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BIOELECTROCHEMICAL SYSTEMS TO INVESTIGATE THE EXOELECTROGENIC ACTIVITY OF HYDROCARBON-DEGRADING BACTERIA

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Anna Speranza Espinoza Tofalos

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AUTOR:

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TRABAJO DE TESIS, presentado en cumplimiento parcial de los requisitos para el Grado de Doctor en Ciencias, mención Química de la Universidad Técnica Federico Santa María y de la Universidad de Valparaíso y el Grado de Doctor en Ciencias Químicas, Geológicas y Ambientales de la Università degli Studi di Milano-Bicocca.

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Abstract

Bioelectrochemistry and, more specifically, microbial electrochemistry, are technologies based on the connection between microbes (named as exoelectrogens or, focusing only on bacteria, electrochemically active bacteria) and electrodes. The exchange of electrons to and from the electrode has been studied primarily in mixed cultures but also with pure strains, mostly using model species such as *Geobacter* and *Shewanella*; however, more efforts are needed to elucidate the interaction between microbes and electrode and to find new interesting niches of application for these microorganisms. A field of application is bioelectrochemical remediation, an effective strategy in environments where the absence of suitable electron acceptors limits classic bioremediation, and in which bioelectrochemical systems are used for the removal of pollutants from environmental matrices. Bioelectrochemical remediation of hydrocarbons with pure strains and microbial communities has been reported; however, only few exoelectrogenic hydrocarbonoclastic bacteria have been characterized, so far.

The degradative potential of several hydrocarbon-degrading strains has been extensively studied, in terms of pollutants removal and mechanism of contaminant mineralization, but not much is known about their exoelectrogenic capacity and possible application for bioelectrochemical remediation. Bioelectrochemistry and its application for bioremediation purposes, has primarily focused on testing the hydrocarbonoclastic capacities of already known exoelectrogenic strains. In this study we took a different approach, and we aimed at studying the exoelectrogenic activity of three strains that showed great potential for bioremediation applications: *Cupriavidus metallidurans* CH34, and *Pseudomonas* sp. strains DN34 and DN36. *C. metallidurans* CH34 is a model metal-resistant strain, whose hydrocarbonoclastic capacities have recently been individuated, and *Pseudomonas* sp. strains DN34 and DN36 that are two hydrocarbon-degrading strains isolated from an oil-polluted site in central Chile. By analyzing current production, bacterial growth and substrate consumption in bioelectrochemical systems (BES), we determined that the three strains possess exoelectrogenic

activity. Moreover, *C. metallidurans* CH34 showed the most promising results with a non-recalcitrant substrate and was selected to assess bioremediation experiments with toluene as model hydrocarbon. We demonstrated for the first time that strain CH34 is able to degrade toluene under denitrifying conditions. Further experiments in Microbial Fuel Cells (MFC) linked toluene degradation to current production by strain CH34, showing current peaks after toluene respike (maximum current density 0.24 mA/m^2). Moreover, a Microbial Electrolysis Cell (MEC) was operated by applying an external voltage (800 mV) between anode and cathode to stimulate microbial metabolism of strain CH34 and to observe the behavior of the strain in terms of toluene removal and current generation. Current outputs increased by two orders of magnitude in comparison with MFC (up to 47 mA/m^2), and coulombic efficiency raised up to 77%, demonstrating that the bacterial cells adjusted progressively to the system conditions and that electrochemical losses were, at least partially, overcome. In order to evaluate the effect of an electron carrier on current production, Neutral Red (NR) was selected as external transporter and amended in a MEC containing toluene and inoculated with strain CH34, but no relevant effect was observed on current production nor coulombic efficiency. Hence, we concluded that NR had no influence on current generation in our system and that a mediated mechanism with this electron carrier is not probable. The mechanism of extracellular electron transport (EET) is a key feature in BESs and the efficiency of the microorganism to exchange electrons with an electrode and to connect the EET to the cellular carbon metabolism, significantly influences the overall process performance. We demonstrated that the first step of the denitrification pathway is activated by nitrate reductases when NO_3^- was the only electron acceptor, but we also aimed at studying whether the pathway of denitrification is still active in absence of nitrate, if a solid the anode is potentiostetically-polarized at the same redox potential of nitrate reductase. Our results indicate that nitrate reductase is not involved in the transport of electrons in BES and that strain CH34 follows a different pathway of electron transport to the anode. However, current production and cells viability demonstrated that strain CH34 was actively performing oxidative phosphorylation, thus that, in a

mechanism that has not been elucidated yet, an extracellular electron transfer takes place, either in a direct or indirect way.

Keywords

Toluene, biodegradation, bioelectrochemical systems, exoelectrogenic, *Cupriavidus metallidurans* CH34, *Pseudomonas*, hydrocarbonoclastic

Abstract

Le tecniche bioelettrochimiche microbiche sono basate sull'interconnessione tra microorganismi ed elettrodi. Lo scambio di elettroni da e verso l'elettrodo è stato studiato sia in colture miste e con ceppi puri (*e.g.*, *Geobacter* e *Shewanella).* Tuttavia, sono necessari ulteriori sforzi per chiarire l'interazione tra microbi ed elettrodo e per trovare nuovi interessanti campi di applicazione per questi microrganismi. Un campo di applicazione è il trattamento biologico di matrici contaminate attraverso l'uso dei Sistemi Bioelettrochimici (BES), una strategia efficace in ambienti in cui l'assenza di accettori di elettroni limita il biorisanamento classico. Il biorisanamento di matrici contaminate da idrocarburi utilizzando tecnologie bioelettrochimiche è stato riportato in letteratura con ceppi puri e comunità microbiche. Tuttavia, finora solo pochi batteri idrocarbonoclastici esoelettrogeni sono stati caratterizzati.

In questo studio è stata analizzata l'attività esoelettrogena di tre ceppi che hanno mostrato un potenziale per applicazioni di biorisanamento: *Cupriavidus metallidurans* CH34, *Pseudomonas* sp. DN34 e DN36. Analizzando la produzione di corrente, la crescita batterica ed il consumo del substrato in sistemi bioelettrochimici (BES), è stato determinato che i tre ceppi possiedono attività esoelettrogena.

Il ceppo *C. metallidurans* CH34 ha mostrato i risultati più promettenti con un substrato non recalcitrante, ed è stato selezionato per valutarne la capacità di biorisanamento con il toluene come idrocarburo modello. Per la prima volta in questa tesi è stato dimostrato che il ceppo CH34 è in grado di degradare il toluene in condizioni denitrificanti. Ulteriori esperimenti in celle a combustibile microbiche (MFC) con il ceppo CH34 hanno correlato la degradazione del toluene con la produzione di corrente (densità massima di corrente 0.24 mA/m^2). Una cella a elettrolisi microbica (MEC) è stata utilizzata applicando un voltaggio esterno (800 mV) per stimolare il metabolismo microbico. Le correnti generate sono aumentate di due ordini di grandezza rispetto alla MFC (fino a 47 mA/m^2) e l'efficienza coulombica è aumentata fino al 77%, dimostrando che le cellule batteriche si sono

ambientate progressivamente alle condizioni del sistema e che le perdite elettrochimiche sono state, almeno parzialmente, superate. Con il fine di valutare l'effetto di un trasportatore di elettroni sulla produzione di corrente, il composto Neutral Red (NR) è stato utilizzato come trasportatore esterno in una MEC contenente toluene e inoculata con il ceppo CH34. Tuttavia, non è stato osservato nessun effetto rilevante riguardo alla produzione corrente e all'efficienza coulombiana. È stato concluso che il NR non ha alcuna nessun effetto sulla generazione di corrente e che un meccanismo mediato da questo trasportatore di elettroni è improbabile.

Il meccanismo di trasporto elettronico al di fuori della cellula è una caratteristica fondamentale dei BES. L'efficienza del microorganismo di scambiare elettroni con l'elettrodo incide in modo significativo sulla performance complessiva del processo. In questo studio è stato dimostrato che la prima fase del pathway della denitrificazione è attivata dalla nitrato reduttasi quando il nitrato è l'unico accettore di elettroni presente. L'espressione dell'enzima nitrato riduttasi è stata studiata in un BES con il fine di determinare se il pathway della denitrificazione è attivo in assenza di nitrato. I nostri risultati indicano che la nitrato riduttasi non è coinvolta nel trasporto elettronico nel BES. Tuttavia, la produzione di corrente e il fatto che le cellule siano metabolicamente attive, dimostrano che il ceppo CH34 sta eseguendo fosforilazione ossidativa. Attraverso un meccanismo che non è stato ancora chiarito, il ceppo CH34 esegue il trasferimento elettronico al di fuori della cellula.

Parole chiave

Toluene, Sistemi Bioelettrochimici, esoelettrogeni, *Cupriavidus metallidurans* CH34, *Pseudomonas*

INDEX

GLOSSSARY

1,2-DCA:	1,2-Dichloroethane
BES:	Bioelectrochemical System
BTEX:	Benzene, toluene, ethylbenzene and xylene
cDNA:	Complementary DNA
CE:	Coulombic Efficiency
CFU:	Colony forming unit
cis-DCE:	cis-Dichloroethene
CHC:	Chlorinated hydrocarbons
CV:	Cyclic voltammetry
EAB:	Electrochemically active bacteria
EET:	Extracellular electron transfer
EU:	European Union
LD:	Rich culture medium
LPG:	Liquefied petroleum gas
M9:	Rich culture medium
MCL:	Maximum Contaminant Level
MDC:	Microbial Desalination Cell
MEC:	Microbial Electrolysis Cell
MES:	Microbial Electrochemical Snorkel
MFC:	Microbial Fuel Cell
MSC:	Microbial Solar Cell
NR:	Neutral Red
OCP:	Open circuit potential
OECD:	Organisation for Economic Co-operation and Development
PAHs:	Polycyclic aromatic hydrocarbons
PCR:	Polymerase chain reaction
RNA:	Ribonucleic acid
RT-PCR:	Reverse transcriptase-polymerase chain reaction
SB:	Serum bottles
SHE:	Standard hydrogen electrode
TCE:	Trichloroethene
TMT:	Thousands of metric tons
TPH:	Total petroleum hydrocarbons
TSB:	Rich culture medium Tryptic Soy Broth
UN:	United Nations
US-EPA:	United States Environmental Protection Agency

1. INTRODUCTION

1.1. Petroleum hydrocarbons as pollutants

Petroleum and its refined products are the principal energy source for industry and people [1]. Due to a growing economy, during 2015, world oil demand growth was 1.54 million barrels per day higher than the previous year, especially in non-OECD countries [2]. According to the UN Energy Statistics Yearbook of 2014 [3], United States is the major producer and consumer of gasoline, a fraction of petroleum, with 384,828 thousands of metric tons (TMT) produced, followed by the rest of the world (34%) and China (11%) (Fig. 1). In comparison, in 2014 Europe produced 168,564 TMT and South America only 61,065 TMT [3].

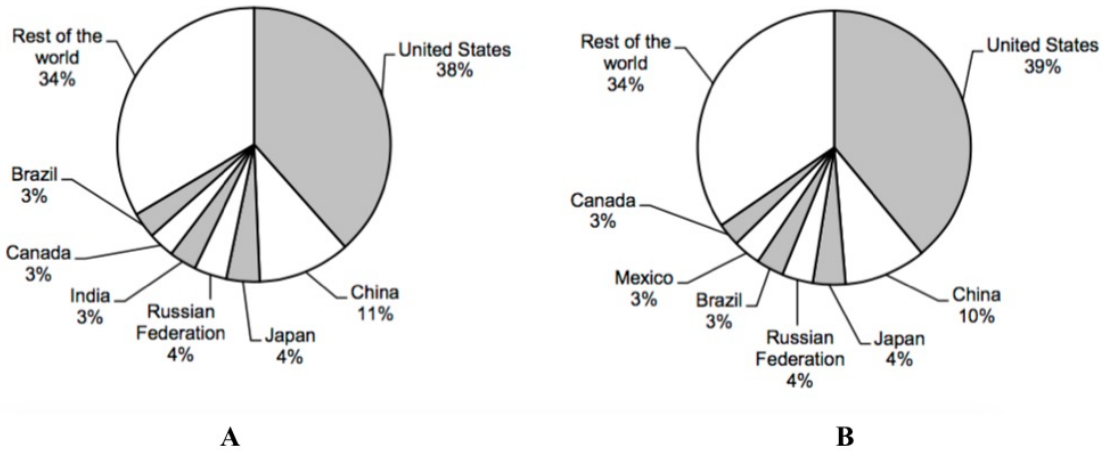

Figure 1. Major motor gasoline producing countries (A) and major motor gasoline consuming countries (B), 2014. Source: 2014 Energy Statistics Yearbook, United Nations [3]

Crude oil is a mixture of hydrocarbons that are present as a liquid in underground geologic formations. Petroleum products are produced from the processing of crude oil and other liquids. Petroleum-based products that can be obtained by refining and comprise refinery gas, ethane, liquefied petroleum gas (LPG), naphtha, gasoline among others [4]. Speight (2002) defines petroleum as a naturally occurring mixture of hydrocarbons, generally in a liquid state, which may also include compounds containing sulfur, nitrogen, oxygen, metals, and other

elements [4]. Petroleum is composed mainly by carbon and hydrogen, and smaller quantity of non-hydrocarbon elements, where sulfur is the most abundant (0.1 – 8% w/w), followed by nitrogen (0.1 – 1.0% w/w), and oxygen (0.1 – 3% w/w). Trace elements are also present at the part per million (ppm) level (vanadium, nickel, iron, and copper) [4]. Based on their structures, hydrocarbons in crude oil are classified as alkanes, cycloalkanes, and aromatics. Alkenes, which are the unsaturated analogs of alkanes, are not often present in crude oil but can be found in many refined petroleum products [5].

Oil spills and leaks are frequent events during routine operations such as exploration, production, refining, transportation and storage [1, 5, 6], and spills in marine environments, even if more probable, constitute less than 10% of total hydrocarbon releases. 90% of total discharge to the environment is represented by routine activities [7]. A review on polluted areas in Europe identified around 1,170,000 Possible Contaminated Sites (PCSs) and 127,000 actually Contaminated Sites (CSs) of which around 45% have already been remediated [8].

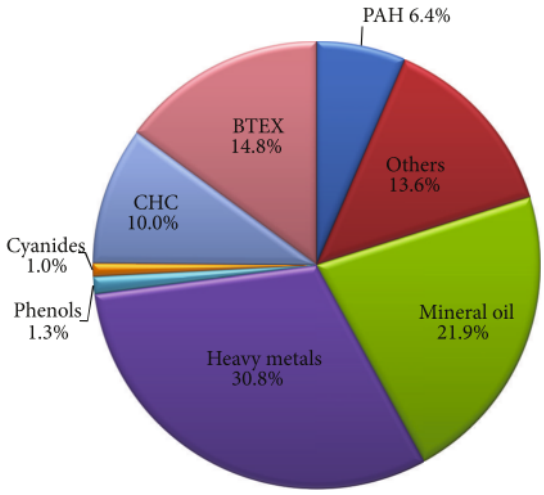

Figure 2. Distribution of contaminants affecting soil and groundwater in Europe. Source: Panagos *et al.,* (2008) [8]

Considering that chlorinated hydrocarbons (CHC); benzene, toluene, ethylbenzene, and xylene (BTEX); polycyclic aromatic hydrocarbons (PAHs) and

minerals oil are petroleum components or derivatives, the distribution of contaminants in groundwater shows two main pollutants: hydrocarbons and heavy metals, where petroleum pollution contributes jointly to 53.1% of groundwater contamination (Fig. 2) [8].

The discharge of these compounds into the environment is the principal cause of water and soil contamination [6, 9]. Even small releases of petroleum into surface and subsurface waters can lead to concentrations of dissolved hydrocarbons that exceed regulatory limits [10]. In Table 1, maximum contaminant levels in drinking water for model hydrocarbons for the United States Environmental Protection Agency (US-EPA) [11], the European Union (EU) [12] and Chile [13] are shown.

The fate and distribution of oil into the environment depends on several biotic [14] and abiotic factors as physical processes related to weathering [15]. It has been reported that petroleum components cause mutations and death of water and soil biota [16], due to its high toxicity [17]. Some oil components have been recognized as being carcinogenic and neurotoxic, such as benzene, toluene, xylene, naphthalene, *n*-hexane [18].

Table 1. Maximum Contaminant Level (MCL) that is allowed in drinking water

Pollutant	Maximum limit permitted in drinking water (mg/L)	Agency	Reference
Benzene	0.005	US-EPA	[11]
	0.001	EU	[12]
	0.01	Chile	[13]
Toluene	1	US-EPA	[11]
	0.7	Chile	[13]
Ethylbenzene	0.7	US-EPA	[11]
Xylene	10	US-EPA	[11]
	0.5	Chile	[13]
Polycyclic aromatic hydrocarbons	0.0001	EU	[12]

It has been reported that the toxicity of petroleum hydrocarbons is correlated with specific boiling-point fraction ranges. Especially, the C_{10}–C_{19} range appears to be degraded preferentially [17]. Hydrocarbons are highly toxic for soil biota and animals, plants and microorganisms [17]. As petroleum spills in water, it affects not only the biota but also physical and chemical processes, because it prevents sunlight to pass through it. Waters, soils and sediments polluted with hydrocarbons cannot be used in agriculture, urbanization and as water source for animals and people. The removal of hydrocarbon pollutants from the environment involves physical, chemical and biological processes [5].

1.2. Remediation of petroleum hydrocarbon contaminated sites

Environmental decontamination of polluted sites is one of the main challenges for sustainable development, and bioremediation is an attractive technology for the clean-up of polluted waters and soils [19]. Remediation technologies can be *in situ* or *ex situ.* Physicochemical and biological processes have been applied for pollutants removal from contaminated environments [19]. Physical strategies include extraction, thermal desorption, soil washing and filtration techniques; while chemical treatments involve the addition of strong oxidant or reducing agents to lower the toxicity of the pollutants. Bioremediation is an eco-friendly, cost-effective and efficient technique for the removal of pollutants from the environment [20] that relies on the activity of microorganism to metabolize the pollutants into safe, or at least less-toxic, compounds, causing minimal (if any) ecological effects [21]. The most common strategies in bioremediation are biostimulation and bioaugmentation, where biostimulation consists in the stimulation of indigenous microorganisms with degradative activities through the addition of nutrients and/or electron acceptors, and bioaugmentation is the application of microorganisms isolated from the same site, with selected and required metabolic activities [22].

To bioremediate hydrocarbon-contaminated water, the most common biostimulation approaches are: bioventing, water circulation systems, air sparging

and biobarriers. Bioventing is used mainly to stimulate aerobic degradation processes by pulling air above the water course. In water circulation systems, water is extracted and amended with electron acceptors and nutrients and back injected into groundwater. With air sparging, compressed air is injected and oxygen is provided to enhance the natural aerobic degradation of oil compounds. With biobarriers, a permeable and biologically active fence is located perpendicularly to the plume, creating a zone of high microbial density [23].

Microorganisms have been vastly used to bioremediate hydrocarbon-polluted environments [20], including soils [24], sediments [25] and aquifers [14, 26]. For the hydrocarbon degradation, microorganisms use several biochemical pathways [27]. Pollutants can be used by microorganisms as carbon and energy sources, leading to their complete degradation (mineralization) or, through detoxification processes, can be converted into harmless compounds [24]. It has been described that microorganisms are the main biocatalysts for hydrocarbon bioremediation [27].

Many microorganisms, including bacteria, are able to metabolize diverse hydrocarbons through evolved mechanisms that activate these compounds generating metabolic intermediates that funnel to central pathways [27, 28].

Hydrocarbon degradation by bacteria under aerobic metabolism has been extensively studied, and the key enzymes involved in these processes are oxygenases, which catalyze the addition of molecular oxygen to the substrate [27]. In the case of toluene, component of the mixture BTEX and one of the most common groundwater contaminants [29], different aerobic degradation pathways have been reported. Bacteria can use monooxygenases or dioxygenases to catalyze the formation of dihydroxylated intermediates; alternatively, the methyl group of toluene may undergo oxidation prior to conversion to a catechol as in the TOL pathway (Fig. 3). Catechols are then cleaved by dioxygenases, with oxygen participating as a substrate, and then transformed to substrates of the Krebs Cycle.

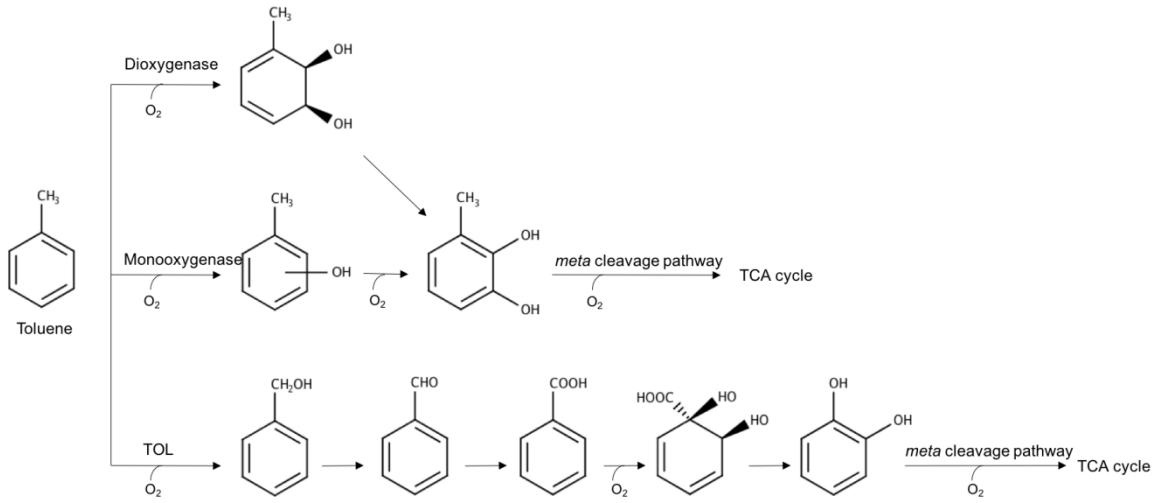

Figure 3. Aerobic toluene degradation pathways in bacteria. Adapted from Laskin *et al.,* (2008) [30]

On the contrary, anaerobic degradation of hydrocarbons in complete absence of oxygen has been discovered only a few decades ago [31] and anaerobic bacteria have been found to be useful in degrading halogenated hydrocarbons, that are usually recalcitrant under oxic conditions [32]. Regarding aromatic hydrocarbons, three different mechanisms have been identified for the initial oxygen-independent attack of the aromatic ring by anaerobic bacteria, using Fe(III), sulfate or nitrate as terminal electron acceptors: fumarate addition, O_2-independent hydroxylation and carboxylation [33]. The mechanism used by bacteria depends on the aromatic hydrocarbon consumed as substrate and by the strains involved in the process. The anaerobic degradation of toluene, as model aromatic hydrocarbon, starts with the addition of fumarate, and subsequent ß-oxidation of the intermediate benzylsuccinate to benzoyl-CoA (Fig.4) [33]. Benzoyl-CoA, that is a central intermediate of anaerobic degradation of aromatic hydrocarbons, is then metabolized to Krebs Cycle intermediates [30].

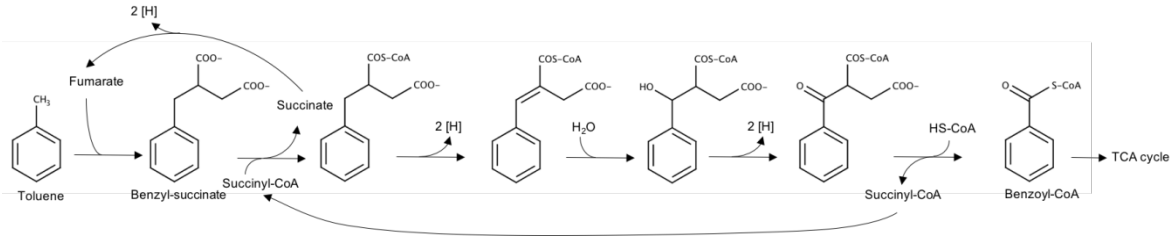

Figure 4. Anaerobic toluene degradation pathway in bacteria. Adapted from Fuchs *et al.,* [33]

Pseudomonadaceae and *Burkholderiaceae* are bacterial families that have been found in hydrocarbon-contaminated environments and that possess degradative abilities [34, 35]. The *Cupriavidus* and *Pseudomonas* genera, respectively belonging to the *Burkholderiaceae* and *Pseudomonadaceae* families, have been found in oil-contaminated sites [35] and the mechanisms involved in the degradation of diverse hydrocarbons are known [27].

In *Cupriavidus metallidurans* CH34, the genes for the aerobic degradation of BTEX and phenol are described, however the capacity of *C. metallidurans* CH34 to degrade these compounds, thus the expression of such genes, has not been studied so far [36].

The ability of Pseudomonas species to aerobically degrade BTEX is well known [37]. *Pseudomonas stutzeri* has been found in a large number of different natural [38] and polluted [34] environments and, like other *Pseudomonas* species (*e.g.*, *P. putida*), is involved in environmentally important metabolic activities. *P. stutzeri* is able to degrade biogenic and xenobiotic compounds (oil derivatives, aromatic and nonaromatic hydrocarbons and biocides) [37] and, during the last decade, *P. stutzeri* has been related to aromatic hydrocarbons degradation, including polycyclic aromatic hydrocarbons (PAHs) [38–40]. *P. stutzeri* is a facultative anaerobe, thus, if available, uses oxygen as the terminal electron acceptor. However, all strains can use nitrate as an alternative electron acceptor and can carry out denitrification [37]. *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36, that are closely related to *Pseudomonas stutzeri*, were isolated from two soil samples collected next to a petroleum refinery located at the Aconcagua River estuary in Chile and have been studied in bioremediation for their ability to degrade naphthalene, fluorene and phenanthrene [34].

1.3. Energetic metabolism of microorganisms

To live, grow and reproduce, living organisms must perform work, channeling energy into biological work and carrying out a significant variety of energy transductions. Chemical energy is employed by organisms to accomplish the synthesis of complex macromolecules from simple precursors [41]. In order to produce energy for living processes, when carbon source is enzymatically oxidized, hydrogen atoms from the hydrogen transport molecules (such as NADH or $FADH_2$) are separated into electrons and protons. Electrons are released and a spontaneous flow through a series of electron carriers located in the cell membrane to a final electron acceptor (*e.g.* O_2, nitrate, nitrite, sulfate, ferric iron). The electron carriers, which are located in the cell membrane according to successively increasing reduction potentials, accept and transfer electrons [41]. Conversely, protons are pumped out of the cell in Gram positive bacteria and to the periplasm in Gram negative bacteria, generating the proton-motive force that drives protons back into the matrix providing the energy for ATP synthesis (Fig.5) [42].

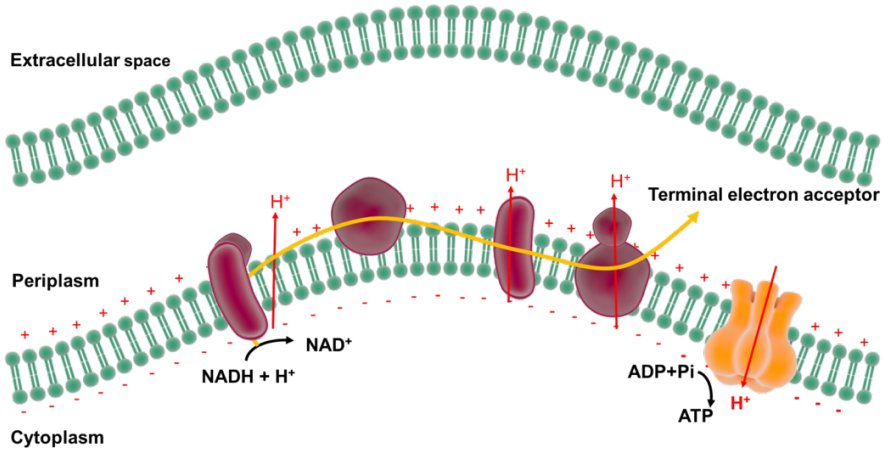

Figure 5. Schematic representation of bacterial electron transport chain. Adaptation from Madigan *et al.,* (2012) [42]

Regarding electron acceptors, microorganisms can anaerobically oxidize hydrocarbons (*i.e.* benzene) to CO_2 with the reduction of Fe(III), nitrate or sulfate

[43]. Denitrification has been coupled with aromatic hydrocarbon biodegradation [44]. Several denitrifying bacteria have been isolated from hydrocarbon-polluted environments [45] and characterized for the removal of benzene [46] and toluene [45, 47]. Denitrification is a key part of this study with the strains under investigation, which will then lead into the more detailed description of denitrification.

Respiratory reduction of nitrate to N_2 via nitrite (Eq.1/Eq.2), nitric oxide (Eq.2/Eq.3) and nitrous oxide (Eq.3/Eq.4) is termed denitrification. The standard reduction potentials (*E°')* for the four N-oxide couples are: nitrate/nitrite +420 mV, nitrite/nitric oxide +375 mV, nitric oxide/nitrous oxide +1175 mV and nitrous oxide/nitrogen +1355 mV (Table 2) [48].

Table 2. Denitrification steps and standard reduction potentials for each reaction

$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$	E°' = +420 mV	(1)
$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$	E°' = +375 mV	(2)
$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$	E°' = +1175 mV	(3)
$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$	E°' = +1355 mV	(4)

Several works individuated bacterial strains able to degrade toluene in anaerobiosis following the path of denitrification [45, 47, 49]. Zhou *et al.,* (2017) isolated new toluene-degrading denitrifying strains belonging to the phylum Proteobacteria and with high similarity to the *Azoarcus* genus [49]. The strains were isolated from different environmental matrices, such as petroleum-polluted soil, freshwater and marine water [49]. Dou *et al.,* (2008) bioaugmented a soil with a BTEX-adapted denitrifying consortia [50]. In this study toluene, the BTEX that was most quickly degraded, achieved over 98% of pollutant removal in 40 days starting with a toluene concentration of 50 mg/kg of soil and its degradation was coupled to nitrate reduction.

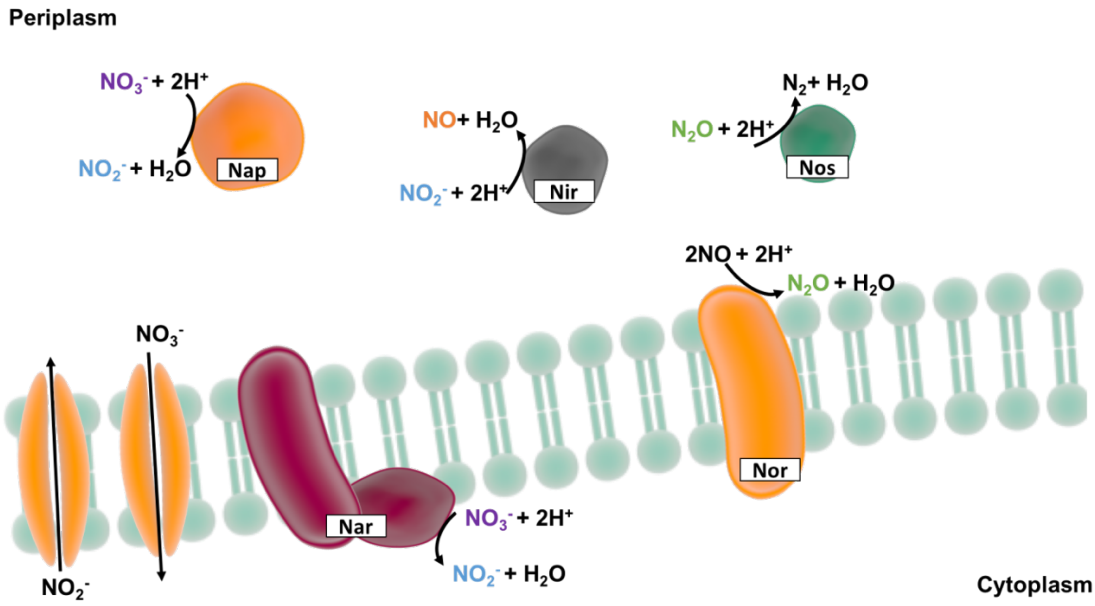

Figure 6. Schematic representation of denitrification enzymes. Adapted from Berks *et al.,* (1995) [48]

C. metallidurans CH34 possesses the genes for denitrification. Distinct types of nitrate reductases have been annotated (NAS, NAR and NAP) and seemingly full denitrification from nitrate to molecular nitrogen is possible in CH34 due to the presence of key genes: *narG* or *napA* encoding a nitrate-reductase, *nirS* gene encoding a nitrite-reductase, the *norB* gene encoding a nitric-oxide reductase, and a *nosZ* gene coding for a nitrous-oxide reductase (Fig.6) [36]. However, the expression of these genes in *C. metallidurans* CH34 has never been studied.

1.4. Microbiological aspects of current production

As mentioned above, microorganisms, as all living organisms, transfer electrons through the electron-transfer chain from an electron donor (lower potential) to an electron acceptor (higher potential) [51]. Some microorganisms are able to use several compounds as electron acceptor, thus when more than one of these electron acceptors are available, a microorganism normally regulates its metabolism in order to use the electron acceptors with the highest potential. For facultative anaerobes oxygen is the best electron acceptor, with a reduction

potential (E_0') of the ½O_2/H_2O couple of +820 mV. However, compounds with lower redox potentials can be used as electron acceptors, as the ion couple F^{3+}/Fe^{2+} ($E^{0'}$=+771 mV), ferricyanide $[Fe(CN)_6]^{3+}/[Fe(CN)_6]^{2+}$ ($E^{0'}$=+360 mV) or nitrate NO_3^-/NO_2^- ($E^{0'}$=+420 mV), among others [52]. Under anoxic conditions, the lack of oxygen limits the biodegradation rate and leads to the need of its replacement [53]. Oxygen can be introduced into groundwater for example by a process called air sparging, however, this technology presents some limitations. Oxygen consumption can be very fast and thus rate limiting. The efficacy of air sparging is determined principally by the degree of contact between the injected air and the contaminated soil and groundwater [54]. Moreover there is the possibility of microbial growth overstimulation leading to biofouling and the subsequent obstruction of the aeration wells. In addition, ferrous ions (Fe^{2+}) present in the aquifer can be oxidized to ferric ions (Fe^{3+}), resulting in the formation of iron-containing precipitates [55]. Some microorganisms (like iron-reducers), possess the ability to transfer electrons outside their cell membrane using an external electron acceptor through a mechanism called extracellular electron transfer (EET) [56]; and those bacteria that can perform this process and that are able to use an electrode as terminal electron acceptor are defined as exoelectrogens [57]. In a Bioelectrochemical System, the anode function as insoluble electron acceptors [51], and can be used as an electron acceptor alternative to oxygen to stimulate biodegradation.

1.5. The concept of Bioelectrochemical System

Bard and Faulkner (2001) defined electrochemistry as the branch of chemistry concerned with the interrelation of electrical and chemical effects [58].

An electrochemical cell is a device capable of generating electrical energy from chemical reactions or to conduct a non-spontaneous reaction by applying electricity for its realization. An electrochemical cell consists of two half-cells formed by a solid electrode and an electrolyte. In electrochemical systems, charge

is transported across the interface between an electronic conductor (an electrode) and an ionic conductor (an electrolyte) [58].

A Bioelectrochemical System (BES) is an electrochemical system in which electro-chemically active microorganisms catalyze the anode and/or the cathode reaction [56].

When the aim is to oxidize a reduced substrate, exoelectrogenic bacteria catalyze the transfer of electrons from the substrate (electron donor) to a solid anode (electron acceptor). When a cathode is located to close the circuit, thus to provide a path for the electron flow, electrical power is generated in a microbial fuel cell (MFC) [59].

Redox reactions occur in these systems, where in one half-cell the semi-reaction of oxidation occurs, while in the other half-cell is carried out a reduction. When a potential difference is generated between the anode and the cathode, electrons flow from one half cell to the other through an external circuit. The standard reduction potential, E°', is a measure (in volts) of the affinity of each electron acceptor for electrons, in standard conditions (25ºC, pH 7, each solute at 1M) [41]. Electrons tend to flow spontaneously from the half-cell with lower Eº` to the half-cell with higher Eº' and the strength of that tendency is proportional to ΔE, the difference in reduction potential. The Gibbs Free Energy Change (ΔG) for the redox reaction is the energy made available by this spontaneous electron flow and is proportional to ΔE (Eq.5):

$$\Delta G = -n \cdot F \cdot \Delta E \quad or \quad \Delta G° = -n \cdot F \cdot \Delta E° \tag{5}$$

where *n* is the number of electrons transferred in the reaction and *F* is Faraday constant [41]. The Gibbs Free Energy Change of the overall reaction determines how the bioelectrochemical system needs to be operated. When the Gibbs Free Energy Change of the overall reaction is negative, electrical energy can be produced, and the BES is operated as a Microbial Fuel Cell. Conversely, when the Gibbs Free Energy Change of the overall reaction is positive, electrical energy must be provided, and the BES is operated as a Microbial Electrolysis Cell (MEC)

(Fig. 7) [56]. The potential of an electrode (anode or cathode) can be determined by measuring the voltage against a reference electrode (an electrode with a known potential) [60]. The standard hydrogen electrode (SHE) is the universal reference for reporting relative half-cell potentials and consists of a platinum electrode in a hydrogen saturated acidic solution (all components at unit activity). SHE is not a very practical reference electrode to use in an experimental set up. Therefore, other reference electrodes are often used. The silver-silver chloride (Ag/AgCl) reference electrode is considered the most popular reference electrode in BES experiments, due to its simplicity, stability, and nontoxicity. In a saturated potassium chloride solution at 25 °C the Ag/AgCl reference electrode develops a potential of+0.197 V against the SHE [60].

A measure of the efficiency of the electrode as electron acceptor is the Coulombic efficiency (CE) that is calculated as the ratio of total coulombs actually transferred to the anode and theoretical coulombs produced if all substrate consumed would have produced current. The factors that affects CE are i) the utilization of an alternate electron acceptor by the bacteria, either those that are present in the medium (*e.g.* nitrate, sulfate, oxygen, among others) or those that diffuse from the membrane (*e.g*. oxygen), ii) competitive processes and iii) bacterial growth [60]

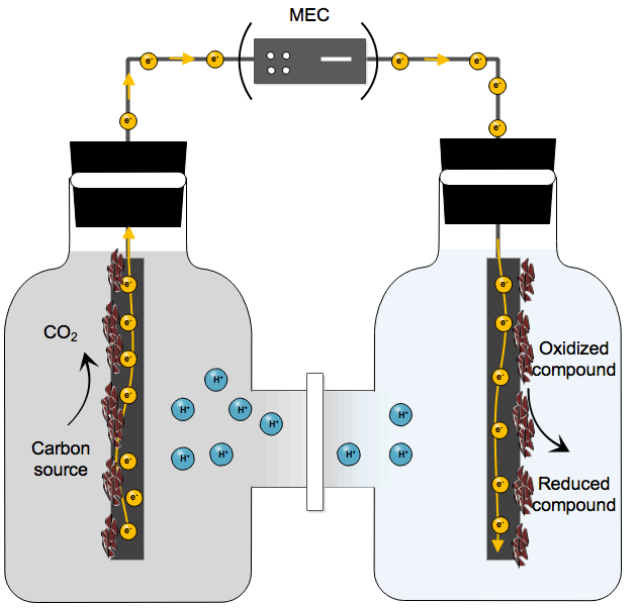

Figure 7. Scheme of a double-chamber Bioelectrochemical System. When a power source is placed, the BES works as a MEC.

1.6. Electron transfer from microorganisms to the anode

The ability of certain bacteria to transfer electrons outside their membrane and to interact with a solid electrode liberating an electrical current was first discovered by Potter in 1911 [61]. Several works have identified exoelectrogenic strains, but only a few have studied the mechanisms of electron transfer in depth. It has been identified that microorganisms can transfer electrons to the electrode directly, through the contact between the electrochemically active microorganisms and the electrode, or indirectly by using redox mediators [56]. These mediators can either be redox-active organic (*e.g.,* humic acids [62]) or inorganic (*e.g.,* sulfur species [63]) compounds that are present in the medium, or are in some cases produced by the microorganisms (*e.g.,* phenazines [64]).

Metal-reducing bacteria are very common in EET processes, and *Geobacter sulfurreducens* and *Shewanella oneidensis* are two of the most studied microorganisms capable of extracellular electron transfer [52]. For both of them outer-membrane cytochromes are extensively involved in the electron transfer process, however *Shewanella* can follow a direct and an indirect, electron carrier-mediated mechanism, while *Geobacter* transfers the electrons directly from the cell to the electrode surface. *G. sulfurreducens,* the model organism for direct transfer mechanism, shows an extensive biofilm formation and the thickness of the biofilm is directly linked with the amount of current produced [65]. The mechanism for electron transport through the biofilm to the anode can be achieved either via mediators, such as cytochromes, into the biofilm matrix that acts as electron shuttle [66], or with “nano-wires” that permit the physical connection between the cells and the electrode [65]. Instead, indirect electron transfer has been extensively studied in the model microorganism *Shewanella oneidensis* that possess large number of cytochromes mainly located in the outer membrane and that can achieve whether direct and indirect electron transfer. The electron transfer mechanism in this bacteria begins with the oxidation of small electron carriers (quinols), followed by an electron flow through a series of cytochromes (CymA or TorC). These cytoplasmic proteins interact with a variety

of redox molecules in the periplasm that finally achieve the reduction of sulfite, nitrate, nitrite and fumarate. In the case of direct electron transfer, *S. oneidensis* possess nano-wires; their structure is different from those expressed in *Geobacter* but that presumably follow the same mechanism. Regarding the indirect electron transfer mechanism, *S. oneidensis* secrets flavin molecules that interact with cytochromes located in the outer membrane and that act as small diffusible shuttle molecules. These flavins transfer the electrons produced by the bacterium from the cytochromes to the electrode [52]. Besides *Shewanella,* other species are able to produce their own electron shuttles thus to use the electrode as ultimate electron acceptor.

Rabaey and Boon (2005) used an isolate from a microbial fuel cell, *Pseudomonas aeruginosa* strain KRP1 that naturally produces phenazinic compounds and a mutant strain without the capacity to synthesize these compounds to demonstrate that electron-shuttles production and current generation were correlated and that bacteria can use mediators produced by other strains as electron mediator [64]. Moreover, it has been demonstrated that if artificial electron shuttles are introduced, not naturally electrogenic bacteria can achieve current generation in bioelectrochemical systems [67]. Park and Zeikus (2000) studied the effect of the addition of a phenazinic mediator, Neutral Red (NR), on current generation in a microbial fuel cells containing either *Escherichia coli* or *Actinobacillus succinogenes* [68]. The authors compared NR with the electron mediation thionin, observing that the addition of NR led to a 10-fold higher current production (3.5 mA) than thionin (0.4 mA) [68].

1.7. Types of Bioelectrochemical Systems

Bioelectrochemical Systems operate in different ways, depending on the biocatalyst used and/or the mode of application. If the BES is operated with microorganisms, it can be classified as a Microbial Fuel Cell. Instead, if enzymes function as the bio-catalyst, the fuel cell will be classified as Enzymatic Fuel Cell (EFC). Moreover, depending on the operating mode, BESs can be classified as

Microbial Fuel Cells, Microbial Electrolysis Cells, Microbial Desalination Cells (MDCs) and Microbial Solar Cells (MSCs) [59]. Another technology is the Microbial Electrochemical Snorkel (MES), a device that does not produce current but that has been designed to maximize the oxidation of organic matter. The simple set up of the device, with two electrodes connected by conductive material, permits the anode respiring bacteria located in an anoxic environment to transfer the electrons to oxygen through the circuit [69]. The two most studied technologies operated with microorganisms are MFCs and MECs.

In MFCs, current is produced by the thermodynamically spontaneous oxidation of a substrate at the anode, and electrons flow through an external circuit to a terminal electron acceptor (*e.g.*, oxygen) that is reduced at the cathode. During the process, protons generated at the anode by the breakdown of organic substrate, are transferred to the cathode, through a proton/hydrogen selective membrane in double chamber MFCs (or directly through the bulk in single-chamber MFCs), and are reduced on the cathode surface [59].

MECs are a more versatile technology in which an external power source is applied to achieve a non-spontaneous reaction. In MECs the current can be produced by the microorganism-catalyzed oxidation of substrates at the anode and used to produce H_2 [70] and other value added chemicals at the cathode (*e.g.* hydrogen peroxide [71]). However, in MECs biocathodes are also very common and are used to enhance the production of certain compounds (*e.g.* methane [72]) using the catalytic abilities of microorganisms and, in some cases, bioanode and biocathodes were used concurrently to achieve de oxidation of wastewaters and the production of methane [73]. Moreover, biocathodes have found a very interesting niche in the bioremediation of chlorinated organic pollutants [74].

1.8. Main applications of BESs

Since the capacity of certain microorganisms to transfer electrons to a solid electrode (thus to generate a flow of electrons) has been discovered in 1911, the primary goal for scientists has been to generate electrical power in BESs [75].

BESs are able to couple bioelectricity generation with wastewater treatment, compensating the large amount of energy spent in conventional wastewater treatments and the problem of sludge disposal. Even so, to date energy recovery from wastewaters in BESs are 3.5 times lower than traditional methane generation with domestic waters [75]. However, if the goal is not electrical energy production, other applications of bioelectrochemical systems are of interest owing to this technology versatility and vast action field. BESs have been recently applied for the production of chemical compounds at the cathode (*e.g.* hydrogen [70], hydrogen peroxide [71], methane [72, 73]), bioremediation of organic pollutants at the anode (*e.g.* diesel [76], toluene [77]), bioremediation of organic (*e.g.* trichloroethene and *cis*-dichloroethene [74]) and inorganic (*e.g.* heavy metals [78]) compounds at the cathode and for biosensoring purposes [75].

1.9. Bioelectrochemical reductions at the cathode

A wide range of processes can be realized at the cathode, such as chlorinated compounds reductions [74], metal reductions [78, 79], and production of chemical compounds. The production of high value compounds includes hydrogen synthesis [70] and methane [72], among others.

Aulenta *et al.,* (2010) studied the reductive dechlorination of the industrial solvent trichloroethene (TCE) to non-toxic ethane on the cathode of a MEC using a dechlorinating culture, originally enriched from a brackish sediment. By polarizing the cathode at -550 mV (vs SHE), in 4 days they achieved the reduction of TCE (> 96%) to the less-toxic cis-dichloroethene (cis-DCE) coupled with a 70% of coulombic efficiency (CE). Moreover, the authors achieved > 79% of cis-DCE reduction to the harmless ethane/ethane mixture but with a lower (20%) coulombic efficiency [74].

BESs have also been used for the reduction of metals at the cathode. Tandukar *et al.,* (2009) achieved the total reduction of Cr(VI) in the cathodic chamber of a MFC inoculated with an anaerobic mixed culture [79]. Luo *et al.,* (2014) investigated the feasibility to produce H_2 and remove Cu^{2+}, Ni^{2+}, Fe^{2+} from a

synthetic acid mine drainage. Electrons produced by the oxidation of sodium acetate at the anode by a mixed culture and supplemented with 1.0 V from a power supply, abiotically reduced copper, nickel and iron in the cathodic chamber. Reduction efficiencies varied depending on the metal but were always >87%. Hydrogen formation was also observed coupled with metal reduction, and a maximum of 59 ± 5.2 mL of H_2 were produced from the solution containing mixed ions (Cu^{2+}, Ni^{2+}, Fe^{2+}) [78].

1.10. Degradation of organic compounds at the anode

Bioelectrochemical Systems were used for the first time in the field of energy production for the degradation of organic compounds at the anode. Since then, bioelectrochemical wastewater treatment for the production of electrical energy has been an attractive and promising technology. It is estimated that the energy that could be recovered through the oxidation of organic matter in urban sewage is 10% of residential electricity [51]. During the last decade, efforts are being made to improve BESs designs [80], materials [81, 82] and operative parameters [83].

Several works investigated the performance of different simple carbon sources (not recalcitrant) or complex substrates (urban and industrial wastewater effluents) in BESs and best performances were obtained with complex substrates from real wastewaters. However, even the best result in power density (93 W/m^2 obtained from an ethanol stillage, Table 3) is not comparable with the 333 W/m^2 that can potentially be obtained from the methane gas generated in anaerobic digestion [75].

If the goal is not to recover electrical current from wastes or substrates, BESs can be used as efficient bioreactors for the treatment of recalcitrant pollutants in diverse environments. Conventional strategies to bioremediate environments polluted by recalcitrant organic compounds often relies on the stimulation of the microbial population (biostimulation). A common approach is to provide electron acceptors in order to accelerate the natural fate of the pollutant [23]. For the removal of hydrocarbons, aerobic microorganisms are stimulated by adding

oxygen (*e.g.,* air sparging) [84]. This strategy is more effective in comparison with anaerobic degradation because the breakdown of hydrocarbons mediated by oxygen is faster in comparison with other electron acceptors (*e.g.,* sulfate, nitrate, Fe(III)) [85]. Anaerobic degradation of hydrocarbons can be also achieved by adding sulfate or nitrate, but the main deficiency of the mentioned strategies is that electron acceptors (whether oxygen, nitrate or sulfate) are rapidly consumed. Moreover, diffusion phenomena inside the polluted matrix affect negatively the effectiveness of such compounds [85]. Bioelectrochemical systems are a novel technique for the decontamination of aquatic sediments, soil and water, because the electrode can serve as electron acceptor and support for microbial growth.

The degradation of organic compounds using these systems was first proposed in 2002 by Bond *et al.,* [86] and the effectiveness of BESs has been demonstrated for the oxidation of hydrocarbons [43, 53, 77, 87, 88] and derivatives (e.g, chlorinated hydrocarbons [74, 89], pyridine [90] and phenol [91, 92]).

Pham *et al.,* (2009) efficiently degraded 1,2-Dichloroethane (1,2-DCA), a recalcitrant chlorinated ground-water pollutant, in the anodic chamber of a MFC using a well-performing anodic microbial community that was pre-acclimated in a MFC with acetate. Removal of 95% of 1,2-DCA occurred within two weeks, with a highest removal rate of 102 $mg_{1,2\text{-}DCA}/L \cdot day$ in the first week and 43±4% of coulombic efficiency [89].

Table 3. Performance of MFC with different substrates. Adapted from Pandey *et al.,* (2016) [93]

Substrate		**Maximum Power Density (W/m^2)**
Simple substrates	D-Glucose	2.160
	D-Xylose	2.330
	D-Glucuronic acid	2.770
	Ribitol	2.350
	L-Serine	0.768
	Acetic Acid	0.835
	Glycerol	0.600
	Indole	0.229
	Furfural	0.361

Complex substrates	Ethanol stillage	93
	Winery wastewater	31.7
	Steroidal drug industrial effluent	22.3
	Saline seafood wastewater	16.2
	Real dye wastewater	8
	Wine	3.82
	Rice mill wastewater	2.3
	Animal carcass	2.19
	Chocolate industry	1.5
	Real field dairy wastewater	1.10

Pyridine, another well-known recalcitrant contaminant that is classified as an hazardous substance by the US-EPA has been used as a substrate by Zhang *et al.,* (2009) in the anodic chamber of a MFC using a mixed culture as inoculum from a wastewater treatment plant. After 90 days of acclimation where a mixture of glucose-pyridine was used as substrate, a maximum of 64.0 mA/m^2 were achieved coupled with a full degradation of pyridine (500 mg/L) within 12 h. The bioelectrochemical-driven degradation of this pollutant resulted to be more efficient than conventional aerobic and anaerobic biodegradation [90].

MFCs have been used for the removal of phenol in water, coupled with the generation of electricity [91, 92]. Biodegradation of phenol was studied in MFCs by Luo *et al.,* (2009) using, as inoculum, sludge from a wastewater treatment plant and phenol, glucose or a mixture of phenol/glucose as substrate. Gradually, MFCs were operated with phenol as sole carbon source and co-occurring with electricity generation, the degradation efficiencies of phenol in all the MFCs reached above 95% within 60 h [92]. The results indicate that, using a MFC, the biodegradation of recalcitrant contaminants such as phenol, can be enhanced in practical application. Friman *et al.,* (2013) used a pure culture of *Cupriavidus basilensis* for the removal of phenol in a BES operated as a MEC using an overpotential of +322 mV (vs SHE). An increase in bacterial cell growth was observed with 86% phenol removal and current production up to 310 mA/m^2, in comparison to the control BES where the electrodes were not connected and thus the anode did not serve as an electron acceptor [91].

1.11. BES for the removal of hydrocarbons in water, soil and sediments

Morris and Jin (2008) for the first time used a BES to couple hydrocarbons removal with electric power production [94]. Since then, BESs have been used to study the electrochemical-driven biodegradation of hydrocarbons in water [87], soil [95] and sediments [53]. The degradation of diesel was studied in a double-cell MFC, inoculated with wastewater from a refinery, and the removal of diesel range organics (DRO) was enhanced in the MFC (81% of DRO removal) compared to controls (31% of removal). An increase of sulfate concentration (naturally a compound of refinery wastewater) was observed in the MFC and, in less proportion, in the control at open circuit potential (OCP). This could be explained, on one hand, due to a desorption process of the sulfate content of the refinery sludge. On the other hand, a higher concentration of sulfate in the MFC suggest that microorganisms preferred the anode as terminal electron acceptor over sulfate. Moreover, a well-known genus of sulfate reducers, *Desulfovibrio*, was found in the single-cell MFC that was inoculated with the same material. The anodic biofilm community was dominated by bacteria with high similarity to nitrate reducers, even if no nitrate was present in the inoculum. This suggests that denitrification may be a default pathway for organic waste degradation at the anode of an MFC [87]. It has been reported that the sulfur cycle plays a leading role in the anaerobic degradation of oil in environments where sulfate is present [96]. In a study with oil-amended microcosms, approx. 77% of n-alkanes were biodegraded in 302 days, with concomitant reduction of sulfate [96]. In BESs amended with petroleum hydrocarbons and where sulfate is present, a mechanism has been proposed: hydrocarbons are oxidized by sulfate-reducers, producing sulfide that is biologically oxidized to S_0 on the anodic surface. Elemental sulfur can be microbiologically reduced back to sulfide or oxidized back to sulfate, closing the cycle [85].

BESs were also studied for the remediation of oil-contaminated saline soils, by analyzing the rate of petroleum removal in reactors with different water content (23%, 28% and 33%) and at different distances from the anode [95]. It was

observed that, with sufficient water content in soil (33%), the degradation of total petroleum near the anode (<1 cm) was enhanced in this system. Moreover, the number of microorganisms (quantified as colony forming unit per gram of soil; CFU/g-soil) increased significantly in the soil with 33% of water content and in the proximity of the anode. A Sediment Microbial Fuel Cell was designed to determine if an anode placed in the anoxic sediment and connected with a cathode located in the overlying aerobic water could enhance total petroleum hydrocarbons (TPH) degradation. With a pollutant concentration of 16,000 mg/kg of TPH, a potential of 190 mV was generated through the circuit and, after 66 days, the MFC achieved a 24% of degradation rate, in comparison with the system at OCP. The authors concluded that BES is a suitable technology to enhance the degradation in sediments, especially when the lack of electron acceptors impede natural bioremediation [53].

BTEX have been used as model hydrocarbons and their degradation in BESs has been observed both with pure cultures [43, 88] and communities [77, 97, 98]. *Geobacter metallireducens,* an extensively studied exoelectrogenic microorganism, was studied in a BES for the oxidation of toluene, where the anode was poised at +497 mV (vs SHE). After a period of adaptation in which the BES was operated with acetate and then benzoate, toluene was provided as sole carbon source. Current peaks were observed after every respike and an acceleration of toluene removal in the BES operated at +497 mV (vs SHE) compared with the control (operated at OCP). Moreover, toluene removal rates increased for every batch period, suggesting that microorganisms adapted subsequently to the conditions (both toluene as carbon source and the anode as electrode acceptor) [43]. Toluene removal in BESs was also studied with another hydrocarbonoclastic strain, *Pseudomonas putida* F1 in a two-chamber MEC with different overpotentials applied on the anode. Operating the MEC under external voltages led to increased bacterial cell growth compared with the control where the electrodes were not connected to each other. It was observed that in experiments in which overpotential was applied, toluene removal was about 80%, in comparison with the control MEC, in which toluene removal ranged between 20

and 25% [88]. Toluene degradation was also studied with microbial consortia in a double-chamber MFC using an oil cracking wastewater treatment plant inoculum that was previously enriched in a single-chamber MFC. The system was tested with different toluene concentrations (11 mg/L, 33 mg/L and 55 mg/L) and it was observed that maximum voltage (mV) and maximum power density (mW/m^2) had a lineal correlation to toluene concentrations. However, CE decreased with increasing toluene concentrations, indicating that toluene is a refractory carbon source for the consortia. The authors also aimed at evaluating the performance of toluene degradation and electricity generation when pyocyanin was used as electron shuttle. Results suggested that adding pyocyanin greatly enhanced the generation of electricity by toluene biodegradation in MFC. In addition, maximum power density and coulombic efficiency increased 3.64-fold and 13-fold respectively [98]. When toluene was used as sole carbon source in a BES with the anode polarized at 197 mV and +497 mV (vs SHE) and inoculated with a marine contaminated sediment, a maximum current density of 283 and 431 mA/m^2 for +197 mV and +497 mV respectively was reported, linked with a toluene degradation rate of 1 mg/L·day regardless of the tested potential. The microbial community in the reactor was dominated by members of the families *Desulfobulbaceae* (anodes), *Desulfobacteraceae* (anodes and bulk) and *Desulfuromonadaceae* (anodes and bulk). The authors related the families *Desulfobulbaceae* and *Desulfobacteraceae* to hydrocarbon degradation, and *Desulfuromonadaceae* to electron transport and sulfur cycle. Moreover, the authors linked the presence of sulfate-reducing bacteria (*Desulfobulbaceae*) with low current outputs at the end of the experiment by proposing a competition mechanism between these microorganisms and electroactive ones. The proposed mechanism is that sulfate-reducing bacteria, enriched on the anode, oxidize toluene using sulfate as electron acceptor and reducing it to sulfide that is further electrochemically oxidized to elemental sulfur on the anode surface. Finally, *Desulfuromonadaceae* could be involved in the reduction of elemental sulfur to sulfide, that can be re-oxidized to S_0 on the anodic surface [77].

2. HYPOTESIS AND OBJECTIVES

2.1. Hypothesis

Employing a bioelectrochemical system where external voltage is applied, will improve the biodegradation of aromatic hydrocarbons in water by hydrocarbonoclastic exoelectrogenic strains. This process of biodegradation will be enhanced with the application of 3-amino-7-dimethylamino-2-methyl-phenazine as electron carrier.

2.2. General Objective

Developing a bioelectrochemical system (BES), where external voltage is applied, to achieve the biodegradation of hydrocarbons in water by hydrocarbonoclastic exoelectrogenic strains of the genera *Pseudomonas* or *Cupriavidus.*

2.3. Specific Objectives

1. Determine the exoelectrogenic capacity of hydrocarbon-degrading *Pseudomonas* and *Cupriavidus* strains.

2. Select the hydrocarbonoclastic bacterial strain grown in succinate with the highest exoelectrogenic activity in a BES where external voltage is applied between the anode and the cathode.

3. Determine the effect of external voltage application on the degradation of a model hydrocarbon by an exoelectrogenic hydrocarbonoclastic strain.

4. Evaluate the effect of the addition of the compound 3-amino-7-dimethylamino-2-methyl-phenazine (Neutral Red) as an electron carrier on current generation by an exoelectrogenic hydrocarbonoclastic strain in a BES where external voltage is applied.

5. Investigate the role of nitrate reductase in the electron transfer mechanism by an exoelectrogenic hydrocarbonoclastic strain in a BES.

3. MATERIAL AND METHODS

3.1. Chemicals

Succinate (>99.0% purity), toluene (>99.9% purity) and 3-amino-7-dimethylamino-2-methyl-phenazine (Neutral Red) were obtained from Sigma-Aldrich (St. Louis, MO).

Reagents used for molecular biology experiments: PCR Green Taq, DNAse free water were obtained from Promega (Madison, WI). RNA was extracted using Fast RNA Pro-Soil-Direct kit (MP Biomedicals, Illkrich, France), treated with DNase using the RNase-Free DNase Set (Promega, Madison, WI) to degrade any residual DNA, and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA). Reverse Transcriptase polymerase chain reaction (RT-PCR) analysis were performed with GoScript Reverse Transcription System (Promega, Madison, WI). Distilled water was deionized to a quality of 18 MΩ/cm.

3.2. Bacterial strains and culture conditions

Exoelectrogenic activity of three hydrocarbonoclastic bacterial strains were studied. The pure cultures used in this study are part of the culture collection of the Laboratory of Molecular Microbiology and Environmental Biotechnology, Universidad Técnica Federico Santa Maria, Valparaiso, Chile. The hydrocarbonoclastic strains that were tested are: *C. metallidurans* CH34 (BTEX, phenol-degrading strain) [36], *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 (naphthalene, fluorene, phenanthrene-degrading strains) [34]. *Pseudomonas putida* F1 was used as negative control. Previously, the capability to degrade hydrocarbons of these strains has only been tested under aerobic conditions.

C. metallidurans CH34 is a metal-resistant bacterium which genome has been completely sequenced and that has been extensively studied for its resistance to Cd(II), Zn(II), Co(II), Ni(II), Cr(VI), Hg(II), Pb(II), Ag(I) and Cu(II) [19], [99–101].

However, strain CH34 owns the pathway for BTEX and phenol aerobic degradation and a previous work (unpublished) demonstrated the ability of this strain to degrade benzene as sole carbon source in aerobic conditions.

Pseudomonas sp. DN34 and *Pseudomonas* sp. DN36, two hydrocarbonoclastic strains able to degrade linear and cyclic alkanes and aromatic hydrocarbons (naphthalene, fluorene, and phenanthrene) have been isolated from a petroleum-polluted soil next to a petroleum refinery located at the Aconcagua River estuary in Chile [34].

Hydrocarbonoclastic strains were grown in M9 minimal medium supplemented with succinate (16 mM) or toluene (0.65 mM).

For the M9 minimal medium the following regents were used: Na_2HPO_4 (7 g/L); KH_2PO_4 (3 g/L); NaCl (0.5 g/L); NH_4Cl (1 g/L); 2.7 mL of $MgSO_4$ (1M); 0.13 mL of $CaCl_2$ (1M) and 2.5 mL of a trace stock salts solution supplemented with: MgO (10.75 g/L); $CaCO_3$ (2 g/L); $FeSO_4 \cdot 7H_2O$ (4.5 g/L); $ZnSO_4 \cdot 7H_2O$ (1.44 g/L); $MnSO_4$ (1.12 g/L); $CuSO_4 \cdot 5H_2O$ (0.25 g/L); $CoSO_4 \cdot 7H2O$ (0.28 g/L); H_3BO_3 (0.06 g/L) [102].

3.3. Electrodes and electrode chambers

Glass material was pre-soaked for at least 12 h with 10% HCl and subsequently washed and dried. Graphite electrodes (anodes and cathodes) were pre-soaked according to the following cycle in an ultrasonic bath: 5 min in 1N HCl, 5 min in distilled water, 5 min in 1N NaOH and finally washed twice in distilled water.

3.4. Bioelectrochemical reactors set-up and configuration

Dual-chamber custom-made three-neck glass reactors separated by a cation exchange membrane (Ultrex CMI-7000S, Membranes International Inc., USA) with 320 mL total volume were used for all bioelectrochemical experiments (Fig.8). Graphite rods of 8 cm^2 (geometric area) were used as anodes and graphite rods of 11 cm^2 as cathodes. No catalyst was intentionally used on the cathode in order

to better reproduce in-field applications with low cost materials. The system was autoclaved at 120 ºC for 30 min, filled with M9 minimal medium and flushed for 30 min with sterile-filtered N_2 to eliminate dissolved oxygen. Preliminary abiotic tests with resazurin as indicator for absence of dissolved oxygen were performed to ensure that no external oxygen was introducing into the system.

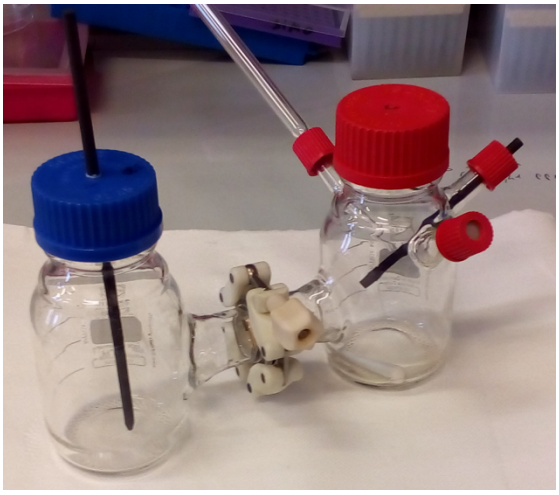

Figure 8. Two-chambers BES used in all the experiments.

According to the experiment, BESs were operated as MFC (without connection to a power supply, Fig. 9A), two-electrodes MEC (with connection to a power supply, Fig. 9B), and three-electrodes MEC using an Ag/AgCl (3M NaCl) reference electrode (Bio-Logic, Seyssinet-Pariset, France) with connection to a potentiostat (Amel mod. 549, Milano, Italy, Fig. 9C). Tektronix PWS2323 Bench Power Supply was used for the experiments performed in Chile (specific objectives 1-2) whereas a self-made power supply was used for the experiments performed in Italy (specific objectives 3-5). Current production was measured directly with a UT60A Digital Multimeter, setting the 40,000 Ω resistance scale (for the experiments performed in Chile) or indirectly with a data logger (Grant-Logger Type 2010), using 1,000 Ω or 10,000 Ω resistances.

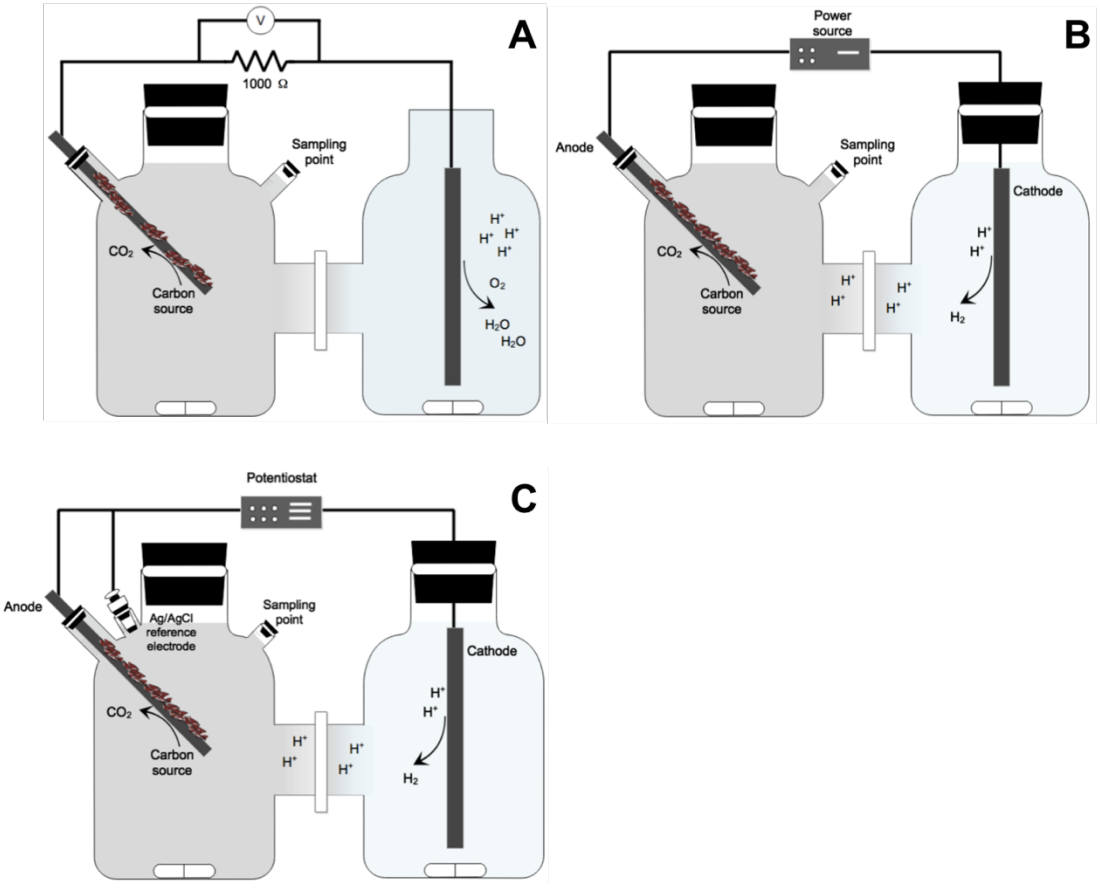

Figure 9. BESs configuration during the experimental procedures. Current production was measured directly with a UT60A Digital Multimeter, setting the 40,000 Ω resistance scale (for the experiments of specific objectives 1-2) or indirectly with a data logger (Grant-Logger Type 2010), using 1,000 Ω or 10,000 Ω resistances (for the experiments of specific objectives 3-5). **A**. MFC configuration: current was measured between the anode and the cathode. **B**. Two-electrodes MEC configuration: a power source was used to apply an external voltage between the anode and the cathode; current was measured with a 10,000 Ω resistance. **C**. Three-electrodes MEC configuration: a potentiostat was used to polarize the anode, current was measured through a 1,000 Ω resistance.

3.5. Succinate analysis

Succinate analysis was performed using an Agilent 1290 Infinity HPLC, provided with a refractive index detector and an Aminex HPX-87H column (Bio-Rad Laboratories Inc., USA) at 55°C using 5 mM H_2SO_4 as eluent at a flow rate of 0.7 mL/min.

3.6. Toluene analysis

Toluene concentration was determined by gas chromatography with a Agilent 6890N instrument with split injection and a flame ionization detector equipped with a headspace autosampler (Agilent 7697A) (EPA method 5021A) [103]. Compounds were separated on a Stabilwax capillary column (0.32mm I.D., 0.5 μm film thickness, Restek, USA). Helium (2 mL/min) was used as carrier. The injection port was held at 250 ºC and used in split mode (50:1). The oven temperature was held at 50 ºC. The total time for one GC run was 20 min. The FID temperature was 250 ºC. Standard curve for toluene quantification were obtained with high purity toluene (>99.9% purity) purchased from Sigma-Aldrich (St. Louis, MO).

3.7. Scanning electron microscopy

Scanning electron microscopy analysis (SEM) (Carl Zeiss, EVO MA-10), were performed on the biotic anode of a BES. Bacteria were fixed on samples with 5% (v/v) glutaraldehyde and dehydrated by washing with a graded ethanol series (from 10 to 100%), followed by critical-point drying and gold coating [104].

3.8. RNA extraction, reverse-transcription and PCR amplification

The protocol for RNA extraction from soil was optimized for bacterial cells, using a soil RNA-extraction kit (Fast RNA Pro-Soil-Direct kit). 3 mL of liquid culture ($turbidity_{600}$ between 0.1-0.6) were centrifuged for 5 min at 500 rpm and 4ºC to preserve RNA integrity. Resulting cell pellet was resuspended in the lysing matrix solution and the protocol was followed according to the manufacturers' recommendations. DNase treatment was carried out using the RNase-Free DNase Set (Promega, Madison, WI) to degrade any residual DNA, and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA) reaching a RNA concentration of ~200 μg/mL. RNA was retro-transcribed to cDNA using the

reverse transcriptase-polymerase chain reaction (RT-PCR) technique with sequence-specific primers. cDNA was finally amplified by PCR with specific primers (Table 4) and PCR products were analyzed by electrophoresis on a 1% (w/v) agarose gel stained with GelStar, and compared with mass standards.

In this study, the expression of three genes was analyzed: *narG* (RMET_RS10375), *napA* (RMET_RS21010) and *rpoB* (RMET_RS16720).

Genes that code for nitrate reductase *narG* and *napA* are present in the genome of *C. metallidurans* CH34 but its expression was never tested before. A literature survey was performed to search for PCR primers designed to target *narG*, *napA* and *rpoB* sequences and selected primers were adapted, correcting minor mismatches. *rpoB,* a housekeeping gene that encodes the β subunit of bacterial RNA polymerase, was used as positive control for RNA extraction and retrotranscription.

Each gene was amplified by polymerase chain reaction (PCR) using adapted primers and adapted PCR programs (annealing temperature reported in literature was replaced with the average of melting temperatures for each couple of primers) (Table 4). PCR were performed in 20 µl volume containing 10 µl of GreenTaq, 4 µl of nuclase-free water, 2.5 µl of each primer (10 pmol/µl) and 1 µl of DNA.

Table 4. Primers set used for the RT-PCR assays

Gene	Primer name	Fragment length (bp)	Primers sequence (5'→3')	Reference
narG	1960f	650	TACGTCGGCCAGGAAAA	Philippot *et al.,* (2002) [105]
	2650r		TTTTCGTACCACGTGGC	
napA	V16f	707	GCGCCCTGCCGCTTCTGCGG	Flanagan *et al.,* (1999) [106]
	V17r		GTGCTGGTTGAAACCCATGGTCCA	
rpoB	1698f	343	AACATTGGTCTGATCAAC	Dahllöf *et al.,* (2000) [107]
	2041r		CGCTGCATGTTCGCGCCC AT	

narG was amplified by primers 1960f and 2650r (Table 4) and thermal cycling was carried out by an initial denaturation step of 4 min at 94°C; 38 cycles consisted of a denaturation step 30 s at 94°C, a primer annealing step of 30 s at 53°C, and an elongation step of 45 s at 72°C; cycling was completed by a final elongation step of 6 min at 72°C. *napA* gene was amplified by primers V16f and V17r (Table 4). Thermal cycling was carried out by an initial denaturation step of 4 min at 94°C; 30 cycles of 1 min at 94°C, 1 min 62°C, 2 min at 72°C; and and final elongation step of 10 min at 72°C. *rpoB* gene was amplified by primers 1698f and 2041r (Table 4) and thermal cycling was performed with an initial melting step at 94ºC for 4 min, followed by 27 cycles of denaturation at 94ºC for 30 s, annealing at 56 ºC for 30 s, and elongation at 72 ºC for 40 s, and a final elongation step at 72 ºC for 10 min. PCR products were analyzed by electrophoresis on a 1% (w/v) agarose gel and compared with mass standards.

Due to mismatches on the original primers, modified primers were tested by PCR on *C. metallidurans* CH34 genomic DNA to determine proper amplification. The results from gel electrophoresis of the PCR-amplified DNA products (Fig.10) primers resulted appropriate for the desired target gene.

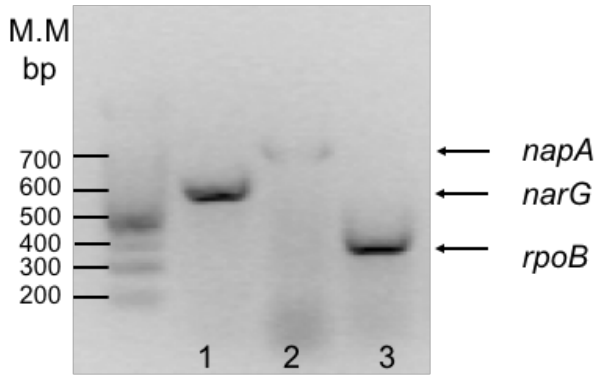

Figure 10. Detection by PCR of *narG*, *napA* and *rpoB* genes in genomic DNA of *C. metallidurans* CH34. Detection of *narG* (lane 1), *napA* (lane 2) and *rpoB* (lane 3) gene in genomic DNA.

3.9. Statistical Analysis

Two-tailed significance test and ANOVA test of succinate removal in the MFC and in the MEC (Fig. 18) was conducted at the 95% confidence level ($\alpha = 0.05$), using the Analysis Toolpak of Microsoft Excel software.

3.10. Experiments with succinate as electron donor

3.10.1. Anaerobic respiration

C. metallidurans CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 were preincubated for 12 h at 30 ºC in rich medium (TSB) in aerobic conditions, reaching a $turbidity_{600}$ of 0.6-0.8 and then washed 4 times with sterile M9 medium to eliminate any trace of rich medium. Strains were previously incubated for 50 h at 30 ºC in 8 mL sealed tubes with M9 mineral medium, succinate (16 mM) and nitrate (62.4 mM). Anaerobic conditions were obtained by flushing the medium with sterile N_2 for 30 min. Bacterial growth was determined periodically by measuring turbidity at 600 nm. *P. putida* F1, a strict aerobe, was used as negative control and incubated under the same conditions described above.

3.10.2. Determination of exoelectrogenic activity

Hydrocarbonoclastic strains were assessed for their exoelectrogenic capacity following a modified version of the methodology of Ribot-Llobet *et al*., 2013 [108].

Experiments were carried in a two-chamber MFC (previously described) with succinate (16 mM) as carbon source. BESs were inoculated with an exponential phase culture of the strain under study (*Pseudomonas* sp. DN34, *Pseudomonas* sp. DN36, *Cupriavidus metallidurans* CH34) with initial $turbidity_{600}$ = 0.1, pre-incubated in anaerobic conditions with nitrate and succinate, operated at 30 °C and under shaking with a magnetic stirrer (150 rpm). An abiotic control MFC was

constructed and maintained under the same conditions but without being inoculated.

Anodic samples of the bulk were collected periodically for 40 h and the turbidity at 600 nm was measured. Current was measured every 60 s with a UT60A Digital Multimeter (Uni-Trend, Dongguan City, China).

Succinate analysis was performed at the beginning and at the end of every experiment for each strain.

Coulombic efficiency (CE) was calculated following the methodology of Liu and Logan (2004) [109]. The Coulombic efficiency (Eq. 6) was calculated as:

$$CE = \frac{C_r}{C_t} \cdot 100\% \tag{6}$$

where C_r *(C)* is the total coulombs collected by the anode and channeled into the electrical circuit, calculated by integrating the current over time. C_t *(C)* is the theoretical amount of coulombs produced if all substrate consumed would have produced current, calculated as (Eq. 7):

$$C_t = \frac{F \cdot b \cdot S \cdot V}{M} \tag{7}$$

where *F* is Faraday's constant (98,485 C/mol of electrons), *b* is the number of mol of electrons produced per mol of substrate (as function of the substrate consumed), *S (g/L)* is the concentration of the degraded substrate, *V (L)* the liquid volume, and *M* the molecular weight of the substrate.

A scanning electron microscopy (SEM) (Carl Zeiss, EVO MA-10), was performed on the biotic anode of a BES inoculated with strain *C. metallidurans* CH34 after 72 h.

3.10.3. Voltage application

To stimulate microbial metabolism, an external voltage was applied between anode and cathode with a power supply. The exoelectrogenic strains were grown in two-chamber MECs as previously described. Different voltages (200, 400, 600, 800 mV) were applied between the anode and the cathode using succinate (16 mM) as sole carbon source. An abiotic MEC (without bacterial cells in the anode chamber) was operated under external voltage (200, 400, 600, 800 mV). Current was measured every 60 s with a Uni-Trend UT60A Digital Multimeter.

Samples for bacterial turbidity measurements were taken periodically for 40 h and samples for succinate concentration were taken at the beginning and at the end of each experiment (0 and 40 h).

3.11. Bioremediation of toluene by *C. metallidurans* CH34

3.11.1. Anaerobic toluene degradation in serum bottles

The capacity of *C. metallidurans* CH34 to metabolize toluene in anaerobiosis was studied.

Strain CH34 was pre-incubated for 15 h at 30 ºC in M9 medium with succinate (16 mM) and nitrate (62.4 mM) in anaerobic conditions to acclimate the cells to anaerobiosis, then pelleted and washed 4 times with sterile M9 medium to eliminate any trace of succinate and nitrate. Strains were inoculated in 110 mL serum bottles (SB) crimp-sealed with a butyl rubber stopper, filled with M9 mineral medium and resazurin (0.001 g/L) as indicator for anaerobic conditions, and N_2-flushed for 30 min. $NaNO_3$ (7.8 mM or 664 ppm) was used to provide nitrate as electron acceptor and toluene (0.65 mM or 60 ppm) was added at last as sole carbon source. The bulk was sampled periodically to determine toluene concentration by GC-FID and bacterial growth by $turbidity_{600}$.

To determine if strain CH34 metabolizes toluene under denitrifying conditions, the expression of nitrate reductase genes present in the genome of strain CH34

(*narG* and *napA*) and *rpoB* (as constitutively expressed control for RNA extraction and retrotranscription) was studied. RNA was extracted and retrotranscribed with specific primers after 8 and 15 days from inoculation.

3.11.2. Toluene degradation in BESs: reactors set-up, operation and experimental conditions

C. metallidurans CH34 was inoculated (final $turbidity_{600}$ of 0.1) in the anodic chamber of a MFC filled with M9 minimal medium and supplemented with toluene (60 ppm) as sole carbon source, to test the capacity of the strain to degrade toluene and to use the anode as electron acceptor. An abiotic MFC was maintained under the same conditions during the whole experiment.

In order to stimulate microbial metabolism, two MECs, one inoculated and one abiotic control, were operated and an external voltage (800 mV) between the anode and the cathode was applied. Samples were taken periodically for toluene analysis.

Coulombic efficiency (CE) was calculated following the methodology of Liu and Logan (2004) [109].

3.11.3. Effect of the addition of Neutral Red as electron carrier

To elucidate if the addition of an electron carrier enhances current production and the efficiency of the toluene removal process, the compound 3-amino-7-dimethylamino-2-methyl phenazine (Neutral Red, NR) was used as electron transporter, following a modified version of the methodology of Park & Zeikus (2000) [68].

Strain CH34 was grown in a BES with toluene as sole carbon source, supplemented with Neutral Red (100 μM) and operated as a MEC by applying 800 mV between the anode and the cathode. An abiotic control BESs was operated under the same conditions. Voltage was measured with a data logger and current density was indirectly calculated thorough a 1,000 Ω resistance.

Periodical sampling was performed in order to measure toluene concentration by GC-FID.

3.12. Mechanism of electron transport: expression of genes coding for denitrification enzymes in BESs

To determine if the pathway of denitrification is involved in the mechanism of electron transfer in BES by *C. metallidurans* CH34, the standard reduction potential of nitrate reductase (+420 mV vs SHE; +224 mV vs Ag/AgCl [3M nACl] reference electrode) was applied by polarizing the anode with a potentiostat and RNA extractions were performed after 2 and 16 days. The sampling volume was calculated to have a final OD of 0.6 in 3 mL volume after centrifugation at 4ºC for 5 min. RNA was extracted and then retrotranscribed to cDNA by RT-PCR using specific primers for *narG, napA* and *rpoB* genes. Finally, the retrotranscribed genes were PCR-amplified. Succinate was used as sole carbon source.

A positive control was included: strain CH34 was grown in anaerobic conditions (sealed serum bottle fluxed with N_2) with succinate as sole carbon source and nitrate as sole electron acceptor. Bacterial cells were harvested after 15 h from inoculation, in a mid-exponential phase and RNA was extracted and retro-transcribed.

4. RESULTS

The exoelectrogenic ability of three hydrocarbonoclastic strains, previously characterized for their capacity to degrade aromatic hydrocarbons in aerobiosis, was tested in BESs. Since the anodic chamber of a BES must be kept under anoxic conditions, the capability of hydrocarbonoclastic strains to grow in anaerobiosis with nitrate as sole electron acceptor and with a non-recalcitrant carbon source (succinate) was studied. Strains that exhibited this facultative metabolism were further tested in MFCs to determine their ability to perform exoelectrogenic electron transfer to an anode. Voltage was then applied in MECs to stimulate microbial metabolism. The strain that showed the best performances in terms of current generation, growth and carbon source removal was used for bioremediation assays in anaerobic conditions in both BESs and serum bottles. Finally, the mechanism of electron transfer was studied in this strain, both with the addition of an electron carrier and by studying the expression of genes that are related to electron transport under denitrifying conditions.

4.1. Strains

Cupriavidus metallidurans CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36, have been applied for bioremediation in previous studies.

C. metallidurans CH34 is a metal-resistant bacterium isolated in 2001 from the wastewater of a zinc factory in Belgium that was polluted with high concentrations of several heavy metals [110]. Strain CH34 contains two megareplicons and two large plasmids, harboring a variety of genes conferring resistance to Cd(II), Zn(II), Co(II), Ni(II), Cr(VI), Hg(II), Pb(II), Ag(I) and Cu(II) [19], 99, 100, 101]. Moreover, the genome of this organism has been sequenced [36], making this strain a model for heavy metal resistance mechanisms. However, there are a large number of uncharacterized genes or Open Reading Frames that may play an important role in metal resistance and detoxification [111]. From the genome of strain CH34, interesting other possibilities for bioremediation have been individuated [36] and

the ability of strain CH34 to degrade benzene as a sole carbon source in aerobic conditions has been demonstrated (Luis Rojas, unpublished results). Possibly, strain CH34 possess much more interesting capabilities that have not been studied yet, such as the ability to degrade aromatic hydrocarbons in anaerobic conditions.

Pseudomonas sp. DN34 and *Pseudomonas* sp. DN36 are two strains that are able to degrade alkanes (linear and cyclic) and aromatic hydrocarbons (naphthalene, fluorene, and phenanthrene) [34].

4.2. Alternative electron acceptors and exoelectrogenic activity

4.2.1. Growth under denitrifying conditions by hydrocarbonoclastic strains

The first condition to determine if a strain is exoelectrogenic is its ability to survive in absence of oxygen using alternative electron acceptors.

C. metallidurans CH34 possesses the genes for full dissimilatory nitrate reduction [36], even if their expression had never been tested. On the other hand, *Pseudomonas* sp. DN34 and DN36 are phylogenetically related to *Pseudomonas stutzeri* species [34], that are known in literature to harbor the pathway of dissimilatory nitrate reduction [37]. For these reasons nitrate was used as sole terminal electron acceptor to determine if strains CH34, DN34 and DN36 are facultative anaerobes. Experiments were conducted in sealed tubes flushed with sterile N_2. The strictly aerobic bacterium *Pseudomonas putida* F1, was used as negative control.

C. metallidurans CH34 showed the highest biomass production ($turbidity_{600}$=1.1), followed by *Pseudomonas* sp. DN36 (0.9) and *Pseudomonas* sp. DN34 (0.6) (Fig. 11). Therefore, *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 are able to grow in anaerobic conditions using nitrate as sole electron acceptor.

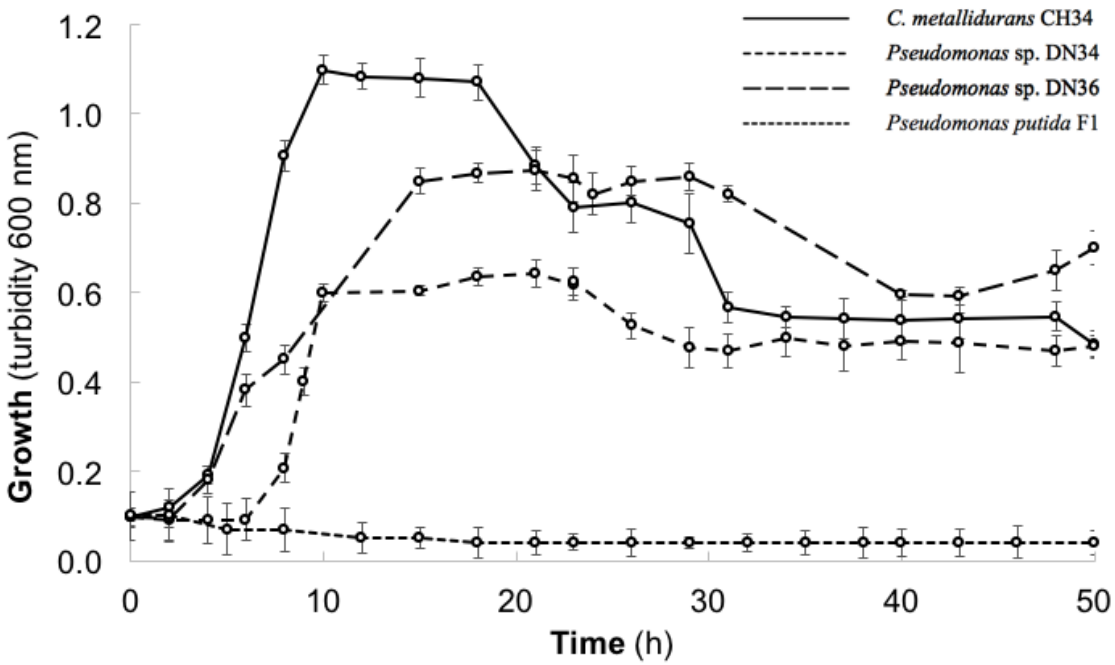

Figure 11. Growth curve of hydrocarbonoclastic strains *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 on succinate as sole carbon source and nitrate as sole electron acceptor. *C. metallidurans* CH34, *Pseudomonas* sp. DN34, *Pseudomonas* sp. DN36 and *Pseudomonas putida* F1 were cultivated in liquid M9 minimal medium supplemented with succinate (16 mM), and NO_3^- (62.4 mM). Growth was monitored measuring turbidity at 600 nm. Each value is an average ± SD of at least three independent experiments.

4.2.2. Anode as electron acceptor in a Microbial Fuel Cell: exoelectrogenic activity of hydrocarbonoclastic strains

The ability of the strains *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 to use the anode of a Microbial Fuel Cell as terminal electron acceptor, thus their exoelectrogenic capacity, was tested in MFCs for 42 h.

Maximum current densities obtained were 0.7, 0.2 and 1 mA/m^2 for *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36, respectively (Fig. 12). In contrast, in the abiotic control very low currents were detected (0.003 mA/m^2) (Fig. 12).

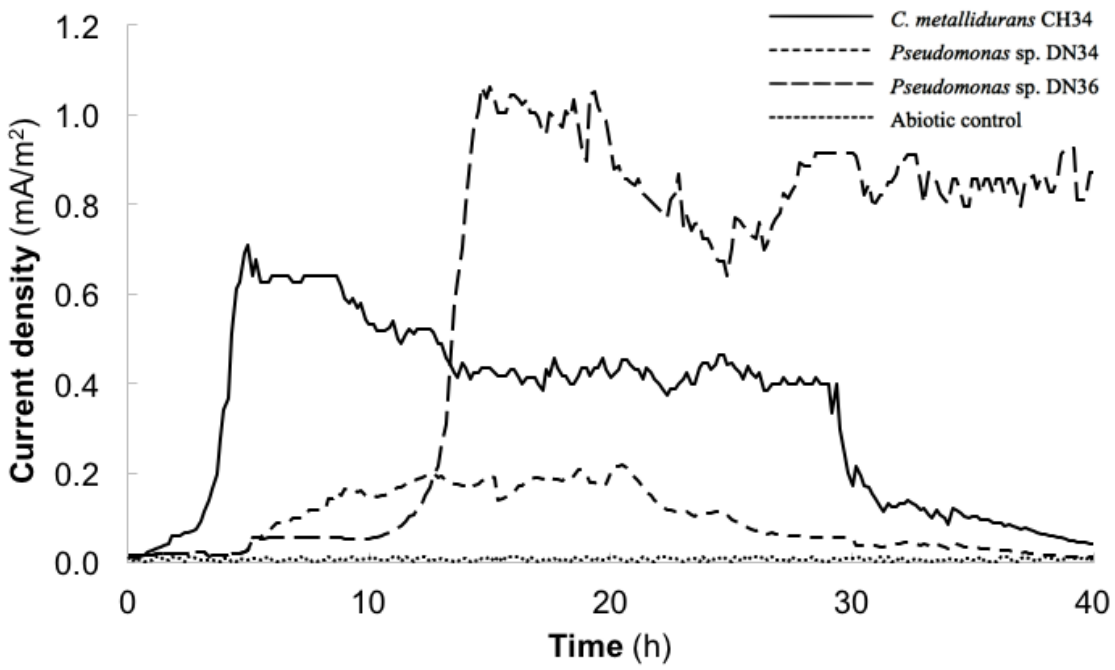

Figure 12. Current density in MFC inoculated with pure cultures of *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 growing on succinate as sole carbon source. Defined current profiles of these strains in comparison with the abiotic control demonstrate the exoelectrogenic activity.

Succinate removal by each strain was determined using a HPLC coupled with an IR detector. *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 reached 91.7±0.4%, 89.4±0.5% and 35.3±0.3% of succinate removal after 42 h, respectively. Coulombic efficiencies were 1.2%, 1.3% and 5.9%, respectively. Low current outputs correlated with low CE but high substrate removal suggest the presence of alternative electron acceptors (*e.g.* oxygen) in the anodic chamber of the BES. However, the shape of current density (Fig.12) resembles a bacterial growth curve, especially if compared with the anaerobic growth curves of the strains CH34, DN34 and DN36 (Fig. 11).

Pseudomonas strains have been described to follow a mediated mechanism of electron transfer [64, 112] but not much is known about the *Cupriavidus* genus. Only one study with a pure strain of *Cupriavidus basilensis* in a MFC indicated that the current obtained with biofilm-covered electrodes in sterile medium were higher than those obtained with planktonic cells [113]. Based on this result in

literature, we performed a microscopy of the biotic anode with a scanning electron microscope (SEM) after 42 h (turbidity$_{600}$ of 0.5) in the MFC inoculated with *C. metallidurans* CH34. The SEM image shows bacterial cells biofilm on the anode surface (Fig. 13).

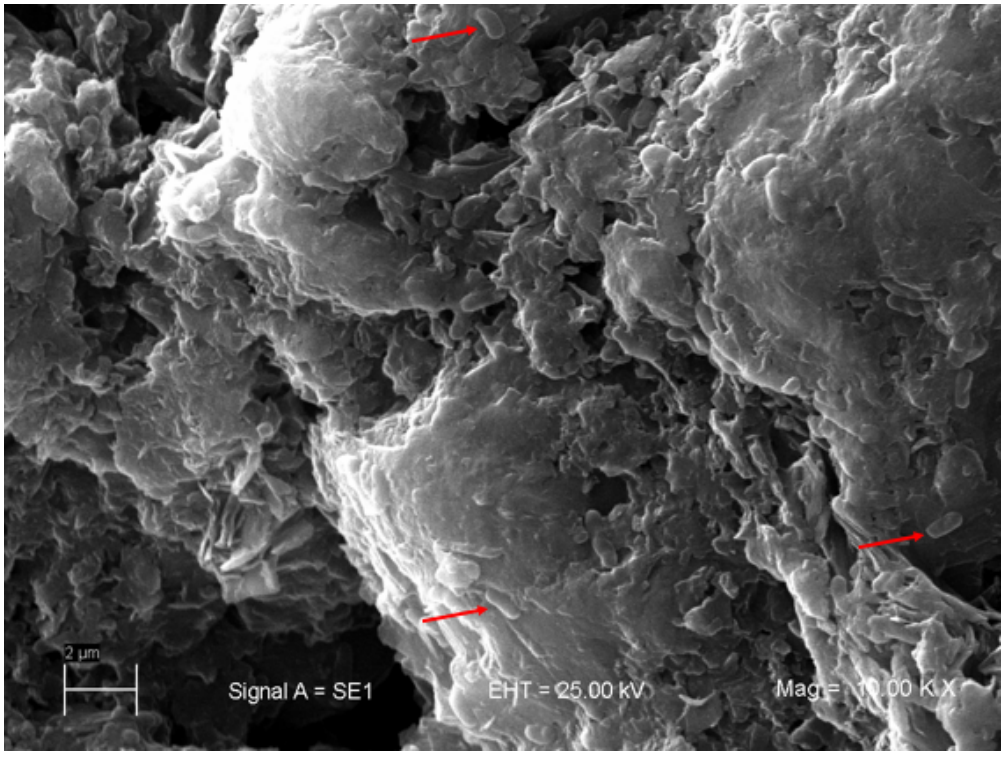

Figure 13. *C. metallidurans* CH34 biofilm formation after 42 h at the graphite anode in MFC observed by SEM microscopy. Bacterial cells are indicated with an arrow.

4.2.3. Anode as electron acceptor in a Microbial Electrolysis Cell: microbial stimulation through voltage application

Due to the low current outputs obtained in MFCs, microbial metabolism was stimulated in MECs where a set of voltages was applied between the anode and the cathode. Prior to experiments with inoculated MECs, different voltages (200 mV, 400 mV, 600 mV, 800 mV) were applied between the anode and the cathode in an abiotic MEC to define current outputs in reactors without microbial activity (Fig. 14). After approximately 30 h, current densities stabilized at less than 10 mA/m^2 (depending on the voltage applied) and remained constant.

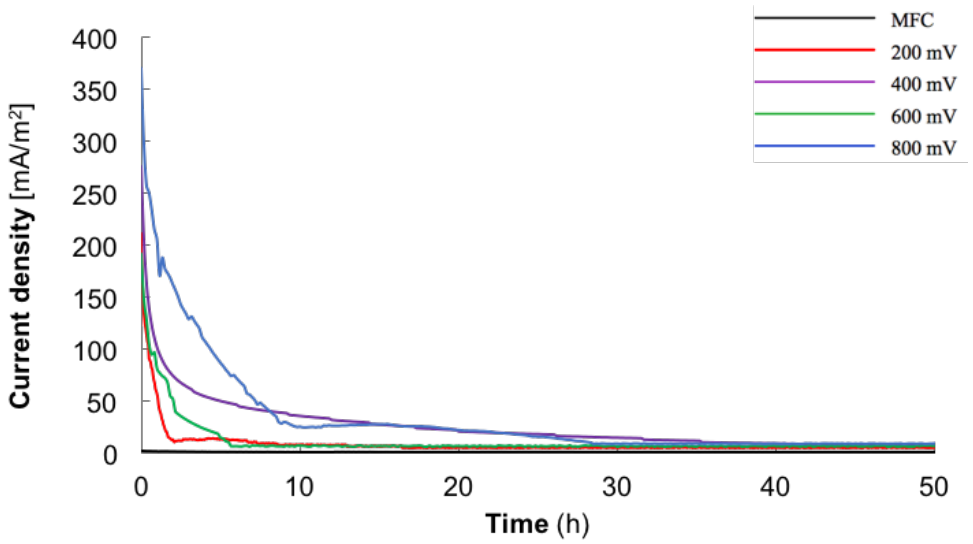

Figure 14. Current density in abiotic MECs at different voltages applied between the anode and the cathode.

Strains CH34, DN34 and DN36 showed different behaviors in terms of current and biomass production depending on the voltage applied.

When an external voltage of 400 mV was applied between the anode and the cathode in the MEC inoculated with *C. metallidurans* CH34, higher biomass production was observed ($turbidity_{600}$=1.3) compared with the control MFC ($turbidity_{600}$=1.1) (Fig. 15B). Under this condition, succinate removal was 95.2±0.2%, while the control BES, operated as a MFC, was 91.7±0.4% (Fig. 18A). Coulombic efficiency increased significantly, from 1.2% in the MFC to 96.8% in the MEC. Even if 400 mV showed the highest biomass production, the best performances in terms of current production were achieved with 800 mV, reaching ~100 mA/m^2 (Fig. 15A).

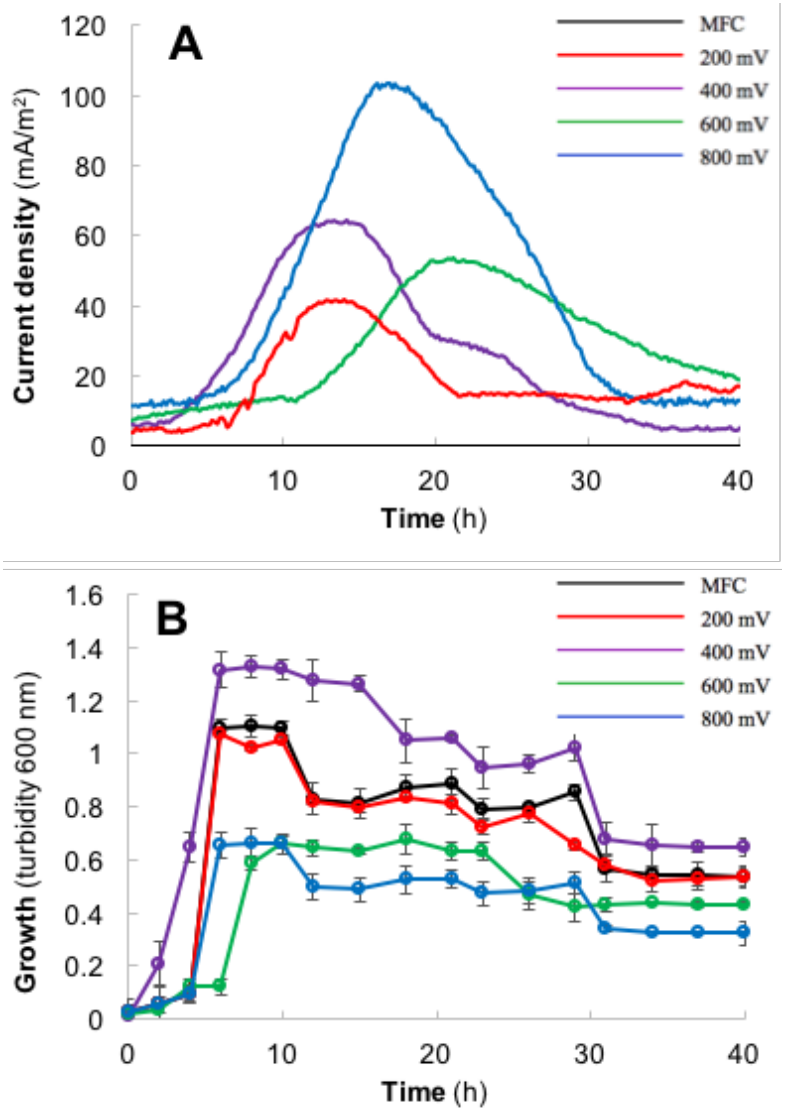

Figure 15. Current density and growth curve on succinate as sole carbon source of *C. metallidurans* CH34 in a MEC, at different voltages applied between the anode and the cathode. A. current density. B. growth curve.

For *Pseudomonas* sp. DN34, the highest biomass production was observed in the MEC with an applied voltage of 800 mV (Fig. 16B). Under this configuration, succinate removal was 91.2±0.2% and coulombic efficiency 49.5%, whereas the control BES (operated as a MFC) only reached 89.4±0.5% of succinate removal (Fig. 18B) and 1.3% of CE. For strain DN34, the application of 800 mV also lead to the highest current densities (~30 mA/m^2, Fig. 16A), but with similar results with 600 mV.

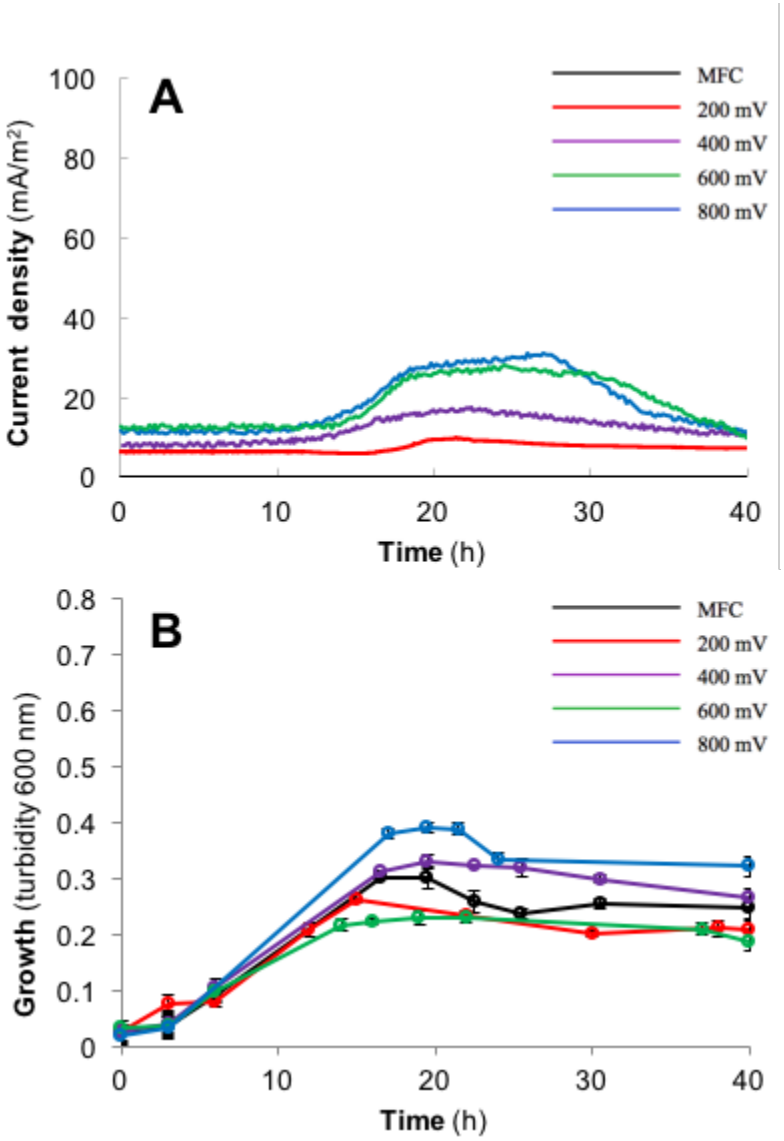

Figure 16. Current density and growth curve in a MEC inoculated with *Pseudomonas* sp. DN34 grown on succinate as sole carbon source, at different voltages applied between the anode and the cathode. A. current density. B. growth curve.

When an external voltage of 200 mV was applied in the MEC inoculated with *Pseudomonas* sp. DN36, a higher biomass production was observed (Fig. 17B). Under this condition, succinate removal and CE were 48.3±0.3% and 90.5% respectively. In the control BES (operated as an MFC) the removal was 35.3±0.3% (Fig. 18C) and CE 5.9%. Current densities as the result of the application of 200 mV, 600 mV and 800 mV were similar, with a slight improvement for 600 mV (Fig. 17A). The differences of succinate removal before

and after the application of external voltage are significant ($p<0.05$) for the three strains.

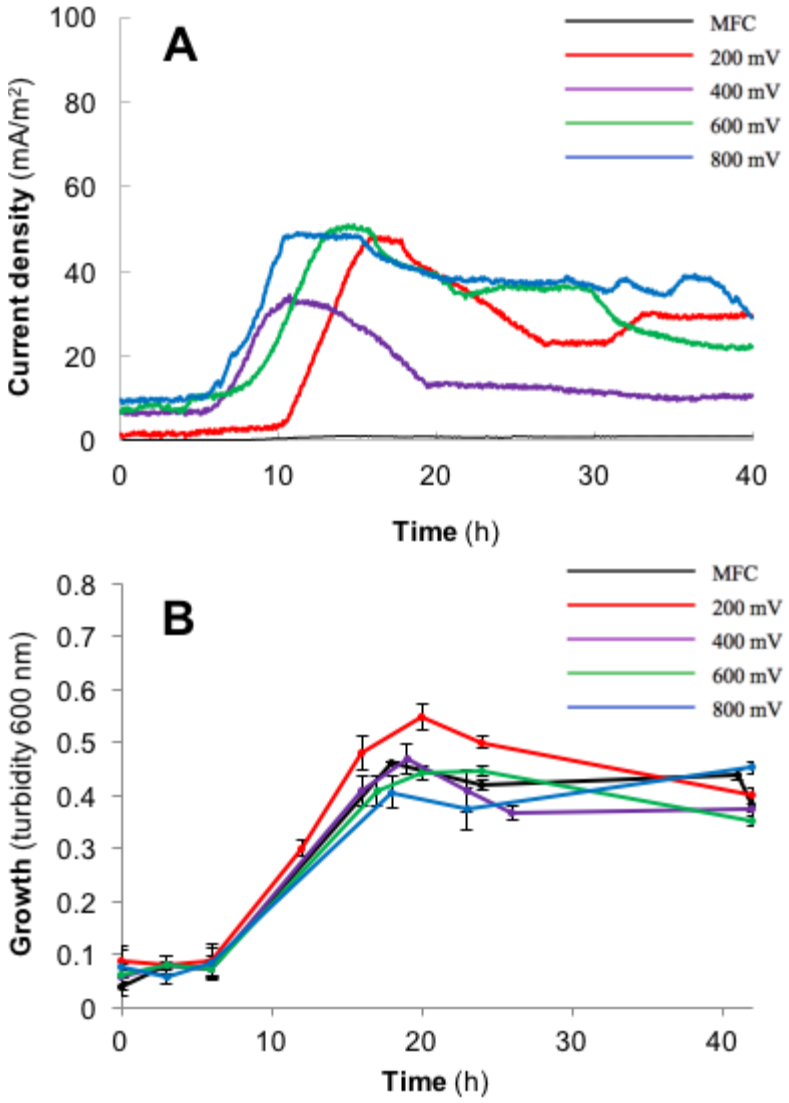

Figure 17. Current density and growth curve in a MEC inoculated with *Pseudomonas* sp. DN36 grown on succinate as sole carbon source, at different voltages applied between the anode and the cathode. A. current density. B. growth curve.

For each strain, a given applied voltage had a positive influence in bacterial growth. However, the application of other voltages influenced cellular growth negatively. Nevertheless, the application of 800 mV led to the highest current production for all strains. Among the three strains, *C. metallidurans* CH34

produced the highest currents (~100 mA/m^2) at 800 mV. *Pseudomonas* sp. DN34 and DN36 did not perform as well and reached maximum current densities of ~30 mA/m^2 and ~50 mA/m^2 (with 800 mV applied), respectively.

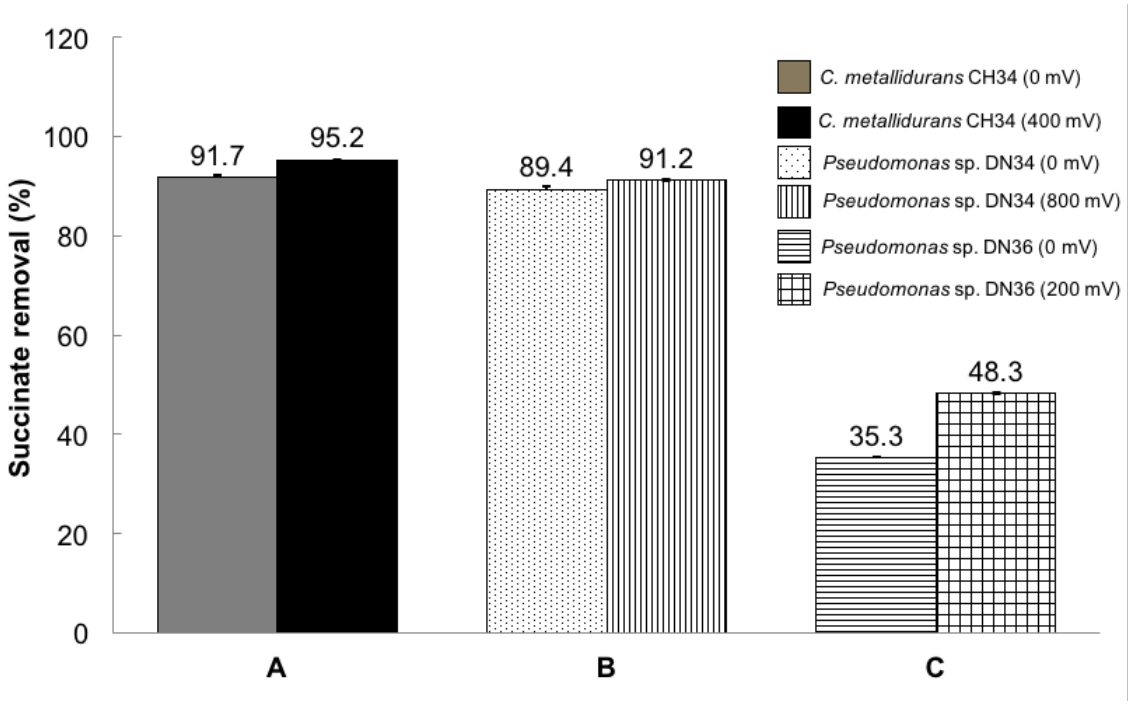

Figure 18. Succinate removal by *C. metallidurans* CH34 (A) and *Pseudomonas* sp. strains DN34 (B) and DN36 (C) in MFCs and MECs after 42 h. MFCs (first column in A, B, C). MECs with applied voltage between the anode and the cathode (second column in A, B, C). The difference between the two treatments is significantly different ($p<0.05$). Each value is an average ± SD of two independent experiments.

4.2.4. Expression of genes coding for denitrification enzymes in BESs by *C. metallidurans* CH34

Among the three strains studied, *C. metallidurans* CH34 showed the best performances in terms of current generation, biomass production and substrate (succinate) consumption in MECs. Moreover, the potential of this strain could be inferred by its genome that is completely sequenced [36]. We aimed at studying if the enzymatic pathway of denitrification could have a role in the transfer of electrons to a solid electrode (anode). Genes that code for nitrate reductase *narG*

(RMET_RS10375) and *napA* (RMET_RS21010) are present in the genome of *C. metallidurans* CH34 but its expression was not tested before.

In the first place, in order to determine if *narG* and *napA* genes are expressed in strain CH34 under denitrifying conditions, RNA was extracted from a liquid exponential-phase culture of *C. metallidurans* CH34 grown in a N_2-flushed SB amended with succinate as sole carbon source and nitrate as sole electron acceptor. The expression of *narG* gene was observed (Fig.19). From the obtained results it seems that strain CH34 lives and grows with nitrate as sole electron acceptor, expressing the genes coding for nitrate reductases.

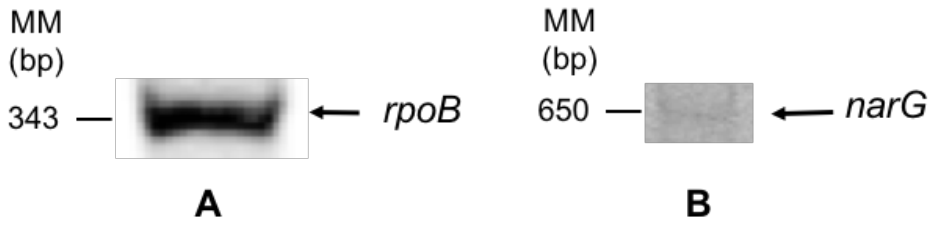

Figure 19. Expression of *narG*, *napA* and *rpoB* genes from *C. metallidurans* CH34 under denitrification conditions. The expression of *rpoB* (A) and *narG* (B) is shown. RT-PCR assays were performed using RNA from CH34 cells grown until exponential phase with succinate as sole carbon donor and nitrate as sole electron acceptor.

We aimed at studying if nitrate reductases are involved in the electron transfer mechanism by strain CH34 in BESs. To reproduce the standard redox potential of nitrate reductase ($E^{o'}$(pH7) = +0.420 V vs SHE) in a BES, the anode was polarized at this potential. RNA was isolated twice during the experiment; at day 2 (t_2) to evaluate the acclimatization of the strain, and at day 16 (final sampling time, t_{16}). Current outputs of the reactor in which RNA was sampled and extracted are shown in Fig.20. Current production was initiated after 6 days of lag phase (Fig.20), that can be associated to an acclimation phase, but reached high current outputs (~250 mA/m^2) after approximately a week.

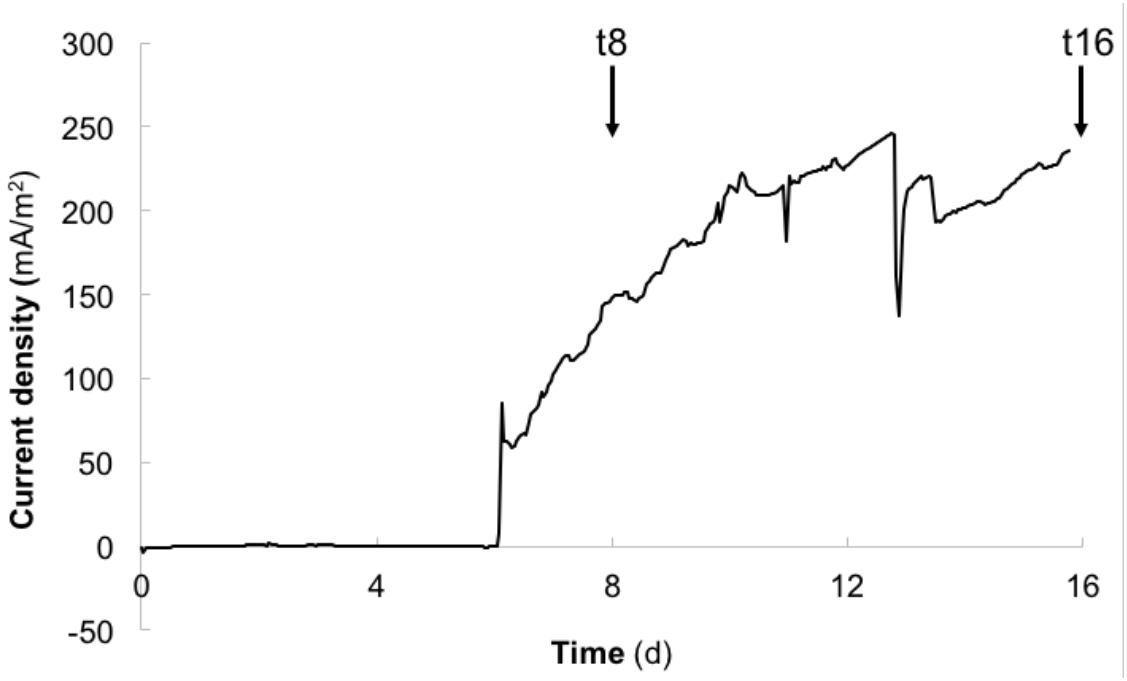

Figure 20. Current density profile in bioanodes inoculated with strain CH34 and polarized at +224 mV (versus an Ag/AgCl [3 M NaCl] reference electrode). Succinate was used as sole carbon source. The reactor was maintained under abiotic conditions from day 0 to day 6 and was inoculated at day 6. Bulk was sampled for RNA analysis on day 8 and day 16. Sampling times are indicated with an arrow.

At t_8, no expression of *narG*, *napA* and *rpoB* genes was detected (data not shown). These results suggest that bacterial cells were not performing denitrification at this time. However, at day 16, nitrate reductase genes were still not expressed (data not shown), but *rpoB* transcripts were detected (Fig. 21). These results indicate that nitrate reductase is not involved during growth in MEC, whereas current generation (Fig. 20) and *rpoB* expression suggest that strain CH34 is active, using a different electron transport mechanism.

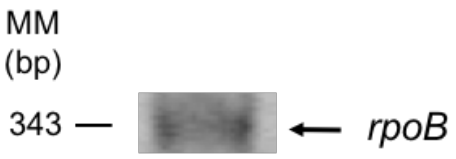

Figure 21. Expression of *rpoB* gene from *C. metallidurans* strain CH34 in a BES. CH34 cells were harvested after 8 days (t16) from inoculation from the anodic-bulk of a BES. Succinate was used as sole carbon source and the anode was polarized at +224 mV (vs Ag/AgCl). RT-PCR assays were performed on the housekeeping gene *rpoB*.

4.3. Toluene degradation by *C. metallidurans* CH34 with different electron acceptors

Based on the results of current generation, growth and substrate consumption in MECs, we selected *C. metallidurans* CH34 as model strain for bioelectrochemical remediation of hydrocarbons. Moreover, from the genome of this strain, we individuated attractive niches for bioremediation. Indeed, it has been described that strain CH34 possesses the genes for the degradation of recalcitrant organic compounds (*e.g.* BTEX, phenol) in aerobiosis [36]. Our goal was to determine if strain CH34 is able to degrade toluene in anaerobic conditions, first with nitrate as electron acceptor and, depending on the results, in the anodic chamber of a BES.

4.3.1. Under denitrifying conditions

Toluene degradation by *C. metallidurans* CH34 under anaerobic conditions was studied. Bacterial cells were cultured anaerobically in mineral medium using toluene (60 ppm) as sole energy and carbon source and 398 ppm of $NaNO_3$ as terminal electron acceptor. A decrease of toluene concentration concurrently with cellular growth was observed in N_2-flushed serum bottles (with resazurin as indicator for anaerobic conditions) (Fig. 22). Under these conditions, strain CH34 was able to degrade up to 73 ppm of toluene within 17 days, corresponding to 3 mg/L·d. Overall, toluene degradation was attributed to bacterial activity, since insignificant losses were observed in the abiotic control (Fig. 22a). The highest

biomass was achieved after the second respike (day 23) and the maximum cell count was $68 \cdot 10^4$ CFU/mL (Fig. 22b).

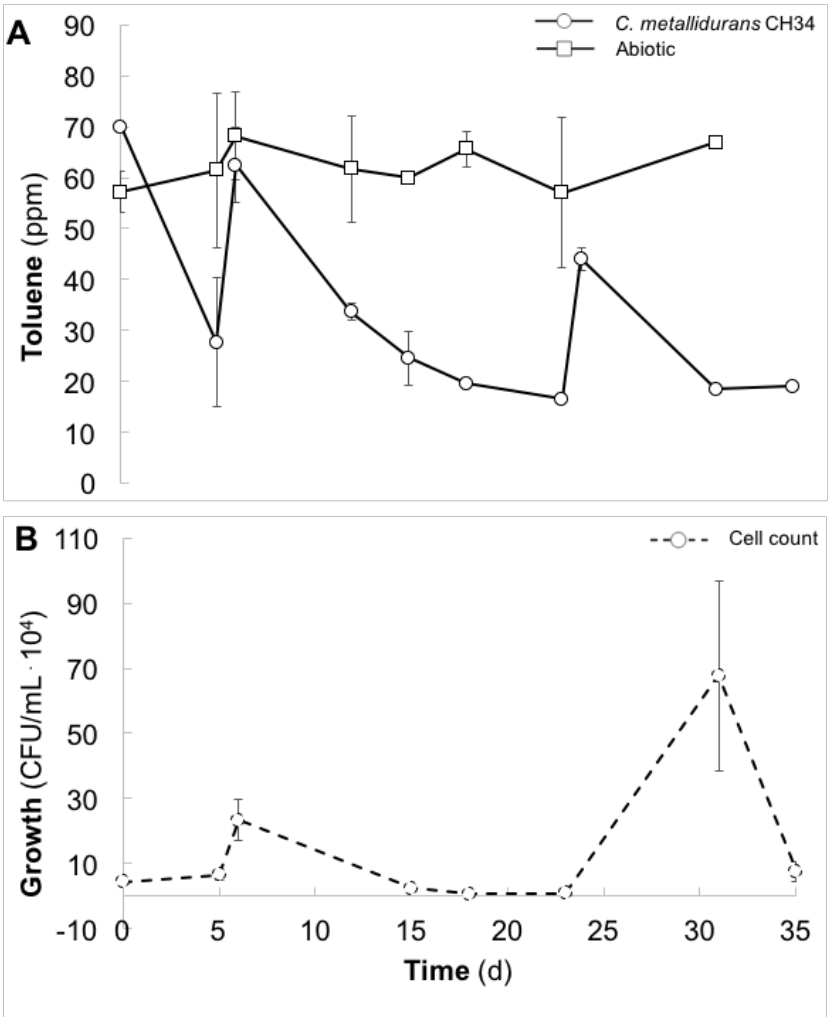

Figure 22. Anaerobic degradation of toluene by *C. metallidurans* CH34. Toluene concentration and bacterial growth in anaerobic serum bottles inoculated with strain *C. metallidurans* CH34 and abiotic control. Toluene concentration decreased after every respike, while the abiotic control maintained toluene concentration at ~ 65 ppm. Values were calculated as the mean ± SD of three independent experiments.

The expression of nitrate reductase genes (*narG* and *napA*) and the housekeeping *rpoB* gene, was studied by RT-PCR after 8 and 15 days. At day 8, *narG* and *rpoB* genes were transcribed (Fig. 23 A,B). At day 15, only *rpoB* showed expression (Fig. 23B). These results suggest that after 8 days, during the

exponential phase of growth, bacterial cells were acclimated and started to metabolize toluene using nitrate as electron acceptor. At day 15 toluene was no longer degraded, bacterial cells reached the stationary phase (Fig. 22) and nitrate reductase genes were not expressed. However, *rpoB* is still expressed at day 15 showing cells activity. These results indicate that strain CH34 is able to metabolize toluene under denitrifying conditions.

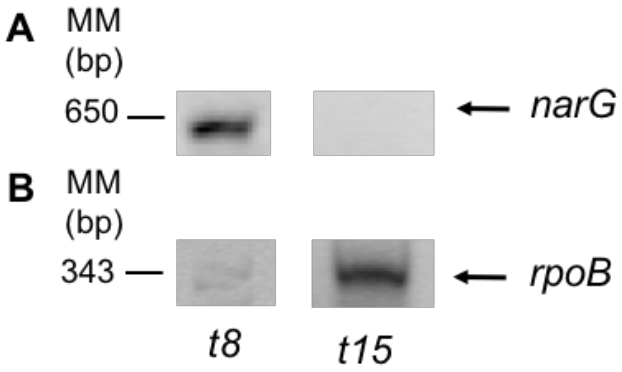

Figure 23. Expression of *narG* and *rpoB* genes from *C. metallidurans* strain CH34 grown on toluene as sole carbon source under denitrifying conditions. The expression of *narG* (A) and *rpoB* (B) is shown. RT-PCR assays were performed using RNA extracted from CH34 cells grown in SB with toluene (60 ppm) as sole carbon source and nitrate (664 ppm) as sole electron acceptor, and collected after 8 and 15 days.

4.3.2. In a Microbial Fuel Cell

Once confirmed that CH34 can grow and degrade toluene under anaerobic conditions, we aimed at testing the biodegradation ability of toluene by strain CH34 in a BES with the anode as sole electron acceptor. To analyze the capacity of *C. metallidurans* CH34 to degrade toluene and produce current, two MFCs (one inoculated and one abiotic) were set. Current density in the inoculated MFC ranged between 0.05 and 0.24 mA/m^2 (Fig. 24) while in the abiotic control the current was 0.01 mA/m^2. Toluene concentration during the first three days decreased in both biotic and abiotic MFCs but after the respike at day 3, toluene concentration in the control remained stable. In inoculated MFC toluene decreased from 61 ppm to 37 ppm (39% of toluene removed) within 17 days,

corresponding to a rate of degradation of 1 mg/L·d. CE calculated after the respike (day 3) until the end of the experiment was 1.0%.

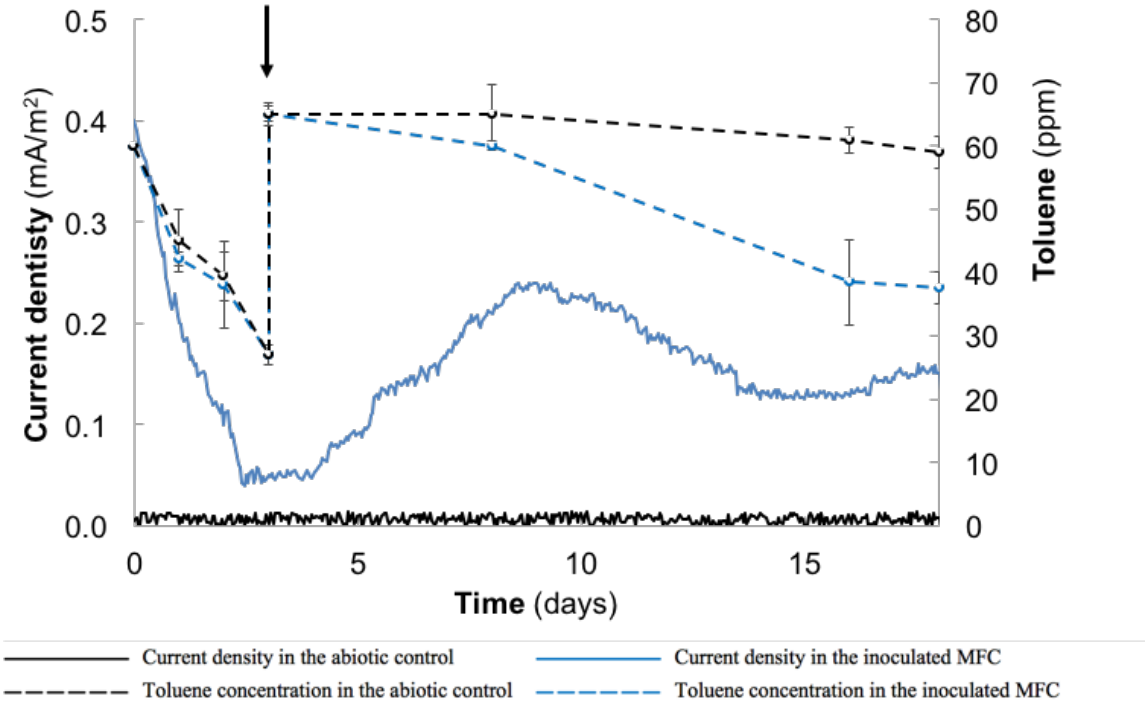

Figure 24. Current density during toluene degradation by *C. metallidurans* CH34 in MFC. Current density (solid line) and toluene concentration (dashed line) in MFC inoculated with *C. metallidurans* CH34 (blue line) and abiotic control (black line). Toluene respike is highlighted with an arrow.

4.3.3. In a Microbial Electrolysis Cell

Low current outputs related to low coulombic efficiencies led us to repeat the experiment in MECs. From the results with succinate in MEC (Fig. 25) and based on a previous experiment in BESs with BTEX and a mixed consortium, we applied a voltage of 800 mV between the anode and cathode.

Two microbial electrolysis cells with toluene as sole carbon source were set and operated for 120 days: one was inoculated with a pure culture of *C. metallidurans* CH34 and the other was an abiotic control. An external voltage of 800 mV was applied between the anode and the cathode to stimulate microbial

metabolism. Toluene respikes were performed in both biotic and abiotic MEC when toluene concentration decreased significantly. At day 90 a last respike was performed only to the biotic MEC, reaching a toluene concentration in the reactor of 60 ppm (Fig. 25).

Batch cycles and coulombic efficiencies are illustrated in Table 5.

Table 5. Coulombic efficiencies for batch cycles before each toluene addition in MEC inoculated with *C. metallidurans* CH34

	Batch cycle 1	Batch cycle 2	Batch cycle 3	Batch cycle 4	Batch cycle 5
Days	0-11	11-22	22-72	72-90	90-120
Coulombic efficiency (%)	ND[a]	11	45	49	77

[a]ND, not determined

CE from days 0 to 11 were not determined due to toluene adsorption processes on the abiotic reactor that could affect the CE calculation. In 55 days, current densities in the biotic MEC increased from a minimum of 13 mA/m^2 to 47 mA/m^2, while in the abiotic control current densities reached a steady state of about 10 mA/m^2 after 8 days. In the biotic reactor, a decrease of toluene concentration was observed after every respike. Strain CH34 was able to degrade up to 87% ppm of toluene within 18 days (batch cycle 4), removing up to 3 mg/L·d. Coulombic efficiencies of the toluene mineralization process increased with time. CE during the first batch cycle was 11%, while the coulombic efficiency of the last batch cycle increased to 77%. Toluene concentration decreased in the abiotic reactor after every respike during the first 10 days due to adsorption processes. However, toluene removal rate in the abiotic MEC decreased from 27 mg/L·d on the first batch cycle (0-2 days) to 0 mg/L·d of the last batch cycle. After this first period of adjustment due to adsorption, toluene in the control abiotic MEC decreased slightly but remained stable between 50 and 60 ppm during 110 days.

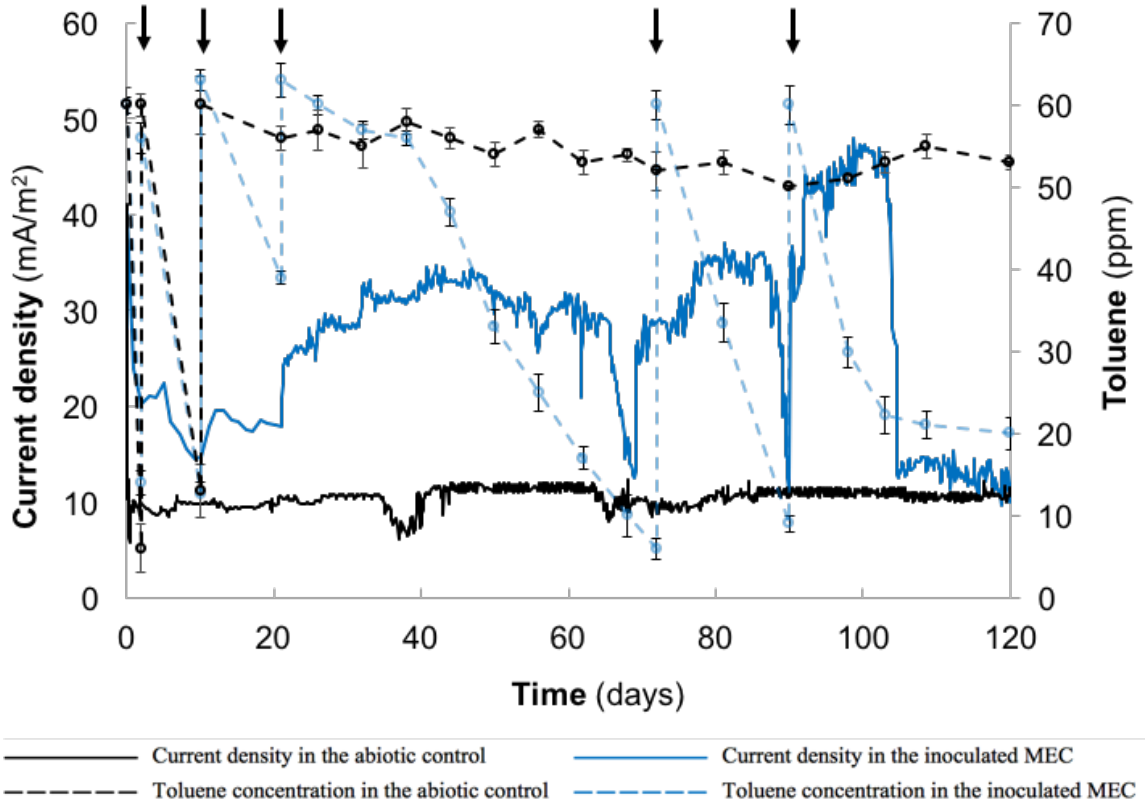

Figure 25. Effect of toluene addition on current density produced by *C. metallidurans* CH34 in MEC. Current density (solid line) and toluene concentration (dashed line) in MECs inoculated with *C. metallidurans* CH34 (blue line) with toluene as sole carbon source and abiotic control MEC supplemented with toluene (black line). Respike is highlighted with an arrow. Maximum current densities obtained in the MEC (48 mA/m^2) increased by two orders of magnitude compared with the MFC (0.23 mA/m^2), demonstrating that microbial metabolism was stimulated. Toluene concentration was measured as an average ± SD of three independent samplings.

These results suggest that strain CH34 may perform extracellular electron transfer to a solid anode, that the rate of electrons transferred to the electrode increases by applying an external voltage and that toluene is efficiently removed in a BES by *Cupriavidus metallidurans* CH34.

4.3.4. Neutral Red as electron carrier in BES

To determine if the application of an external electron carrier has an influence on current production, two MECs with toluene as sole carbon source and Neutral Red as electron transporter were set and operated for 105 days: an abiotic control

and a MEC inoculated with a pure culture of *C. metallidurans* CH34. An external voltage of 800 mV was applied between the anode and the cathode to stimulate microbial metabolism. Toluene was added in both biotic and abiotic MEC when toluene concentration decreased significantly. At day 80 a last respike was performed only to the biotic MEC, reaching a toluene concentration in the reactor of 60 ppm (Fig. 26). Maximum toluene removal (82%) was achieved during the fifth batch cycle (56 to 80 days), corresponding to 2 mg/L·d. A clear trend between current production and toluene consumption was observed during each batch cycle, suggesting that current peaks are related to substrate consumption. Current density peaks reached 54 mA/m^2 during the second and third batch cycle, and diminished during the last two cycles (up to 50 mA/m^2). Current outputs obtained in the BES amended with Neutral Red are very similar to those reported in the reactor without electron carrier (Fig. 25), demonstrating that NR had no impact in current production. In the abiotic reactor, current density maintained stable at ~10 mA/m^2 during the whole experiment, as previously reported for abiotic conditions in BESs where 800 mV were applied (Fig. 25).

During the experiment, toluene was added five times in the biotic reactor; however, the first cycle has not been considered to calculate coulombic efficiency due to toluene adsorption on graphite. CE are shown in Table 6 and, in contrast to the results of the experiment without Neutral Red (Table 5), in this experiment coulombic efficiency was relatively high since batch cycle 2 (42%). During batch cycle 3, CE increased to 66%, but decreased during the last two periods (Table 6).

Table 6. Coulombic efficiencies for batch cycles before each toluene addition in MEC inoculated with *C. metallidurans* CH34 and amended with NR as electron carrier

	Batch cycle 1	Batch cycle 2	Batch cycle 3	Batch cycle 4	Batch cycle 5
Days	0-9	9-33	33-56	56-80	80-105
Coulombic efficiency (%)	ND[a]	42	66	38	60

[a]ND, not determined

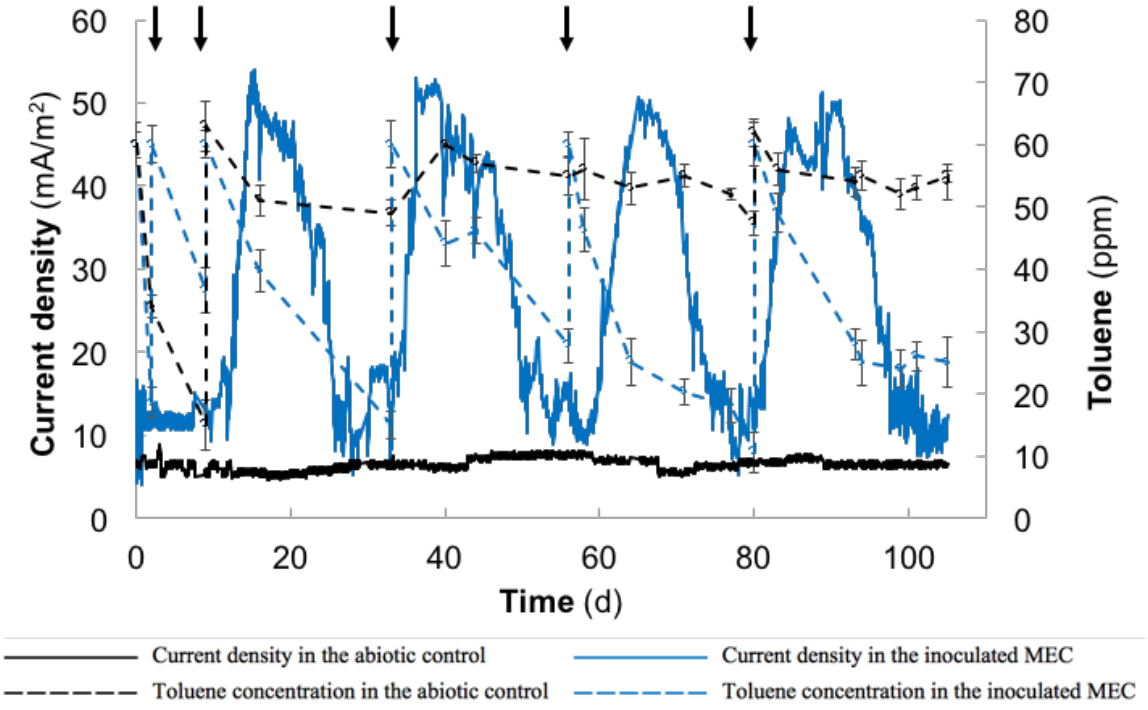

Figure 26. Effect of Neutral Red as electron carrier on current density produced by *C. metallidurans* CH34 in a MEC where 800 mV were applied between the anode and the cathode. Current density (solid line) and toluene concentration (dashed line) in MECs inoculated with *C. metallidurans CH34* (blue line) with toluene as sole carbon source and abiotic control MEC supplemented with toluene (black line). Respike is highlighted with an arrow. Maximum current densities obtained in the MEC (54 mA/m^2) are comparable with those obtained in the MEC without Neutral Red (Fig.25), suggesting that Neutral Red has no direct role in electron transport in strain CH34. Toluene concentration was measured as an average ± SD of three independent samplings.

5. DISCUSSION

5.1. Alternate electron acceptors and exoelectrogenic activity

5.1.1. Denitrification by hydrocarbonoclastic strains

The exoelectrogenic ability of three hydrocarbonoclastic strains: *C. metallidurans* CH3, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36, previously characterized to degrade different hydrocarbons in aerobic conditions, was tested in BESs. One critical point for bioelectrochemical systems, is that the anodic chamber must be kept under anoxic conditions, in order to allow the electrode to function as electron acceptor. Therefore, the ability of the hydrocarbonoclastic strains to grow in anaerobiosis was studied. Nitrate was selected as sole electron acceptor for the three strains because the genes of complete dissimilatory nitrate reduction are present in the genome of strain CH34 [36], and strains DN34 and DN36 are phylogenetically related to *Pseudomonas stutzeri* that are well-known denitrifying bacteria [37].

Growth curves for strain CH34, DN34 and DN36 in anaerobic conditions are illustrated in Fig. 11. Facultative anaerobic metabolism of *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 was demonstrated for the first time in this study and is of particular interest for possible applications of these strains in bioelectrochemical remediation and other fields.

5.1.2. Anode as electron acceptor in a Microbial Fuel Cell: exoelectrogenic activity of hydrocarbonoclastic strains

Strains CH34, DN34 and DN36 were tested in MFCs to determine their ability to perform exoelectrogenic electron transfer to an anode. Maximum current densities obtained in the MFCs inoculated with *C. metallidurans* CH34 (0.7 mA/m^2), *Pseudomonas* sp. DN34 (0.2 mA/m^2) and *Pseudomonas* sp. DN36 (1 mA/m^2) (Fig. 12) are low if compared with other works in the literature with pure cultures, that exceeded our value for more than an order of magnitude (~ 10-100

mA/m^2) [114]. However, first studies with pure cultures obtained current densities similar to this study. Kim *et al.,* (1999) [115] reported a maximum current of 0.0033 mA (~0.02 mA/m^2) in a two-chamber BES inoculated with a pure culture of *Shewanella putrefaciens* IR-1. Low maximum current densities (6 mA/m^2) were also obtained with *S. putrefaciens* IR-1 in a double-chamber MFC with graphite felt as anode and cathode. So far, the *Cupriavidus* genus was employed only once in BES, achieving maximum current outputs of 902 mA/m^2 with acetate as carbon source in a double-chamber MFC [113]. *Pseudomonas* strains have been described for their exoelectrogenic capacity in several reports, using self-produced mediators to carry out the process of electron transfer [64, 112] and pure cultures have been used in the anodic chamber of BES to achieve hydrocarbon remediation [88, 116]. *Pseudomonas stutzeri* has been reported in the diesel-degrading microbial community of the anodic chamber of a MFC [87], but also on the cathode of a BES, demonstrating the cathodic mediator-based EET ability of this species [117]. High succinate removal (> 89% for *C. metallidurans* CH34 and *Pseudomonas* sp. DN34 and 35% for *Pseudomonas* sp. DN36 in 72 h) coupled with low coulombic efficiencies (1.2%, 0.3% and 2.9% for *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36, respectively) suggest that important electrochemical losses and probable oxygen diffusion from the cathodic chamber to the anodic chamber occurred during the experiment. The diffusion of oxygen into the anode chamber has been described as an important issue that leads to a limitation of the coulombic efficiency [51]. We hypothesize that oxygen diffused from the cathodic chamber, creating two layers, one aerobic and one anaerobic, and that only a part of the cells which are present in the anodic chamber, used the electrode as electron acceptor, while the others metabolized succinate aerobically. This is consistent with high succinate removal but low CE.

Based on the results of current production and cell growth in MFCs, considering that current curves follow the trend of bacterial growth, and by taking into account the causes of low current outputs, we concluded that *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 are electrochemically

active bacteria. Moreover, biofilm formation is a common property of electroactive microorganisms [118] and SEM microscopy analysis revealed that a *C. metallidurans* CH34 biofilm was formed on the anode surface, which might be responsible for current generation. Several works indicated the formation of a biofilm on the electrode surface (both anode [119] and cathode [74]) with pure strains [57, 113] and mixed cultures [120]. For some strains, biofilm formation elucidated the mechanism of electron transfer, as for *Shewanella oneidensis* MR-1, that interacts directly with the electrode through nanowires [57]. However other pure strains and bacterial consortia have been found attached to the anode. A bacterial biofilm of *Cupriavidus basilensis* was reported on the graphite anode of a MFC and by comparing current production obtained with biofilm-covered electrodes in sterile medium in comparison with current outputs generated only with planktonic cells, the authors indicated that the biofilm mainly contributed to current generation [113].

5.1.3. Anode as electron acceptor in a Microbial Electrolysis Cell: microbial stimulation through voltage application

Due to low currents obtained with MFCs and based on the hypothesis of oxygen diffusion from the cathodic chamber, strains CH34, DN34 and DN36 were inoculated in MECs where the cathode was maintained under anoxic conditions. A set of voltages was applied to study how the strains respond to external stimulation, in terms of bacterial growth and current production. For the three strains, when a specific voltage was applied between the anode and the cathode, an increase in biomass production and succinate removal was observed. For *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN 36 the application of 400 mV, 800 mV and 200 mV, respectively, lead to a higher biomass production (Fig. 15B, 16B and 17B) and succinate removal (Fig. 18) compared with the conditions where no voltage was applied (MFC configuration). However, the best results in terms of current densities were obtained with 800 mV for all strains*. C. metallidurans* CH34 showed the best performance, achieving a current

peak of ~100 mA/m^2 after 15 h from inoculation, followed by strain DN36 (~50 mA/m^2) and strain DN34 (~38 mA/m^2) with succinate as carbon source. Similar results (current densities between 120±30 mA/m^2 and 290±40 mA/m^2) were obtained in a dual-chamber MEC (anode polarized at -103 mV vs SHE) using spent yeast as substrate and a mixed culture as inoculum [121]. In another report, slightly higher currents (average measured current density in 4 h was 470±74.3 mA/m^2) were obtained in dual-chamber MEC (anode and cathode made of graphite felt and titanium mesh with platinum coating respectively), with 500 mV of applied voltage (vs SHE) and a mixed culture as inoculum [122]. Current densities obtained by Rozendal *et al.,* [122] are slightly higher but in the same order of magnitude compared to our study, and lower values registered in our experiments might be attributed to the lack of Pt catalyzer and probable H_2 diffusion (and its possible microbially-driven consumption) from the cathodic chamber into the anodic one. Similar current densities were also obtained in a report that studied the influence of spacing between anode and cathode in single-chamber MECs [123]. With 3.5 cm of spacing ~100 mA/m^2 (~250 A/m^3) were obtained, but this value increased significantly (up to ~1400 A/m^3) when the distance between electrodes diminished to 2 cm. This result demonstrates that the separation between electrodes and the geometry of the cell have great influence on current production. Pure strains of *Geobacter* were also tested in MECs [124] with two different applied potentials (400 mV and 700 mV) obtaining current densities that ranged between 31±5 A/m^3 (corresponding to ~800 mA/m^2) and 57±2 A/m^3 (corresponding to ~1.466 mA/m^2) with 400 mV of voltage applied, and 31±0 A/m^3 (corresponding to ~800 mA/m^2) and 160±2 A/m^3 (~4.144 mA/m^2) with 700 mV. The results obtained in this report are higher than in our study, but the different reactor configuration and the use of Pt catalyst on the cathode might have had a significant influence of current yield. Even if oxygen reduction is a very slow reaction on the cathode without catalysts [125], this tend to be quite expensive and in our work we intentionally left the cathode not-catalyzed in order to better reproduce further in-field applications with low-cost materials. One study with biotic anodes and cathodes demonstrated that, after biofilm was grown on

the cathode surface, Pt-free cathodes achieved similar power generation as that with the Pt-based cathodes [126], supporting our choice to use a not-catalyzed cathode.

In this study, 800 mV of voltage applied resulted in the highest current densities and cellular growth just for *Pseudomonas* sp. DN34. However, *C. metallidurans* CH34 and *Pseudomonas* sp. DN36 showed the highest performance, in terms of biomass production, with 400 mV and 200 mV of voltage applied, respectively. Moreover, we observed a set of potentials that led to a reduction of biomass production compared with the control where no external voltage was applied and that functioned as a MFC. For instance, the application of 600 mV and 800 mV almost halved $turbidity_{600}$, thus growth, for strain CH34 in comparison with the MFC control where no voltage was applied (Fig. 15B). A similar situation occurred for *Pseudomonas* sp. DN34, where less biomass production was observed with 200 mV and 600 mV, in comparison with growth in the MFC (Fig. 16B). In contrast, *Pseudomonas* sp. DN36 does not seem to be influenced negatively by the application of a voltage (Fig. 17B). These differences might be linked with the potential of the electron acceptor, because, to pursuit a thermodynamically more favorable reaction, the anode must have a more positive potential than the electron carrier, either it is a cell membrane protein or a mediator [127]. However, the potential that determines the optimal growth conditions is not necessarily the highest, since it depends on the composition of the medium, the growth of biofilm on the anode and the inner process of electron transfer [127]. Moreover, in every microbial process, a fraction of the organic substrate is expected to be consumed for growth and thus lost for current generation. In our case, 800 mV was the most enhancing voltage both in terms of current generation and cell growth only for strain DN34; conversely for strain CH34 and DN36, when 400 mV and 200 mV was applied, bacterial growth was supported at the expense of current production.

In this study, succinate was almost completely removed (> 89% for *C. metallidurans* CH34 and *Pseudomonas* sp. DN34) in MFCs, thus the application of a voltage in MECs did not increase carbon source removal. For *Pseudomonas* sp. DN36, succinate removal in the MFC was quite low (35.3±0.3%) and the

application of a voltage in the MEC showed a significantly higher response of the system on succinate removal (48.3%±0.3) compared with the MFC.

The application of a specific voltage for each strain showed a slight increase in biomass production, suggesting that the electron transfer process is specific for each microorganism. Current outputs obtained with MECs are comparable with other studies in the literature and confirm the strains *C. metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36 are electroactive bacteria; however, to reach much higher current densities, improvements in term of reactor configuration and catalyst on the cathode should be achieved.

5.1.4. Role of nitrate reductases during growth of *Cupriavidus metallidurans* CH34 in a bioelectrochemical system

Krake *et al.,* (2015) proposed a model for the extracellular electron transfer mechanism in *Geobacter sulfurreducens* and *Shewanella oneidensis* [52], two metal-reducing bacteria that have been utilized as electroactive model organisms for EET investigations. In *Shewanella oneidensis*, it has been suggested that electrons are transported, through the electron transport chain, from the inner membrane to the outer membrane, where an extracellular cytochrome MtrC transfers the redox equivalents to final exogenous electron acceptors [52]. However, even if it has been demonstrated that cytochromes play an important role in the extracellular electron transport process from the bacterial cell to a solid electrode, each strain can follow different pathways that must be studied independently. In the same review, the electron transport chains of eight organisms studied in BESs were analyzed and five of them shared the ability of using nitrate as terminal electron acceptor [52]. To determine if nitrate reductases play a role in electron transfer during the growth of the strain in BESs, we potentiostetically applied the standard redox potential of nitrate reductase (+420 mV vs SHE) and evaluated if the genes which encodes for these enzymes are expressed in the cells that are present in the anodic chamber. In this experiment, succinate was used as carbon source because our goal was to investigate the

mechanism of electron transfer, independently of the carbon source. In the first place, we confirmed, at a molecular level, that nitrate reductase *narG* is expressed under denitrifying conditions in strain CH34 (Fig. 19) and proceeded in studying the expression of these genes in a BES.

In comparison with the results obtained in the MEC where voltage was applied with a power source between anode and cathode, when the anode was potentiostetically controlled, much higher current was recorded (up to ~250 mA/m^2) (Fig. 20). To have a controlled potential over the anode positively influenced the electron transport from the cells to the electrode, because the potential of the anode determines the redox potential of the final electron acceptor, either a mediator or membrane protein and metabolism routes can be distinguished based on the anode potential [114]. When a voltage is applied non-potentiostetically, the potential of the anode constantly changes, making the channeling on a single metabolic route more difficult.

Current production and the expression of the *rpoB* gene as control for cells viability at day 16, when currents were still at their maximum value, demonstrate that cells were metabolically active. However, no expression of genes *narG* nor *napA* was detected during the experiment (Fig. 21).

The active role of nitrate reductases was demonstrated in this study with nitrate as sole electron acceptor, but we also aimed at studying if the genes that encode for nitrate reductase are still expressed if a solid electron acceptor (the anode) is polarized at the same redox potential of nitrate reductase. Our results suggest that nitrate reductase is not involved in the transport of electrons in a BES and that strain CH34 follows a different mechanism of electron transport to the anode. However, current production and the expression of the *rpoB* gene as control for cells viability demonstrate that cells are actively performing oxidative phosphorylation, thus that, in a mechanism that has not been elucidated yet, an extracellular electron transfer takes place, either in a direct or indirect way.

Some other pathways might be involved in the EET mechanism by strain CH34 that must be elucidated. Several types of cytochromes and quinones have been described to play an important role in the extracellular electron transport

mechanism in model strains [52] and various membrane-associated protein complexes that are present in the genome of *C. metallidurans* CH34 may be important in the extracellular electron transport process (*i.e.* succinate:quinone reductases, bc1-type cytochrome complexes, bb3-type cytochrome c oxidases, quinol oxidases); thus, further studies are needed to elucidate the EET mechanism in this strain.

5.2. Toluene biodegradation by *Cupriavidus metallidurans* CH34

5.2.1. Under denitrifying conditions

Based on the results of current production, bacterial growth and substrate consumption by strains CH34, DN34 and DN36 we observed that *C. metallidurans* CH34 achieved the best performances, obtaining the highest current density (~100 mA/m^2), growth (1.4 of $turbidity_{600}$) and succinate removal (95.2±0.2%) in MEC. Moreover, the genome of *C. metallidurans* CH34 has been sequenced [36], which may be useful to gain insight about the mechanisms of electron transfer in this particular bacterium.

C. metallidurans CH34 possesses the genes encoding pathways for the aerobic degradation of various recalcitrant organic compounds, including benzene, toluene, *o*-xylene, and phenol. However, anaerobic hydrocarbons degradation pathways have not been reported so far [36]. Strain CH34 harbors the genes encoding for the benzoyl-CoA reductase (*boxAB*) [36]. Since benzoyl-CoA is an intermediate of the anaerobic toluene catabolic pathway [128], the anaerobic pathway (at least partial) for toluene biodegradation under denitrifying condition might be present in this strain. Based on the results of our research so far and by analyzing the metabolic possibilities of strain CH34 to degrade hydrocarbons, we aimed at studying the capacity of this strain to degrade toluene, in liquid culture with nitrate as electron acceptor and in BESs, with the anode as electron acceptor.

In our study, the degradation of toluene in anaerobic serum bottles (anaerobic conditions were tested by the addition of the redox dye resazurin), inoculated with *C. metallidurans* CH34 and with nitrate as sole electron acceptor was reported (Fig. 22A). Bacterial growth was observed during the experiment (Fig. 22B) and gas production was reported during sampling (but not analyzed). The expression of two different types of nitrate reductase genes (*narG* and *napA*), that differ in their location and biochemical properties (*narG* is a membrane-bound enzyme and *napA* a periplasmic one) was studied [129]. The expression of nitrate reductase gene *narG* was observed at day 8 (Fig. 23A), when a consumption of toluene was also observed (Fig. 22). However, at day 15, only *rpoB* showed expression (Fig. 23B), indicating that cellular metabolism was still active but that nitrate was no longer used as electron acceptor. Strain CH34 was not able to metabolize all toluene (maximum toluene removal was 73% of toluene was removed within 17 days). Even though toluene has been described to be fully degradable under denitrifying conditions in 20-40 days (depending on the amount of toluene added) with mixed culture [50], more time was probably needed to achieve complete mineralization. Another possible explanation for slow degradation reported in our study is that one or more products (metabolites) formed during toluene metabolism inhibited further toluene removal. It has also been demonstrated that hydrocarbon-degrading bacteria may undergo a physiological stress response that can affect the efficiency of the process [130]. However, our goal was to determine if *C. metallidurans* CH34 is able to degrade toluene under denitrifying conditions. The results of toluene removal, bacterial growth and nitrate reductase expression suggest that strain CH34 metabolizes toluene using nitrate as electron acceptor. Since we demonstrated that strain CH34 is able to degrade toluene in anaerobiosis, its capacity to remove toluene in BESs was studied.

5.2.2. In a Microbial Fuel Cell

When *C. metallidurans* CH34 was inoculated in a MFC containing toluene as sole carbon source, current densities up to 0.24 mA/m^2 and CE of 1.0% were observed. A decrease in toluene concentration in both MFCs during the first three days from set-up was observed, probably due to an adsorption process, a phenomenon that has been reported on graphite electrodes in BESs [43, 77]. However, after the respike, toluene concentration in the control remained stable (around 60 ppm) while in the inoculated MFC, toluene decreased from 61 ppm to 37 ppm in 17 days (corresponding to 39% of toluene removed). Maximum current densities in the MFC with toluene as sole carbon source (Fig. 24) are lower (0.24 mA/m^2) compared with those obtained in the MFC supplemented with succinate (0.7 mA/m^2) but the degradation of toluene is more complex and yields less energy than the metabolism of succinate. A similar behavior was observed in a MFC inoculated with *Cupriavidus basilensis* where current densities obtained with acetate as sole carbon source were significantly higher (902 mA/m^2) compared with those obtained with phenol (310 mA/m^2) [113]. Low current densities and CE obtained in our study in MFC with toluene are much lower in comparison with most studies in the literature [85], however similar current outputs (300-550 μA, corresponding to ~0.003-0.006 mA/m^2) have been previously obtained in a two-chambered MFC fed with groundwater containing benzene [131] and in a dual-chamber MFC inoculated with a mixed culture (current outputs between 0.86±0.14 mA/m^2 and 8.87±0.85 mA/m^2 and current efficiencies between 0.37±0.10 (%) and 1.94±0.23 (%), depending on the operating temperature) with phenanthrene and benzene [132]. As for the experiments in MFCs with succinate, low currents and coulombic efficiency might be attributed to the absence of catalyst on the cathode and oxygen diffusion from the cathodic chamber. However, based on our results of concurrent current generation and toluene removal in MFC, we confirm the capacity of *C. metallidurans* CH34 to degrade toluene using the anode as electron acceptor.

5.2.3. In a Microbial Electrolysis Cell

Due to our promising results obtained with voltage application in MECs with succinate, and by considering that MECs operated with a power supply have been suggested for the treatment of hydrocarbon-contaminated wastewaters [133] and that a broad range of voltages have been used to stimulate microbial metabolism [5], we aimed at studying the degradation of toluene by *C. metallidurans* CH34 in MECs. Based on previous experiments with toluene in BESs using a power supply, and by considering that our best results in terms of current generation with succinate were obtained with 800 mV, we applied this voltage between the anode and the cathode in a double-chamber MEC that was operated for 120 days. In the abiotic control, toluene concentrations decreased during the first 13 days but after the second respike, a decrease in the rate of toluene removal from 27 mg/L·d in the first batch cycle to 0 mg/L·d in the last batch cycle was achieved. As mentioned for the MFC configuration, an initial toluene loss in the abiotic BES may be attributed to adsorption processes, but constant current densities (10 mA/m^2) during the whole experiment demonstrate that no electrochemical oxidation of toluene was achieved in the control BES. Toluene removal was enhanced in the MEC, reaching a 87% of toluene removal within 18 days. In comparison, the MFC only reached 39% of toluene removed within 17 days. This result is consistent with the fact that, by setting the potential of the anode at more positive voltages will provide more energy for the microorganisms [127]. The use of higher anode potential to support current production and bacterial growth has been demonstrated. Busalmen *et al.,* (2007) polarized the anode of a MEC inoculated with *G. sulfurreducens* at +800 mV and +300 mV (vs SHE) and reported that higher current outputs were produced at +0.8 V [134]. Higher current outputs were also obtained with a mixed culture in a benthic-MFC. The anode submerged in marine sediment was polarized at three different potentials (+142 mV, +303 mV, and +818 mV vs SHE). The application of +818 mV led to the highest current and fastest substrate oxidation rate. Moreover, faster microbial colonization at an anode poised at a more positive potential was observed.

Maximum current densities obtained in the biotic MEC (48 mA/m^2) (Fig. 25) increased by two orders of magnitude compared with the MFC (0.24 mA/m^2), demonstrating that electrochemical losses related to oxygen diffusion and the lack of catalyst were, at least partially, overcome. Comparable maximum current outputs (86 mA/m^2) were achieved in MECs by Lu *et al.* (2014) [135] during hydrocarbon degradation in soil and by Venkidusamy *et al.* [136], who achieved a maximum power of 21±3 mA/m^2 in a BES inoculated with a pure culture of *Rhodopseudomonas palustris* strain RP2 using diesel as carbon source. In our study, coulombic efficiency increased with time, reaching a maximum value of 77% after the last batch cycle. We hypothesize that the increase in coulombic efficiency is due to a cellular adaptation to the environmental condition and to the biofilm formation that enhances electron transfer [137]. Similar values (46.7 ± 1.3%) have been reported by Venkidusamy *et al.* [136]. In accordance with our study, low coulombic efficiency values with hydrocarbons as carbon source are reported in literature [98].

5.2.4. Neutral red as electron carrier in BES

Results of current production, coulombic efficiency and toluene removal obtained in MECs by *C. metallidurans* CH34 show the capacities that this strain has for bioelectrochemical remediation of hydrocarbons. However, current densities can be improved, and artificial redox mediators have been used in BESs to allow or improve the production of electricity by bacteria that cannot efficiently use the anode as external electron acceptor, therefore increasing the viability and performance of these systems [138]. Neutral red, a phenazinic compound, has been used for enhancing electricity production in MFCs inoculated with pure strains [68, 139], and with a microbial consortium [138]. The application of an external electron carrier might also elucidate the mechanism involved in the extracellular electron transfer. It has been demonstrated that *Pseudomonas aeruginosa* KRP1 produces its own soluble redox mediator (pyocyanin), a phenazinic compound that function as electron carrier not only for *P. aeruginosa*,

but also for other bacteria that are not capable of producing their own mediators [64]. The *Cupriavidus* genus was studied in BESs with acetate or phenol in MFC [113] and MEC [91] and, based on the results of CV in MEC, the authors suggested that an unidentified self-produced mediator (with a potential of 137 mV vs SHE) could be involved in the mechanism of electron transfer [91]. However, in the study in MFCs with this strain, the majority of current produced was obtained from the biofilm attached to the anode [113].

We aimed at studying the effect of the addition of a well-studied electron carrier, Neutral Red, in terms of current production, coulombic efficiency and toluene removal in a MEC (800 mV of voltage applied) inoculated with strain CH34. In the abiotic control, NR did not have any influence on current densities that maintained constant at ~10 mA/m^2 (Fig. 26) as in the previous experiment without NR (Fig. 25). Maximum current outputs obtained in the inoculated MEC (54 mA/m^2) are very similar to those obtained in the reactor without electron carrier (47 mA/m^2, Fig. 25), which suggests that NR had a mild impact on current production. Moreover, maximum toluene removal was not improved (82%, corresponding to 2 mg/L·d) by the addition of NR in comparison with the MEC not amended with NR (87% toluene removed corresponding to 3 mg/L·d). However, by comparing CE obtained with and without NR, these values increased during the first two batch cycles (42% and 66% with NR, in comparison to 11% and 45% without NR) but diminished in the last two batch cycles (38% and 60% with NR, in comparison to 49% and 77% without NR). Same current densities but higher CE during the first two batch cycles indicate that NR had some influence on electron channeling to the electrode. However, lower CE in the third and fourth cycle indicate a loss of effectiveness of the electron carrier. Only one report investigated the effectiveness of NR in a MFC for the biological oxidation of toluene at the anode using a sludge from the oil cracking wastewater treatment plant as inoculum [97]. The authors reported that the addition of 200 μM of NR in the anodic chamber that contained 11.09 ppm of toluene led to an increase of output voltage from 53.5 mV (corresponding to ~124 mA/m^2) to 109.2 mV (corresponding to ~253 mA/m^2). However, toluene removal was much slower in

the MFC containing NR and the time for complete removal (34.1 h for complete mineralization) almost doubled in comparison with the removal time in the unmediated MFC (16.2 h). This decrease of the efficiency of the process might be attributed to a toxic effect of NR, that in high concentrations, has been reported to inhibit cell growth, especially for gram positive microorganisms [97]. Our results indicate that NR had no influence on current generation in the system, suggesting that a mediated mechanism with this electron carrier does not occur. However, the addition of this mediator had a slight positive impact on coulombic efficiency during the first 60 days (batch cycle 2 and 3) that might be related with an increase of the conductivity due to the presence of NR; and a negative impact on the last 45 days of the experiment (batch cycle 4 and 5). By assuming that our system has design limitations that hinder current outputs, NR could have had a toxic effect on strain CH34 that resulted in a decrease of toluene uptake, thus a minor efficiency of the process. Moreover, if we assume that strain CH34 follows a self-mediated mechanism of electron transfer, NR might have interfered with the supposed-mediator, decreasing the efficiency of the electron transport mechanism.

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Microbial electrochemistry is a novel technology that has been applied in bioremediation during the last decade and the search for new exoelectrogenic strains is a challenge in this field. Bioelectrochemical remediation of polluted sites has been developed during the last decade and has proven to be effective to enhance the removal of hydrocarbons from polluted environmental matrices.

Some bacterial cultures, which were well known for their exoelectrogenic capacity, were studied in bioelectrochemical systems to determine their potential application in bioremediation. However, several hydrocarbonoclastic strains that have extensively been studied for their capabilities in bioremediation, might have an application in bioelectrochemistry. Their exoelectrogenic capacity and biodegradation performances in BES should be studied.

In this study, the exoelectrogenic capacity to use the anode as solid electron acceptor was demonstrated for the hydrocarbon-degrading strains *Cupriavidus metallidurans* CH34, *Pseudomonas* sp. DN34 and *Pseudomonas* sp. DN36, which are known for their capacity to degrade aerobically aromatic hydrocarbons. This conclusion is supported by current outputs and bacterial growth observed in MFCs amended with a non-recalcitrant substrate. Oxygen presence in the anodic chamber due to its migration from the cathode in MFC configuration might have acted as electron acceptor competitor, lowering current production and coulombic efficiency. Oxygen a more suitable electron acceptor than the anode, therefore, oxygen would have favored cell growth and substrate consumption. In future studies, oxygen concentration in the anodic chamber should be monitored.

The application of an external voltage in MECs increased current densities, stimulating bacterial metabolism. Based on the results of current production, an optimum applied voltage of 800 mV for bioremediation experiments was selected.

C. metallidurans CH34 showed the best performance in terms of current generation, growth and substrate consumption in MEC and was selected for bioremediation assays of the model hydrocarbon toluene in BES.

Genome analysis of *C. metallidurans* CH34, indicated potential applications of

this strain in bioremediation than those already described so far, as the presence of the genes encoding the complete denitrification pathway. However, other interesting possibilities that are not reported in the genetic baggage of strain CH34 should be studied, as the possible degradation of aromatic compounds in anaerobiosis.

The capacity of *C. metallidurans* CH34 to degrade toluene under denitrifying conditions was demonstrated for the first time in this thesis. Toluene removal coupled with current generation by *C. metallidurans* CH34 was observed in MFC and improved in MEC.

The addition of Neutral Red did not show a significant influence on the performance of toluene removal coupled with current generation, indicating that the mechanism of electron transfer in strain CH34 is not mediated by Neutral Red. To investigate the role of the denitrification pathway in BES by strain CH34, we potentiostetically-applied the redox potential of this enzyme over the anode to simulate its behavior. Nitrate reductase was not involved in the *C. metallidurans* CH34 pathway of electron transport in BES.

Results of current production, coulombic efficiency and toluene removal obtained in MECs by *C. metallidurans* CH34 showed its capacity for bioelectrochemical hydrocarbon remediation. The removal of toluene was studied in this thesis, but the heavy metal resistance and the autotrophic metabolism in strain CH34 offer interesting possibilities for the bioremediation of heavy metals at the cathode. As strain CH34 metabolizes toluene in anaerobiosis, the degradation of other hydrocarbons (BEX) should be studied on the anode.

The first hypothesis of this thesis indicates that well-known hydrocarbonoclastic bacteria possess exoelectrogenic activity and their metabolism can be stimulated in a MEC by applying an external voltage. In the thesis we demonstrated that the hydrocarbonoclastic bacteria *Pseudomonas* sp. DN34*, Pseudomonas* sp. DN36 *and Cupriavidus metallidurans* CH34 possess exoelectrogenic activity. Toluene removal by model hydrocarbonoclastic strain *C. metallidurans* CH34 in a MEC where 800 mV were applied (87% of toluene removal in 18 days, corresponding to 3 mg/L·d) is higher compared with toluene removal in the MFC (33% of toluene

removal in 17 days, corresponding to 1 mg/L·d), confirming the first hypothesis.

The second hypothesis indicates that the biodegradation process will be enhanced by the addition of Neutral Red as electron mediator. The addition of NR did not improve the removal of toluene in MEC, refusing the second hypothesis.

The general and specific objectives were achieved. A bioelectrochemical system has been developed for the biodegradation of the model hydrocarbon toluene in water by the hydrocarbonoclastic exoelectrogenic *Cupriavidus metallidurans* CH34.

Bioelectrochemical systems is a novel technology for the bioremediation of polluted waters and several hydrocarbon-degrading bacteria might own the exoelectrogenic capability and can be used to remediate hydrocarbon-polluted environments. For in-field applications low-cost and low-maintenance materials are required. The search for exoelectrogenic bacteria that are capable to remediate co-contaminated environments (*e.g.,* hydrocarbons and heavy metals) is a challenge for bioelectrochemical technology. In this study, the exoelectrogenic capacity of two hydrocarbonoclastic strains: *Pseudomonas* sp. DN34 and DN36, and the model metal-resistant and hydrocarbonoclastic *Cupriavidus metallidurans* CH34 was demonstrated. Further studies with other pollutants by *C. metallidurans* CH34 will be assessed and the mechanism of electron transfer in this strain will be studied through transcriptomic analysis.

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8. ANEXXES

8.1. Scientific production

Espinoza A, Daghio M, González M, Franzetti A, Seeger M. Exoelectrogenic activity during toluene and succinate degradation by hydrocarbonoclastic *Cupriavidus metallidurans* CH34 and *Pseudomonas* sp. DN34 and DN36 in Bioelectrochemical Systems. In preparation.

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8.2. Participation at conferences

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