operation in spite of repeated toluene spiking. The role of members of the *Desulfobulbaceae* in the electron transfer to the anode can thus be inferred. On the basis of these observations, it is possible to speculate that bacteria related to the families *Desulfobulbaceae* and *Desulfobacteraceae* were involved either in hydrocarbon degradation and (or) in sulfide/sulfur oxidation with concomitant electron transfer to the anode.

Microorganisms of the family *Desulfuromonadaceae* were enriched mainly in R0a and to minor extent in R300b. *Desulfuromonas acetoxidans* has been reported to be able to reduce elemental sulfur and use the anode of a BES as final electron acceptor (53).Thus the abundance of this family could suggest that metabolism related to elemental sulfur reduction might play an important role during toluene removal in our reactors. However, the role of sulfur cycle on hydrocarbons degradation in BESs still has to be completely elucidated.

Members of the family *Bacillaceae* (phylum *Firmicutes*) were relatively abundant in the bulk of reactor R300c. Their ability to degrade aromatic hydrocarbons in aerobic conditions has been previously described (54). Members of the phylum *Firmicutes* were also selected during the electrochemical degradation of petroleum hydrocarbons in soil (26). Another recent study reported the presence of *Bacillus* species in the anodic biofilm during *cis*-dichloroethene oxidation, however in this work the microbial degradation was triggered by the presence of oxygen produced due to high anodic potential (+1300 mV) (55). Another study showed that the *Bacillus subtilis* was able to use an electrode as electron acceptor by excreting external mediators (56).

Interestingly, the presence of iron reducing microorganisms, which usually dominate the anodic communities in BES fed with more degradable substrates, such as acetate, was scarce (57, 58).

In addition, SRB, closely related to *Desulfobulbus rhabdofromis* and *Desulfobulbus propionicus* were selected at the anode and were significantly more abundant at the anode of the reactors at 0 mV. *Desulfobulbus rhabdoformis* was isolated from a water-oil separation system (59) and the presence of this genus was widely described in petroleum reservoirs (60, 61). *Desulfobulbus propionicus* was able to grow using an electrode as sole electron acceptor (62).

Mechanisms of toluene degradation and final conclusion

No differences were observed both in terms of current density production and toluene degradation rate for both the tested potentials (0 and +300 mV). The most abundant microorganisms enriched both in the anodic and in the bulk communities were SRB that are known hydrocarbon degraders. The high organic matter input from oil spill in marine environments lead to a quick oxygen depletion and hydrocarbon removal by sulfate reduction is typically enhanced (63). Controversial information is available about toluene degradation rate in sulfate reducing conditions. In microcosms set up to assess toluene removal in soil a degradation rate of ~1 µg kg^{-1} d^{-1} has been estimated (64), while a more recent work calculated a toluene removal rate between 1 and 4 mg L^{-1} d^{-1} in microcosms amended with sulfate as electron acceptor (65), which is similar to the value calculated in the current study (~1 mg L^{-1} d^{-1}). Thus, further experiments will be required to understand if the application of BES during hydrocarbon removal in marine environments lead to an effective enhancement of the treatment efficiency, or if it results only in a sink for sulfide removal. It is clear from our results that an important role exist for sulfur species in the bioelectrocatalytic process studies here. Rakoczy and colleagues suggested that bioelectrochemical benzene degradation in sulfate rich environments could occur in the bulk rather than on the anodic surface. The authors hypothesized that the electron transfer was mediated via an electrode shuttle related to the sulfur cycle (22), which corroborates earlier findings of Dutta *et al.* in which acetate consumption was accelerated in the presence of sulfide due to improved cycling (27). A similar mechanism was described during ferric iron reduction, where ferrihydrite was reduced to ferrous iron by sulfide produced during microbial thiosulfate reduction (66). The cyclic voltammetry, however, indicates that also direct bioelectrocatalytic activity may exist. Assuming that only sulfur mediated electron transfer occurred, we estimate that between 700 and 1100 mg L^{-1} of sulfate should have been consumed. This value is much higher than the sulfate reduction experimentally observed, suggesting that direct electron transfer may have been the prevalent mechanism during the first weeks of operation.

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Chapter 3 – Development of BES-based biobarriers for gasoline contaminated groundwater treatment.

Preliminary results

ABSTRACT

BTEX compounds (benzene, toluene, ethylbenzene and xylenes) and fuel oxygenates, such as MTBE (methyl *tert*-butyl ether) are the main constituents of gasoline. In this study the bioelectrochemical degradation of a BTEX and MTBE mixture was assessed using single chamber microbial electrolysis cell reactors connected to a power source. Current production was coupled to hydrocarbon removal. Toluene and *p*-xylene degradation was stimulated but the other components of the mixtures appeared to be more recalcitrant to the microbial degradation.

INTRODUCTION

The results reported in Chapter 1 showed that controlling the electron acceptor delivery during microbial biodegradation is crucial. Oxygen releasing compounds were provided to stimulate the aerobic biodegradation of hydrocarbons. Although, during the test without the selected microbial inoculum, the average dissolved oxygen concentration was about 5 mg L^{-1} throughout the column, aerobic microorganisms were enriched mainly near the inlet. Farther, anaerobic facultative bacteria, belonging to the genus *Thauera*, were enriched. Furthermore, in the column experiment inoculated with a selected inoculum, oxygen was quickly depleted and continuous amendment with oxygen releasing compounds was required.

We hypothesized that the microbial activity in the BB might also be stimulated by a BES. Both the anode and the cathode might be buried in the support material and connected to a power supply to stimulate the electron transfer to the anode (Fig. 1).

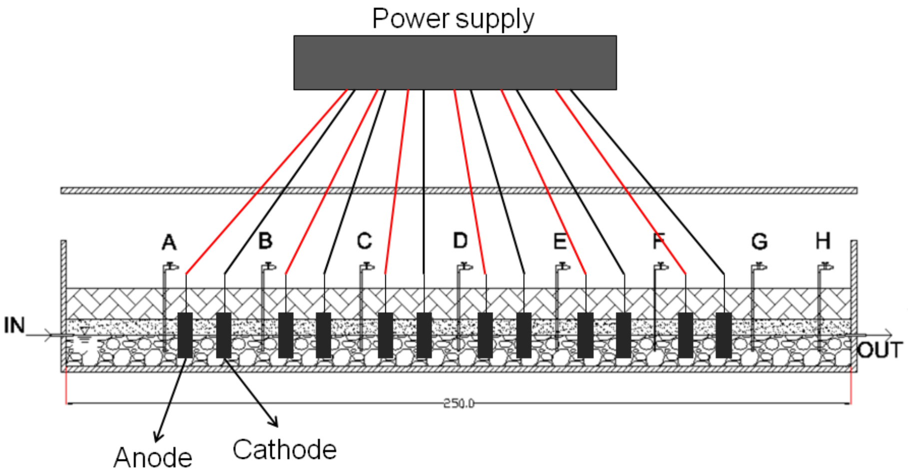

Fig. 1 – Scheme of a BES-based biobarrier for gasoline contaminated groundwater treatment.

However, parameters like electrode material, electrode spacing and voltage applied might strongly affect the overall performance of the biobarrier. Laboratory-scale batch experiments could be used to gain useful information to further develop a bench scale experiment under flow conditions

A new microbial electrolysis cell (MEC) reactor design was recently proposed as a suitable technology when cheap screening analysis are required (1). This reactor design was successfully used to assess the treatability of refinery wastewater with bioelectrochemical systems (BES) (2). It consists in a single chamber MEC constructed by introducing two electrodes in a serum bottle. The electrodes can then be connected to a power source or to a potentiostat (Fig. 2) (1).

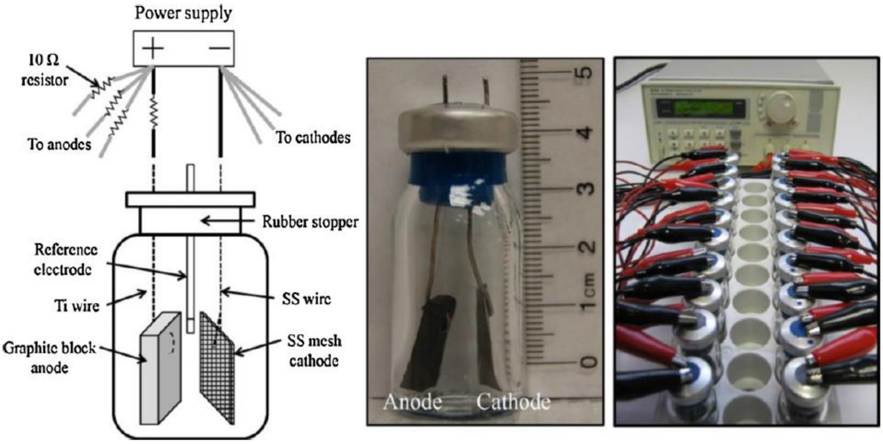

Fig. 2 - BES reactor design. From Call and Logan 2011 (1).

BTEX compounds (benzene, toluene, ethylbenzene and xylenes), and additives, such as the fuel oxygenates methyl *tert*-butyl ether (MTBE) and ethyl *tert*-butyl ether (ETBE) are some of the main constituents of gasoline (3, 4). The degradation of toluene and benzene in BES was previously studied both with pure cultures and mixed cultures (5–9) but no information about bioelectrochemical degradation of xylenes, ethylbenzene and fuel oxygenates is available. Furthermore hydrocarbon degradation might be different when they occur in mixtures instead then as single compounds. Inhibitory or stimulating effects can reduce or enhance respectively the removal rate of the single hydrocarbons (10–14). However, a lack of knowledge about gasoline degradation in BES still exists. The main goal of this preliminary study was to assess whether the degradation of a BTEX and MTBE mixture can be stimulated by using a BES-based approach. The reactor design proposed by Call and Logan was used in order to allow a further screening of different voltages to understand the effect on the hydrocarbon degradation rate.

MATERIALS AND METHODS

MEC reactors were constructed using 120 mL serum bottles filled with 30 g of pumice (2-5 mm) as support material for the microbial growth. Each reactor was flushed with N_2 for 30 minutes in order to remove oxygen and ensure anaerobic conditions. After flushing, each reactor was sealed with a thick rubber stoppers (Rubber BV, Hilversum, Netherlands) and an aluminum cap. Refinery wastewater (85 mL) was added as microbial inoculum. Graphite plates (10 cm^2) connected to stainless steel wire (1 mm) were used both as anode and cathode. The stainless steel wire and the electrical connections were covered with an heat shrinkable tube to prevent corrosion. A voltage (1 V) was applied with a power supply and the current profile was monitored over time by a data logger (Squirrel SQ2010, Grant, Cambridge, UK). During the first weeks of operation the organic matter added with the refinery wastewater (COD 415 ± 78 mg O_2 L^{-1}) was the sole carbon source. When the current decreased, due to the depletion of the biodegradable matter, a mixture of BTEX and MTBE was spiked (Table 1).

Hydrocarbon	Concentration (mg L^{-1})
Benzene	8.1 ± 0.9
Toluene	21 ± 2
Ethylbenzene	1.7 ± 0.1
o-Xylene	2.3 ± 0.1
m-Xylene	1.7 ± 0.1
p-Xylene	1.9 ± 0.1
MTBE	73 ± 6

Table 1 – BTEX and MTBE concentrations measured at the beginning of the degradation experiment.

Two open circuit controls (OCa and OCb) were set up in order to assess the extent of the biodegradation without electrodes serving as electron acceptors (Table 2). A sterile control (1VAb) was set up in order to quantify the abiotic hydrocarbon removal.

Reactor	Voltage applied	Inoculum
1Va, 1Vb	Yes	Yes
OCa, OCb	No	Yes
1VAb	Yes	No

Table 2 – Reactor and experiment details.

BTEX and MTBE concentration were analyzed by GC-FID (Agilent 6890N) equipped with an Headspace Autosampler (Agilent 7697A) (EPA method 5021A) (15).

RESULTS AND DISCUSSION

The maximum current density during the first weeks of operation was 60 mA m^{-2} and 100 mA m^{-2} respectively in reactor 1Va and 1Vb (Fig. 3A, 3B). During this phase the current production was due to the presence of organic compounds in the refinery wastewater used as inoculum source. When the degradable organic carbon was depleted the current slowly decreased and a mixture of BTEX and MTBE was spiked in each reactor (first batch cycle, day 38). Immediately after the addition, the current increased to 370 mA m^{-2} (1Va) and 420 mA m^{-2} (1Vb). The current production was coupled to hydrocarbon removal and slowly decreased when the most degradable compounds were depleted reaching negligible valued at day 60. The mixture of BTEX and MTBE was spiked again (second batch cycle) and a peak of about 310 mA m^{-2} was reached in both 1Va and 1Vb. After 20 days (day 80) a third batch cycle was repeated and the same behavior was observed: the current increased immediately and decreased after the degradation of the most degradable hydrocarbons in the mixture. During the whole experiment negligible current was observed in the abiotic control (Fig. 4).

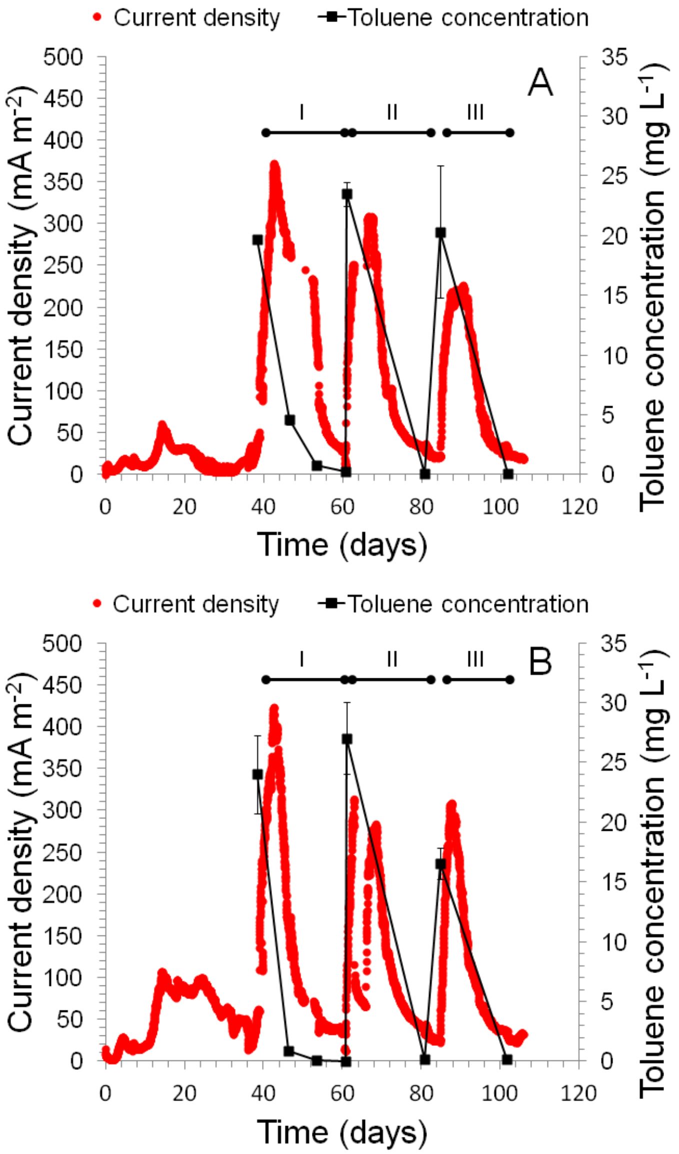

Fig. 3 - Toluene and current density profiles in reactors 1Va (A) and 1Vb (B) during the first batch cycle (I), the second batch cycle (II) and the third batch cycle (III).

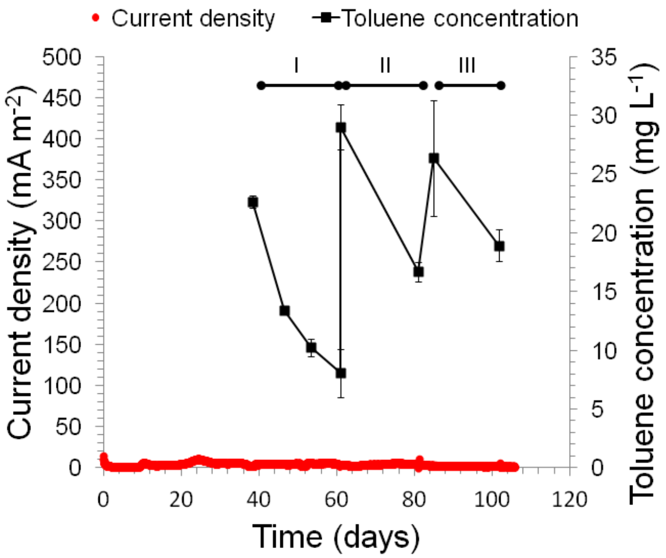

Fig. 4 - Toluene and current density profile in the abiotic control (1VAb) during the first batch cycle (I), the second batch cycle (II) and the third batch cycle (III).

Current production was coupled to hydrocarbon removal even though during the first days the adsorption on the electrodes played a crucial role. However, hydrocarbon adsorption on the electrodes did not affect the microbial mineralization as previously demonstrated (6, 7). The current densities measured in this study were higher than the current densities usually reached with BES for hydrocarbons degradation. Current densities ranging from 11 mA m^{-2} to 150 mA m^{-2} were obtained during bioelectrochemical toluene degradation using pure cultures of *Geobacter metallireducens* and *Pseudomonas putida* F1 as microbial inoculum (5, 7). Lower current densities were obtained with mixed cultures during the degradation of hydrocarbon mixtures. Lu and colleagues obtained a maximum current density of 86 mA m^{-2} during petroleum hydrocarbon degradation in soil with an electrode serving as electron acceptor (16). Higher currents (up to 2.1 ± 0.2 A m^{-2} corresponding to about 190 mA m^{-3}) were obtained during refinery wastewater treatment in single chamber MEC (2). However, refinery wastewater treatment in a dual chamber microbial fuel cell (MFC) led to lower current densities (about 10 mA m^{-3}) (17). In this study, considering toluene as the sole carbon source, and the number of electrons passed into the circuit 149 mg L^{-1} (first batch cycle) 94 mg L^{-1} (second batch cycle) and 71 mg L^{-1} (third batch cycle) of toluene should have been oxidized in reactor 1Va. Similarly in reactor 1Vb the amount of toluene oxidized should have been 102 mg L^{-1} (first batch cycle) 80 mg L^{-1} (second batch cycle) and 68 mg L^{-1} (third batch cycle). Thus, the total amount of hydrocarbon removal could not explain the current produced, unless other reactions (i.e. H_2 oxidation) occurred at the anode. It was demonstrated that, when bioelectrogenic bacteria oxidize a substrate at the anode of a single chamber MEC, the current can result in H_2 evolution at the cathode. H_2 can then be consumed at the anode by the bioelectrogenic microorganisms in a process called H_2 recycling (1, 18, 19). The higher current densities measured in this study, compared to current densities usually observed with other BES architectures, might thus be due to the anodic oxidation of H_2 produced at the cathode.

The most recalcitrant compounds were MTBE and benzene (Fig. 5A, 5B). MTBE was not removed and accumulated into the reactors after each batch cycle. In contrary, benzene was slightly removed, but no difference were observed in the reactors with the microbial inoculum compared to the abiotic test, suggesting that benzene removal occurred because of adsorption on graphite electrodes. Ethylbenzene and *o*-xylene showed a behavior similar to benzene (data not shown). *m*-Xylene was partially degraded only during the second batch cycle but no differences in the removal efficiency were observed within the different conditions during the first and the third batch cycle (data not shown). Toluene and *p*-xylene were almost completely removed after each batch cycle in 1Va and 1Vb (Fig. 5C, 5D), while they were only partially removed in the open circuit controls (OCa and OCb) and in the abiotic experiment (1VAb). This latter depletion was probably due to adsorption on the electrode. The higher removal of both toluene and *p*-xylene in reactors 1Va and 1Vb compared to the open circuit controls demonstrated that the bioelectrochemical treatment enhanced the degradation rate.

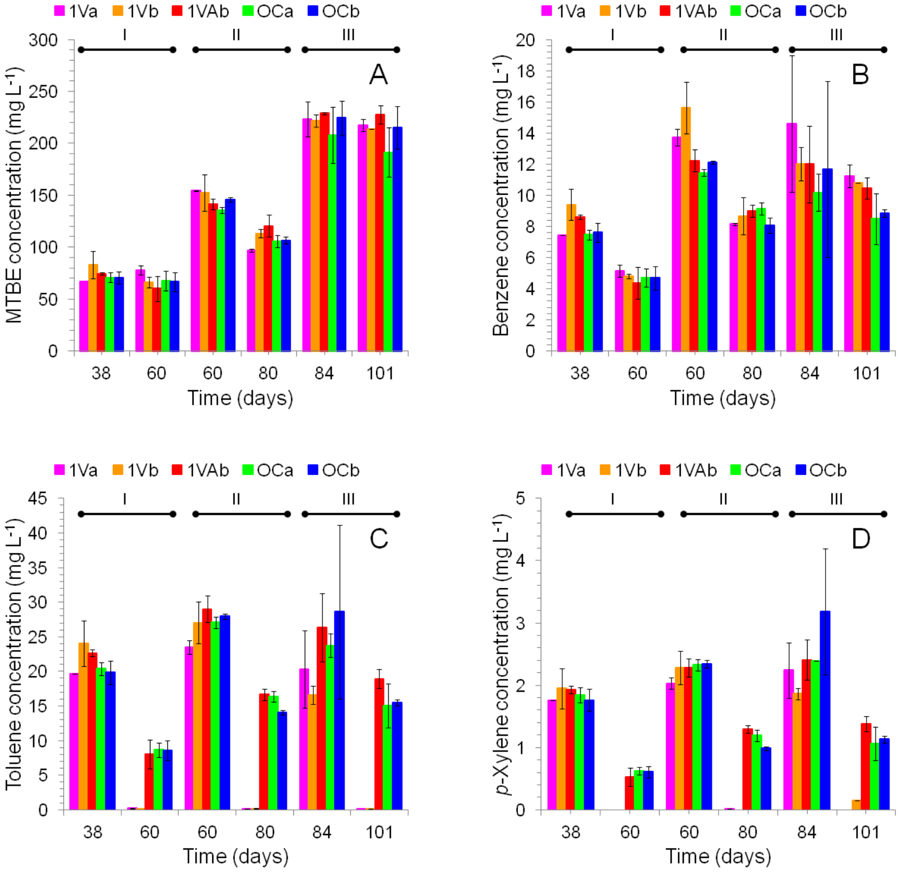

Fig. 5 - MTBE (A), benzene (B), toluene (C) and *p*-xylene (D) concentrations measured during the first batch cycle (I), the second batch cycle (II) and the third batch cycle (III).

These data are consistent with the reported biodegradability of BTEX compounds and MTBE. The fuel oxygenate MTBE is known to be recalcitrant in anaerobic conditions. No pure cultures able to mineralize MTBE without oxygen have been described so far. Furthermore, little reproducibility was observed during anaerobic MTBE biodegradation in mixed cultures, indicating thus that this metabolism is rare (20, 21). Conversely, toluene can be degraded in anaerobic conditions with NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-} and CO_2 as terminal electron acceptors both by isolates or mixed cultures (22). Toluene removal rate was 1.1 ± 0.2 mg L^{-1} d^{-1}, slightly higher than the values previously reported in both MFCs and MECs inoculated with mixed cultures (Table 3) (7, 9). The removal rates calculated in this study, however, might be overestimated due to adsorption on graphite electrodes, as previously reported in Chapter 2. *p*-Xylene removal rate was 0.25 ± 0.04 mg L^{-1} d^{-1}. *p*-Xylene was often reported to be the more recalcitrant among the xylenes in anaerobic conditions, but its degradation was observed in several mixed cultures with NO_3^-, Fe^{3+}, SO_4^{2-} (22). Benzene is the most recalcitrant among the BTEXs, however mixed cultures able to remove benzene have been described with NO_3^-, Fe^{3+}, SO_4^{2-} (22, 23).

Degradation rate	Redox mediator	Reactor	Reference
0.2 mg L^{-1} day^{-1}	Not detected	MEC	(7)
0.7 mg L^{-1} day^{-1}	Not detected	MFC	(9)
0.3 mg L^{-1} day^{-1}	Neutral red	MFC	(9)
0.3 mg L^{-1} day^{-1}	Ferricyanide	MFC	(9)
1 mg L^{-1} day^{-1}	Self produced	MEC	Chapter 2

Table 3 – Toluene removal rates previously reported in BES.

While several studies addressed the interactions occurring during the degradation of BTEX and MTBE mixtures in aerobic conditions (24, 25), little knowledge about the interactions in anaerobic conditions is reported so far. Generally, the presence of toluene had a detrimental effect on the biodegradation of other BTEX compounds (10–14). In this study only toluene and *p*-xylene degradation were observed, however it is not possible to clearly understand if the other compounds are not degraded due to inhibition effects, or because of lacking of specific degradation pathways in the microbial community.

In conclusion single chamber MEC were successfully applied to stimulate hydrocarbon degradation, but only toluene and *p*-xylene were removed, while the other components of the mixture appeared to be more recalcitrant. Further studies are needed to assess the effect of different voltages (i.e. 0.8 V and 1.2 V) on the degradation efficiency and on the microbial community composition. In addition, the use the cheap and simple reactor architecture proposed by Call and Logan (1), and presented in this chapter, could allow the elucidation of the complex substrate interactions occurring during bioelectrochemical degradation of hydrocarbon mixtures.

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Final remarks

The number of contaminated sites worldwide is increasing due to the growing use of petroleum hydrocarbons both for fossil fuel and chemical compounds production. These contaminants are a concern due to the high toxicity and their recalcitrance in anaerobic conditions. Owing to the high abundance of organic matter, the favorable electron acceptors are quickly depleted in contaminated sites, and the development of strategies to stimulate the microbial activity and promote the biodegradation of the contaminant is required. The most widely used approach involves the addition of oxygen in order to stimulate the aerobic degradation. Aerobic biobarriers (BBs) are a relatively new technology for the treatment of contaminated groundwater. During the first part of the PhD the aerobic treatment of gasoline contaminated groundwater was studied in a bench scale biobarrier both with and without the addition of selected bacteria. The results showed that the microbial inoculum was outcompeted by the native microorganisms on the pumice used as filling material. However, the presence of a selected inoculum was not necessary to ensure the removal of the contaminants. Furthermore, it was highlighted that oxygen concentration plays a crucial role in determining the composition of the microbial community, and therefore the metabolism, during the treatment. Even the addition of oxygen releasing compounds did not ensure the enrichment of aerobic microorganisms throughout the whole column.

The non-homogeneous distribution of oxygen in the reactive area is not the only drawback of the aerobic bioremediation strategies. Oxygen can easily diffuse away from the contaminated area, and be consumed for the oxidation of reduced species (i.e. Fe^{2+}). Continuous oxygen supplying is required, thus increasing the cost of the remediation. To overcome these limitations, the stimulation of degradative microorganisms by bioelectrochemical systems (BES) was proposed. In a BES-based biobarrier the electrodes could be buried in the support material and the anode could act as a solid electron acceptor during the microbial respiration. Many parameters (i.e. electrode potential, voltage applied, substrate interactions) can affect the efficiency of the process thus, a preliminary evaluation of these parameters can help the further development of a BES-based bench scale BB for gasoline treatment. We investigated the degradation of a BTEX and MTBE mixture using a MEC and applying a voltage (1V). The electrodes were buried in pumice, in order to simulate a BES-based BB. The experiment showed that current production was coupled with hydrocarbon degradation and that the biolectrochemical treatment enhanced the contaminants removal. However, only toluene and *p*-xylene were degraded, while the other contaminants appeared to be more recalcitrant. These preliminary results are encouraging and further studies will be performed in order to assess the effect of different voltages. Furthermore, the experimental apparatus could be used also to investigate the influence of substrate interactions during hydrocarbon removal.

During this PhD work hydrocarbon degradation in marine environments was also studied. The experiments were performed by applying two different anodic potentials (0 mV and +300 mV vs Ag/AgCl). Current production was coupled to toluene degradation, but the anodic potential did not affect toluene removal rate. However, after renewed toluene spiking, the current production decreased and toluene was removed by sulfate reduction. This experiment allowed gaining more insight about toluene bioelectrochemical degradation in sulfate-rich environments. Comparing our observations with the results obtained in other studies we could hypothesize that two main degradation mechanisms occurred in the reactors. Toluene degradation by direct electron transfer to the anode (Fig. 1A) and by sulfate reduction producing sulfide (Fig. 1B). Sulfide acted as an electron transfer shuttle to the anode and was oxidized to elemental sulfur which covered the anode (Fig. 1B).

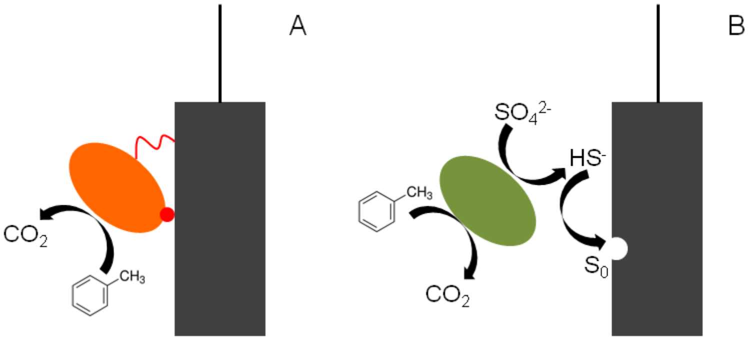

Fig. 1 - Toluene degradation mechanisms proposed. Direct electron transfer to the anode (A), or electron transfer mediated by sulfide (B).

BES is a rapidly growing technology and its application for the bioremediation of sites contaminated by petrochemical compounds was recently proposed to overcome the limitations of the current microbial stimulation strategies. The research in the field of the BES-based remediation technologies is still in its infancy. However during the past years several advances have been made. Different studies reported the proof of concept about the applicability of BES-based strategies for the bioremediation of several matrixes (i.e. sediment, groundwater, marine water) contaminated by a large number of petroleum hydrocarbons. The next efforts will be undertaken to elucidate the parameters that could influence the applicability and the bioremediation performance during *in situ* operation. Scaling up the reactors and switching from batch experiments to continuous flow reactors will play a crucial role in understanding the applicability of this technology.

Annexes

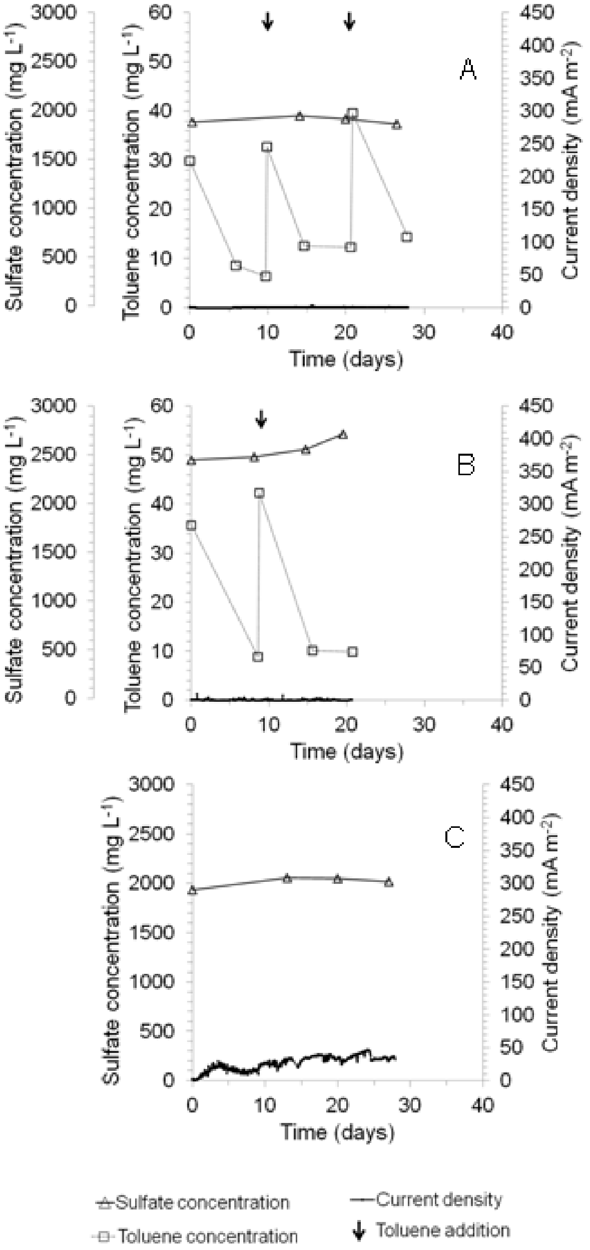

Fig. S1 - Current density, toluene concentration and sulfate concentration profiles in the abiotic control polarized at +300 mV (vs Ag/AgCl) - Ab300 (A) -, in the abiotic control polarized at 0 mV (vs Ag/AgCl) - Ab0 (B) - and in the control without toluene - Sed300 (C).

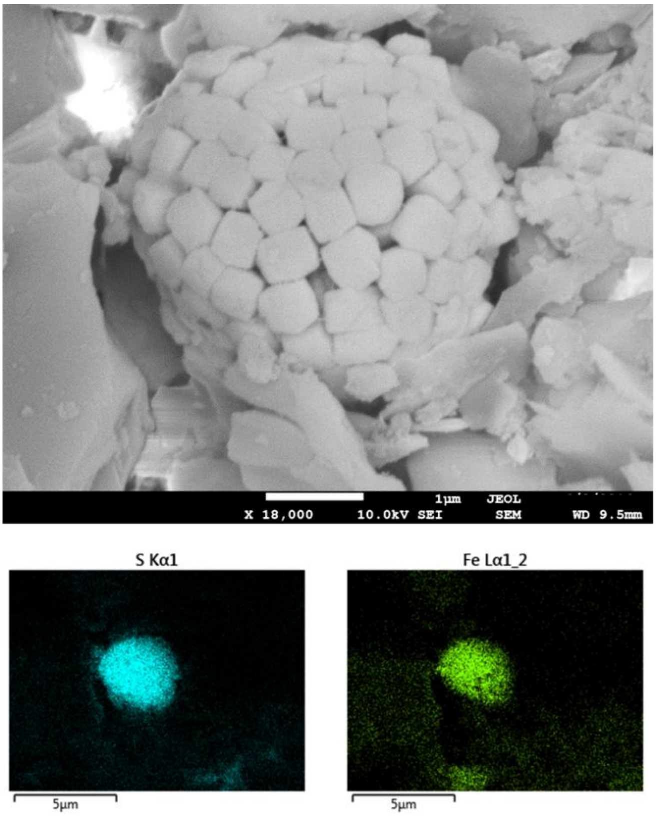

Fig. S2 - FeS particle detected by SEM-EDS.

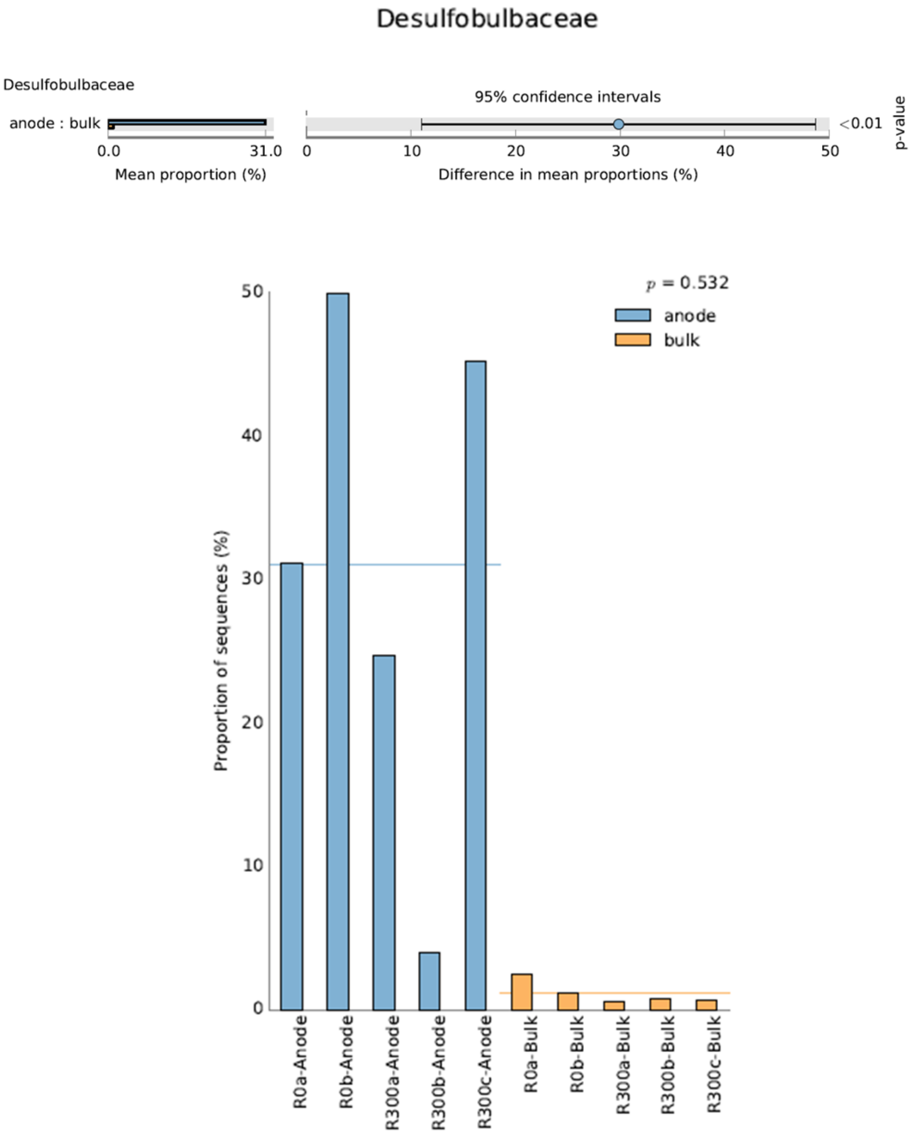

Fig. S3 - Post-hoc test performed at family level indicating that the family *Desulfobulbaceae* is more abundant in the microbial communities selected at the anodes compared to the bulk of the reactors.

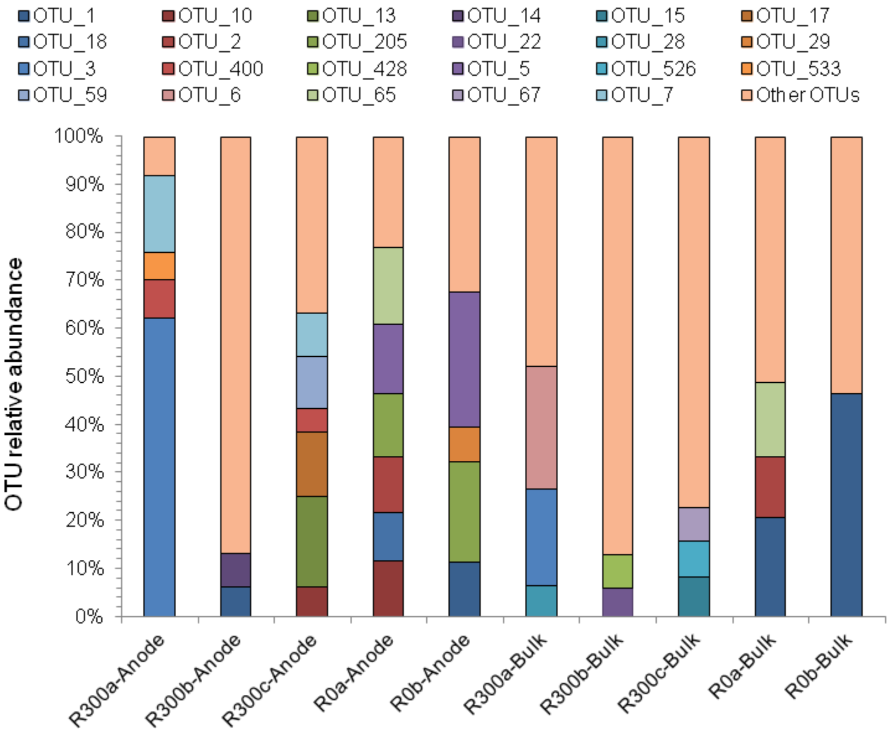

Fig. S4 - Taxonomic composition at OTU level of the microbial communities selected at the anodes (polarized at 0 mV and +300 mV *vs* Ag/AgCl) and in the bulk of the reactors. The OTUs with a relative abundance of 5% (or higher) are reported.

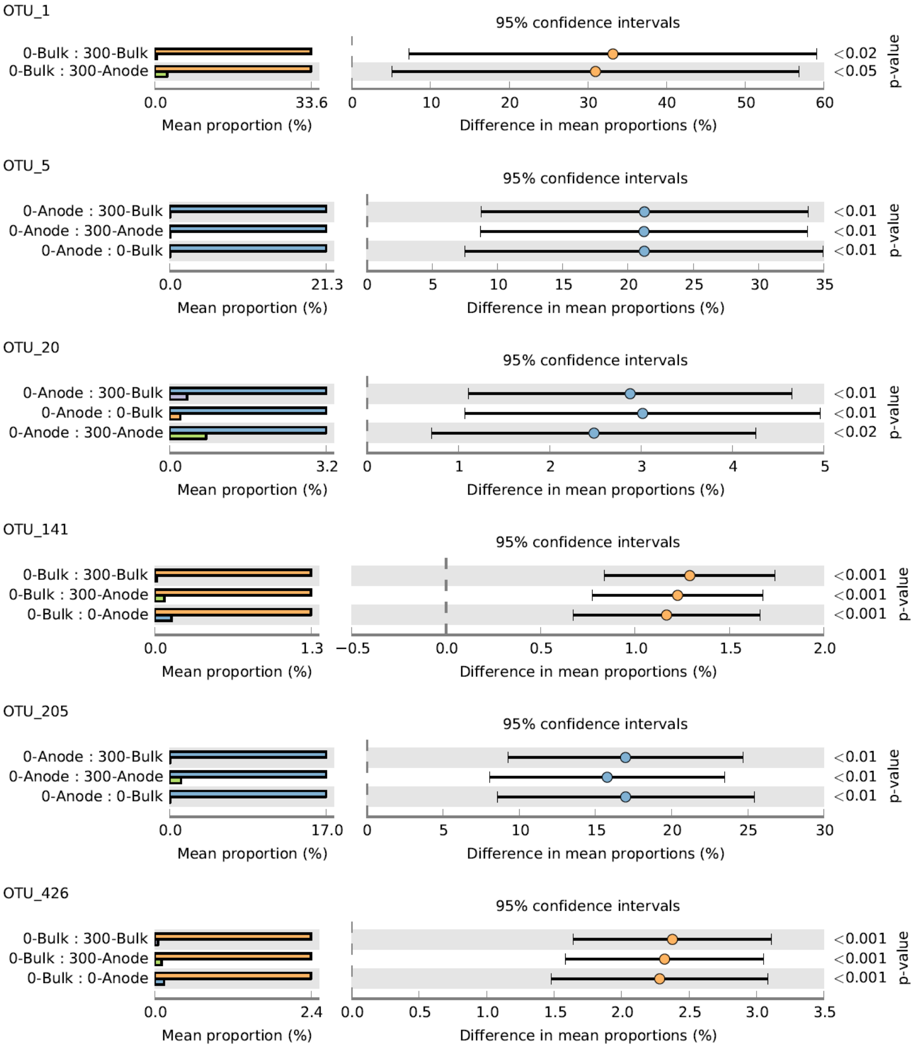

Fig. S5 - Post-hoc test performed at OTU level revealing that the OTU_1, OTU_141 and the OTU_426 were more abundant in the bulk of the reactors polarized at 0 mV (vs Ag/AgCl) (0-Bulk). The OTU_5, OTU_20 and OTU_205 were more abundant at the anodes of the reactors polarized at 0 mV (vs Ag/AgCl).

family	R300a-Anode	R300b-Anode	R300c-Anode	R0a-Anode	R0b-Anode	R300a-Bulk	R300b- Bulk	R300c- Bulk	R0a- Bulk	R0b- Bulk	Inoculum
Streptomycetaceae	0%	0%	1%	0%	0%	0%	0%	9%	0%	0%	0%
Rhodobacteraceae	1%	4%	1%	0%	0%	2%	10%	1%	2%	2%	13%
Lactobacillaceae	0%	0%	1%	0%	0%	0%	0%	8%	0%	0%	0%
Helicobacteraceae	0%	6%	2%	0%	4%	13%	2%	1%	2%	3%	10%
Flavobacteriaceae	0%	3%	0%	0%	0%	1%	2%	0%	1%	1%	15%
Desulfuromonadaceae	0%	13%	4%	56%	3%	0%	27%	1%	40%	2%	7%
Desulfobulbaceae	25%	7%	76%	40%	69%	1%	2%	1%	3%	2%	11%
Desulfobacteraceae	73%	50%	6%	3%	21%	77%	39%	0%	44%	79%	16%
Chromatiaceae	0%	2%	0%	0%	0%	0%	2%	0%	1%	1%	5%
Bacillaceae 2	0%	1%	0%	0%	0%	0%	0%	7%	0%	0%	0%
Bacillaceae 1	0%	4%	0%	0%	0%	1%	2%	21%	0%	1%	1%
Alteromonadaceae	0%	1%	0%	0%	0%	2%	1%	0%	2%	3%	7%
Other families	0%	1%	0%	0%	0%	2%	1%	0%	2%	3%	7%

Table S1 - Taxonomic composition at family level of the microbial communities selected on the anodes and in the sediment in the microbial inoculum and in reactors polarized at 0 mV (vs Ag/AgCl) and +300 mV (vs Ag/AgCl). The families with a relative abundance of 5% (or higher) in at least one sample were reported.

Scientific production

LIST OF PUBLICATIONS

Daghio M, Vaiopoulou E, Patil SA, Franzetti A, Rabaey K. Bioelectrochemical toluene degradation in marine environments. In preparation.

Daghio M, Tatangelo V, Franzetti A, Gandolfi I, Bestetti G, Careghini A, Sezenna E, Saponaro S. Hydrocarbon degrading microbial communities in a bench scale aerobic biobarrier for gasoline contaminated groundwater treatment. Under revision.

Careghini A, Saponaro S, Sezenna E, Daghio M, Franzetti A, Gandolfi I, Bestetti G. Lab-scale tests and numerical simulations for *in situ* treatment of polluted groundwater. J. Hazard. Mater. Accepted.

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BOOK CHAPTERS

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